Putative extrinsic blood coagulation pathway inhibitors from the tick *Ornithodoros savignyi*

by

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Submitted in partial fulfillment of the requirements for the degree

Magister Scientiae

in the

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

December 2001

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List of Abbreviations

aPTT	activated partial thromboplastin time
arcsec	arc seconds
Asx	aspartic acid or asparagine
BSA	bovine serum albumin
BSAP1	$BaSO_4$-adsorbing protein 1
BSAP2	$BaSO_4$-adsorbing protein 2
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CP4	collagenous surfactant-associated protein
CPC	1-hexadecylpyridinium chloride
CRP	C-reactive protein
2D	two-dimensional
Da	Dalton
DBS	4-diazobenzene sulphonic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
ESMS	electro-spray mass spectrometry
FAO	Food and Agriculture Organization of the United Nations
fVII	factor VII
fVIIa	activated factor VII
fIXa	activated Factor IX
fX	factor X
fXII	factor XII
g	gram
g	acceleration due to gravity
Gas6	growth-arrest-specific gene 6
Gla	γ-carboxyglutamic acid
Glx	glutamine or glutamic acid
GRAB	Gauteng Regional Association of Biochemistry
HIC	hydrophobic-interaction chromatography
HLys	hydroxylysine
HMK	high-molecular-weight kininogen
HPLC	high-performance liquid chromatography
HPro	hydroxyproline

IFN-γ	interferon-γ
IL-4	interleukin-4
IL-5	interleukin-5
IL-10	interleukin-10
IPG	immobilized pH gradient
IUBMB	International Union of Biochemistry and Molecular Biology
K_d	affinity constant
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MSA	methanesulphonic acid
PBS	phosphate buffered saline
PEC	(4-pyridylethyl) cysteine
pI	isoelectric point
PITC	phenylisothiocyanate
PRGP 1	proline-rich γ-carboxyglutamic acid-containing protein 1
PRGP 2	proline-rich γ-carboxyglutamic acid-containing protein 2
PT	prothrombin time
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin
rma	relative molar abundance
RP	reversed-phase
RP-HPLC	reversed-phase high-performance liquid chromatography
SAP	surfactant associated protein
SASBMB	South African Society of Biochemistry and Molecular Biology
SDS	sodium dodecylsuplhate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SP-A	surfactant protein A
SPR	surface plasmon resonance
TEMED	N, N, N', N'- tetramethyl-ethylene diamine
TF	tissue factor
TFA	trifluoroacetic acid
TFPI	tissue factor pathway inhibitor
Th1	T-helper 1 lymphocytes
Th2	T-helper 2 lymphocytes
TMG	transmembrane Gla protein
tricine	N-[Tris(hydroxymethyl)methyl] glycine
Tris	Tris(hydroxymethyl) aminomethane
UV	ultraviolet light

Acknowledgements

My sincere appreciation to the following people:

Prof. A.W.H. Neitz , Head of the Department of Biochemistry, University of Pretoria, my supervisor for this dissertation, and co-supervisor Dr. A.R.M. Gaspar, Department of Biochemistry, University of Pretoria, for their support.

Ben Mans, for his innumerable valuable suggestions, unfailing interest and advise in all research matters.

Dr. M.J. van der Merwe, Department of Biochemistry, University of Stellenbosch, for performing electro-spray mass spectrometry.

Prof. W.F. Brandt, Department of Biochemistry, University of Cape Town, for performing amino acid sequencing and matrix-assisted laser desorption ionization mass spectrometry.

Prof. J. Verschoor, Department of Biochemistry, University of Pretoria, Gilbert Siko and Pieter Vrey for their advice and assistance in the use of the IAsys biosensor.

Mr. N.J. Taljaard, Department of Biochemistry, University of Pretoria, for performing amino acid analysis of numerous samples.

Ms. E. Fourie, Department of Haemotology, University of Pretoria, for the use of, and her assistance with, a fibrintimer.

Mr. A. Botha, Laboratory for Microscopy and Microanalysis, University of Pretoria, for supervising the scanning electron microscopy and performing the energy dispersive X-ray spectrometry.

Mr. A.J. Thorne, a good friend, for his expert assistance with graphics and computer problems.

In particular, all my friends and family for their patience in listening to my many woeful stories and complaints.

All my fellow student who have supplied friendly advice.

And finally, the South African National Research Foundation and the University of Pretoria for their financial support.

Chapter 1

Literature overview

1.1 Introduction

Ticks are obligate ectoparasites that infest most terrestrial vertebrates. Their biogeographic range extends over most of the world and they are classified into three major families, the Ixodidae, Argasidae, and Nuttalliellidae. The latter family has only one extant representative species. Following mosquitoes, ticks are the second most important arthropod vectors, but surpass even them in the diversity of pathogens they transmit. Among these are viruses, rickettsias and spirochetes, pathogenic fungi and protozoa (Sonenshine, 1993). The route of transmission for most of these pathogens is through the salivary glands, which also serve as a breeding ground for some. This, together with the fact that some ticks possess toxins that can cause paralysis and other toxicoses, illustrate the substantial health risks posed by ticks, both for humans and domestic animals.

The worldwide economic losses incurred due to tick infestations are enormous. In 1979 and again in 1984 the United Nations Food and Agriculture Organization (FAO) estimated the global cost of tick control and productivity loss caused by tick infestations to be US$ 7 billion per year (Pegram *et al.*, 1993; Sauer *et al.*, 1995). In 1993, a report from the same agency stated that approximately 80% of the world's cattle population of 1281 million are at risk from ticks and tick-borne diseases (Pegram *et al.*, 1993). In Africa, ticks are the most serious constraint to increased production leading in cattle to longer calving times, later weaning, slower daily live-weight gain and less milk production (Pegram *et al.*, 1993). Effective means of tick control are therefore of the utmost importance.

As haematophagous parasites, ticks obtain a blood meal by lacerating host epidermis with their cheliceral digits and then inserting the capitulum into the wound. During this process of attachment and penetration many blood capillaries are damaged, supplying the blood the tick will feed on. Normally such damage would induce the activation of various coagulation proteins and thrombocytes whose task it is to prevent excessive blood loss by plugging the wound with a blood clot. At the same time, vessels should constrict to limit blood flow to the wound. To feed successfully, ticks suppress these host defences using a wide variety of potent vasodilators, anticoagulants, antithrombotic and fibrinolytic agents present in their saliva (Stark & James, 1996).

Furthermore, tick feeding induces a complex array of host immune responses many of which are actively suppressed by the tick. Lymphocytes have a reduced ability to proliferate, there is a diminished primary antibody response to T-cell dependent antigens, decreased elaboration of macrophages and Th1-lymphocyte cytokines (IFN-γ) (Wikel, 1996), whereas Th2 cytokine production (IL-4, IL-5 and IL-10) is enhanced (Brossard & Wikel, 1997). It is known that IL-10 inhibits Th1 cell development and reduces the *in vitro* T-lymphocyte proliferative response to stimulators like concanavalin A. Also, a number of tick-secreted antihistamines have been described (Paesen *et al.*, 1999). Ticks therefore posses a variety of immunosuppressive and anti-inflammatory agents.

1.2 Methods of tick control

A common strategy for disease-prevention is the eradication of the disease vector. In the Americas this has been the method of choice. Success has been achieved in the eradication of *Boophilus* spp. in the United States and in areas of Argentina (McCosker, 1993). This approach is however limited by the need of relatively isolated infested areas, as reinfestation is a common occurrence. Prime examples of such isolated areas are oceanic islands. Once ticks have been eradicated, reinfestation can be prevented through appropriate legislation. An example of the success of this method is the total eradication of *Boophilus microplus* from Caribbean islands (Pegram *et al.*, 1996).

The agents predominantly used to eradicate or control tick populations are acaricides. Weekly or even twice-weekly applications of these chemical pesticides are still common practice. This approach has a number of shortcomings. Firstly, the extremely wide distribution of most tick species necessitates the spraying of large areas, causing widespread environmental contamination (Sonenshine, 1993). The secretive habits of many ticks complicate the use of chemical toxicants as these often do not reach ticks hiding in caves or crevices, burrows, cracks and other shelters. Their immense reproductive capacity implies that animals or habitats must be repeatedly treated, at great cost, to prevent reinfestation. The longevity of some species, which can be many years, the fact that they acquire resistance to acaricides and can survive particularly harsh environmental conditions (can survive without food for months or years) present yet further obstacles to tick control. It is important to note, that resistance to acaricides has increased the spread of tick-transmitted pathogens (Sonenshine, 1993). For these reasons, McCosker, the senior officer of the Infectious and Parasitic Disease Group of the FAO wrote in a 1996 review of tick control measures: '...it is probably too late for the widespread eradication of ticks using acaricides to be economically or environmentally acceptable'.

There is therefore a need for novel approaches to tick population control. Biological control, use of tick parasites, pathogens or predators, and molecular methods, vaccines, hormonal disruption of development or reproduction, have been proposed as alternatives to chemical control (Obenchain & Galun, 1982; Brown *et al.*, 1984). Most molecular approaches centre on the utilization of host resistance by immunization or by acquired immunity attained through repeated tick infestations (Janse van Vuuren *et al.*, 1992). One example is the induced immunity attained by cattle against *Boophilus microplus* when immunized with a membrane-bound glycoprotein antigen from the gut of that tick (Willadsen *et al.*, 1989). When ticks ingest the blood from an immunized animal, the antibodies directed against the gut protein cause gross damage to the tick. Among the observed effects is a reduction in engorgement weight, a decline in egg production and death.

Similar effects have been observed when immunization is carried out with salivary gland proteins. It is well established that vertebrates respond immunologically to the macromolecules in tick saliva (Wozniak *et al.*, 1995). The nature of this response appears to be complex and involves cellular and humoral components. In one experiment, salivary gland antigen was administered to guinea pigs never previously exposed to ticks; this induced a significant degree of resistance to infestation (Obenchain & Galun, 1982; Lee & Opdebeeck, 1999). The development of host resistance reduced the number of ticks engorging and the volume of the blood meals obtained. It is therefore evident, that if a host can mount a successful immune response when challenged with salivary gland components, tick feeding and possible population growth could be controlled. The use of salivary gland antigens as a vaccine is therefore plausible.

Studying salivary gland proteins will contribute to the development of further experimental vaccines (Wikel, 1996). This study hopes to make such a contribution. Studying salivary gland secretions has also lead to the discovery of numerous anti-haemostatics, which have allowed the detailed examination of the coagulation pathways and the pleotropic nature of some clotting factors (Stark & James, 1996). Furthermore, knowledge of anticoagulant structure should be useful in the development of new therapeutic agents (Sauer *et al.*, 1995).

1.3 *Ornithodoros savignyi* as a model for the study of anti-haemostatics

Ornithodoros savignyi, commonly known as the sand tampan, occurs in the north-western part of South Africa and large areas of Namibia and Botswana (Howell *et al.*, 1978). Potential hosts include humans, domestic animals and a number of large wild animal species. The experimental transmission of African swine fever virus by *O. savignyi* (Mellor & Wilkinson, 1985) and the presence of the spirochaete *Borrelia crocidurae*, which causes a generally mild relapsing fever in humans, has been demonstrated (Gaber *et al.*, 1984). Despite this evidence, under natural conditions the tick is not known to act as a vector for the transmission of any pathogenic microbes. Nevertheless, the tick is undoubtedly a major pest of domestic livestock. Its bites are

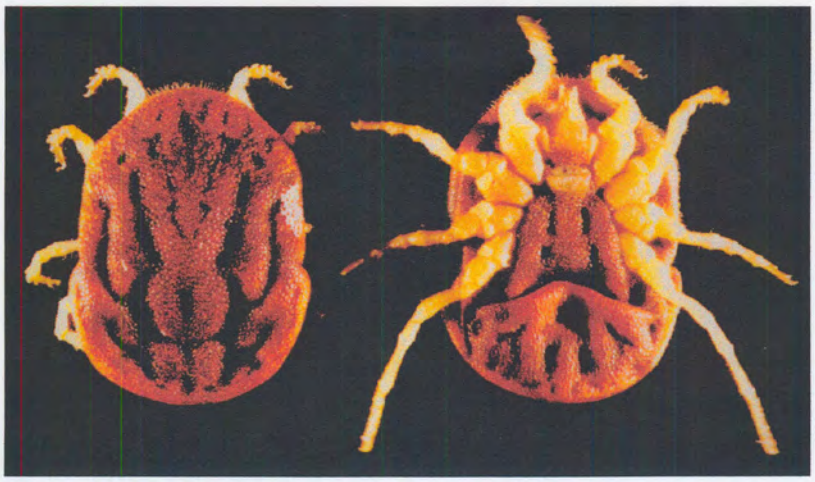
extremely painful and it secretes salivary toxins that frequently cause the death of the hosts, especially young animals such as calves and lambs. Adult animals may also die from these toxins, if infested with a large number of ticks. Humans are known to become sensitized to these toxins and the bite of even one tick can then provoke a severe allergic reaction, which will require immediate medical attention (Howell *et al.*, 1978). The study of *O. savignyi* is therefore of medical importance. The toxin it secretes has been isolated and characterized in terms of its isoelectric point, molecular mass (Neitz, 1976) and portions of its amino acid sequence are known (Neitz *et al.*, 1983; Mans *et al.*, 2001).

In addition, *O. savignyi* is a rich source of anti-haemostatics that may be of future therapeutic use. Two anticoagulant proteins have been isolated and characterized; they are factor Xa inhibitor (Gaspar *et al.*, 1995 and 1996) and savignin, a thrombin inhibitor (Nienaber *et al.*, 1999). The gene encoding the factor Xa inhibitor has been identified and sequenced (Joubert *et al.*, 1998). Two antithrombotics, both platelet aggregation inhibitors, one apyrase (Mans *et al.*, 1998a; 1998b & 2000a) and the other savignygrin (Mans *et al.*, 2000b), have been identified and characterized. Recently, the presence of a fibrinogenolytic agent in the crude salivary extract was established (Mahlaku *et al.*, 1998).

O. savignyi belongs to the tick family Argasidae, which in the vernacular is referred to as the soft ticks. These are so named because they lack a dorsal shield and have a uniform leathery integument that can fold in on itself (Figure 1.1a), allowing the tick to store a large quantity of host blood when fully distended. Soft ticks are fast feeders, often spending no more than 15-30 minutes on the host. Female ticks can be identified by the presence of a large genital opening on the ventral side, an organ that is lacking in males.

These ticks can be kept in the laboratory with great ease, as they require little care. They are quite abundant in the field and can be collected in large numbers. Their salivary glands are large enough to be removed by dissection (Figure 1.1b) making this tick an easy model for biochemical investigations of anti-haemostatics.

a



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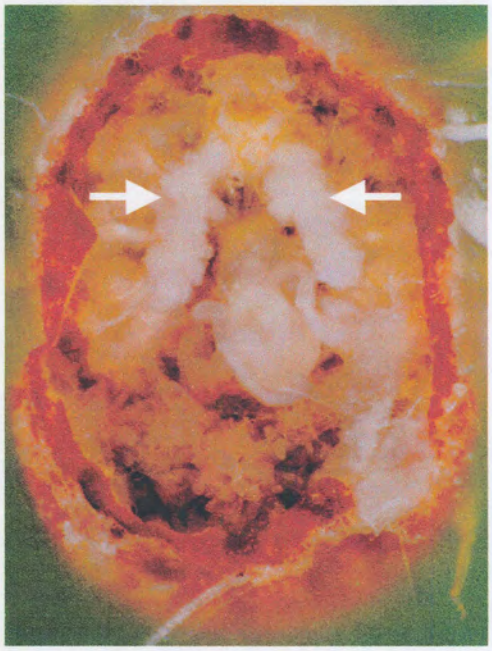
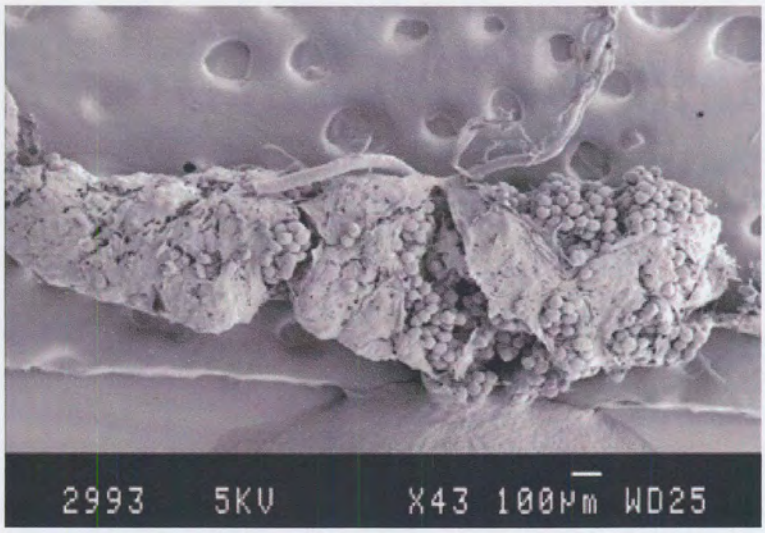


Figure 1.1: **a** The dorsal (left) and ventral (right) view of a female tick of the species *Ornithodoros savignyi*. **b** The internal organs of the tick *Ornithodoros savignyi*. The paired salivary glands lie in an anterolateral position in the body cavity and resemble two bunches of white grapes (indicated by arrows). Photographs: courtesy of B.J. Mans (Department of Biochemistry, University of Pretoria).

1.4 Salivary gland morphology

The salivary glands are the largest glands in the tick body. These paired glands are anterolaterally positioned in the body cavity and each resembles a truss of white grapes (Figure 1.1b). A central salivary duct extends through the length of each gland and these fuse to form a common salivarium at their distal ends. The salivary glands of Argasid ticks comprise large clusters of agranular and granular acini (alveoli) (Figure 1.2a). The former are connected directly to the main duct by short acinar ducts. The latter are connected by similar acinar ducts to secondary intralobular ducts that open into the main duct. The agranular acini play a role in water sorption (uptake of atmospheric moisture), the granular acini, by far the most abundant type of acinus, contain secretory granules (Figure 1.2b) (Sonenshine, 1991). Among the content of these secretory granules are anti-haemostatic, anti-inflammatory, and, in some cases, immunosuppressive compounds.

a



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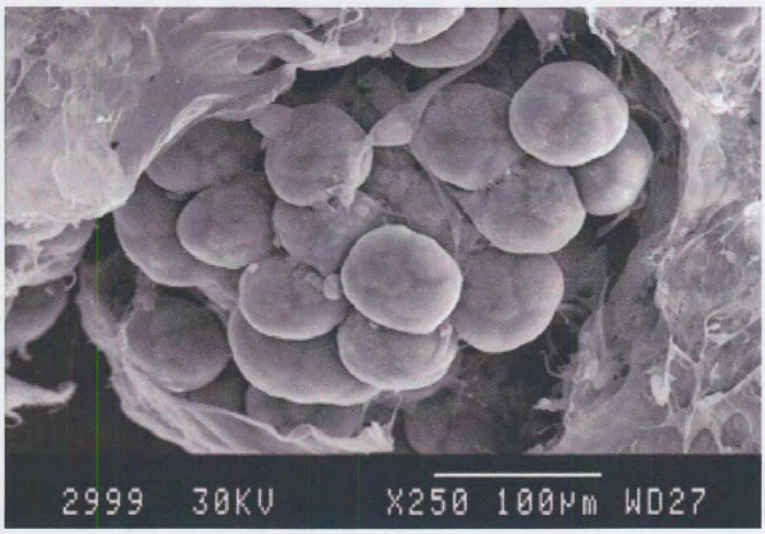


Figure 1.2: **a** Scanning electron micrograph of an *Ornithodoros savignyi* salivary gland magnified 43x. To the right of the image is the gland's distal and to the left its proximal end. Clusters of acini are exposed by ruptures in the salivary gland sheath. The image was taken at an accelerating voltage of 5kV. **b** Scanning electron micrograph of a portion of the salivary gland in **a**, showing a cluster of granular acini magnified 250x. Cells filled with secretory vesicles are visible under the acini membranes. The image was taken at an accelerating voltage of 30kV.

In Argasid ticks, only one type of granular acinus occurs (Sauer *et al.*, 1995), with three different cell types, which can be distinguished morphologically and histochemically (El Shoura, 1985; Mans *et al.*, 1998c). In contrast ticks of the Ixodidae family have much more complicated salivary glands. There are as many as three types of granular acini, with between 7 and 9 cell types (Roshdy & Coons, 1975).

1.5 The haemostatic system

Haemostasis is the cessation of bleeding at the site of cut or scarred vessels. There are three phases to haemostasis: (1) vasoconstriction to limit blood flow to the site of injury, (2) the formation of a haemostatic plug by loosely aggregated platelets and (3) the formation of a fibrin mesh that binds the platelets into a more stable clot. During all stages of haemostasis, regulatory processes are active that will counteract all procoagulant and prothrombotic activity. The formation of a haemostatic plug depends on the dynamic equilibrium between the haemostatic and anti-haemostatic processes (Murry *et al.*, 1998). Following is a description of platelet aggregation, the coagulation cascades and how they are regulated, as well as an evaluation of the potential of factor VII and tissue factor as targets for anti-haemostatics.

1.5.1 Platelet aggregation and activation

When subendothelial tissue is damaged, platelets adhere immediately to exposed collagen fibrils, microfibrils of elastin and basement membrane like material. Collagen is a strong platelet agonist that induces platelet activation, leading to secretion of additional agonists. These include ADP, thromboxane A_2 and serotonin (Marcus & Safier, 1993). These compounds recruit other platelets that aggregate upon the initial layer of adherent platelets, thereby forming a haemostatic plug. Platelets are linked together by fibrinogen, which binds the GPIIb/IIIa membrane receptors of adjacent platelets. Adhesion to exposed subendothelium is mediated by the plasma glycoprotein von Willebrandt factor, which binds the platelet receptor GP1a-IIa (Murry *et al.*, 1998).

During aggregation, platelets become activated and undergo a shape change from a disk to a sphere. During this process, the platelet membrane composition changes. Phosphatidylserine is translocated from the cytosolic to the exoplasmic membrane face (Zwaal *et al.*, 1986). This generates a procoagulant surface on which coagulation factor can associate into multi-enzyme complexes, a process that is essential for coagulation.

Furthermore, during activation, platelets degranulate releasing numerous haemostatic molecules. Among them are fibrinogen, factor V, high-molecular weight kininogen and von Willebrand factor. Also released is a range of protease inhibitors like α_2-macroglobulin, α_1-antitrypsin and C1 inhibitor.

1.5.2 Blood coagulation: An Overview

Table 1.1 lists all the components of the coagulation cascades. The blood coagulation proteins are present as zymogens in blood plasma. When proteolytically cleaved, they become active. With the exceptions of fibrinogen, tissue factor and the accessory factors V and VIII, all are serine proteases. The coagulation cascade is bifurcated (Figure 1.3); a blood clot can form via either the intrinsic or extrinsic pathways. These pathways converge in a final common pathway involving the activation of prothrombin and the subsequent conversion of fibrinogen to fibrin. The intrinsic pathway is initiated when factor XII (fXII) is activated by plasma prekallikrein and high-molecular-weight kininogen (HMK), in the presence of exposed negatively charged surfaces like collagen. This is known as contact phase activation and like the activation of factor IX occurs in the plasma. The activation of factor X by factor IXa, in complex with factor VIIIa, and the activation of prothrombin by factor Xa, in complex with factor Va, are reactions that occur on the anionic phospholipid membrane surface of aggregated platelets (Figure 1.4). The factor IXa-factor VIIIa complex is referred to as the tenase complex and the factor Xa-factor Va complex as the prothrombinase complex.

The extrinsic pathway is initiated by the association of factor VIIa (fVIIa) with the integral plasma membrane protein tissue factor (TF), also known as factor III or thromboplastin. The reactions of this cascade are limited to the plasma membrane surface of subendothelial cells, which constitutively express TF. Due to the location of TF, this cascade becomes active only when the subendothelium is exposed to blood, as during an injury.

Table 1.1: Human blood coagulation factors. (Source: Voet & Voet, 1995)

Factor Number	Common name	Subunit mass (kDa)
I	Fibrinogen	340
II	Prothrombin	72
III	Tissue factor or thromboplastin	37
IV	Ca^{2+}	
Va	Proaccelerin	330
VII	Proconvertin	50
VIII	Antihaemophilic factor	330
IX	Christmas factor	56
X	Stuart factor	56
XI	Plasma thromboplastin antecendent	160
XII	Hageman factor	80
XIII	Fibrin-stabilizing factor	320
	Prokallikrein	88
	High-molecular weight kininogen	150

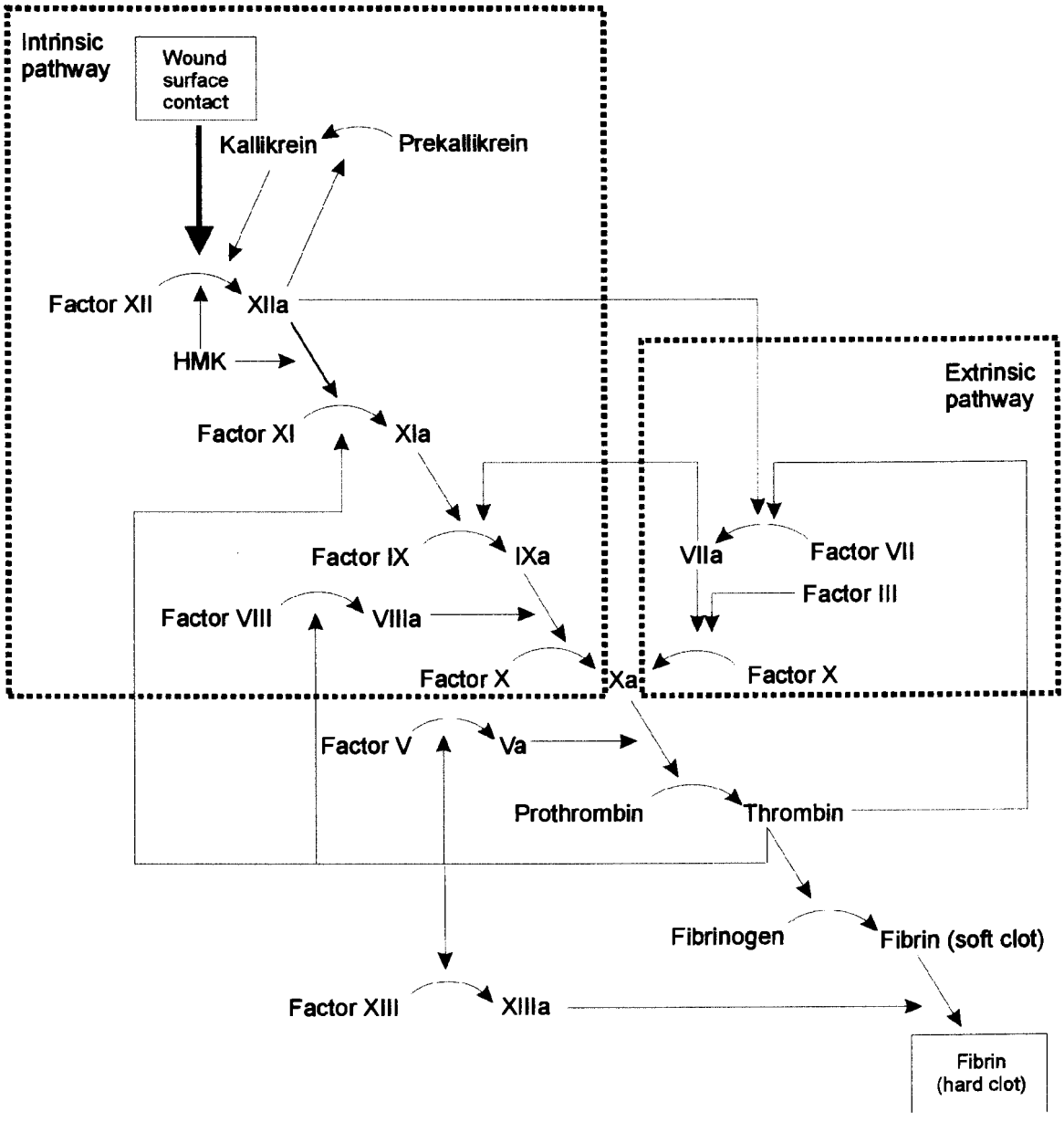


Figure 1.3: The blood coagulation cascade. The components of the intrinsic and extrinsic pathways are enclosed in the large rectangles with dotted lines. The intrinsic pathway is activated when Factor XII binds to negatively charged surfaces like collagen, indicated as 'Wound surface contact' in the diagram. The extrinsic pathway is activated upon tissue damage, when blood becomes exposed to Factor III, an integral membrane protein of subendothelial cells. HMK = high molecular weight kininogen. (Source: Voet & Voet, 1995)

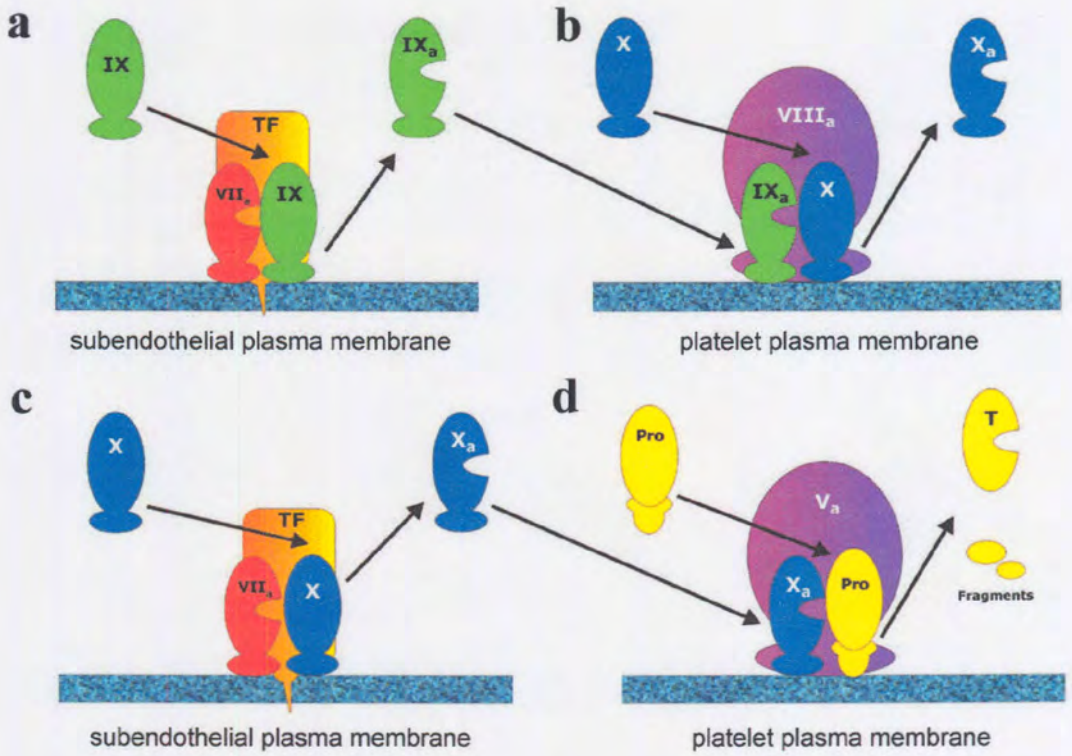


Figure 1.4: The various complexes formed during blood coagulation. **a**. The tissue factor-factor VIIa complex activating factor IX. **b**. Tenase-complex activating factor X. **c**. The tissue factor factor VIIa complex activating factor X. **d**. Prothrombinase complex activating prothrombin. (Diagram adapted from Esmon, 1993)

1.5.3 Regulation of coagulation and fibrinolysis

Plasma contains several serine protease inhibitors whose presence suppresses clot formation and limits the spread of a clot beyond the vicinity of an injury. Antithrombin III inhibits all active proteases of the coagulation cascade except fVIIa. Heparin, released by an injury from mast cells lining blood vessels, enhances the activity of antithrombin III several hundredfold. Protein C, a plasma zymogen until activated by thrombin, in the presence of its cofactor protein S, proteolytically inactivates factor Va and VIIIa. The vascular endothelial cell-surface membrane protein, thrombomodulin, binds thrombin and

converts it into a form with a decreased ability to catalyze clot formation, but with an enhanced capacity for protein C activation. Further anticoagulants are α_2-macroglobulin, heparin cofactor II and α_1-antitrypsin, which act as minor inhibitors under physiological conditions (Murry *et al.*, 1998).

In addition to these regulatory molecules, there exist agents that will degrade an already formed clot. Plasmin, another serine protease, will actively cleave polymerized fibrin. It is formed when plasminogen is proteolytically cleaved by a variety of activators, such as urokinase, tissue-type plasminogen activator and streptokinase.

1.5.4 Potential of factor VII and tissue factor as targets for anti-haemostatics

Fibrin is the end product of both the intrinsic and extrinsic pathways. These pathways converge at the stage of activation of factor X (fX). The only coagulation factors unique to the extrinsic pathway are factor VII and tissue factor (Murry *et al.*, 1998).

Factor VII (fVII) is a single-chain zymogen, which upon activation forms factor VIIa (fVIIa), a two-chain enzyme. A single disulphide bond links the individual polypeptide chains. The activation of fVII and the conversion of factor IX and factor X by fVIIa are reactions that are highly dependent on the binding of the zymogen fVII to tissue factor. Basal levels of fVIIa activity in solution have however been reported (Nakagaki *et al.*, 1991).

Tissue factor (TF) is a integral membrane glycoprotein expressed in the adventitial cell layer surrounding blood vessels and on the surface of a variety of other tissues (Kelly *et al.*, 1995 and 1997). Expression of this protein has also been reported in monocytes (Bach *et al.*, 1986). Vascular damage exposes blood to TF, which forms a calcium-dependent, high-affinity complex with fVII from plasma. Factor VII bound to TF can be proteolytically activated by factor XIIa, factor IXa, factor Xa (Nakagaki *et al.*, 1991), thrombin (Mann & Lorand, 1993) and, at least in part, by an autocatalytic process (Nakagaki *et al.*, 1991). It is important that tissue factor be in contact with a phospholipid

membrane. Human placental tissue factor is inactive if not lipidated (Doellgast & Rothberger, 1986).

It is known that small quantities of factor VII (~5% of normal) are adequate to maintain normal haemostasis. However, when factor VII levels drop below a minimum level (2% of normal), severe bleeding occurs, which cannot be alleviated by factor IX_a (Nemerson, 1983). The intrinsic pathway cannot compensate for the loss of activity from the extrinsic pathway. Humans congenitally deficient in factor VII are known to suffer frequent bleeding episodes (Mariani & Mazzucconi, 1983). Whereas patients lacking factor XI have only a mild bleeding tendency and patients lacking factor XII in general have no bleeding tendency (Prydz, 1983). Similarly, individuals with functionally deficient prokallikrein and high molecular weight kininogen do not exhibit haemorrhagic problems comparable to those experienced by individuals with other coagulation factor deficiencies (Jackson, 1984).

The contact phase may therefore play only a minor role in blood coagulation, suggesting that the extrinsic pathway is of primary importance for haemostasis. Targeting the TF-fVIIa complex with an inhibitor could therefore have tangible benefits for a haematophagous organism. The extrinsic pathway produces fibrin before the intrinsic pathway. The former requires approximately 12-30 seconds to produce fibrin, whereas the intrinsic pathway requires a few minutes (Voet & Voet, 1995). Inhibiting the extrinsic pathway may thus prevent this initial rapid formation of fibrin, ensuring the parasite its blood meal. Also of note is the K_d of fVII binding, which is very near its plasma level concentration, 10-20 nM, indicating that changes in either the plasma concentration of this factor or its affinity for tissue factor could have significant effects on the ability of the extrinsic pathway to produce fibrin (Bach *et al.*, 1986).

If the TF-fVIIa complex is indeed a good target for anti-haemostatic agents, some such compounds should have been identified. Anticoagulants against factor VII have been identified in the salivary glands of *Dermacentor andersoni* ticks (Gordon & Allen, 1991) and an inhibitor of the TF-fVIIa complex has been isolated from the haematophagous

nematode *Ancylostoma canium* (Stanssens *et al.*, 1996). Inhibition of the extrinsic pathway, by a crude salivary gland extract from *Ornithodoros savignyi* has also been reported (Gaspar *et al.* 1995).

One approach to improving treatment of thrombotic diseases involves the design and testing of inhibitors that block specific stages of the coagulation cascades (Kelley *et al.*, 1997). Potent inhibitors of the TF-fVII complex would facilitate greater understanding of this complex and aid in the development of potential therapeutic agents.

1.6 γ–Carboxyglutamic acid-containing proteins

γ–Carboxyglutamic acid (Gla) residues are found in a select group of proteins, all of which bind Ca^{2+} (Graves *et al.*, 1994). The residues are formed by the vitamin K-dependent post-translational carboxylation of the γ-carbon of glutamic acid resides, a reaction catalyzed by the membrane-bound enzyme γ-glutamyl carboxylase located on the luminal side of the rough-endoplasmic reticulum (Vermeer, 1990). This enzyme is present in most human tissues and has been found in both vertebrates and invertebrates. The broad distribution of the carboxylase gene seems to indicate that the biosynthesis of Gla is a highly conserved function among the animal phyla (Begley *et al.*, 2000). The structure of the amino acid is given in Figure 1.5.

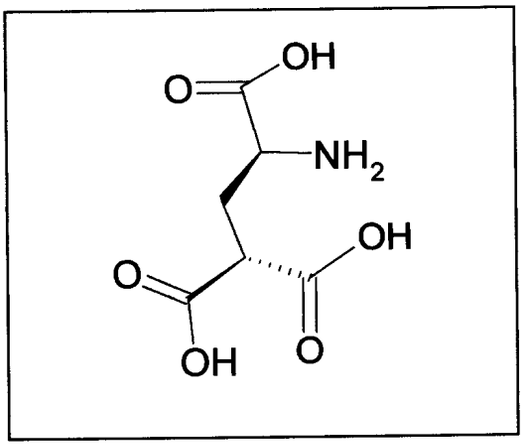


Figure 1.5: The molecular structure of γ–carboxy-L-glutamic acid (Gla) ($C_6H_9NO_6$; molecular weight 191.14 g/mol) (Windholz *et al.*, 1983).

Some of the Gla proteins use their Ca^{2+}-bound Gla residues to bind negatively charged surfaces like activated platelet membranes (Esmon, 1993). The coagulation factors VII, IX, X and prothrombin, as well as the coagulation regulatory proteins Protein C, S and Z, are Gla-containing proteins that use these residues for such a purpose. On the membranes the coagulation factors associate to form protein complexes necessary for conversion from their inactive to their active form. Due to the high degree of homology that these proteins share and the fact that they have a common origin (most are synthesized in hepatocytes), they have been grouped together in one family, the blood plasma Gla proteins (Table 1.2) (Furie *et al.*, 1999).

The other known Gla proteins have been grouped into families according to the same reasoning. Osteocalcin and matrix Gla-protein, having been purified from calcified tissues, form the bone Gla protein family (Vermeer, 1990). Neurotoxins isolated from the venom of the cone snail *Conus geographus*, and related species, constitute the third family and are collectively known as the conopeptides (Olivera *et al.*, 1990). A fourth family contains two proline-rich γ-carboxyglutamic acid-containing proteins (PRGP), PRGP1 and PRGP2 (Kulman *et al.*, 1997), isolated from artherosclerotic plaques and two human transmembrane Gla proteins (TMG), TMG3 and TMG4, which are expressed broadly in fetal and adult tissues (Kulman *et al.*, 2001). The latter family of Gla proteins are all integral membrane proteins and may play a role in a variety of cellular processes, including protein turnover, cell-cycle progression, and signal transduction. In addition to these proteins, pulmonary surfactant-associated proteins (SAP) have been shown to contain Gla residues (Rannels *et al.*, 1987), as has the protein encoded by growth-arrest-specific gene 6, Gas6, (Nakano *et al.*, 1997). The latter protein has been described as a cell survival factor, a growth factor and cofactor in the uptake of apoptotic cells by macrophages (Kulman *et al.*, 2001). Lastly, the presence of Gla within the human γ-glutamyl carboxylase itself has been reported (Berkner & Pudota, 1998).

The known Gla proteins are listed in Table 1.2. To date, no Gla proteins have been discovered in ticks, but γ-glutamyl carboxylase genes are known to occur in the fellow

arthropods *Limulus polyphemus* (horseshoe crab) and *Drosophila* species (Begley *et al.*, 2000).

Table 1.2: Known Gla-containing proteins.

Blood clotting and clotting regulatory proteins	Prothrombin
	Factor VII
	Factor IX
	Factor X
	Protein C
	Protein S
	Protein Z
Bone proteins	Osteocalcin
	Matrix-Gla protein
Conopeptides	Conantokin G
	Conantokin T
Transmembrane proteins	PRGP1
	PRGP2
	TMG3
	TMG4
Other proteins	Gas6
	Pulmonary surfactant-associated proteins
	γ-glutamyl carboxylase (human)

As previously stated, the serum proteins Factor VII, IX, X and prothrombin play an integral role in blood coagulation. All these proteins possess γ-carboxyglutamic acid residues, which after binding Ca^{2+} ions, become internalized, creating a near linear array of Ca^{2+} ions (Figure 1.6) (Furie *et al.*, 1999). This ordering of ions and the resulting structure of the polypeptide backbone causes the exposure of three hydrophobic amino acids on the ω-loop (residues 1-11) (Figure 1.7). This loop region participates in a

specific interaction with the anionic phospholipid membranes of platelets by inserting itself into the hydrophobic part of the membrane (Figure 1.8). It has been proposed that these residues anchor prothrombin (Falls *et al.*, 2001), factor IX (Freedman *et al.*, 1996) and factor VII to membranes, while other sites on the N-terminal domain interact with the head groups of the phospholipids. The N-terminal domain (the first 45 amino acids) of these proteins is known as the Gla-domain and is conserved among the blood Gla proteins (Vermeer, 1990).

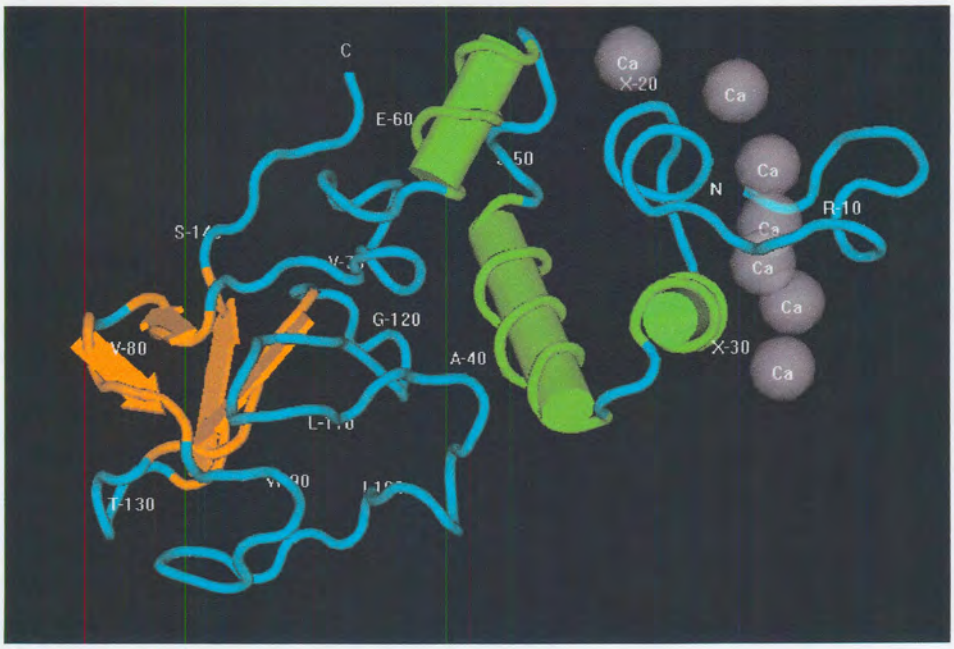


Figure 1.6: The structure of bovine prothrombin fragment 1 (residues 1-156), complexed with calcium (grey). The regular array of Ca^{2+} ions in the Gla domain is shown. The N and C-termini are labelled, as well as every tenth amino acid. Two of those labelled are Gla residues, X-20 and X-30. α-Helices are in green, β-strands in orange and loop region in turquoise. The structure was obtained from the National Centre for Biotechnology Information (NCBI) MMCB structure database under the ID 2853.

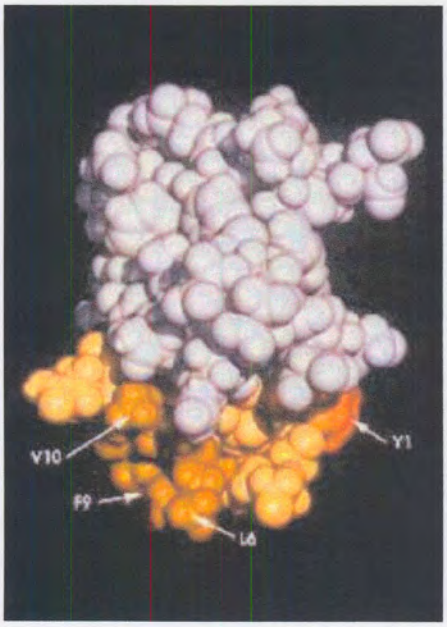


Figure 1.7: The phospholipid-binding site of Factor IX. Residues 1–11 (yellow and shades thereof) include the regions required for phospholipid binding. Hydrophobic residues (dark yellow), including leucine 6, phenylalanine 9, and valine 10, define a hydrophobic patch on the exterior of the protein that likely buries inside the phospholipid bilayer. The N–terminal tyrosine 1 (orange-yellow) interacts with Lys-22. Tyrosine 1 *(Y*1), leucine 6 *(L*6), phenylalanine 9 *(F*9), and valine 10 *(V1*0) are indicated. (Source: Freedman *et al.*, 1996).

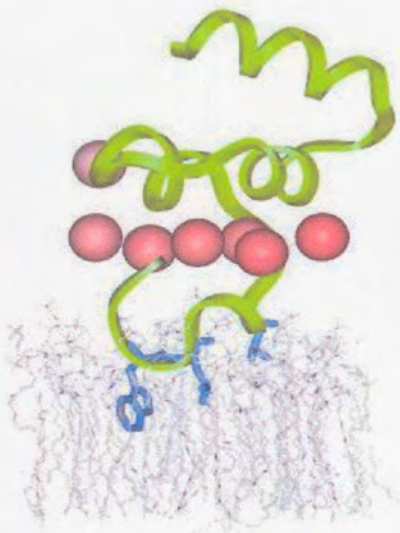


Figure 1.8: Model of prothrombin (residues 1-46) binding to a phospholipid membrane. Interaction between the calcium bound prothrombin Gla domain anchored by Trp 4 at a penetration depth of 7 Å with a gel phase phospholipid membrane surface. The backbone of the prothrombin Gla domain is illustrated as a ribbon, the Trp 4 side chain shown in magenta and the seven calcium ions are shown in red. (Source: Falls *et al.*, 2001)

1.7 Introduction to the present study

In 1997 Ben Mans of the Department of Biochemistry at the University of Pretoria was investigating the platelet aggregation inhibitor, apyrase. He had isolated this protein from the salivary glands of the tick *Ornithodoros savignyi*. His findings led him to predict that apyrase is targeted to platelets by a Ca^{2+}-binding motif similar to those of the platelet membrane-binding coagulation factors (Mans, 1997). To test this hypothesis, he subjected samples of crude salivary gland extract and purified protein to $BaSO_4$ adsorption. All of the membrane-binding coagulation factors are known to selectively adsorb to this insoluble salt. The test for apyrase was positive, it did adsorb to the low-grade form of the salt. The data however indicated the presence of other $BaSO_4$-adsorbing compounds. It is these compounds that were the focus of the current study.

1.8 Objectives of the study

The primary aims of the study were:

- Isolation of two $BaSO_4$-adsorbing proteins from the salivary glands of *Ornithodoros savignyi*.
- Characterization of the isolated proteins, including screening for anticoagulant activity.
- Determination of possible membrane-binding properties

Future aims are to clone and sequence the genes encoding the $BaSO_4$-adsorbing proteins, their structural modelling, and the expression of recombinant proteins and to more fully investigate the native protein's function.

Several aspects of this study were reported at scientific meetings. A poster was presented at the BioY2K Combined Millennium Meeting, the 16th Conference of the South African Society of Biochemistry and Molecular Biology (SASBMB), Grahamstown 2000 (Ehebauer *et al.*, 2000a), and an oral presentation was delivered at the 2nd Gauteng Regional Association of Biochemistry (GRAB) Symposium, Pretoria 2000 (Ehebauer *et al.*, 2000b). Also, a poster was presented at the International Union of Biochemistry and Molecular Biology (IUBMB) Special Meeting on the Biochemical and Molecular Basis of Disease, which coincided with the 17th Conference of SASBMB, Cape Town 2001 (Ehebauer *et al.*, 2001).

Chapter 2

Purification and characterization of $BaSO_4$-adsorbing proteins from the salivary glands of *Ornithodoros savignyi*

2.1 Introduction

2.1.1 Protein purification by $BaSO_4$-adsorption

The Gla-containing coagulation factors VII, IX, X and prothrombin bind Ca^{2+} ions with their γ-carboxyglutamic acid residues in order to associate with membranes. The fact that they are able to bind bications has been usefully employed in their isolation. A variety of bication-containing salts have been used to selectively adsorb these proteins directly from serum. Mostly used are the adsorbents $BaSO_4$, barium citrate and calcium phosphate (Dupe *et al.*, 1975; Deacon & Howell, 1975). Human factor IXa has been purified by adsorption to barium citrate (Bajaj & Birktoft, 1993). Human and bovine factors VII have been purified using $BaSO_4$ (Flengsrud, 1979; Jesty & Nemerson, 1974), as has factor IX and prothrombin (Fujikawa & Davie, 1976).

2.1.2 Protein purification by partition chromatography

Multidimensional chromatography, the use of two or more columns or chromatography techniques to purify a compound, was used to effect the separation and improve the resolution of the $BaSO_4$-adsorbing protein peaks. The chromatographic techniques used in this study were reversed-phase and hydrophobic interaction high-performance liquid chromatography (HPLC).

Reversed-phase (RP) HPLC has become a common method used for the analysis and purification of peptides and proteins. Its popularity is attributed to a number of factors among which is 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 phase modifiers; the generally high recoveries, even at ultramicroanalytical levels; and the excellent reproducibility that can be achieved (Aguilar & Hearn, 1996).

Reversed-phase HPLC usually uses *n*-alkylsilica-based sorbents from which proteins are eluted with gradients of increasing concentrations of an organic solvent such as acetonitrile, methanol or 2-propanol. The most frequently used solvent is acetonitrile, because it is highly optically transparent in the wavelengths used in RP-HPLC and has a low viscosity (Aguilar & Hearn, 1996). The addition of trifluoroacetic acid (TFA) to the mobile phase allows ion pairing with cations of the sample, which can drastically alter the separation (Mant & Hodges, 1991). This hydrophobic anionic ion-pairing agent suppresses the cationic properties of proteins, so that they are separated even more strongly on the basis of their hydrophobicity.

A problem often encountered when purifying a protein with RP-HPLC is the loss of biological activity (Aguilar & Hearn, 1996). This has been attributed to the high-density of *n*-alkyl chains on the column packing material surface and the harsh mobile-phase conditions (like the use of organic modifiers for elution and low pH). This problem can be circumvented by the use of hydrophobic interaction chromatography (HIC), a technique, that like RP-HPLC, relies on hydrophobic interactions with the stationary phase to effect separation (Mant & Hodges, 1991), but is known to maintain the activity of proteins (Wu & Karger, 1996). It is able to do so because of the much lower density of hydrophobic ligands bound to the stationary phase and because the mobile phase is an aqueous buffer of high salt concentration at neutral pH. These conditions tend to be non-denaturing, leaving the protein in its native state (Mant & Hodges, 1991).

The most useful mobile phase eluent for HIC is a solution of ammonium sulphate. It is highly soluble (up to 3.5 - 4 M); a concentrated solution of the salt resists microbial growth and has high UV transparency (Wu & Karger, 1996).

2.1.3 General protein microcharacterization

When individual proteins from a complex sample have been resolved, they are further characterized by three main methods: mass spectrometry, amino acid composition analysis and amino acid sequence analysis (Kellner *et al.*, 1994). The properties so determined, the protein's molecular mass, amino acid composition and at least a partial amino acid sequence, are often enough to identify it. If proteins prove to be particularly difficult to purify, or can be purified in only small quantities, as is the case with the $BaSO_4$-adsorbing proteins, partial amino acid sequences can provide information necessary for the isolation of a protein's gene and allow the subsequent expression of recombinant protein. Structural and functional studies requiring large milligram quantities of protein can then be conducted.

The $BaSO_4$-adsorbing protein's molecular masses were estimated from tricine SDS-PAGE using molecular mass markers as reference. This is a quick and easy method, but provides only a rough estimate of protein mass with accuracies of ±5% to 10% (Wilson & Walker, 2000). To obtain much more accurate data, electro-spray mass spectrometry was performed. Mass spectrometric analyses can give accuracy up to 0.001%. Comparing the mass values obtained from these two methods allows certain predictions regarding protein glycosylation and other post-translational modifications.

Amino acid analysis is achieved by hydrolyzing the protein to yield its component amino acids and then identifying and quantifying them chromatographically. The standard hydrolysis procedure is to heat the protein in a highly concentrated acid solution. Unfortunately, this procedure destroys or chemically modifies asparagine, glutamine, cysteine and tryptophan residues. Asparagine and glutamine are converted to their respective acids, aspartic acid and glutamic acid, and quantified with them. Tryptophan is completely destroyed, but can be quantified by performing methanesulphonic acid hydrolysis. Similarly, 50-100% of cysteine/cystine is lost. This can be overcome by treating a sample with performic acid to form cysteic acid, which is not acid labile, or by reducing the protein and then alkylating the thiol groups prior to hydrolysis (Kellner *et*

al., 1994). Both these techniques were used to quantify Cys in the $BaSO_4$-adsorbing proteins. After hydrolysis the amino acids are derivatized with phenylisothiocyanate (PITC), which produces phenylthiocarbamyl (PTC) derivatives that are detected by their absorbance at 254nm.

Protein sequencing is performed using Edman degradation, a process that sequentially and selectively removes the N-terminal amino acid form the protein. The procedure entails the coupling of PITC to the N-terminal amino acid, followed by the cleavage of the PTC derivative by an anhydrous acid and the concomitant conversion of the PTC derivative into a phenylthiohydantoin (PTH) derivative. The PTH-amino acid is then identified by reversed-phase HPLC. The rest of the polypeptide is recycled to identify the next amino acid. The entire Edman degradation process is invariably carried out in an automated analyser (Wilson & Walker, 2000).

It is often the case that proteins are N-terminally blocked, either by virtue of a natural post-translational modification (e.g. formyl, acyl or pyroglutamic acid group), or due to an accidental side-reaction during sample preparation. Such artificial blocking can result from the use of impure chemicals and detergents. For example, reagent contaminated with acetic acid can cause N-terminal acetylation. Blocked proteins cannot be sequenced, because Edman degradation requires a free amino group for coupling to PITC. To obtain sequence information of a blocked protein, it must be enzymatically or chemically cleaved and the resulting fragments purified and sequenced. During this study it was discovered that the two $BaSO_4$-adsorbing proteins are N-terminally blocked. They were chemically cleaved with cyanogen bromide, the resulting fragments purified and these sequenced.

Polyacrylamide gel electrophoresis has been used to achieve high-resolution separations of proteins. This resolution is greatly improved by making use of two-dimensional gel electrophoresis, a technique that combines the resolving powers of isoelectric focusing and regular SDS-PAGE. During the current study, this technique was used to estimate the purified proteins' isoelectric points (pI). Theoretically, the ideal first dimension makes

use of an immobilized pH gradient (IPG), which provides reproducible, stable pH gradients of any desired pH range (Dunn & Corbett, 1996). Non-linear gradients spanning a wide pH range provide improved protein resolution.

In addition to the above characterizations, the $BaSO_4$-adsorbing proteins were investigated for the presence and number of disulphide bonds as described in Section 2.2.11. Possible anticoagulant activity was assayed by using one-stage clotting assays as described in 2.2.12. This chapter describes the purification and characterization of two $BaSO_4$-adsorbing proteins isolated from *O. savignyi* salivary glands.

2.2 Materials and Method

2.2.1 Materials

All materials used 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, formic acid, guanidinium chloride and bromophenol blue were purchased from MERCK, Darmstadt, Germany. Acrylamide, N,N'-methylene bisacrylamide, glycerol, 2β-mercaptoethanol and $CaCl_2$ were obtained from BDH Chemicals Ltd., Poole, England. The high-grade (commercial) $BaSO_4$ used for adsorption was obtained form Associated Chemical Enterprises. The acetonitrile-R Chromasolv and tricine were purchased from Fluka-Riedel-deHaën, Buchs, Germany. Low molecular weight peptide standards were obtained from Amersham Pharmacia Biotech, Little Chalfont, England. TFA, 4-vinyl pyridine, N-ethylmorpholine and cyanogen bromide were from Sigma-Aldrich, USA. SDS was from Boehringer Mannheim GmbH, Germany. Coomassie Brilliant Blue R-250 was from BioRad, Richmond, USA. Trisodium citrate was from uniVAR, Saarchem Pty. Ltd., Muldersdrift, South Africa. Pathromtin SL, Thromborel-S, human factor VII deficient and human factor X deficient plasma were from Dade Behring, Marburg, Germany. The Spetrapor molecular porous membrane tubing used for dialysis was from Spectrum Medical Industries Inc., Los Angeles, USA.

2.2.2 Preparation of salivary glands

Ornithodoros savignyi ticks were collected in the Upington district of the Northern Cape Province of South Africa by sifting of sand. Only female ticks were dissected 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. Using a binocular stereomicroscope (10x magnification), the salivary glands were removed with forceps and immediately placed in a Petri dish containing a few drops of 0.9% NaCl. After all ticks had been dissected, the

salivary glands were placed in an Eppendorf tube and flash frozen in liquid nitrogen. The glands were then stored at -20°C until needed.

2.2.3 Purification of proteins

Fifty salivary glands were sonified in 500µl H_2O using a Branson Sonic Power Co. Sonifier for 3 x 6 pulses (30% duty cycle and an output control of 3). The cellular debris was removed by centrifugation at 10,000 *g* for approximately 5 minutes. The supernatant was retained and subjected to $BaSO_4$ adsorption.

To the crude extract was added 0.1g of high-grade $BaSO_4$. It was then incubated on ice for 1 hour and the tube inverted every 10 minutes to facilitate maximum adsorption of the protein to the $BaSO_4$. The tube was then centrifuged at 120 *g* for 5 min. The supernatant was discarded and the pellet washed with 0.5M NaCl by first resuspending the pellet in 1 ml of the NaCl solution and then adding another 5 ml. The suspension was centrifuged at 120 *g* for 5 min and the supernatant again discarded. The wash step was repeated twice more. The precipitate was then eluted with 2ml of a 2M NaCl solution for 30 min. This solution was centrifuged at 1100 *g* for 10 min and the supernatant retained. The resulting pellet was eluted at least once more with 2ml of 2M NaCl and centrifuged. The supernatants from each elution were pooled and loaded onto a reversed-phase column.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Waters HPLC, with diode array detector, run by Millenium Chromatography Manager software. Reversed-phase chromatography was performed using a Jupiter 5µ C5 300A (250mm x 4.60mm) column from Phenomenex. Eluent A was a 0.1% TFA; 0.1% CH_3CN solution and eluent B a 0.1% TFA; 100% CH_3CN solution. The column was pre-equilibrated with eluent A. Proteins were eluted by a gradient elution over 42min as set out in Table 2.1, at a flow rate of 1 ml/min. Absorbance was measured at 215nm. The collected fractions were diluted 1:10 with hydrophobic-interaction chromatography (HIC) buffer A (1.7M $(NH_4)_2SO_4$; 20mM Tris-HCl, pH 7.6) in preparation for HIC.

Table 2.1: Reverse-phase chromatography method. Eluent A was a 0.1% TFA; 0.1% CH_3CN and eluent B a 0.1% TFA; 100% CH_3CN solution. A 25min gradient elution was performed followed by a 10min isocratic elution.

Time (min)	% Eluent A	% Eluent B	Duration (min)
Initial	100	0	-
0	100	0	5
5	40	60	25
30	40	60	10
40	100	0	2
42	END		

Hydrophobic-interaction chromatography was performed, using a Beckman HPLC run by System Gold Chromatography software. The column used was a TSK-Phenyl-5-PW (75mm x 7.5mm) column from BIO-RAD. Buffer B was a 20mM Tris-HCl (pH 7.6) solution. The column was pre-equilibrated with buffer A and the proteins eluted as set out in Table 2.2. The flow rate was 1 ml/min and the absorbance was measured at 230nm.

Table 2.2: Hydrophobic-interaction chromatography method. Buffer A was a 1.7M $(NH_4)_2SO_4$; 20mM Tris-HCl (pH 7.6) solution and Buffer B a 20mM Tris-HCl (pH 7.6) solution.

Time (min)	% Buffer A	% Buffer B	Duration (min)
Initial	100	0	-
0	100	0	5
5	0	100	30
35	0	100	20
55	END		

Fractions collected from the HIC column were desalted by loading them onto the C5 reversed-phase column and eluting as described above. Samples were then vacuum-dried and stored at -20°C. $BaSO_4$ adsorption on the crude extract, followed by HIC, was used to purify the proteins for the plasma clotting assays. This avoided possible denaturation and the concomitant loss of protein activity on the reversed-phase column (Aguilar & Hearn, 1996). The samples were desalted by dialysis using Spectrapor molecular porous membrane tubing with a molecular cut-off weight of 3500 Da. Samples were dialyzed for 2 hours in an excess of water and then concentrated by vacuum drying.

2.2.4 Reducing and non-reducing tricine sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out as described by Schägger and von Jagow (1987) using a Biometra electrophoresis system. The gel consisted of a 4% stacking and a 16% separating gel, prepared as set out in Table 2.3. Low molecular weight peptide mass markers (100µg/100µl) and reduced as well as non-reduced HPLC fractions were loaded. The non-reducing sample buffer contained 4.4ml of H_2O; 1.0ml of 0.5M Tris-HCl (pH 6.8); 0.8ml of glycerol; 1.6ml of 10% (w/v) SDS and 0.2ml of 0.05% (w/v) bromophenol blue. In addition to these reagents, the reducing sample buffer contained 0.4ml of 2β-mercaptoethanol and 0.4ml less H_2O. Samples were diluted 1:4 with either sample buffer and heated to 94°C for 5min.

The anode buffer was a 0.2M Tris-HCl (pH 8.9) and the cathode buffer a 0.1M Tris; 0.1M tricine; 0.1% SDS solution. The latter buffer has an approximate pH of 8.25 and was not adjusted. Proteins were stacked using 30V and then separated at 80V. Gels were stained with Coomassie blue [0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% methanol (v/v); 10% acetic acid (v/v)] and destained with a large excess of 40% methanol (v/v), 10% acetic acid (v/v).

Table 2.3: Stacking and separating gel content. The acrylamide solution contained 49.5% T; 3% C acrylamide/N,N'-methylene bisacrylamide. The gel buffer was a 3M Tris-HCl (pH 8.45); 0.3% SDS solution. The gel mixtures were degassed and polymerized with 5µl TEMED and 50µl ammonium persulphate (1g/ml).

	Stacking gel	Separating gel
Acrylamide (ml)	0.5	3.34
Gel buffer (ml)	1.55	3.34
H_2O (ml)	4.2	3.32
Total volume (ml)	6.25	10.0

2.2.5 Electro-spray mass spectrometry

Samples of the purified proteins were analysed using electro-spray mass spectrometry (ESMS). The ESMS was performed by M.J. van der Merwe (Department of Biochemistry, University of Stellenbosch) using a Micromass Quattro triple quadropole mass spectrometer and an electron spray ionization source. Samples were dissolved in 100 µl of 50% acetonitrile containing 0.1% formic acid. The sample was applied by injection of 10 µl into 50% acetonitrile vapour. The instrument was calibrated with horse heart myoglobin.

2.2.6 Amino acid composition analysis

The amino acid composition of the proteins was determined by hydrolysis under acidic conditions (1M HCl) at 110°C for 24 hours. Tryptophan and cysteine were assayed for by methanesulphonic acid hydrolysis and performic acid oxidation, respectively.

The amino acids were derivatized by the addition of 10µl 2:2:1 methanol:water: triethylamine solution. After drying for 5min, 20µl 7:1:1:1 methanol:water:triethylamine: phenylisothiocyanate solution was added and left at room temperature for 20min. To remove excess reagent the sample was dried under vacuum for 1 hour. The dried samples

were then dissolved in 200µl of a solution containing 710mg Na_2HPO_4 in 1 liter of water with 10% H_3PO_4 and 5% CH_3CN at pH 7.40.

Amino acid analysis was performed using the PICO.TAG method. The derivatized amino acids were separated by reversed-phase HPLC. The sample were dissolved in 100µl 5mM Na_2HPO_4; 5% acetonitrile (pH 7.4). Samples were filtered through 0.45µm membranes (Microsep) and 20µl of each sample was applied to the reverse-phase column. The column used was a PICO.TAG 3.9mm x 15cm. A gradient elution was performed using as buffer A 0.14M Na-acetate.$3H_2O$ (pH 5.7) and as buffer B a 60% acetonitrile solution. The conditions for separation were as indicated in Table 2.4. The flow rate was 1ml/min and absorbancy was measured at 254nm. Data was analysed by System Gold software.

Table 2.4: Conditions for separation of derivatized amino acids. Buffer A was a 0.14M Na-acetate.$3H_2O$, pH 5.7 solution and buffer B a 60% acetonitrile solution.

Time (min)	Buffer A%	Buffer B%	Duration (min)
Initial	90	10	-
0	49	51	10
10	0	100	0.5
10.5	0	100	2.2
12.7	90	10	7.5
20.2	END		

2.2.6.1 Methanesulphonic acid hydrolysis

Added to a dry protein sample was 20µl of 4M methanesulphonic acid (MSA) containing 0.2% of tryptamine HCl. To this was added 100µl water. The sample was then hydrolyzed at 100°C for 24 hours. After having cooled, 22µl of 4M KOH was added and the sample dried under vacuum. The sample was redried using 20µl of redrying reagent,

2:2:1 methanol:water:triethylamine. Derivatization was performed using a 7:1:1:1 methanol: water:triethylamine:phenylisothiocyanate solution. The derivatized sample was dried under vacuum and reconstituted in 100-200µl of the HPLC buffer used for the analysis.

2.2.6.2 Performic acid oxidation

The performic acid solution was prepared by adding 19 volumes of 97% formic acid to 1 volume 30% H_2O_2. The solution was left to stand covered for 1 hour. To the dry protein was added 10µl of this fresh reagent. After 30min the sample was vacuum dried. Hydrolysis was performed using 6M HCl at 100°C for 24 hours. Derivatization was carried out with 5µl of 7:1:1:1 methanol:water:triethylamine:phenylisothiocyanate solution.

2.2.7 Cyanogen bromide cleavage

A method adapted from Matsudaira (1990) was used to chemically cleave the proteins. Pure dry protein samples were dissolved in 50µl 70% formic acid and one or a few cyanogen bromide crystals added. The solution was swirled lightly, capped with N_2 (g) and kept in the dark for 12 hours at room temperature. Thereafter, the samples were lyophilized. Upon completion of freeze-drying, 50µl of reducing buffer was added to the proteins and the mixture incubated at room temperature for 1 hour. The reducing buffer contained 6M guanidinium chloride; 0.1M Tris-HCl (pH 8.5); 0.1M DTT. The resulting peptides were purified and separated by reversed-phase chromatography as described in Section 2.2.3 and collected fractions vacuum-dried.

2.2.8 Partial amino acid sequencing

Peptides obtained form the cyanogen bromide cleaved proteins were submitted for automated Edman amino acid sequencing. Sequencing was performed by W.F.B Brandt (Department of Biochemistry, University of Cape Town) using a vapour-phase amino

acid sequencer, modified as described by Brandt *et al.* 1984. Samples were also analysed by matrix assisted laser desorption ionization (MALDI) mass spectrometry.

2.2.9 Theoretical and experimental determination of protein isoelectric points

2.2.9.1 Calculation of isoelectric points from amino acid composition data

The isoelectric point of a particular protein can be predicted from its amino acid composition. The theoretical pI values were calculated according to the '*simple*' method described by Patrickios & Yamasaki (1995). The equation used was:

$$pI = pK_a - \log R$$

where the pK_a is 4.2 (representing the average pK_a value of the acidic amino acid side chains and the terminal carboxyl as defined by Patrickios & Yamasaki (1995)) and R is the ratio between the number of acidic and basic amino acids in the protein:

$$R = (Glu + Asp + C\text{-}t) / (Lys + Arg + N\text{-}t)$$

where C-t represents the C-terminal and N-t the N-terminal group, both of which are taken as 1. The Glu and Asp residues in the protein cannot be accurately determined because Gln and Asn are degraded during acid hydrolysis. However, taking into account the relative molar abundance of Glu and Asp in proteins, the number of Glu and Asp residues in the isolated proteins can be estimated:

$$Glu \text{ or } Asp = T / rma$$

where T is the total number of amino acids constituting a protein and *rma* is the relative molar abundance of the amino acid in question. The *rma* for Glu is 6.2 and that for Asp is 5.5 (Mathews & Holde, 1991).

2.2.9.2 Two-dimensional polyacrylamide gel electrophoresis

Dry protein was dissolved in 125µl sample buffer. The buffer, made up to 5ml, contained 2.4g urea, 0.1g CHAPS, 15mg DTT, 100µl IPG buffer (pH 3-10 NL) and 50µl of 0.1% bromophenol blue. The sample was pipetted into a well of the Immobiline DryStrip Reswelling Tray (Amersham Pharmacia Biotech) and an IPG strip with a non-linear pH gradient from 3-10 laid over it. The strip was covered with 1ml of silicone oil and allowed to swell overnight. The strip was then transferred to the Multiphor II system (Amersham Pharmacia Biotech) for isoelectric focusing under conditions recommended by the manufacturer.

After focusing, the IPG strip was briefly rinsed with water and then placed in 10ml equilibration buffer for 15min in preparation for the second dimension. The solution was continuously agitated. The equilibration buffer contained 3.6g urea, 0.1g SDS, 100mg DTT, 3ml glycerol and 50µl of 0.1% (w/v) bromophenol blue made up to 10ml with 20mM Tris-HCl (pH 6.8).

The second dimension was a tricine SDS-PAGE gel prepared as described in Section 2.2.4. The IPG strip was placed onto the gel surface and covered with stacking gel to allow effective protein transfer. Electrophoresis proceeded at 30V until the samples had stacked, then continued at 80V. The gel was stained with Coomassie Brilliant Blue R-250.

2.2.10 Protein pyridylethylation

The pure protein was pyridylethylated to determine the number of Cys residues in each protein. The alkyl groups added bind to the Cys residue's thiol groups preventing the reformation of disulphide bonds after reduction (Kellner *et al.*, 1994). The protein was first treated with 1ml of a 6M guanidine hydrochloride; 4mg/ml DTT solution made up in 10ml of morpholinium buffer. This denaturing/reducing solution was filtered through a 0.22µm filter before use. The morpholinium buffer contained 0.5M ethylmorpholinium

acetate at pH 8.3 and was prepared by making up 6.4ml of N-ethylmorpholine to 100ml with H_2O. The pH was adjusted with acetic acid. Once the reducing solution was added, the tube was centrifuged briefly and the solution layered with N_2 (g). The mixture was left to react for 120 min in the dark at room temperature. Thereafter 8µl of 4-vinyl pyridine was added to alkylate the protein. The resulting mixture was again spun briefly, layered with N_2 (g) and left to react for 120 min in the dark at room temperature.

The pyridylethylated proteins were purified by reversed-phase chromatography using the C5 column, as described in Section 2.2.3. It is important to separate the proteins from the alkylating reagents immediately as these can cause deleterious side-reactions. Before loading the sample, it was diluted 1:4 with reversed-phase eluent A.

2.2.11 Determining the presence of disulphide bonds

To ascertain whether Cys residues are involved in the formation of disulphide bonds the amino acid composition of pyridylethylated protein, once in a reduced state and once in a non-reduced state, was determined. During the alkylation procedure described in Section 2.2.9, the protein was treated with 1ml of a 6M guanidine hydrochloride; 4mg/ml DTT solution made up in 10ml of morpholinium buffer. Under these conditions disulphide bonds are broken. One such reduced sample was prepared. A second protein sample was treated with the same solution, but lacking DTT. Under these conditions disulphide bonds remain intact.

The alkyl groups protect the Cys residues from degradation during acid hydrolysis. Therefore normal acid hydrolysis (1M HCl; 110°C; 24 hours) of the reduced and non-reduced alkylated protein could be performed and the amino acids derivatized and analysed as in Section 2.2.6.

2.2.12 Plasma-based clotting assays

2.2.12.1 Screening for inhibition of the intrinsic and extrinsic pathways

To test for any anticoagulant activity, samples were added to standard one-stage clotting assays using normal citrated human plasma. Inhibition of the intrinsic coagulation pathway was tested by measuring the activated partial thromboplastin time (aPPT) and inhibition of the extrinsic pathway by measuring the prothrombin time (PT) (Becker *et al.*, 1984). The tests were performed using an automated Behring Fibrintimer A, which operates by passing a light beam through a cuvette containing the sample plasma and records any change in light intensity using a photodetector. Even the most labile clot can be detected, and this method is suitable for clear and turbid plasmas, as well as kaolin-containing reagents.

Blood was collected in sterile Vacutainer tubes containing 0.108 M trisodium citrate as anticoagulant. The blood was obtained from a healthy donor not congenitally deficient in any coagulation factors. Erythrocytes were removed by centrifugation at 120 *g* for 20 min. The plasma supernatant was then collected and centrifuged at 1680 *g* for 30 min to remove thrombocytes. The platelet-poor plasma was divided into 1 ml aliquots and stored at –70°C.

Inhibition of the intrinsic pathway was determined using Pathromtin SL. This reagent sensitively detects factor VIII and IX as well as the contact factors. Test solutions were prepared by mixing equal volumes of the prepared citrated human plasma and the samples. To 100µl of this plasma, pipetted into a tube prewarmed at 37°C, was added 100µl Pathromtin SL and the solution incubated for 2 min at the same temperature. Thereafter, 100µl 0.025 M prewarmed $CaCl_2$ was added and the coagulation time measured. The control was prepared by mixing equal volumes of plasma and buffer.

Inhibition of the extrinsic pathway was determined using Thromborel S, which is used to screen for disorders involving factor II, V, VII and X. Samples and the control were

prepared as for the aPPT test. Into a prewarmed tube was pipetted 100µl of the sample plasma or control plasma and the tube incubated for 1 min at 37°C. To this was added 200µl prewarmed Thromborel S and the coagulation time measured.

2.2.12.2 Determination of anti-factor VII_a and anti-factor X_a activity

Inhibition of any one of the factors comprising the extrinsic pathway will result in a longer prothrombin time. Factor deficient plasmas can be used to identify which of these factors are inhibited (van Dam-Mieras *et al.*, 1984). Plasma in which a particular factor is inhibited will not be able to accommodate the absence of the factor in the corresponding deficient plasma and result in a prolonged assay time. One hundred microliters normal plasma was mixed with 100µl human factor VII deficient or human factor X deficient plasma and incubated at 37°C for 1 min, in the presence of the purified proteins. Then 200µl prewarmed Thromborel S was added to determine the prothrombin time.

2.3 Results

2.3.1 Protein purification

Fifty glands were sonified to prepare the salivary gland extract. The extract was centrifuged to remove cellular debris and subjected to $BaSO_4$ adsorption. After washing out non-absorbent compounds, the proteins were eluted from the salt with 2M NaCl. The eluate was analysed by reversed-phase HPLC. As is evident from the chromatogram in Figure 1.2a, only one peak with a retention time of 22.870 min was observed. This peak was collected and the fraction analysed by SDS-PAGE. The gel indicated the presence of at least two protein components (Figure 1.2b). Separation of these components was achieved by loading the reversed-phase fraction onto a HIC column.

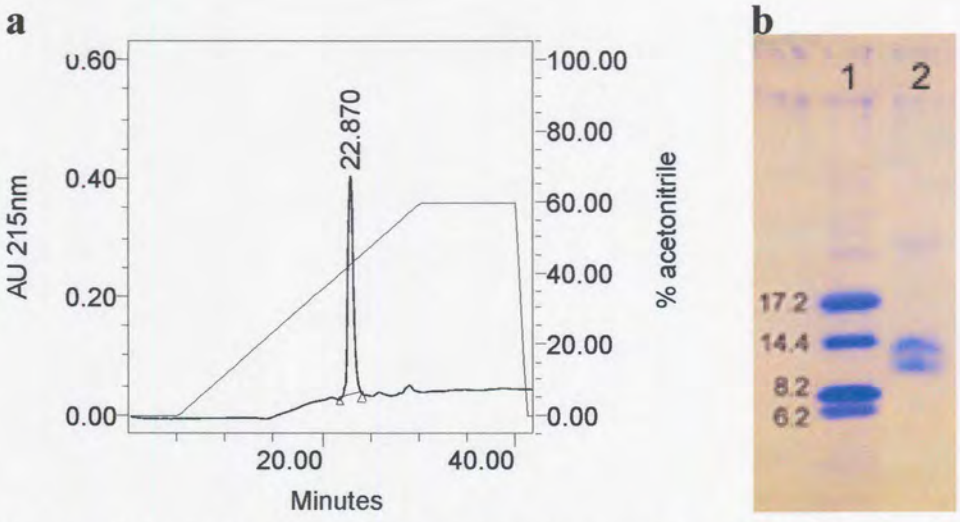


Figure 2.1: **a** Reversed-phase chromatogram of the $BaSO_4$ eluate. Absorbance was measured at 215nm. The percentage acetonitrile used throughout is indicated by the overlaid gradient. **b** The single peak was analysed by tricine SDS-PAGE (lane 2). Lane 1 contained peptide mass markers. Masses are given in kDa.

The HIC chromatogram in Figure 2.2 shows two peaks, the first with a retention time of 25.33 min and the second with a retention time of 29.13 min. These peaks were collected and the fractions desalted by reversed-phase chromatography. Figure 2.3 shows the RP re-chromatograms for the first (**a**) and second HIC peak (**b**), respectively. In both

chromatograms only one peak is observed and these have near identical retention times: 22.816min and 22.830min. These peaks were analysed by tricine SDS-PAGE. From the gel photograph in Figure 2.4 it can be concluded that the reversed-phase co-purifying proteins were successfully separated using HIC. Only single protein bands are visible. To confirm protein purity, a sample of each RP peak was analysed by electro-spray mass spectrometry (Figure 2.6). The mass spectrum of each peak had only one main component, confirming the homogeneity of the isolated proteins. From this data it can be concluded, that the mass of the first HIC peak component was 9333.54 Da, that of the second 9173.50 Da. Forthwith the 9.3 kDa protein will be referred to as *Ba*SO_4-*A*dsorbing *P*rotein (BSAP) 1 and the 9.1 kDa protein as *Ba*SO_4-*A*dsorbing *P*rotein 2. Collectively they will be referred to as the BSAPs.

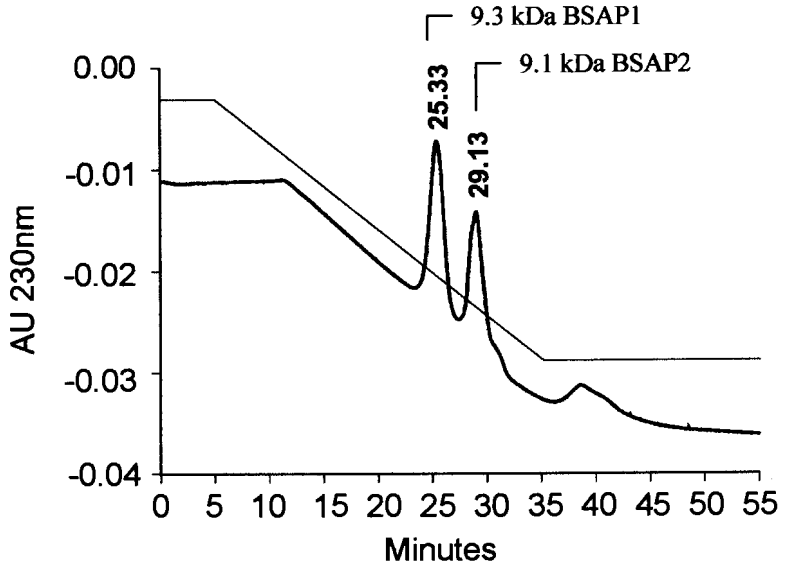


Figure 2.2: Hydrophobic interaction chromatography of the reversed-phase fraction. RP-fraction was diluted 1:10 with HIC buffer A prior to injection. Absorbance was measure at 230nm and a gradient (1.7M – 0M ammonium sulphate) elution was performed as indicated. The molecular mass and designated identity of each peak is given (see text for Discussion).

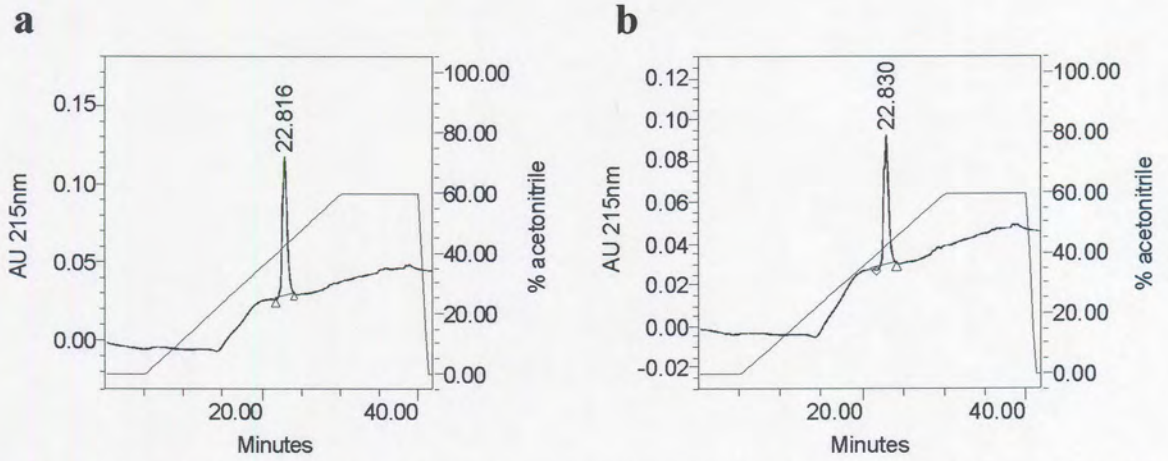


Figure 2.3: Reversed-phase re-chromatography to desalt HIC fractions. **a** The RP chromatogram of the first collected HIC peak. **b** The RP chromatogram of the second collected HIC peak. Absorbance was measured at 215nm. The percentage acetonitrile used throughout is indicated by the overlaid gradient.

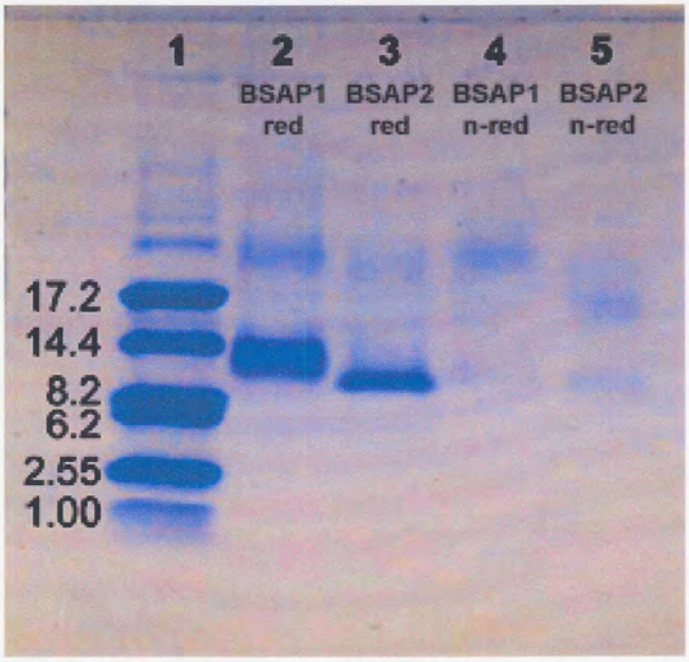


Figure 2.4: Reducing and non-reducing tricine-SDS PAGE analysis of the purified proteins. Lane 1 contained low molecular weight peptide mass markers (masses of individual fragments are given in kDa); lane 2 and 4 contained the protein from the first RP-desalted HIC peak; lane 3 and 5 contained the protein from the second RP-desalted HIC peak. Proteins in lane 1 to 3 were electrophoresed under reducing conditions (red), those in lane 4 and 5 under non-reducing conditions (n-red). The concentration of protein loaded in each lane was not determined due to the low amounts that could be purified. Gel was Coomassie-stained.

The molecular mass of the purified proteins was estimated from the polyacrylamide gel in Figure 2.4, using the low molecular weight peptide mass markers as standards. A calibration curve of relative mobility versus molecular mass was drawn and an equation describing the curve calculated (Figure 2.5). Determining the proteins' relative mass according to the equation, gives a value of 12.1 kDa for BSAP1 (in lane 2 of Figure 2.4) and a mass of 10.0 kDa for BSAP2 (in lane 3 of Figure 2.4) (Table 2.5). These values are not in agreement with the molecular masses obtained by ESMS (Figure 2.6). Such discrepancies are not uncommon when using SDS-PAGE. Anomalous mass values are often encountered with glycoproteins, very hydrophobic and very basic or acidic proteins, as well as with low-molecular mass peptides (Matagne *et al.*, 1991).

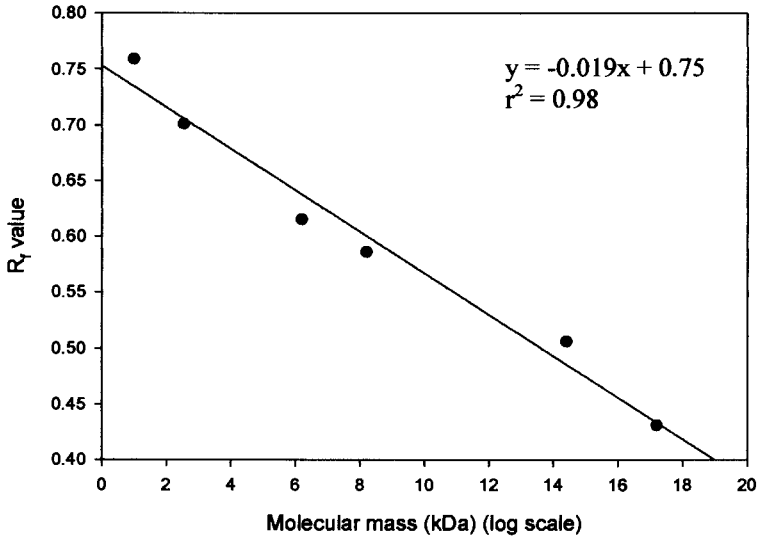


Figure 2.5: Calibration curve for low molecular weight peptide mass markers. The graph shows the relative mobilities of peptides under reducing conditions. The equation describing the regression line is given.

Table 2.5: Molecular masses of purified $BaSO_4$-adsorbing proteins as determined by SDS-PAGE analysis. The peptide mass markers are derived from cyanogen bromide cleaved myoglobin. Fragments were numbered in order of decreasing size (Fasman, 1989). The 14.2 kDa fragment results from incomplete digestion.

Samples	Rf values	Molecular masses of peptide markers & purified proteins (kDa)
Peptide mass markers	0.43	17.2 myoglobin (native)
	0.49	14.4 myoglobin I & II (dimer)
	0.59	8.2 myoglonin I
	0.61	6.2 myoglobin II
	0.70	2.6 myoglobin III
	0.76	1.0 myoglobin fragment*
BSAP1	0.52	12.1
BSAP2	0.56	10.0

* This species has not been characterised.

The polyacrylamide gel in Figure 2.4 shows purified BSAP1 and BSAP2 under reducing and non-reducing conditions. Although there are at least two bands per lane an overall trend is still discernable. The proteins seem to have a slightly greater mobility under non-reducing conditions than under reducing conditions. The difference is an indication of the presence of disulphide bonds, but its small magnitude seems to imply no significant role for these bonds in the protein's tertiary structures. The faintness of the non-reducing bands is due to low protein concentration. Neither BSAP1 nor BSAP2 consist of subunits, since there are no bands with masses lower than those of the two proteins. The presence of bands of significantly higher mass, approximately 20.1 kDa (lane 2) and 18.9 kDa (lane 3) as determined form the calibration curve in Figure 2.6, may indicate the existence of BSAP1 and BSAP2 complexes. The masses of these bands are about twice that of the 12.1 kDa BSAP1 and the 10.0 kDa BSAP2, as determined by tricine SDS-PAGE. It may thus be possible that these proteins can exist as dimers.

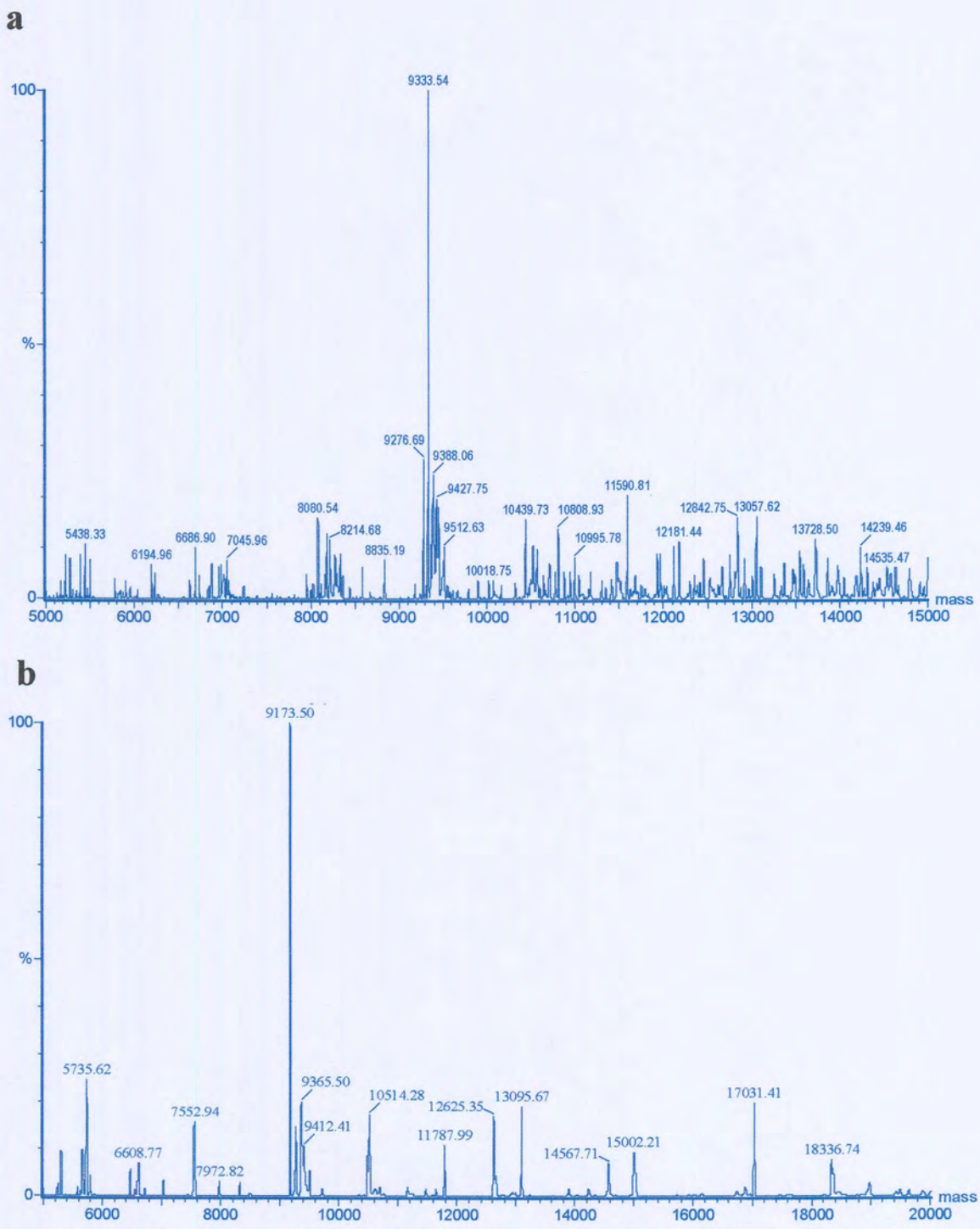


Figure 2.6: Deconvoluted electro-spray mass spectra of the $BaSO_4$-adsorbing proteins. **a** Spectrum of BSAP1 **b** Spectrum of BSAP2. The absence of any other large peaks is an indication of sample purity.

2.3.2 Amino acid composition

The amino acid composition of BSAP1 and BSAP2 differ in 18 amino acid residues (Table 2.6 and Figure 2.7). A noteworthy difference is the absence of histidine and isoleucine from BSAP1 and the presence of one of each of these residues in BSAP2. Also, performic acid oxidation seems to indicate the presence of 5 cysteine residues in the former, but only 4 cysteine residues in the latter protein. Both proteins have 2 tryptophan residues, as determined through methanesulphonic acid hydrolysis. Asparagine and glutamine are converted to aspartic acid and glutamic acid during acid hydrolysis (Kellner *et al.*, 1994). The number of these amino acid residues is therefore added to that of aspartic acid and glutamic acid. BSAP1 and BSAP2 consist of approximately 73 and 71 amino acids, respectively.

Table 2.6: The amino acid composition of the $BaSO_4$-adsorbing proteins. The molar ratios were calculated relative to Leu, which was given a value of 1. If the sum of the molecular masses of the individual amino acids did not add up to the known mass of the respective proteins, the molar ratios were multiplied by an appropriate factor to get to the correct molecular mass of the proteins. Values in brackets are the integers nearest to the molar ratios and are an indication of the number of residues of each amino acid present in the proteins. Asx = aspartic acid & asparagine; Glx = glutamic acid & glutamine.

Amino acid	Molar ratios of the $BaSO_4$-adsorbing proteins		Amino acid	Molar ratios of the $BaSO_4$-adsorbing proteins	
	BSAP 1	**BSAP 2**		**BSAP 1**	**BSAP 2**
Asx	10.98 (11)	9.12 (9)	Tyr	3.06 (3)	4.04 (4)
Glx	13.26 (13)	11.61 (12)	Val	1.93 (2)	0.93 (1)
Ser	6.49 (7)	5.17 (5)	Met	0.87 (1)	1.02 (1)
Gly	9.48 (10)	9.30 (9)	Ile	0.37 (0)	1.20 (1)
His	0.11 (0)	1.13 (1)	Leu	2.17 (2)	2.22 (2)
Arg	3.23 (3)	2.24 (2)	Phe	1.95 (2)	2.04 (2)
Thr	1.80 (2)	3.69 (4)	Lys	3.42 (2)	4.10 (4)
Ala	4.54 (5)	2.73 (3)	Cys	4.47 (5)	4.28 (4)
Pro	3.56 (4)	4.73 (5)	Trp	1.52 (2)	1.60 (2)
			Total	73.22 (74)	71.15 (71)

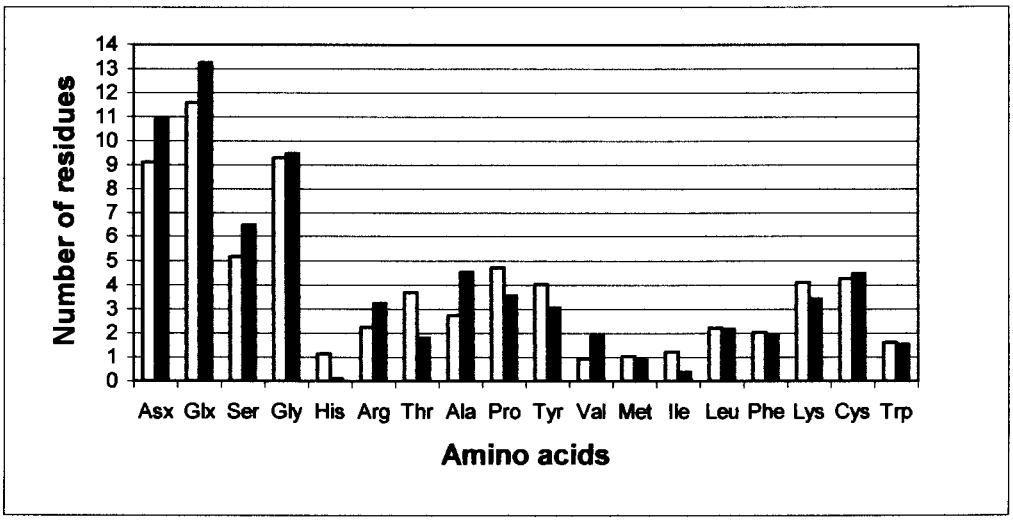


Figure 2.7: Comparative plot of the two $BaSO_4$-adsorbing proteins' amino acid composition, using the molar ratios given in Table 2.6. White bars = BSAP2, black bars = BSAP1.

2.3.3 Cyanogen bromide cleavage of BSAP1 and BSAP2

As no N-terminal sequences were obtained, the proteins may be N-terminally blocked. The purified proteins were therefore cleaved with cyanogen bromide to yield fragments for internal sequence analysis. Only two peptide fragments were expected for each protein since both contain only one Met residue. This prediction proved correct for BSAP1, which shows just such a cleavage pattern when fragments are purified by RP chromatography (Figure 2.8a). The chromatogram shows three peaks, one with retention time 22.51 min, which is presumably, uncleaved protein since this retention time is very similar to that obtained for the native protein in Figure 2.3a. The two other peaks at 23.10 min and 24.21 min were collected and submitted for MALDI mass spectrometric analysis and sequencing.

A different cleavage pattern could be observed for BSAP2. This protein did not yield fragments that could be readily separated by RP chromatography. Figure 2.8b shows

them eluting as one peak. Again, there is a small peak with retention time 22.50 min, which most likely is uncleaved native protein. The entire peak at 23.86 min was submitted for MALDI mass spectrometric analysis and sequencing.

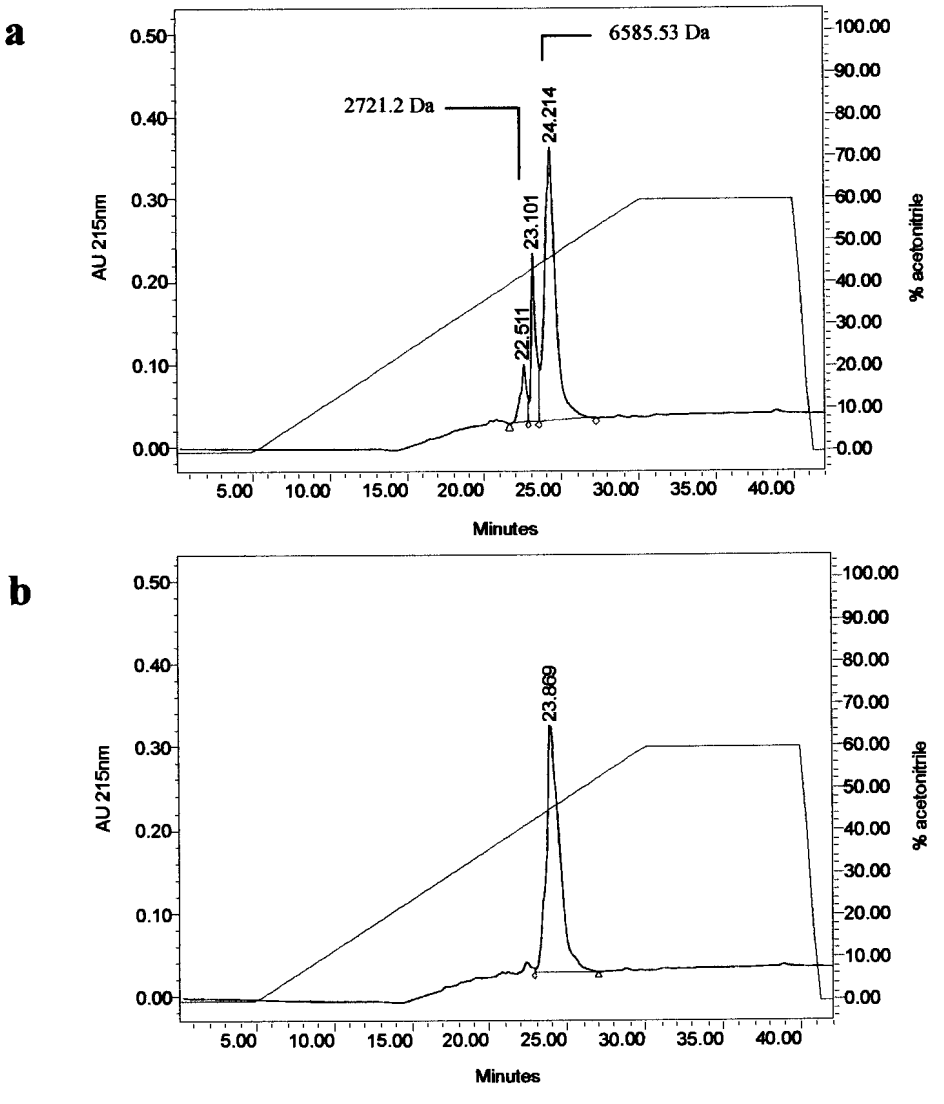


Figure 2.8: Reversed-phase separation and purification of cyanogen bromide cleaved $BaSO_4$-adsorbing protein fragments. **a** The RP chromatogram of cleaved BASP1. The individual peaks were resolved and their masses as determined by MALDI-MS are given (see Figure 2.9). **b** The RP chromatogram of BSAP2. Only one peak with possible adducts was detected. Absorbance was measured at 215nm and the percentage acetonitrile used throughout is given by the overlaid gradient.

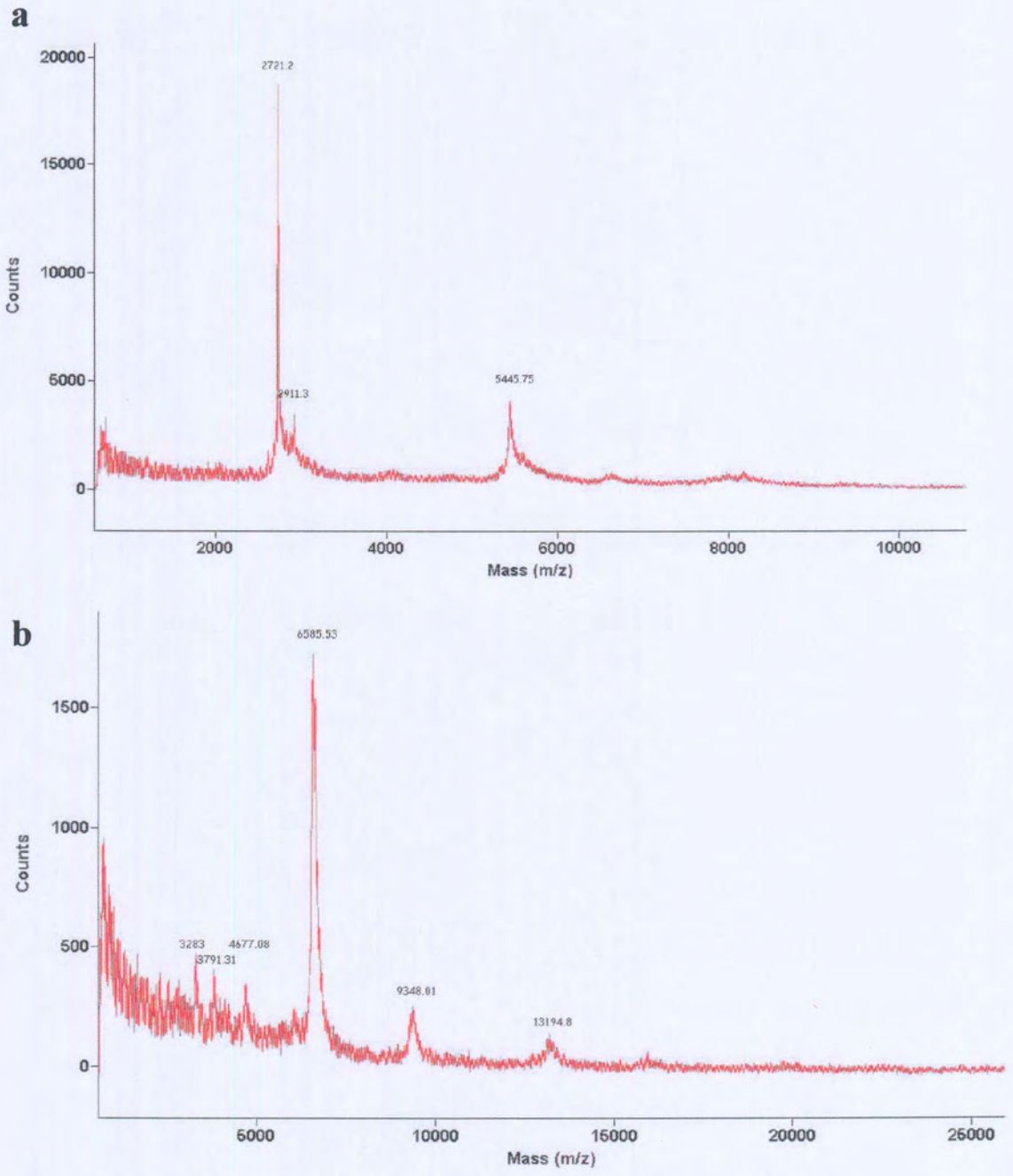


Figure 2.9: MALDI mass spectra of BSAP1 cyanogen bromide-cleavage products. **a** Spectrum of peak 23.10 min in Figure 2.8a. **b** Spectrum of peak 24.21 min in Figure 2.8a.

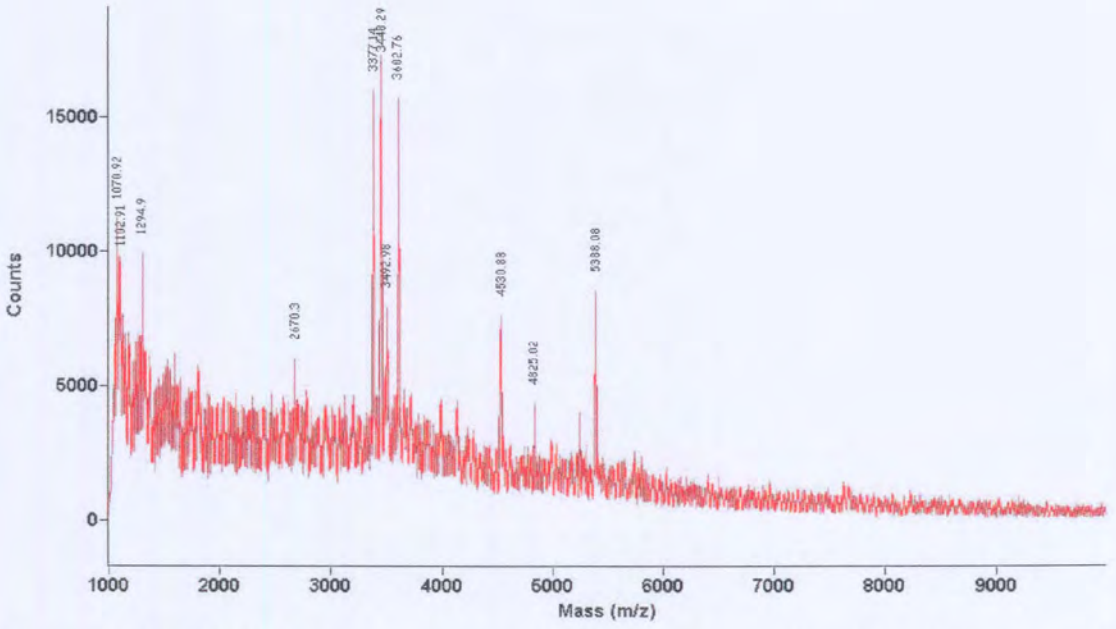


Figure 2.10: MALDI mass spectrum of BSAP2 cyanogen bromide-cleavage products. Spectrum is of peak 23.86 min in Figure 2.8b.

The MALDI mass spectra of the BSAP1 and BSAP2 cyanogen bromide cleaved fragments are shown in Figure 2.9 and 2.10, respectively. The masses of the two RP-separated BSAP1 fragments were 2721.2 Da and 6585.53 Da, which add up to 9306.7 Da, a value near identical to that of the native protein (Figure 2.5a). In both Figure 2.9a and b the $2M^+$ peaks of the mass components are observable. They are the 5445.75 Da and the 13194.8 Da peaks, respectively. As the cleavage pattern of BSAP2 is so different from that of BSAP1, it would be expected that the masses of the protein's cleavage fragments differ from those of BSAP1. This is indeed the case as can been seen in Figure 2.10. A cluster of four components with masses between 3377 Da and 3602 Da are evident. These peaks represent one of the protein fragments (detected by Western blotting; data not shown), whereas the component at 5388.08 Da is presumably the second fragment.

2.3.4 Amino acid sequencing

Several attempts at N-terminal sequencing have failed, which may imply that the proteins are N-terminally blocked. To overcome this problem, internal sequences were sought. The two purified BSAP1 cyanogen bromide cleavage products, the 2721.2 Da and 6585.53 Da fragments, were submitted for sequencing. Since BSAP2 yielded fragments that could not readily be separated, the entire crude mixture of cleavage products was submitted for sequencing. In the latter case it was hoped, since the proteins may be N-terminally blocked, that only the C-terminal fragment would yield a sequence despite the presence of the blocked fragment.

No sequences for either of the BSAP1 fragments were obtained. Table 2.7 shows a sequence obtained from the sample containing the BSAP2 fragments. As already stated, the N-terminal of both proteins may be blocked, implying that the sequence is that of the unblocked C-terminal fragment. The first Edman cycle provided inconclusive data as to the identity of the first amino acid in this fragment. It could be either an aspartic acid or serine residue. Cycles 4 and 5 may have been glutamic acid residues, but the data is questionable.

Table 2.7: Amino acid sequence analysis of BSAP2 fragments obtained by cyanogen bromide cleavage.

Cycle number	PTH amino acid
1	Asp/Ser
2	Gly
3	Gly
4	Xxx
5	Xxx
6	Ile
7	Leu
8	Gly

2.3.5 Protein pI calculation and two-dimensional electrophoresis

The amino acid composition data given in Table 2.6 was used to calculate BSAP1 and BSAP2's isoelectric points. The pI for BSAP1 was calculated to be 3.87 and that of BSAP2 was 4.03. The proteins are thus predicted to be acidic in nature, a property that has been observed when the purified proteins are loaded onto an anion-exchange column (method and results not shown). The 2D polyacrylamide gel in Figure 2.11 confirms the acidic nature of the proteins. Two protein spots corresponding to BSAP1 and BSAP2's known molecular masses are indicated with a white arrow. The lateral streaks and spots may be due to charge micro-heterogeneity. The prominent tail that is present may have arisen due to a combination of poor sample transfer from the IPG strip and incomplete stacking.

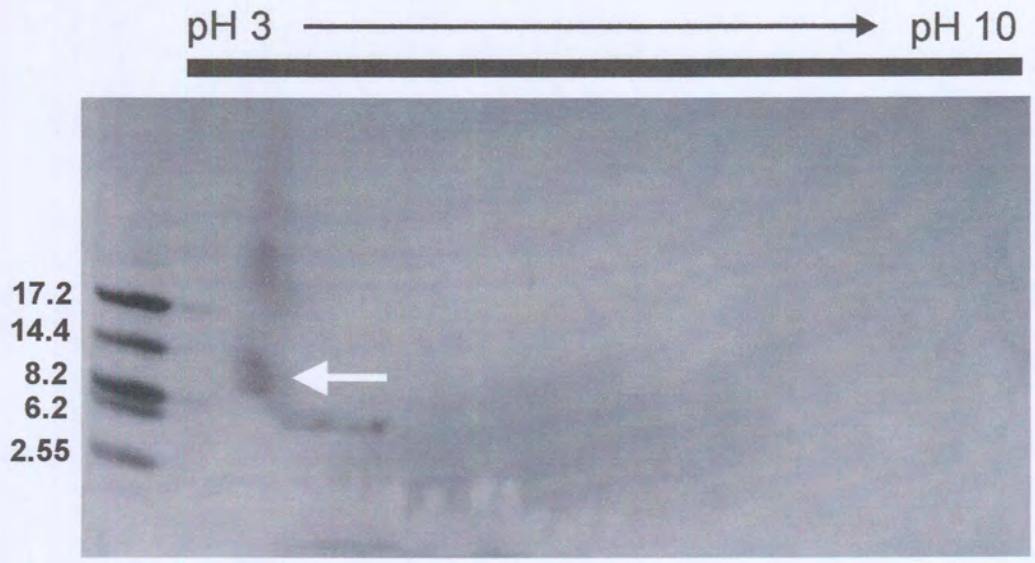


Figure 2.11: Two-dimensional electrophoresis of BSAP1 and BSAP2. Two protein spots corresponding to BSAP1 and BSAP2's known molecular mass are indicated with a white arrow. The IPG strip pH 3-10 (non-linear) is indicated. The molecular masses of the peptide mass markers are given in kDa.

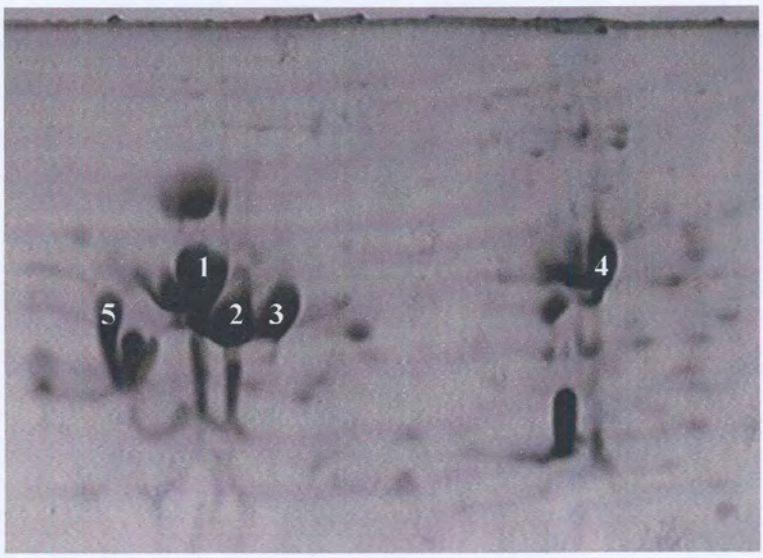


Figure 2.12: Proteome of crude tick salivary gland extract. Known proteins are numbered 1-5: **1** TSGP1; **2** TSGP2; **3** TSGP3; **4** TSGP4; **5** BSAP1 and BSAP2. Acidic proteins are on the left and the anode at the bottom. (Modified from: Mans *et al.*, 2001).

The proteome of the crude salivary gland extract of *O. savignyi* was described by Mans *et al.* (2001). The authors identified four proteins putatively involved in granule biogenesis, designated TSGP1-4 (Figure 2.12). In addition to these proteins BSAP1 and BSAP2 can now be identified.

2.3.6 Presence of disulphide bonds

As stated earlier, two samples of pyridylethylated protein were prepared. One was prepared under reducing conditions and the other under non-reducing conditions. All the Cys residues in the reduced protein were alkylated with 4-vinyl-pyridine to form (4-pyridylethyl) cysteine (PEC). Under the acidic conditions used for hydrolysis, PEC is stable. The non-reduced protein has its disulphide bonds intact and consequently, no alkyl group can bind. These Cys residues are therefore not protected from degradation during acid hydrolysis. By comparing the number of PEC residues in the reduced and non-reduced protein, the number of Cys residues involved in the formation of disulphide

bonds can be established. Table 2.5 shows the amino acid composition of the reduced and non-reduced pyridylethylated proteins. The molar ratios were calculated relative to Leu.

Table 2.8: Amino acid composition of the reduced and non-reduced pyridylethylated BSAP1 and BSAP2. Asx = aspartic acid & asparagine; Glx = glutamic acid & glutamine; PEC = (4-pyridylethyl) cysteine

Reduced proteins			Non-reduced proteins		
Amino acid	BSAP1	BSAP2	Amino acid	BSAP1	BSAP2
Asx	12.57	8.32	Asx	10.90	9.22
Glx	13.24	11.39	Glx	13.30	11.41
Ser	6.74	4.94	Ser	6.38	5.05
Gly	9.50	9.31	Gly	9.39	9.30
His	0.18	0.93	His	0.10	1.13
Arg	2.88	2.18	Arg	3.21	2.24
Thr	1.71	3.74	Thr	1.89	3.69
Ala	4.76	2.56	Ala	4.54	2.73
Pro	3.10	4.50	Pro	3.45	4.73
Tyr	2.95	5.01	Tyr	3.18	4.33
Val	1.73	0.39	Val	1.92	0.88
Met	1.42	1.65	Met	0.86	1.02
PEC	**6.89**	**8.15**	**PEC**	**0.49**	**0.50**
Ile	0.16	0.95	Ile	0.37	1.20
Leu	1.81	1.91	Leu	2.06	2.12
Phe	1.69	1.88	Phe	1.95	2.10
Lys	3.19	4.08	Lys	3.39	4.21

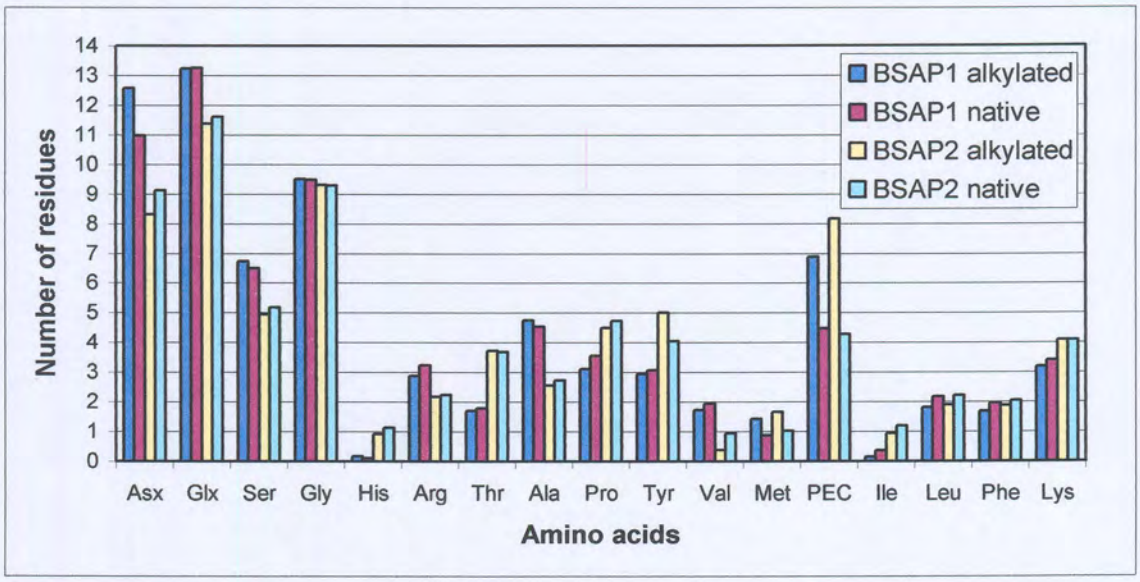


Figure 2.13: Amino acid composition of reduced and alkylated BSAP1 and BSAP2, compared to that of the reduced native proteins. Tryptophan was omitted because this was not separately determined in the alkylated proteins.

The absence of His and Ile in BSAP1 is confirmed by the results in Table 2.8. With the obvious exception of PEC, the amino acid analysis of the pyridylethylated proteins confirms the results of the native protein analysis in Table 2.6 and Figure 2.7. Reduced BSAP1 had approximately 6, possibly 7 PEC residues, while reduced BSAP2 had about 8. The non-reduced proteins had practically none. This implies that all Cys residues in both proteins are involved in the formation of disulphide bonds. Since two Cys residues are required for disulphide bond formation, the number of Cys residues in BSAP1 is probably 6. Figure 2.13 compares the composition of the reduced and native proteins.

2.3.7 Screening for inhibition of the intrinsic and extrinsic pathways and determination of anti-$fVII_a$ and anti-fX_a activity

The reagents used in the one-stage clotting assays can display some batch-to-batch variation and are sensitive to differences in the salt concentration, pH, or concentration of inhibitors in the plasma (Kelley *et al.*, 1995). It is therefore recommended by Kelley *et al.*

(1995), that only clotting times increased by at least 1.5 times the control value should be considered significant.

The proteins for these experiments were prepared by $BaSO_4$-adsorption followed by HIC and desalting by dialysis. No reversed-phase was used. The HIC chromatogram was similar to that in Figure 2.2. The coagulation times of normal plasma in the presence of BSAP1 and BSAP2 are given in Table 2.9. The aPTT of the control and the samples differ by 9.2 sec for BSAP1 and 2.4 sec for BSAP2. The difference between the control and the sample aPPT values is 1.12 and 1.02, respectively. According to Kelly *et al.* (1995) these differences are too small to be considered significant and subsequently it appears that these proteins have no effect on the intrinsic pathway. The PT of the control and the samples differ by 13.5 and 11.2 sec for BSAP1 and BSAP2, respectively. The difference between the control and the sample PT values is 1.56 and 1.46. This is closer to the 1.5 limit set by Kelley *et al.* (1995). The coagulation times of the extrinsic pathway therefore seem somewhat prolonged. The low levels of inhibition that are observed may be due to very low concentrations of the presumptive inhibitors in the samples tested. The concentration of the protein used in each experiment was not determined due to the low amounts that could be purified.

Table 2.9: The activated partial thromboplastin time (aPPT) and the prothrombin time (PT) of normal citrated human plasma in the presence of BSAP1 and BSAP2. Also given are the PT values of normal plasma mixed with fVII or fX deficient plasma in the presence of the same proteins. Coagulation times were measured in seconds and values in the table are the mean time of two assays. The control contained buffer instead of protein.

Test	Mean coagulation times (sec)		
	Control	BSAP 1	BSAP 2
aPPT	74.4	83.6	76.6
PT	23.9	37.4	35.1
fVII-deficient	30.4	29.3	29.2
fX-deficient	37.5	35.7	35.9

Since the coagulation times of the extrinsic pathway were somewhat extended, the proteins may act on factor II, V, X or VII. As the intrinsic pathway showed no significant inhibition, factor II, V and X can be ruled out as possible targets of inhibition, leaving factor VII as potential target. The coagulation times of plasmas, consisting of a mixture of normal and factor VII deficient plasma, in the presence of BSAP1 and BSAP2 were near identical (Table 2.9). The largest difference in time was 1.2 sec. It would therefore appear that factor VII is not the target of these proteins. To confirm that they also have no effect on the intrinsic pathway, activity of factor Xa was assayed. There was almost no difference in the coagulation times. This experiment independently rules out factor X as the target of inhibition. The somewhat prolonged PT of the extrinsic pathway thus remains unexplained. Possibly tissue factor could be the target of the isolated proteins.

2.4 Discussion

Two $BaSO_4$-adsorbing proteins have been isolated to apparent homogeneity from the crude salivary gland extract of the tick *Ornithodoros savignyi*. Both proteins selectively adsorb to $BaSO_4$, while all other proteins do not, a fact that becomes evident when examining the reversed-phase chromatogram in Figure 2.1. The proteins co-purify during reversed-phase chromatography. The absence of any other peaks is an indication of the selectiveness with which proteins adsorb to $BaSO_4$. Initially, it was assumed that this peak contained only one protein component. Electrophoresis of a sample in a polyacrylamide gel however showed the presence of two proteins. These were separated by hydrophobic-interaction chromatography (Figure 2.2). The high salt content of the collected HIC fractions necessitated desalting, if the proteins were to be used in further experiments. This was achieved by re-chromatography of the proteins on the reversed-phase column.

Since it is known that reversed-phase chromatography can lead to protein denaturation, samples to be used in the one-stage clotting assays were prepared by loading the crude salivary extract directly onto the HIC column. The column used and the conditions applied are deemed non-denaturing, as discussed in the introduction of this chapter. The proteins were separated (chromatogram similar to Figure 2.2 – not shown), but the yield was low when compared to that of the HIC separated reversed-phase fractions. It is possible that this was a batch-specific effect. Instead of reversed-phase re-chromatography, the collected fractions were desalted by dialysis.

By comparison, the high hydrophobic ligand density and the use of organic modifiers in RP-HPLC tend to denature proteins and separation is, therefore, based on the overall hydrophobicity of the protein rather than the surface hydrophobicity as is the case in HIC (Mant & Hodges, 1991). The fact that the proteins separate on the HIC column indicates that they differ in terms of their surface hydrophobicity, since this is the property on which separation on HIC columns is based (McNay & Fernandez, 1999; Regnier, 1987).

Contrary to this is the fact that these proteins have identical retention times on the reversed-phase column pointing to identical total hydrophobicities.

The homogeneity of the protein samples was assessed by means of electro-spray mass spectrometry and reducing and non-reducing polyacrylamide gel electrophoresis. The molecular masses of the proteins were estimated from the tricine SDS-PAGE gel using the low molecular weight mass markers as standards for the plotting of a calibration curve (Figure 2.6). The calculated mass of BSAP1 was 12.1 kDa and that of BSAP2 10.0 kDa (Table 2.5). These masses are in reasonable agreement with the more exact values obtained by electro-spray mass spectrometry, which gave values of 9333.54 Da and 9173.50 Da, respectively (Figure 2.5).

The proteins seem to have a somewhat higher mobility under non-reducing conditions, indicating a more compact structure. This is most probably due to the presence of disulphide bonds. The slight increase in mobility however implies a minor role for these bonds in the protein's tertiary structure. Neither protein contains any subunits, as no peptides with masses lower than those of the native protein were observed.

Molecules with roughly twice the mass of the native proteins seem to be present in electrophoresed samples of BSAP1 and BSAP2. This may indicate the existence of dimers, but most likely is the result of incomplete SDS binding. This is known to occur with highly acidic proteins, where the many negative charges on the polypeptide backbone prevent the binding of SDS in its expected 1.4g/g protein ratio (Matagne *et al.*, 1991). A large portion of the amino acid composition of the isolated BSAPs may be acidic amino acids, as indicated by the amino acid analysis in Table 2.6. The proteins are also known to elute late on an anion-exchange column indicating an acidic nature (data not shown). It may thus be that low levels of SDS-binding are responsible for the observed high molecular-weight bands. Alternatively, the presence of the disulphide bonds could have reduced the quantity of bound SDS (Pitt-Rivers & Impiombato, 1968).

The purified proteins differ in composition by 18 amino acid residues (Table 2.5). The nature of these differing amino acids is not conserved, some are uncharged and polar, some non-polar and others charged. Noteworthy differences in their composition are the absence of histidine and isoleucine in BSAP1 and the presence of one of each of these residues in BSAP2 and, as determined by performic acid oxidation, the presence of 4 cysteine residues in the latter and 5 in the former protein. Overall, the aspartic acid, asparagine, glutamic acid and glutamine content are high, accounting for roughly 30% of the proteins composition. Likewise, both proteins have a high serine and glycine content. Methanesulphonic acid hydrolysis identified 2 tryptophan residues per protein.

Amino acid analysis of pyridylethylated protein identified 6, possibly 7, cysteine residues in BSAP1 and 8 residues in BSAP2. The discrepancy in the number of cysteine residues as determined by performic acid oxidation and protein pyridylethylation, is probably due to the greater accuracy of the latter method. This view is supported by the fact that the overall amino acid composition of the native and the alkylated proteins is the same (Figure 2.11). The likely presence of disulphide bonds was first indicated by non-reducing electrophoresis of the purified proteins. Comparing the molar ratios of the (4-pyridylethyl) cysteine residues in the reduced and non-reduced protein samples showed that all cysteine residues are involved in the formation of disulphide bonds.

Each protein contains only one methionine residue. It could thus be predicted that both proteins, when cleaved with cyanogen bromide, would yield two peptide fragments. This seems to have been the case. Their cleavage pattern however differed as is evident when examining the chromatographic profiles in Figure 2.8. Predictably, the mass spectrometric analysis of the fragments showed different masses for the individual fragments (Figure 2.9 and 2.10). From this it can be concluded that the methionine residues occupy different positions in the polypeptide chains of the two proteins.

No amino acid sequences for either of the two BSAP1 cyanogen bromide cleavage fragments were obtained. The BSAP2 cleavage products did however yield a partial sequence. Since the N-terminal of both proteins may be blocked, the obtained sequence

may represent the amino acid sequence of the unblocked C-terminal fragment. The sequence reads:

Asp/Ser – Gly – Gly – Xxx – Xxx – Ile – Leu – Gly

The first Edman cycle provided inconclusive data as to the identity of the first amino acid in this fragment. It could be either an aspartic acid or serine residue. No definitive amino acid could be identified in cycles 4 and 5. A homology search comparing the above amino acid sequence with those in protein sequence databases yielded no results.

The isoelectric points of BSAP1 and BSAP2 were determined theoretically and experimentally. It was theoretically determined by calculation using the proteins' amino acid composition. According to these calculations, BSAP1 has a pI of 3.87 and BSAP2 a pI of 4.03. Experimentally, the pIs were confirmed by two-dimensional gel electrophoresis. The overall position of the proteins in the gel is a clear indication of their acidic nature. The theoretical predictions and the experimental results are therefore in agreement.

The one-stage clotting assays provided no definitive results. The two proteins probably do not inhibit the intrinsic coagulation cascade, but low-level inhibition of the extrinsic pathway was detected. Factor VII does not seem to be the target. Tissue factor is the only other unique component of the extrinsic pathway. Perhaps it could be the site of inhibition. Currently there do not appear to be any known agents that inhibit or bind directly to tissue factor. The regulatory protein tissue factor pathway inhibitor (TFPI), which reversibly inhibits TF-fVIIa, binds fXa and fVIIa; there is no interaction with tissue factor itself (Dennis & Lazarus, 1994a). Also antithrombin III, which has been shown to inhibit TF-fVIIa in the presence of heparin, acts on fVIIa, not on TF (van 't Veer & Mann, 1997). Synthetic anticoagulants that have been developed to inhibit the extrinsic pathway mostly act on factor VIIa alone or on fVIIa in complex with TF (Dennis *et al.*, 2000; Dennis & Lazarus, 1994a and 1994b). Targeting tissue factor with an anticoagulant would thus be a novel function.

The low levels of inhibition may be due to the low concentration of the proteins in the tested samples. As stated above, the yield of protein was severely reduced when crude salivary extract was loaded onto the HIC column without prior desalting by reversed-phase chromatography. The yield was estimated by comparing peak areas. The protein concentrations were not determined experimentally. Alternatively, the detected inhibition may only be due to non-specific associations, although it has been reported that crude *O. savignyi* salivary extract exhibited significant inhibition of the extrinsic pathway (Gaspar *et al.* 1995).

Since both proteins seem to have similar anticoagulant activity, it may be that they are iso-forms. The proteins' mass difference is relatively small (160.04 Da), they co-purify during reversed-phase chromatography, pointing to similar hydrophobic properties, and both may be acidic in nature. Their most striking similarity is the fact that both adsorb to $BaSO_4$, a property shared with few other proteins. Despite this evidence, the different number of disulphide bonds, as well as the different cyanogen bromide cleavage patterns, seems to indicate the opposite.

The following chapter explores the proteins' adsorption to $BaSO_4$ more fully and investigates the possible means by which this interaction is formed.

Chapter 3

The nature of protein adsorption to $BaSO_4$

3.1 Introduction

In addition to the blood coagulation factors that have been purified using barium salts, there are some non-Gla-containing proteins that have been purified using the same methodology. Examples are the human C-reactive protein (CRP) isolated from plasma, a phagocytosis-promoting and complement activating factor (Kindmark & Williams, 1989). The CP4, a collagenous surfactant-associated protein from rat pulmonary epithelium (Persson *et al.*, 1989), human factor VIII (McCarroll *et al.*, 1981) and human factor XI (Bajaj & Birktoft, 1993). All these proteins selectively adsorb to $BaSO_4$. The vitamin K-dependent coagulation factors are believed to associate with barium salts through their bication-binding Gla residues. Like these proteins, CRP and CP4 possess calcium ion-binding sites. The former protein uses this site to associates with membranes, binding phosphocholine in pneumococci plasma membranes (Shrive *et al.*, 1996). Presumably, the residues that bind the Ca^{2+} ions in both these proteins can bind Ba^{2+}, providing a possible explanation for their selective adsorption. To summarize, most $BaSO_4$-adsorbing proteins bind calcium and most use these bound ions either directly or indirectly to interact with membranes.

While purifying CRP, it was noted that adsorption to commercial $BaSO_4$ is not as efficient as that achieved by laboratory-prepared $BaSO_4$ (Kindmark & Williams, 1989). The adsorption of the BSAPs to both forms of the salt was therefore evaluated. This chapter examines some of the properties of the adsorbent $BaSO_4$ and the molecules that bind to it.

3.1.1 Properties of $BaSO_4$

It is known that $BaSO_4$ possesses two different crystal forms, α and β-$BaSO_4$ (Kotowski, 1960). The α crystals form only at temperatures above 1010°C. All the $BaSO_4$ used in this study is the kind prevalent at room temperature, β-$BaSO_4$.

The β-$BaSO_4$ can exist in different 'grades' depending on the way it is produced. So-called high grade $BaSO_4$ is composed of mostly smooth-surfaced rhombic or prism shaped crystals, most of which are roughly 5μm in diameter (Howell & Deacon, 1975 and 1976; Dupe *et al.* 1975). The low grade $BaSO_4$, produced by precipitation, consists of large aggregates up to 40μm in size. These aggregates are composed of many miniature rhombic crystals providing the low grade $BaSO_4$ with a much larger surface/gram ratio than the high-grade variety. It can therefore act as a better adsorbent.

The low-grade $BaSO_4$'s porous nature allows proteins and lipoproteins to be completely included in the aggregates. It had been proposed that this form of the salt could be used as a packing material for size-exclusion columns (Howell & Deacon, 1975). However, the pores did not consist of the same degree of fineness as those of gel filtration materials and the use of low-grade $BaSO_4$ for this purpose was abandoned.

The examination of the electrophoretic mobility of different grades of $BaSO_4$ particles at different pH values provides a parameter, which reflects the surface charge of the salt in suspension (Howell & Deacon, 1975). In all cases the electrophoretic mobility at low pH values showed that the particles were positively charged. Mobility decreased towards the cathode as the pH increased and reached zero at pH 5-7. This remained the same until pH 9, after which the mobility was reversed. The main factor contributing to the characteristic shape of the pH-mobility curve is the presence of free barium ions attached at the surface of the salt's crystal lattice. This provides the otherwise insoluble $BaSO_4$ with a net positive charge. Presumably, it is this positive charge that the Gla residues of the vitamin K-dependent clotting factors adsorb to.

3.1.2 Protein adsorption to $BaSO_4$

As stated previously, the vitamin K-dependent coagulation proteins bind Ba^{2+} with their calcium-ion-binding Gla residues. The other known $BaSO_4$-adsorbing proteins however have no such amino acids. Glycosylated hydroxyproline and hydroxylysine residues may mediate the binding of the non-Gla-containing protein CP4, since it is known to contain a high percentage of these substituted amino acids (Persson *et al.*, 1989). Presumably, the carbohydrates provide the interface between the protein and the Ba^{2+} ions. The protein CRP coordinates its binding of two Ca^{2+} ions per subunit through several amino acids. Two aspartic acid residues, an asparagine residue, a glutamic acid residue and the main chain carbonyl of a glutamine residue bind the fist ion (Shrive *et al.*, 1996). The second calcium is coordinated by two glutamic acid, one aspartic acid and one glutamine residue. These residues may thus facilitate the binding of $BaSO_4$ by CRP.

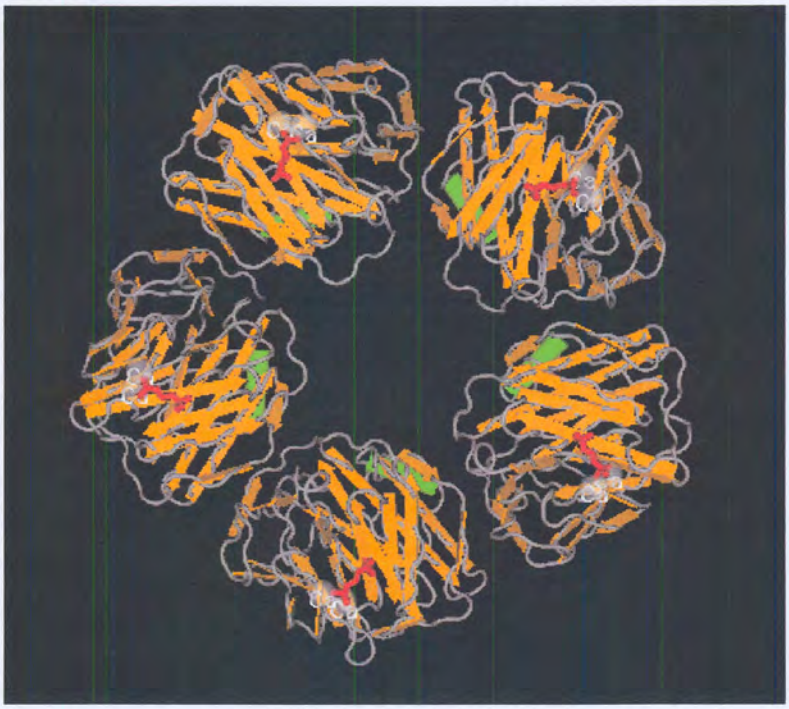


Figure 3.1: The structure of human C-reactive protein complexed with phosphocholine. The protein consists of five subunits, each of which binds two calcium ions (grey spheres). Bound to the Ca^{2+} ions is phosphocholine from pneumococci plasma membranes (red). α-Helices are in green, β-strands in orange and loop region in grey. The structure was obtained from the National Centre for Biotechnology Information (NCBI) MMCB structure database under the ID 11487.

Since the proteins isolated during this study adsorb to $BaSO_4$, like some of the Gla-containing coagulation factors, or the hydroxylysine and hydroxyproline-rich CP4, it may be possible that these proteins also contain such modified residues. The presence of hydroxyproline and hydroxylysine can be established through amino acid analysis. The detection of Gla residues is however more difficult.

A wide variety of methods have been developed for the quantitative or qualitative identification of γ-carboxyglutamic acid. These include specific stains, modified protein sequence analysis, analysis of alkaline hydrolysates by amino acid analysis (Smalley & Preusch, 1988) or using capillary electrophoresis (Britz-Mckibben *et al.*, 1999), anion-exchange, reversed-phase, gas or thin layer chromatography and identification by monoclonal antibodies (Brown *et al.*, 2000). However, most of these methods, excluding the latter, require large amounts of pure protein. Mass spectrometry is a useful alternative, being able to detect proteins in the low nanomolar range.

3.2 Materials and Methods

3.2.1 Materials

All materials used were of analytical grade and deionized water was used in all experiments. Na_2SO_4, $Ba(OH)_2$, HCl, NaOH, $NaNO_2$, anhydrous Na_2CO_3, methanol, acetic acid and *p*-aminobenzenesulphonic acid were purchased from MERCK, Darmstadt, Germany. EDTA was obtained from BDH Chemicals Ltd., Poole, England. The $BaSO_4$ used for adsorption was purchased form Associated Chemical Enterprises. The factor IXa was from ICN Biochemicals Inc., Aurora, USA and the bovine serum albumin was from Boehringer Mannheim GmbH, Germany.

3.2.2 Scanning electron microscopy and energy dispersive X-ray spectrometry

Dry samples of the commercial and the synthesized $BaSO_4$ were prepared for scanning electron microscopy by lightly tapping some of each onto adhesive carbon paper affixed to a copper plate. These plates were placed onto a sample tray and plated with gold to dissipate electrical charge and heat. The samples were then placed in a JEOL 6000FE scanning electron microscope operated at an accelerating voltage of 5kV.

Duplicates of the above samples were prepared for energy dispersive X-ray spectrometry, performed by A. Botha (Laboratory for Microscopy and Microanalysis, University of Pretoria). Since the individual particles were large enough to perform this experiment, it was not necessary to coat the samples with carbon. Three separate analyses were performed of each sample, each time at a different location on the plate. The accelerating voltage was 20kV and the lifetime of each sampling was 100sec.

3.2.3 Protein adsorption to commercial and laboratory-synthesized $BaSO_4$

The $BaSO_4$ used for adsorption was purchased form ACE (batch 6541), or was synthesized in the laboratory. The latter was prepared by slowly adding 0.061g Na_2SO_4,

dissolved in 1ml water, to a continuously vortexed 1ml suspension containing 0.073g $Ba(OH)_2$. Before addition of the Na_2SO_4 the $Ba(OH)_2$ was placed on ice for at least 10 min. Roughly 0.1g $BaSO_4$ precipitate is formed, which is collected by centrifugation. The supernatant is discarded.

The salivary gland extracts were prepared and subjected to $BaSO_4$ adsorption as described in Section 2.2.3, using the commercial or the synthesized salt. The obtained eluate was analysed and desalted by reversed-phase chromatography as described in the same section, but using a Beckman HPLC run by System Gold Chromatography software.

3.2.4 Electro-spray mass spectrometry

Electro-spray mass spectrometry was performed to determine the identity of the reversed-phase peak components. Samples of each peak were submitted for analysis, which was carried out as described in Section 2.2.5.

3.2.5 Detecting γ-carboxyglutamic acid residues

The presence of Gla residues was qualitatively evaluated by staining a tricine SDS-PAGE gel with 4-diazobenzene sulphonic acid (DBS), a compound known to selectively bind to Gla-containing proteins in polyacrylamide gels (Jie *et al.*, 1995). In addition, native and decarboxylated protein samples were analysed by ESMS.

3.2.5.1 Synthesis of 4-diazobenzene sulphonic acid

Since 4-diazobenzene sulphonic acid could not be purchased commercially, it therefore had to be synthesised. This was done according to the method set out in Vogel's Textbook of Practical Chemistry (Furniss *et al.*, 1989). A 100ml solution containing 8.66g *p*-aminobenzenesulphonic acid and 2.65g anhydrous Na_2CO_3 was prepared. The solution was heated until all the reagents had dissolved and all the formed CO_2 was

released. The mixture was then cooled under running tap water, until its temperature was about 15°C. To it was added 3.7g $NaNO_2$ dissolved in 10ml water. The resulting solution was then poured into ice cold HCl (10.5ml HCl/60g ice) while stirring. The presence of free nitrous acid was tested with potassium-iodide-starch paper. A blue colour is a positive test. Once an adequate amount of yellow DBS crystals had formed they were filtered and washed in ice-cold acetone. The crystals were then dissolved in water and stored at -20°C, until needed. Just before use, the DBS was washed with ice-cold acetone and then centrifuged briefly. This was repeated until the supernatant, after centrifugation, was colourless. The crystals were then dried by blowing N_2 (g) over them.

3.2.5.2 DBS staining

The reversed-phase fractions (pure protein), a peptide mass marker, Factor IXa (positive control) and BSA (negative control) were loaded into separate lanes of a tricine SDS-PAGE gel and electrophoresed as described in Section 2.2.4. The gels were stained with freshly prepared DBS staining solution consisting of 8.5mM DBS, 6.4mM $NaNO_2$ in 2M acetate buffer (pH 4.6) (Jie *et al.*, 1995). Immediately after gel electrophoresis the gels were washed for 5 min with 50% methanol/10% acetic acid to fix the proteins and remove SDS. This was repeated three times. Thereafter the gels were stained with 50ml of the DBS staining solution under gentle agitation for 2 hours at room temperature in the dark. The staining solution was then decanted and the gel developed with a developing solution consisting of 24ml 12.5M NaOH and 3.4ml 0.5M EDTA. The gels were incubated in this solution in the dark at room temperature until bands were visible.

3.2.5.3 ESMS analysis of native and decarboxylated protein

The method for specifically decarboxylating Gla residues is based on the well-known sensitivity of malonic acid and its derivatives to thermal decarboxylation (Price *et al.*, 1984). It is possible to dry Gla-containing proteins from an acidic solution and then to thermally decarboxylate the protonated γ-carboxyl groups of Gla to Glu without hydrolytic side reactions. Purified protein was decarboxylated by freeze-drying protein samples from 50mM HCl overnight and then heating it to 110°C for 5 hours, under

vacuum. The volume of acid added to the samples was 4µl/µg protein. Native and decarboxylated samples were submitted for ESMS analysis by M.J. van der Merwe (Department of Biochemistry, University of Stellenbosch) as described in Section 2.2.5. The difference in mass of the native and decarboxylated protein would allow the calculation of the number of Gla residues in the protein.

3.2.6 Detecting hydroxyproline and hydroxylysine

The amino acid composition of BSAP1 and BSAP2 was discussed in the previous chapter. To detect hydroxyproline, amino acid analysis was performed as described in Section 2.2.6. Detecting hydroxylysine required a modification of that procedure. Sample preparation and derivatization was carried out as described, but a longer column, PICO.TAG 3.9mm x 300mm, and different buffers and elution conditions were used. Buffer A contained 19.05g sodium acetate dissolved in 2 liters of water, with an 10% acetic acid adjusted pH of 6.45. Of this solution, 1950 ml was filtered and to it was added 50ml 100% acetonitrile. Buffer B contained a mixture of acetonitrile, water and methanol in a ratio of 9:8:3. A gradient over 66 min in length was applied followed by 20 min of equilibration as described in Table 3.1.

Table 3.1: Conditions for the separation of derivatized amino acids for hydroxylysine detection. Time is given in minutes.

Time (min)	Buffer A%	Buffer B%	Duration (min)
0	100	0	-
0	97	3	14.5
14.5	94	6	9.5
24	91	9	6
30	65	35	30
60	0	100	5.7
65.7	100	0	0.3
66	100	0	20
86	END		

3.3 Results

3.3.1 Scanning electron microscopy

The commercial $BaSO_4$ obtained form ACE (batch 6541) is composed of mostly smooth-surfaced angular crystals, most of which are roughly 5-8µm in diameter (Figure 3.2a). The $BaSO_4$ synthesized in the laboratory consisted of large aggregates, 5-30µm in size (Figure 3.2b). These aggregates were made of innumerable micro-crystals that upon closer examination have similar angular shapes to the much larger crystals of the commercial $BaSO_4$. It was not uncommon that several of these aggregates clumped together in conglomerates. These properties provide the synthesized $BaSO_4$ with a much larger surface/gram ratio. Another difference between the $BaSO_4$ samples is the apparent porous nature of the synthesized kind and the solid, non-porous nature of the commercial kind. All these observations are in agreement with already published investigations of low and high grade $BaSO_4$ (Howell & Deacon, 1975 and 1976; Dupe *et al.* 1975). It is therefore safe to conclude that the commercial $BaSO_4$ is the high-grade and the synthesized $BaSO_4$ the low-grade form of the salt.

a **b**

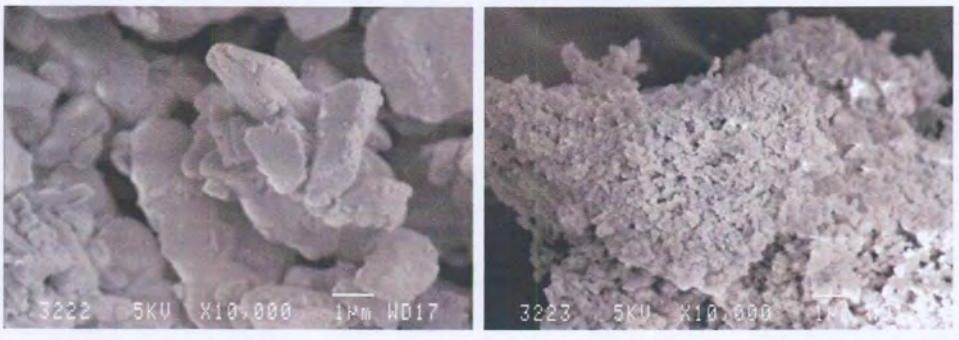


Figure 3.2: **a** Scanning electron micrograph of commercial $BaSO_4$ particles (ACE; batch 6541). **b** Scanning electron micrograph of synthesized $BaSO_4$ particles. Micrographs were taken at an accelerating voltage of 5kV and at 10 000x magnification. Note the crystalline and porous appearance of the particles in **a** and **b** respectively.

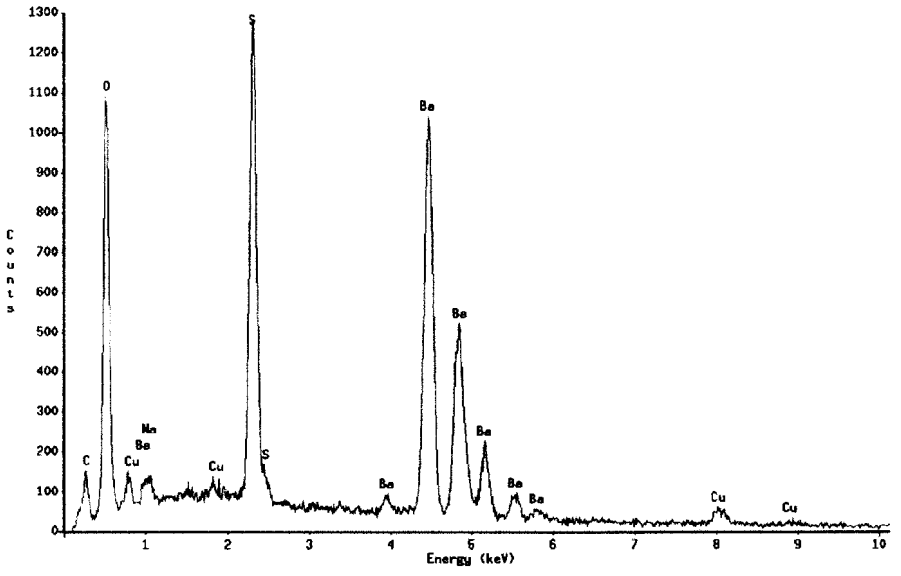
3.3.2 Energy dispersive X-ray spectrometry

The energy of X-rays emitted by different elements when bombarded with electrons is unique to each element. This allows a qualitative and quantitative analysis of a sample's chemical composition. By applying an accelerating voltage of 20kV, the X-rays emitted by the $BaSO_4$ samples were measured. As seen in Figure 3.3a and b, the X-ray spectra of the commercial and the synthesized $BaSO_4$ are similar. Both show the L-peaks of Ba and the K-peaks of S and O. From these spectra and the quantitative data in Table 3.2, it is clear that both samples contained predominantly Ba and S. A small quantity of the Cu constituting the sample plates, on which the samples were affixed, was also detected, as well as some C from the carbon paper adhesive. The small amount of Na that was detected most probably originates from the compounds used to synthesize the $BaSO_4$. From the above analysis it can be concluded, that the compound synthesized by reacting Na_2SO_4 and $Ba(OH)_2$ is definitely $BaSO_4$.

Table 3.2: Quantitative elemental analysis of the commercial and synthesized $BaSO_4$ samples. The lifetime of each observation was 100sec and the accelerating voltage was 20kV. The values were obtained by averaging the measured values of three separate analyses. Wt % = weight percentage.

	Commercial $BaSO_4$		Synthesized $BaSO_4$	
Element	Element Wt %	Wt % Error	Element Wt %	Wt % Error
S	20.7	±0.32	17.87	±0.30
Cl	0.17	±0.11	0.18	±0.11
Na	1.63	±0.19	1.02	±0.14
Ba	73.69	±1.04	77.46	±1.26
Cu	3.81	±0.42	3.47	±0.42

a



b

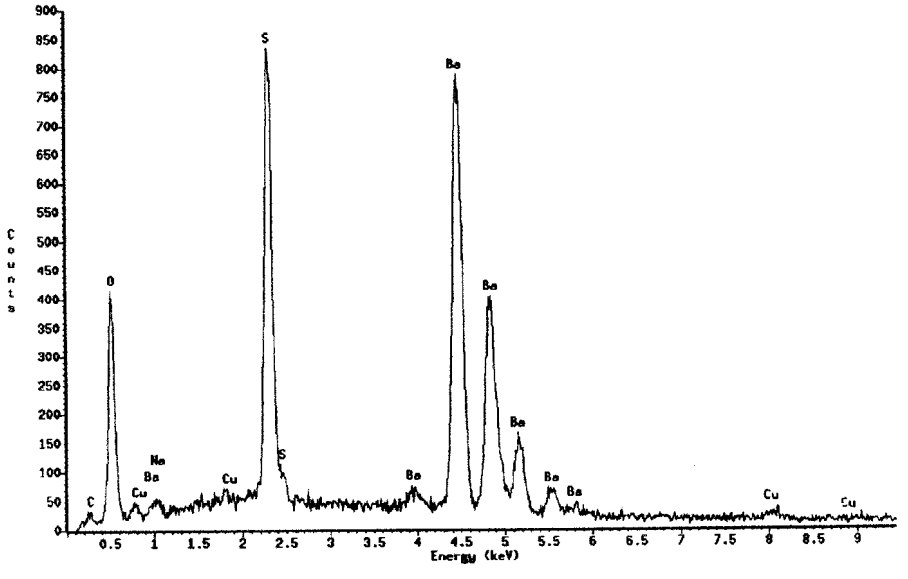


Figure 3.3: **a** X-ray spectrum of commercial (ACE batch 6541) and **b** synthesized $BaSO_4$. The accelerating voltage was 20kV and the lifetime of each observation was 100sec. Each peak is identified as characteristic of a particular element. The element responsible for a peak is given above that peak.

3.3.3 Protein adsorption to commercial and laboratory-synthesized $BaSO_4$

Some published articles describing the isolation of proteins by $BaSO_4$ adsorption-elution noted that low-grade $BaSO_4$ proved to be a better adsorbent. Larger quantities of protein could be purified (Howell & Deacon, 1975). With a larger surface/gram ratio, the low-grade salt has more surfaces for proteins to adsorb to. No such improvement in protein adsorption was detected for the BSAPs. Figure 3.4 shows the RP-chromatograms of protein samples from commercial and laboratory-synthesized $BaSO_4$ eluates. Figure 3.4a shows the chromatogram of the commercial barium-adsorbed protein. It contained only one peak, which when analysed, proved to be BSAP1 and BSAP2 (data not shown). This data conforms to that given in chapter 2. The laboratory-synthesized salt also adsorbed BSAP1 and BSAP2, as was evident from the ESMS analysis of the peak with retention time 20.41 min in Figure 3.4b (Figure 3.5a). The greater adsorption to low-grade $BaSO_4$ reported by other investigators was not evident in this study. It was repeatedly noted that the low-grade form of the salt appeared to adsorb less protein then the commercial salt. The use of the lab-synthesized salt for protein purification would therefore be of no benefit.

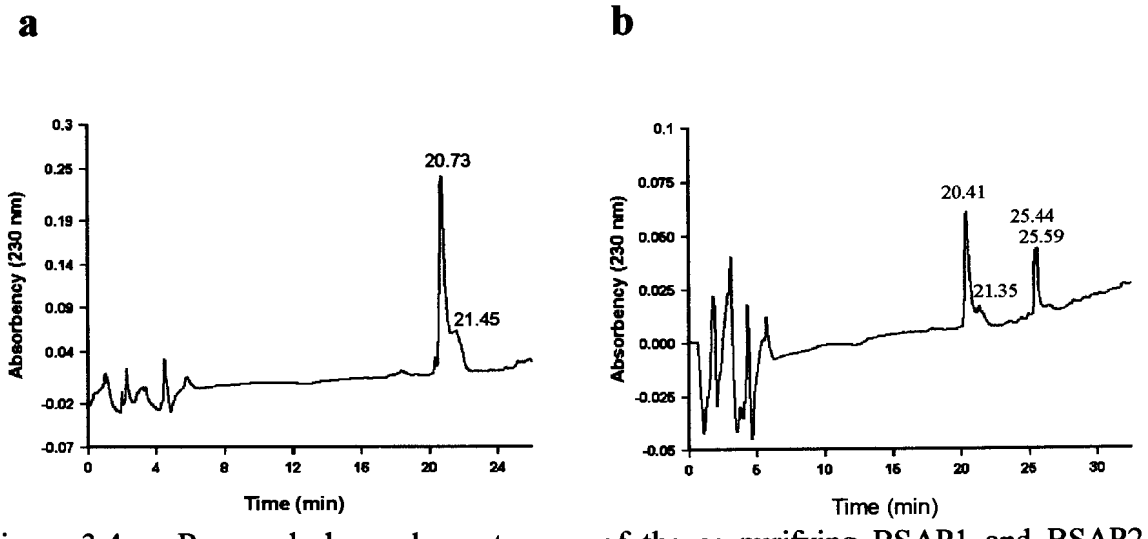


Figure 3.4: **a** Reversed-phase chromatogram of the co-purifying BSAP1 and BSAP2 adsorbed with commercial $BaSO_4$. **b** Reversed-phase chromatogram of proteins adsorbed with laboratory-synthesized $BaSO_4$. All samples were loaded onto a C5 reversed-phase column and eluted at a flow rate of 1ml/min. Absorbance was measured at 230nm.

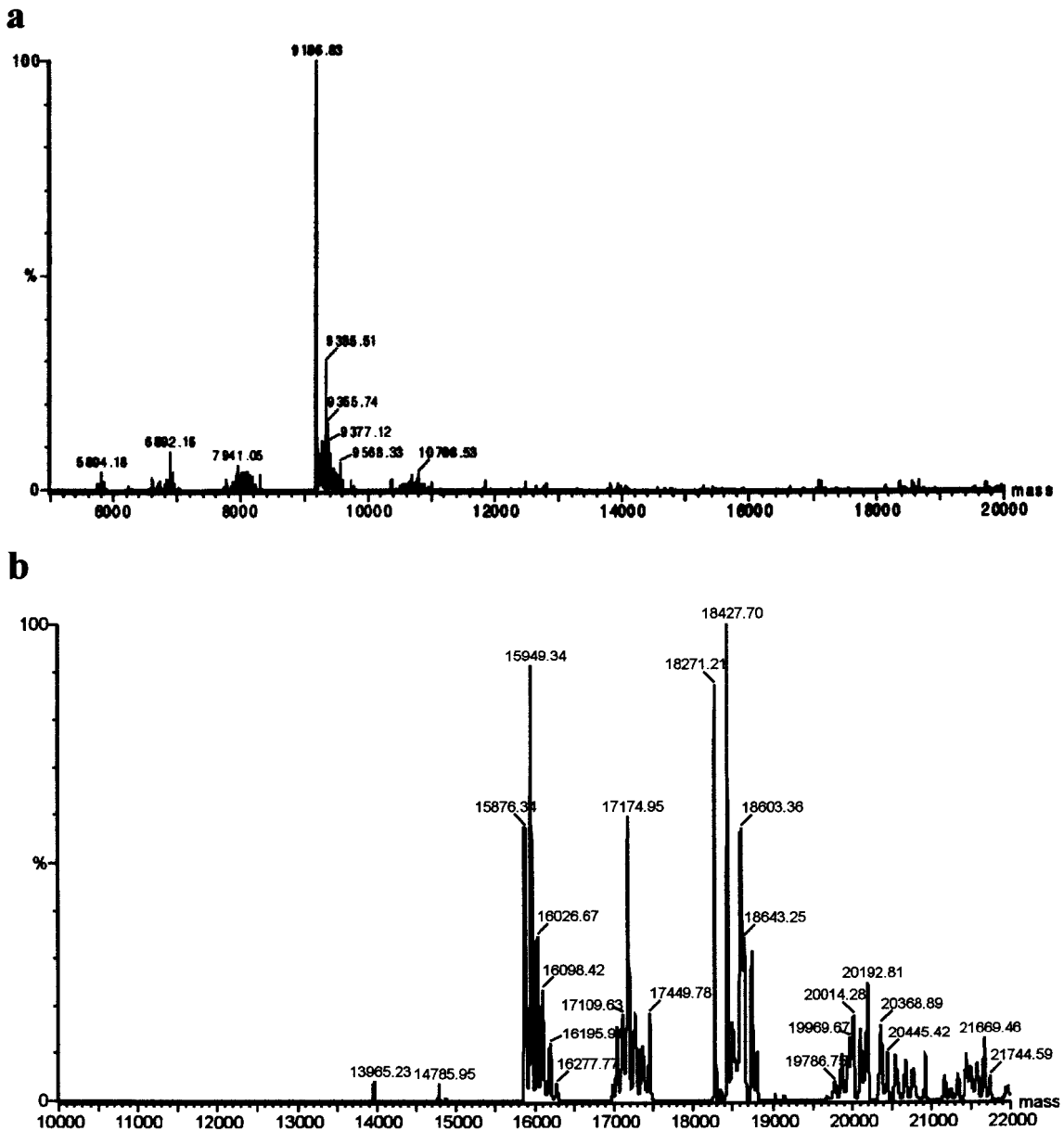


Figure 3.5: Deconvoluted spectra of the ESMS analysis of the RP-purified proteins adsorbing to low grade $BaSO_4$. **a** Two components are present in the 20.41 min peak. The main component is BSAP2 at mass 9186.83 Da and the second component is BSAP1 at mass 9336.51 Da. **b** At least 6 main components are present in the 25.59 min peak. The masses of the main components are 15876, 15949, 17174, 18271, 18427 and 18603 Da.

The presence of a second peak with retention time 25.59 min and its adduct at 25.44 min, in Figure 3.4b was unexpected. Electro-spray mass spectrometry showed at least 6 main components to be present in this peak (Figure 3.5b). They have masses ranging from 15.8 to 18.6 kDa. The molecular masses of three of these components are identical to the molecular masses of the T1, T2, and NT proteins previously isolated from *O. savignyi* by Mans *et al.* (2000c). The T2 toxin has a mass of 15877 Da, NT a mass of 15957 Da and T1 a mass of 17170 Da. The three components found in the second peak of the reversed-phase chromatogram had masses of 15876 Da, 15949 Da and 17174 Da. The identity of the other three main components with masses of 18271 Da, 18427 Da and 18603 Da, as well as those of the components present in smaller quantities, is unknown.

3.3.4 Presence of γ-carboxyglutamic acid residues

3.3.4.1 DBS staining

γ-Carboxyglutamic acid-containing proteins stain red in the presence of DBS, while non-Gla proteins stain yellow (Jie *et al.*, 1995). The background turns a transparent light yellow. The detection limit for this stain is about 1 μg of protein. To qualitatively screen for Gla residues in the purified proteins, a sample of each was electrophoresed together with bovine serum albumin (BSA) and factor IXa (fIXa). The non-Gla-containing BSA served as negative control, while the Gla-containing fIXa served as positive control. As is evident from the DBS stained polyacrylamide gel in Figure 3.6, the BSA stained yellow and the fIXa stained red. The BSAP1 and BSAP2 stained yellow indicating the absence of Gla residues from these proteins. To confirm this result, native and decarboxylated samples were analysed by ESMS.

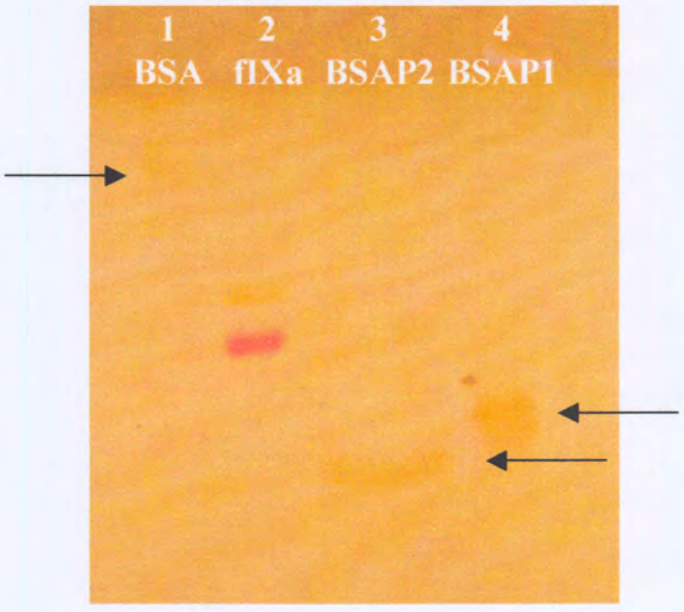


Figure 3.6: 4-Diazobenzenesulphonic acid (DBS) stained polyacrylamide gel. Lane 1 contained BSA, lane 2 factor IXa (fIXa), lane 3 BSAP2 and lane 4 BSAP1. BSA served as negative control (yellow band), fIXa as positive control (red band). The background stains light yellow. The concentration of protein loaded in each lane was not determined due to the low amounts that could be purified Arrows indicate the relative positions of yellow protein bands.

3.3.4.2 ESMS analysis of native and decarboxylated protein

The spectra of the native and the decarboxylated proteins show no significant mass differences (Figure 3.7). The native proteins had a mass of 9196 Da and 9370 Da. These values correspond reasonably well with the masses that were previously determined, 9173 Da and 9333 Da for BSAP2 and BSAP1, respectively (Figure 2.5). The decarboxylated proteins had a mass of 9199 Da and 9332 Da. If only one Gla residue were present in either protein, then there should be a mass difference of at least 45 Da, this being the mass of a protonated carboxyl group. No such difference exists between the native proteins and their decarboxylated forms, confirming the absence of Gla residues in the BSAPs.

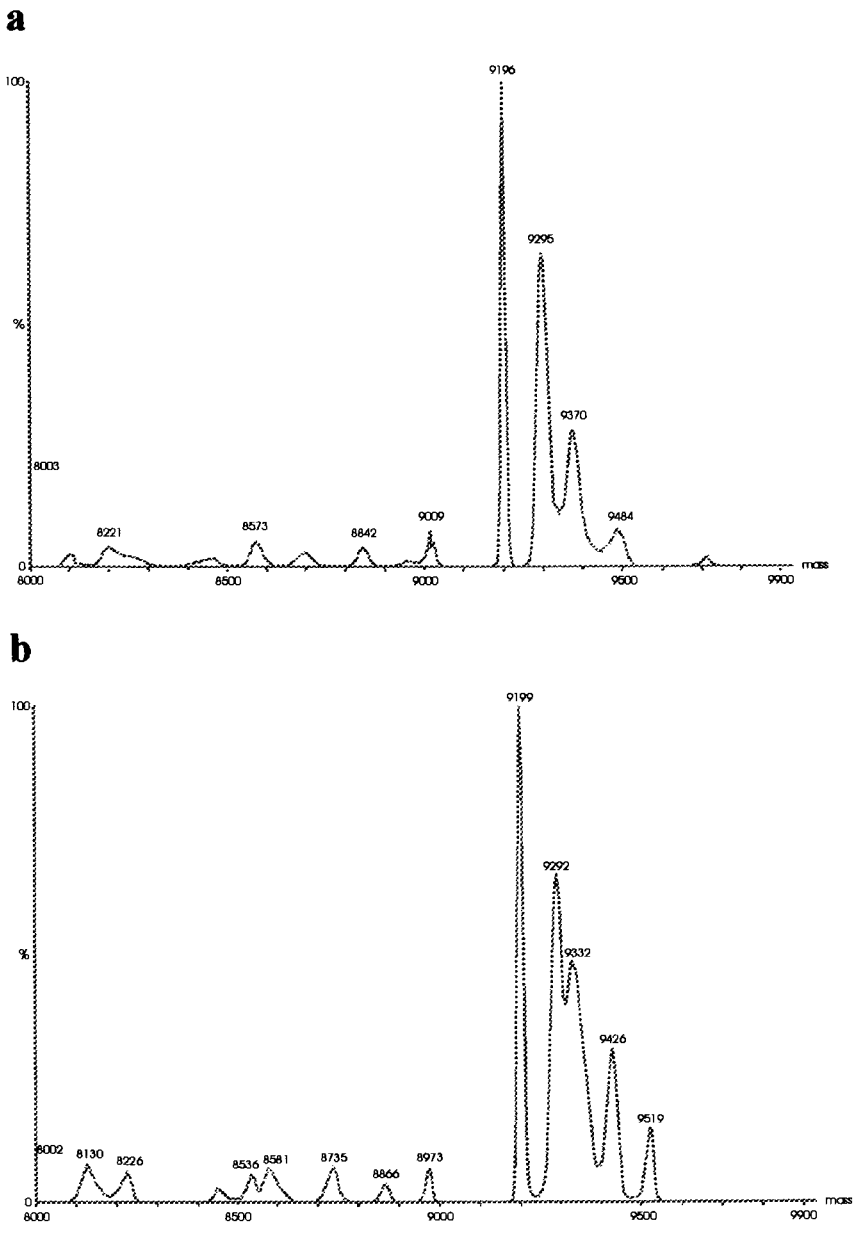


Figure 3.7: Electron-spray mass spectra. **a** The spectrum of the native proteins. Their masses were 9196 Da (BSAP2) and 9370 Da (BSAP1). **b** The spectrum of the decarboxylated proteins. Their masses were 9199 Da (BSAP2) and 9332 Da (BSAP1).

3.3.5 Presence of hydroxyproline and hydroxylysine

Derivatized hydroxyproline elutes between glutamic acid and serine with a retention time of 2.35 min (Figure 3.8). Derivatized hydroxylysine elutes between leucine and phenylalanine at 50.12 min (Figure 3.10a). The analyses for these amino acids in the BSAPs are given in Figure 3.9 and Figure 3.10b and c, respectively. Neither hydroxyproline nor hydroxylysine were detected.

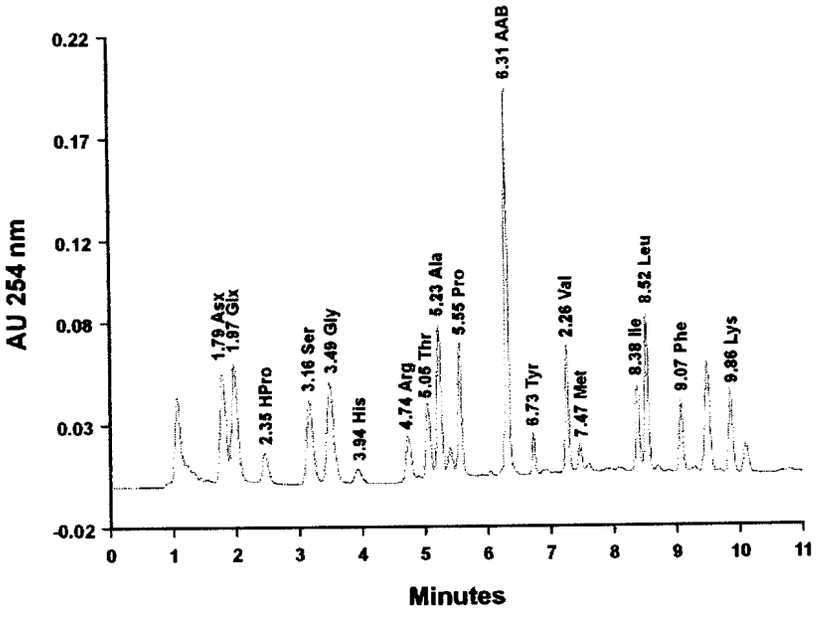


Figure 3.8: Reversed-phase amino acid profile of the hydroxyproline control. The retention times of the amino acids are given in minutes. Absorbance was measured at 254 nm. AAB was an internal standard. HPro = hydroxyproline.

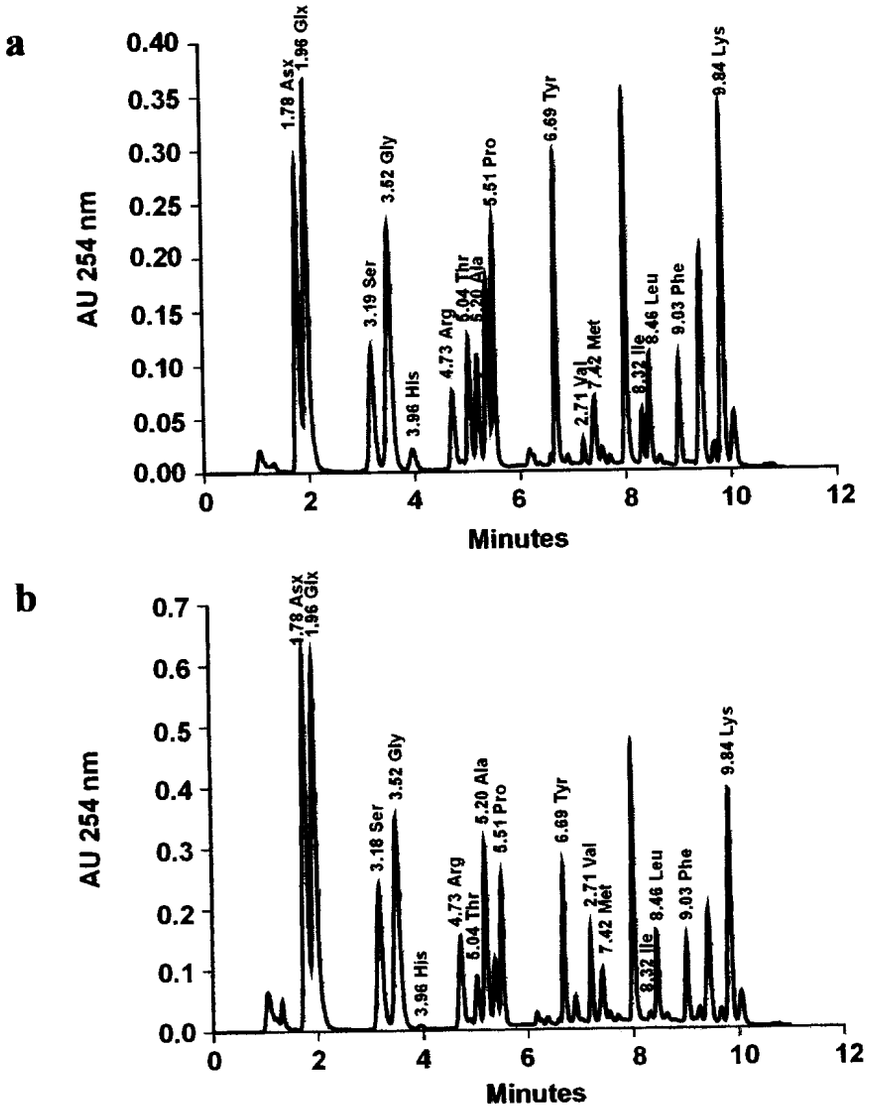


Figure 3.9: Amino acid analysis of BSAP1 and BSAP2 to determine the possible presence of hydroxyproline. **a** Reversed-phase amino acid profile of BSAP2. **b** Reversed-phase amino acid profile of BSAP1. The retention times of the amino acids are given in minutes. Absorbance was measured at 254 nm.

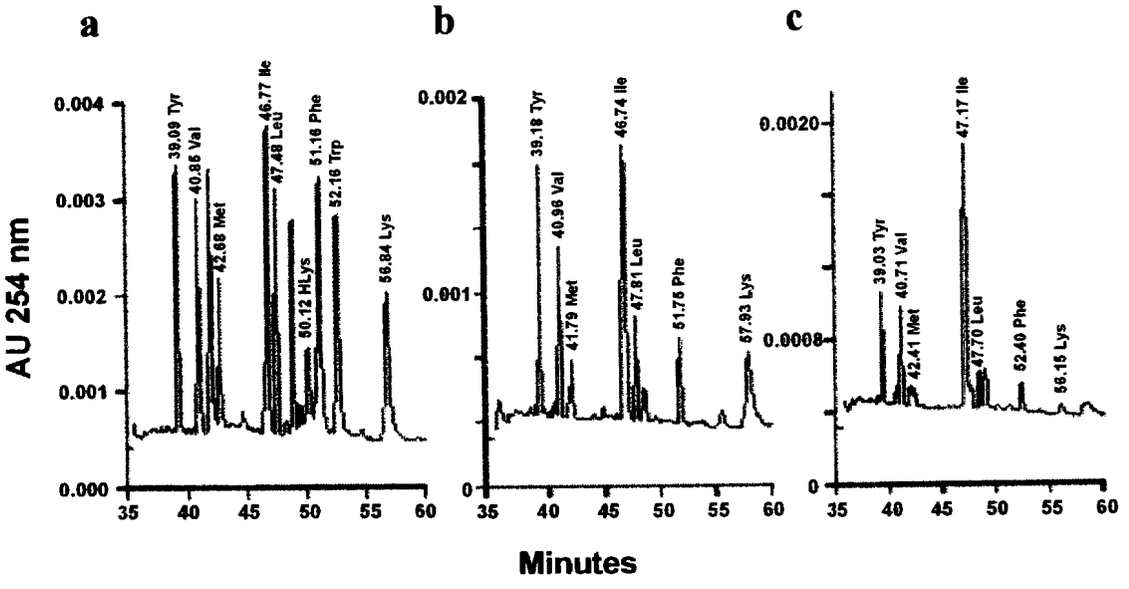


Figure 3.10: Amino acid analysis of BSAP1 and BSAP2 to determine the possible presence of hydroxylysine (HLys). **a** Standard showing HLys at 50.12 min. **b** Reversed-phase amino acid profile of BSAP1. **c** Reversed-phase amino acid profile of BSAP2. The retention times of the amino acids are given in minutes. Absorbance was measured at 254 nm.

3.4 Discussion

Energy dispersive X-ray spectrometry identified the compound synthesized from Na_2SO_4 and $Ba(OH)_2$ as $BaSO_4$ (Figure 3.3). The crystalline natures of this product as well as the commercial salt were investigated by scanning electron microscopy (Figure 3.2). This showed the laboratory synthesized $BaSO_4$ to be the low-grade and the commercial $BaSO_4$ the high-grade form of the salt. Work done by Howell & Deacon (1975; 1976) showed that pH has little effect on either the adsorption of proteins or anionic dyes. The charge nature of the salt differed only slightly with changing pH for both the low and high-grade $BaSO_4$.

As seen in Figure 3.2b, the small size of the low-grade $BaSO_4$ crystals should provide the synthesized $BaSO_4$ with a much larger surface/gram ratio. This means more surfaces are exposed for adsorption. More sites for adsorption translate into more sites for protein adherence. The low-grade salt should therefore adsorb more protein than the high-grade salt. It has been observed that a tenfold increase in the amount of protein, which is adsorbed by the low-grade $BaSO_4$, when compared to the equivalent weight of high-grade form, is not uncommon (Howell & Deacon, 1975). No such improved protein binding was noted in the study of *O. savignyi*'s $BaSO_4$-adsorbing proteins. On the contrary, there was a marked general decrease in the amount of protein that was adsorbed (compare Figure 3.4a and b).

Another difference between the $BaSO_4$ samples is the porous nature of the low-grade form and the solid, non-porous nature of the high-grade form. The low-grade $BaSO_4$'s porous nature allows proteins and lipoproteins to be completely included in the aggregates. The low-grade $BaSO_4$ therefore has size-exclusion properties, which may influence the adsorption and subsequent elution of proteins. This may explain the co-purification of the T1, T2 and NT proteins, and others that remain unidentified, with BSAP1 and BSAP2, when the low-grade $BaSO_4$ is used. If so, these proteins do not truly

adsorb to the salt and could be eluted from the salt by including more wash steps in the $BaSO_4$ adsorption procedure.

Since many of the known $BaSO_4$-adsorbing proteins contain Gla residues, it was of interest to determine whether theses residues are present in BSAP1 and BSAP2. γ-Carboxyglutamic acid readily decarboxylates to glutamic acid at high temperatures in its protonated form. For this reason, amino acid analyses of Gla-containing proteins following acid hydrolysis at 110°C are negative for γ-carboxyglutamic acid. To quantitate Gla in proteins, alkaline hydrolysis has been widely applied (Burnier *et al.*, 1981; Smalley & Preusch, 1988). Samples of the isolated protein were base hydrolyzed, but only poor derivatization of amino acids was achieved (method and results not shown) and consequently, analyses of this form were abandoned.

Instead, the Gla-specific DBS staining of electrophoresed protein samples was conducted. Both BSAP1 and BSAP2 stained negative for the presence of Gla residues. The sensitivity of this result depends on the protein concentration in the gel. To confirm the result, ESMS analyses of native and decarboxylated samples were performed. According to these analyses, there are no Gla residues in either protein. The masses of the native proteins and their decarboxylated forms are near identical (Figure 3.7).

The amino acid analysis of BSAP1 and BSAP2 did not indicate the presence of hydroxyproline or hydroxylysine (Figure 3.8 and 3.9). This data and the confirmed absence of Gla residues, implies that BSAP1 and BSAP2 bind to $BaSO_4$ through means other than those of the blood plasma Gla proteins or CP4. They may possibly associate with $BaSO_4$ in a manner similar to CRP, using a few amino acid residues to coordinate the binding of a Ba^{2+} ion. It would be of interest to determine whether or not the BSAPs are Ca^{2+}-binding proteins, since the existence of such a Ca^{2+}-binding site seems to be a prerequisite for binding $BaSO_4$.

Another striking similarity of the known $BaSO_4$-adsorbing proteins is that many of them associate with membranes. Since the proteins purified from *O. savignyi*'s salivary glands

show the same high degree of selective $BaSO_4$ adsorption, a justifiable hypothesis may be that these proteins likewise bind Ca^{2+} and possibly use these ions to interact with membranes. Such a property may be essential to the proteins function if they are indeed inhibitors of the extrinsic pathway. Coagulation assays described in the previous chapter indicated that the extrinsic pathway is slightly inhibited, but that the observed inhibition is not due to any inhibitory effect on factor VII. The only other unique component of the extrinsic pathway is tissue factor, an integral membrane protein. Should the anticoagulant properties of BSAP1 and BSAP2 be confirmed, one could thus further speculate that they are targeted to tissue factor, either by the indirect conformational or direct influence of a Ca^{2+}-binding site. So far, the most compelling piece of evidence to support such an assumption is the observed adsorption to $BaSO_4$.

The following chapter investigates whether BSAP1 and BSAP2 have the predicted membrane-binding properties and whether or not such an interaction is dependent on Ca^{2+} ions.

Chapter 4

Investigating the possible membrane-binding properties of BSAP1 and BSAP2

4.1 Introduction

The techniques commonly used to study molecular interactions, like equilibrium dialysis, are limited in that they only allow the study of the equilibrium position reached after molecules have bound. No information on the rate constants for the formation and dissociation of molecular complexes can be gained. Further disadvantages are that most such methods are slow and not easily automated. The recent development of optical biosensors has changed that.

Biosensors combine a biological recognition element with an optical transduction element (Thévenot *et al.*, 2001). They can have varying sensing elements such as enzymes, cell membranes, antibodies, nucleic acids, organelles, whole cells and micro-organisms, all of which can be immobilised to the sensor surface. These sensing elements will pick up changes in their immediate environment and transmit those changes in the form of physical signals to a transducer that picks up those signals and transmits them as optical signals that are interpreted by a computer.

Such instruments allow the real-time observation and quantification of biomolecular interactions as they happen without the need for fluorescent or radioisotope labelled analytes and ligands. Rapid quantitative studies of molecular recognition, affinity, kinetics, concentration and multi-molecular interactions are now routinely performed with such instruments. The dynamics as well as the strength of binding between molecules can be easily analysed. There are two instruments that are currently used to perform such real-time binding studies. They are the IAsys cuvette system, which is

based upon the resonant mirror principle, and the BIAcore system, based on surface plasmon resonance (SPR).

Several studies involving coagulation factors and their inhibitors have been conducted using optical biosensors. The inhibition of factor IXa generation and tenase complex formation by the insect salivary protein prolixin-S was investigated using surface plasmon resonance (Isawa *et al.*, 2000). Aspects of the interaction between factor VII and tissue factor were studied using the same technique (O'Brien *et al.*, 1994). The regulatory affect of tissue factor on plasminogen binding and activation was investigated using both surface plasmon resonance and the resonant mirror (Fan *et al.*, 1998).

4.1.1 The resonant mirror

In the IAsys biosensor, the signal transducer is an optical detector. It makes use of a resonant mirror cuvette, which has a prism as its base. Ligand is directly immobilised onto the prism surface. The binding of the analyte to the ligand is monitored continuously, exploiting the changes in refractive index caused by molecules interacting at the cuvette surface.

This occurs more or less as follows. A laser light beam is directed at the prism over a range of angles. In Figure 4.1 only one angle is shown for clarity. At all angles total internal reflection occurs. At a particular angle, the resonant angle, light tunnels through the coupling layer and propagates through the resonant layer. This process is reversible and some light escapes. Phase-detection is used to isolate that component of light that propagates in the resonant layer. An electromagnetic component, called the evanescent field, is formed and propagated into the lower refractive index medium, the analyte solution, where it rapidly decays. Any change in mass within the evanescent field will change the refractive index profile close to the surface, changing the resonant angle. Binding and dissociation events are seen as shifts in the resonance angle arising as one partner or more in free solution binds to, or dissociates from, the other partner

immobilised at the surface of the sensor. This shift is the signal and is expressed in arc seconds.

The evanescent field is extremely sensitive to mass changes within its reach. One of its shortcomings, however, is that the field decays exponentially the further it is from its source. Its intensity is reduced by two thirds within about 100nm (IAsys Plus User's Guide, 2000). Molecular interactions thus need to take place close to the sensor surface in order to be detected.

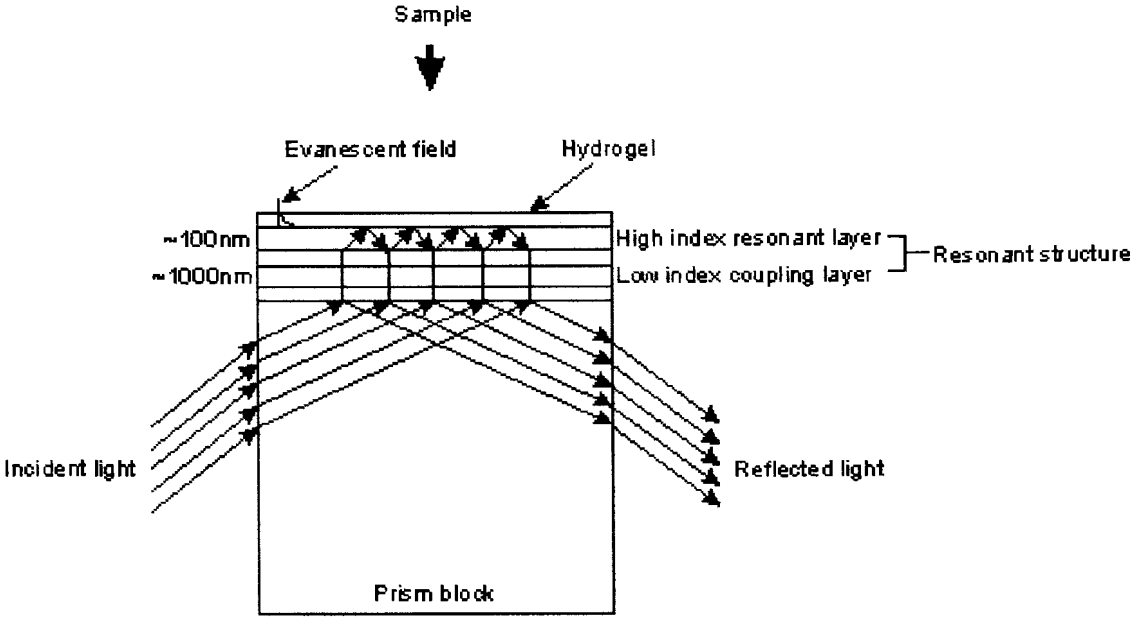


Figure 4.1: Configuration of the resonant mirror sensor device. Only one angle of incident light is shown for clarity. (Adapted from IAsys Plus User's Guide, 2000).

4.1.2 Investigating protein-lipid interactions with the IAsys biosensor

Lipid membranes that are immobilised on a surface can mimic cell membranes for the study of the binding interaction between biomolecules (Fisher & Tjärnhage, 2000). A number of different techniques for the creation of such membranes on solid supports have been described. Among them are the Langmuir-Blodgett method or the self-assembly of

thiol-lipids to gold surfaces (Fisher & Tjärnhage, 2000; Jass *et al.*, 2000). These methods were not always successful when applied to optical biosensor cuvettes. A useful alternative is to prepare membrane-like structures on solid-supports starting with liposomes. This method, called fusion or coalescence, makes use of the fact that liposomes will transfer material to both hydrophilic and hydrophobic surfaces (Fisher & Tjärnhage, 2000).

When a liposome makes contact with a suitable surface it may adsorb, break up, and spread to form a bilayer on a hydrophilic surface or a monolayer on a hydrophobic surface (Sackmann, 1996; Jass *et al.*, 2000). This sequence of events, adsorption, rupture, and spreading, are repeated numerous times as many liposomes come into contact with an appropriate surface. These then unite to form a large, continuous bilayer. This process is illustrated in Figure 4.2, which depicts the adhesion of only one liposome for clarity. Despite overlap between the different liposomes, it is often difficult to get larger areas of confluent, defect-free lipid bilayer (Jass *et al.*, 2000). Large numbers of unfused liposomes may remain after washing and the underlying sensor surface could be exposed in some locations (Fisher & Tjärnhage, 2000). Pre-coating the sensor surface with a cationic detergent can solve that problem.

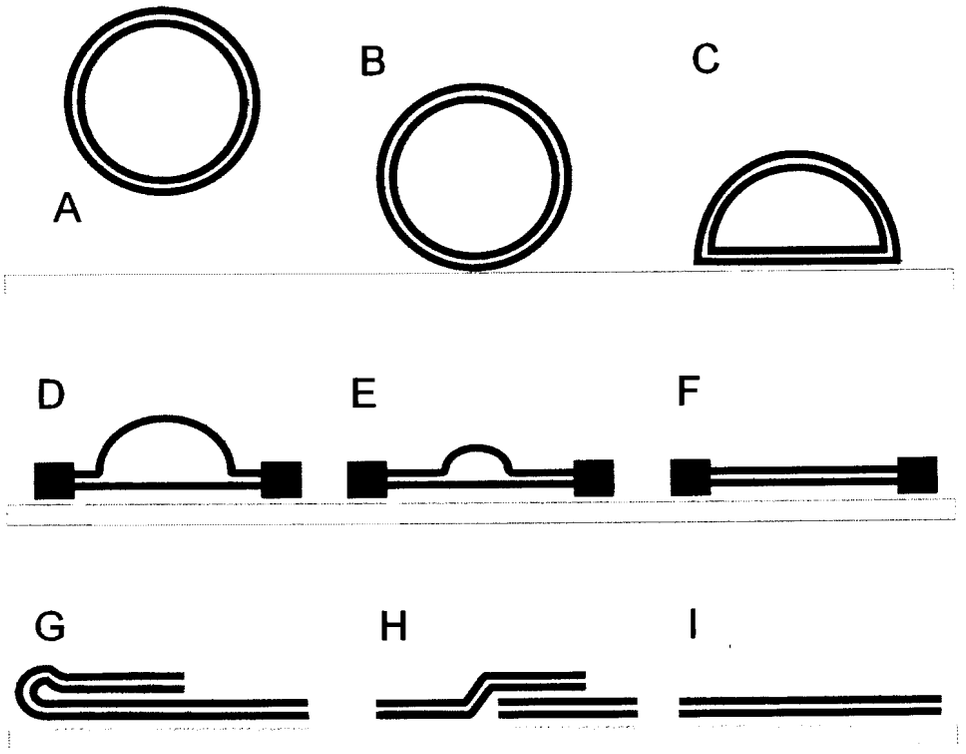


Figure 4.2: Diagrammatic representation of liposome transformation into a supported planar bilayer. The liposome approaches (A), adsorbs (B), and attaches (C) to the surface. It then begins to flatten from the edges (D). The outer flattened areas spread (E) and eventually the liposome collapses to form two bilayers (F) stacked one upon the other. Theoretical calculations suggest that rupture occurs when the contact angle of the liposome edges exceeds a critical value. The upper bilayer then either rolls of the lower (G) or slides off (H), resulting in a single bilayer (I). The edges are marked as boxes as the structure and organization of these boundaries are unknown. (Source: Jass *et al.*, 2000 and Puu & Gustafson, 1997).

Supported membranes created by liposome coalescence are known to be very stable. This makes them ideal surfaces to be used in the monitoring of samples over many months (Fisher & Tjärnhage, 2000). Furthermore, phospholipids immobilised on sensor surfaces represent convenient and physiologically relevant models of membrane surfaces. They have been used to study the kinetics of interaction of coagulation factors with phospholipids and as a model for the assembly of the multi-protein complexes of the coagulation cascade (Saenko *et al.*, 1999). In such a system, the ability of BSAP1 and BSAP2 to associate with membranes, in the presence and absence of bication-containing salts like $CaCl_2$, could be evaluated.

4.2 Materials and Methods

4.2.1 Materials

All materials used were of analytical grade and deionized water was used in all experiments. The KCl, NaCl, HCl and Na_2HPO_4 were from MERCK, Darmstadt, Germany. The $CaCl_2$ and KH_2PO_4 were from BDH Chemicals Ltd., Poole, England. Factor IXa was obtained from ICN Biochemicals Inc., Aurora, USA and the bovine serum albumin from Boehringer Mannheim GmbH, Germany. The phospholipid mixture containing L-α-phosphatidylcholine from fresh egg yolk, the cationic detergent 1-hexadecylpyridinium chloride (CPC) and the pre-liposome formulation 3 containing β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine and dioleoyl-L-α-phosphatidyl-DL-glycerol were purchased from Sigma-Aldrich, USA. The non-derivatised cuvettes and the IAsys instrument were form Affinity Sensors, Cambridge, UK. The Coomassie Protein Assay Reagent used for protein concentration determinations was purchased from Pierce, USA.

4.2.2 Determination of protein concentration

The concentration of proteins was determined with the Pierce Coomassie Protein Assay Reagent as recommended by the manufacturer. A stock solution of bovine serum albumin (BSA) was prepared at a concentration of 0.2mg/ml. A BSA dilution series was prepared in a 96 well microtiter plate. Water was used as the blank. Protein samples were pipetted into separate wells. Absorbance was measured at 620 nm using an SLT 340 ATC ELISA reader (SLT Labsystems). The absorbance of the blank was subtracted from those of the protein samples and the BSA dilutions. The latter corrected absorbance values were used to plot a standard curve and the sample's protein concentration determined. Each assay was performed in duplicate.

4.2.3 Preparation of liposomes

The interaction of BSAP1 and BSAP2 with neutral and negatively charged membranes was investigated. The former experiment would indicate the existence of a hydrophobic binding site possibly analogous to that of the membrane-binding coagulation factors. An example is factor IX (Figure 1.7) (Freedman *et al.*, 1996).

The neutral and negatively charged membranes were formed from liposomes of differing composition. The neutral membranes were formed from liposomes consisting of a mixture of L-α-phosphatidylcholine lipids from fresh egg yolk. The negatively charged membranes were formed from Sigma-Aldrich's pre-liposome mixture containing β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine and dioleoyl-L-α-phosphatidyl-DL-glycerol.

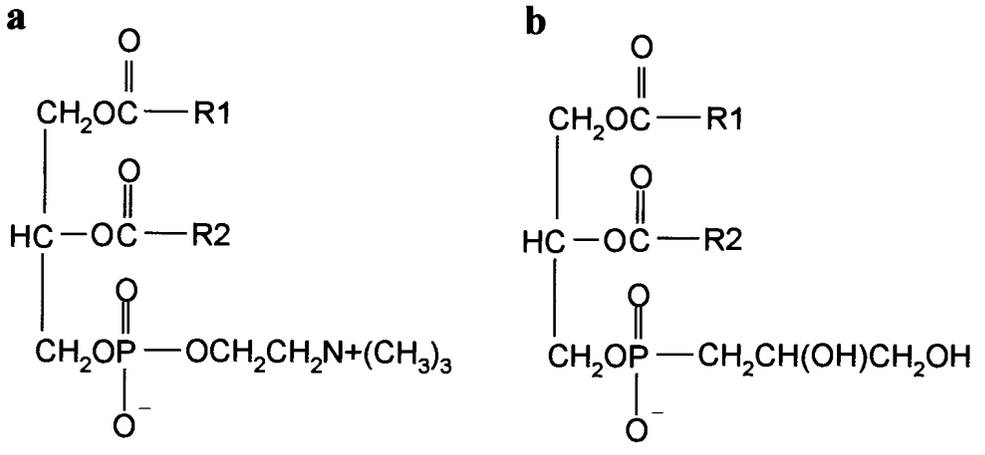


Figure 4.3: The general structures of **a** phosphatidylcholine and **b** phosphatidylglycerol. Note that phosphatidylcholine has no net charge, whereas phosphatidylglycerol is negatively charged.

The phosphatidylcholine from egg yolk was dissolved in chloroform, whereas the pre-liposome mixture was dissolved in water (as recommended by the supplier). Before aliquoting, the chloroform was evaporated and the lipids redissolved in PBS. The pre-liposome mixture dissolved in water was directly aliquoted. Phospholipid aliquots containing 1mg/ml resuspended lipid were prepared and stored at –20°C. Before use, the

volume of an aliquot was adjusted to 500µl with PBS (see Section 4.2.3 for preparation of PBS). The phospholipids were sonicated continuously for 1 min using a Branson Sonic Power Co. Sonifier (20% duty cycle and an output control of 2) to form liposomes.

4.2.3 Immobilisation of liposomes to non-derivatised cuvettes

A 20x stock solution of phosphate buffered saline (PBS) was prepared by adding 160g NaCl, 4g KCl, 4g KH_2PO_4 and 58g Na_2HPO_4 to 1 litre of water. A 1x dilution of the stock with a pH of 7.4 was used in all subsequent experiments.

Resonance scans were conducted at the beginning of each experiment and on occasion monitored throughout. The distribution should show a single clear peak with symmetrical skirts. If the peak is broad, or there are more peaks, this may be an indication of heterogeneous distribution of the sample on the resonant mirror surface (Puu & Gustafson, 1997).

A new dual-well non-derivatised cuvette was inserted into the IAsys biosensor. The temperature was maintained at 25°C and the stirrers were set at 70%. Each well was washed three times with 50µl of PBS and was followed by 10 min of equilibration. Resonance scans were conducted to determine the suitability of the sensor surface for immobilisation. The acquisition of data was begun and approximately 3 min of baseline data collected. The cuvettes were washed three times with 50µl of the cationic detergent 1-hexadecylpyridinium chloride and data collected until a plateau was reached (Verschoor *et al.*, 2001). Thereafter, the content of one well of the cuvette was replaced with 30µl of the prepared 100% phosphatidylcholine liposomes and the other with the phosphatidylcholine and phosphatidylglycerol liposomes and the response monitored until a new plateau was reached. To each well were then added two 30µl quantities of PBS without removing the liposomes. No further additions were made until the response had levelled off. The wells were then washed three times with 50µl of PBS. Before storage, 100µl of PBS was pipetted into each well. The cuvette was then stored in a sealed container at 4°C.

4.2.4 Determining protein-lipid interactions

The BSAP1 and BSAP2 proteins were purified by reversed-phase chromatography as described in Section 2.2.3. Factor IXa and BSA served as positive and negative control, respectively. Unless otherwise specified, 5µg quantities of each protein were used in the binding experiments. The possible Ca^{2+}-dependence of BSAP1 and BSAP2 membrane-association was evaluated by pre-incubating the protein samples and controls with and without 10µl of 5mM $CaCl_2$ for 10 min.

The stored cuvette was placed in the IAsys biosensor and allowed to equilibrate for at least 1 hour. The biosensors internal temperature was maintained at 25°C and the stirrers were set at 70%. Each well was washed three times with 60µl of PBS. Resonance scans were routinely performed to monitor the quality of the sensor surface. Data acquisition was started and approximately 2 min of baseline data collected. Then, 5µg of protein sample in 60µl of PBS was added and the response recorded. After 5 min, the protein sample was removed and 60µl of PBS added to wash off any bound protein. The dissociation was recorded over 2 min. The lipid-covered sensor surface was then regenerated by the addition of 60µl of 20mM HCl (Puu, 2001; Fisher & Tjärnhage, 2000). After 2min, the acid was removed and 60µl of PBS added. The cuvette could then be used for the next experiment.

4.3 Results

4.3.1 Immobilisation of liposomes to non-derivatised IAsys cuvettes

Figure 4.4 shows the immobilisation of phosphatidylcholine liposomes onto a non-derivatised sensor surface. At the time labelled (1) CPC was added to one well of the cuvette generating a response of ~600 arc seconds (arcsec). The CPC molecules are ionic detergents that accumulate on the resonant mirror surface forming distinct layers (Mukerjee & Anavil, 1975). The amphiphilic nature of these molecules causes them to associate in such a way that their hydrophobic ends are directed upward away from the sensor surface. The hydrophilic ends interact with the negative silanol groups of the glass prism. When the liposomes are added at step (2), these will associate with CPC's protruding hydrophobic ends. The liposomes will adhere to the surface of the cuvette eventually spreading to covering it. Unbound liposomes were washed out at step (4). Regular checks of the symmetry in the resonance scans indicated the formation of a smooth surface and the absence of precipitated aggregates.

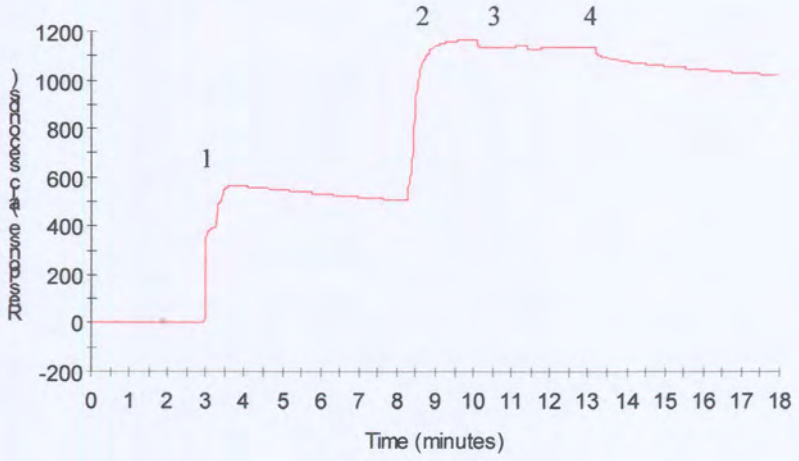


Figure 4.4: Immobilisation of neutral liposomes to a non-derivatised IAsys cuvette. (1) Indicates the three successive additions of CPC; (2) indicates the addition of phosphatidylcholine liposomes; (3) the first PBS wash step and (4) the last PBS wash step.

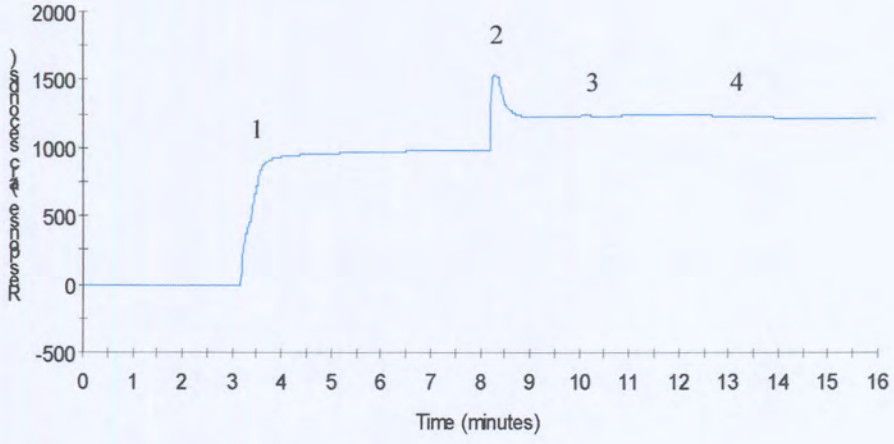


Figure 4.5: Immobilisation of negatively charged liposomes to a non-derivatized IAsys cuvette. (1) Indicates the three successive additions of CPC; (2) indicates the addition of liposomes consisting of β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine and dioleoyl-L-α-phosphatidyl-DL-glycerol; (3) the first PBS wash step and (4) the last PBS wash step

The immobilisation of negatively charged liposomes is shown in Figure 4.5. The addition of CPC to the non-derivatized cuvette gave a response of approximately 900 arcsec. The liposomes prepared from a mixture of phosphatidylcholine and phosphatidylglyserol were then added, which resulted in an increase in the signal to 1500 arcsec. Shortly thereafter, the signal levelled off at 1250 arcsec and remained there even after the PBS wash step. The difference between the CPC and final liposome response is 350 arcsec. The same difference for the immobilisation of neural liposomes was 400 arcsec. Regular checks of the symmetry in the resonance scans indicated the formation of a smooth surface and the absence of precipitated aggregates.

4.3.2 Association of proteins with planar phospholipid membranes

Both BSAP1 and BSAP2 associated with the neutral and negative membranes. Figure 4.6 and Figure 4.7 show typical sensograms obtained for BSAP1 and BSAP2 membrane binding, respectively. For the neutral membrane, the binding response of BSAP1 is roughly 580 arcsec and that of BSAP2 is 270 arcsec. For the negative membrane, the binding response of BSAP1 is 310 arcsec, significantly lower than it is for the neutral membrane. The response of BSAP2 to the negative membrane is practically identical to that of the neutral membrane.

Both proteins dissociated weakly from the membrane as indicated by the shallow response at position (2) in Figure 4.7 and the momentary decrease in signal followed by a resumed associative response in Figure 4.6. If only bulk changes had taken place, the addition of buffer would have resulted in an instantaneous drop in signal (Puu, 2001). In the case of true binding, as is observed for BSAP1 and BSAP2, only a small or no change in signal is noticed.

The sensor's membrane surface could be regenerated successfully with 20mM HCl, as indicated by the return of the signal to the baseline at position (4). It was therefore possible to study several binding experiments on the same surface. The sensor membrane surface was regenerated about 20 times and is expected to remain stable for several months if stored at 4°C (Puu, 2001). It has been reported that protein binding signal values exceeding 1000 arcsec often indicates that proteins stack (Puu, 2001). This does not seem to be the case in the present system. According to the above data it appears that BSAP1 binds neutral membranes more strongly than BSAP2, but has much lower affinity for negative membranes. In contrast, BSAP2 binds both membranes equally strongly.

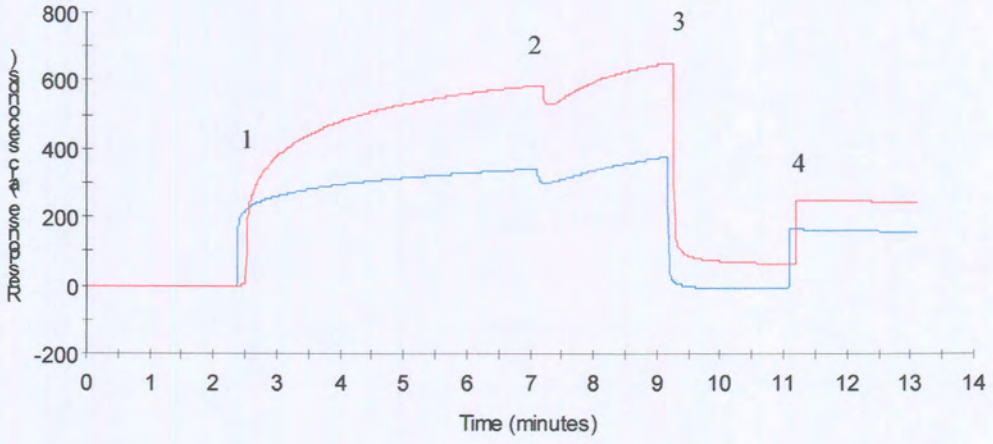


Figure 4.6: Sensogram showing the association and dissociation of BSAP1 to neutral membranes (red) and negative membranes (blue). (1) Addition of 5µg of BSAP1; (2) wash with PBS; (3) regeneration of lipid surface with 20mM HCl and (4) wash with PBS.

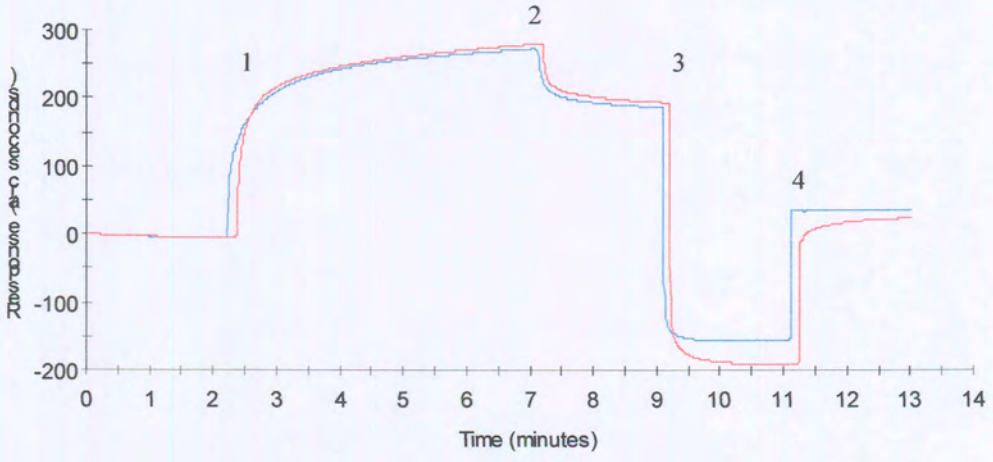


Figure 4.7: Sensogram showing the association and dissociation of BSAP2 to neutral membranes (red) and negative membranes (blue). (1) Addition of 5µg BSAP2; (2) wash with PBS; (3) regeneration of lipid surface with 20mM HCl and (4) wash with PBS.

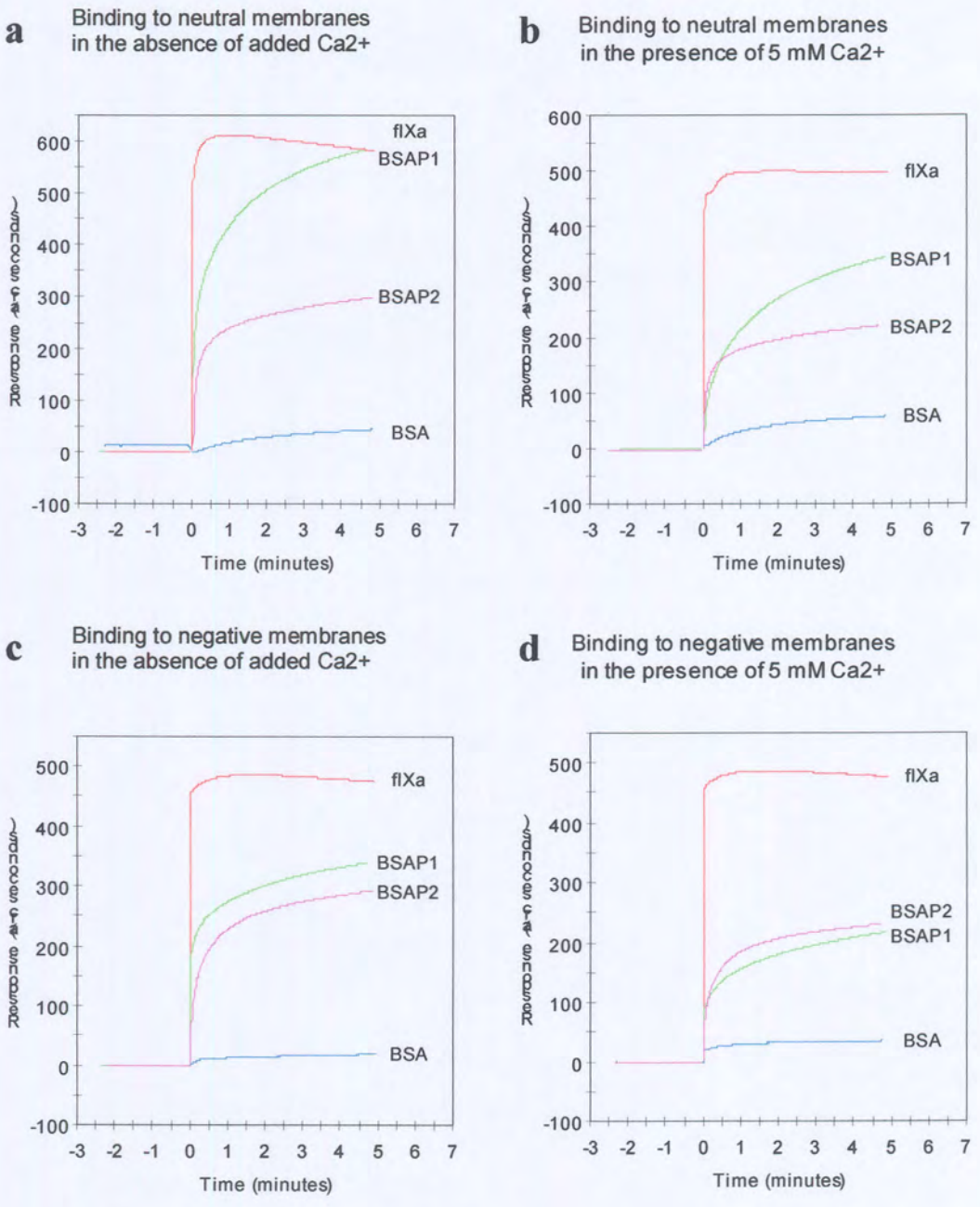


Figure 4.8: The association of BSAP1 (green) and BSAP2 (purple) with neutral and negative membranes in the presence and absence of 5mM Ca^{2+} ions. The positive control was factor IXa (flXa) (red), a membrane-binding coagulation factor. The negative control was bovine serum albumin (BSA) (blue), a blood plasma protein. Approximately 2 min of baseline data was collected at the start of each experiment. The association phase starts at 0 min. In each experiment 5µg of protein was used. (**a**) Binding of proteins to neutral membranes in the absence of Ca^{2+}. (**b**) Binding of proteins to neutral membranes in the presence of 5 mM Ca^{2+}. (**c**) Binding of proteins to negative membranes in the absence of Ca^{2+}. (**d**) Binding of proteins to negative membranes in the presence of 5 mM Ca^{2+}.

The association of BSAP1 and BSAP2 with neutral membranes in the presence and absence of Ca^{2+} is compared in Figure 4.8a & b. In both cases, the BSA negative control had a response of approximately 40 arcsec, an indication that there was no interaction between the protein and the membrane. This is in accordance with published data (Puu, 2001). Factor IXa, a known membrane-binding coagulation factor, interacted quickly with the membranes as indicated by the steep gradient of the binding curve. In the absence and presence of Ca^{2+} its binding response was similar, 600 and 500 arcsec, respectively. Both BSAP1 and BSAP2 interacted with the neutral membranes, but did so to a differing extent as observed in Figure 4.6 and 4.7. In the absence of Ca^{2+}, BSAP1's response reached about 600 arcsec and that of BSAP2 about 280 arcsec. In the presence of Ca^{2+}, the response of both proteins is lower, that for BSAP1 is about 340 arc seconds and that of BSAP2 about 200 arcsec. This is unexpected, since it was assumed that any interaction between the membrane and the proteins would be dependent on Ca^{2+}.

The binding of BSAP1 and BSAP2 to negative membranes in the presence and absence of Ca^{2+} is compared in Figure 4.8c & d. As with neutral membranes, BSA showed no significant interaction. Factor IXa interacted strongly with the negative membrane in both the absence and presence of Ca^{2+}. The magnitude of the interaction is similar to that of the neutral membranes, around 500 arcsec in both cases. The fact that fIXa can bind with the membranes in the absence of Ca^{2+} seems to indicate the presence of this ion in the purchased protein preparation. Only if Ca^{2+} is bound by fIXa can it bind membranes, since these ions are necessary for the formation of the Gla domain that facilitates such binding (see Figure 1.8). The binding response of BSAP1 in the absence of Ca^{2+} was roughly 310 arcsec and that of BSAP2 was 270 arcsec. As observed with the neutral membranes, the response for both proteins was lower in the presence of Ca^{2+}. The response of BSAP1 was 200 arcsec and that of BSAP2 was 210 arcsec.

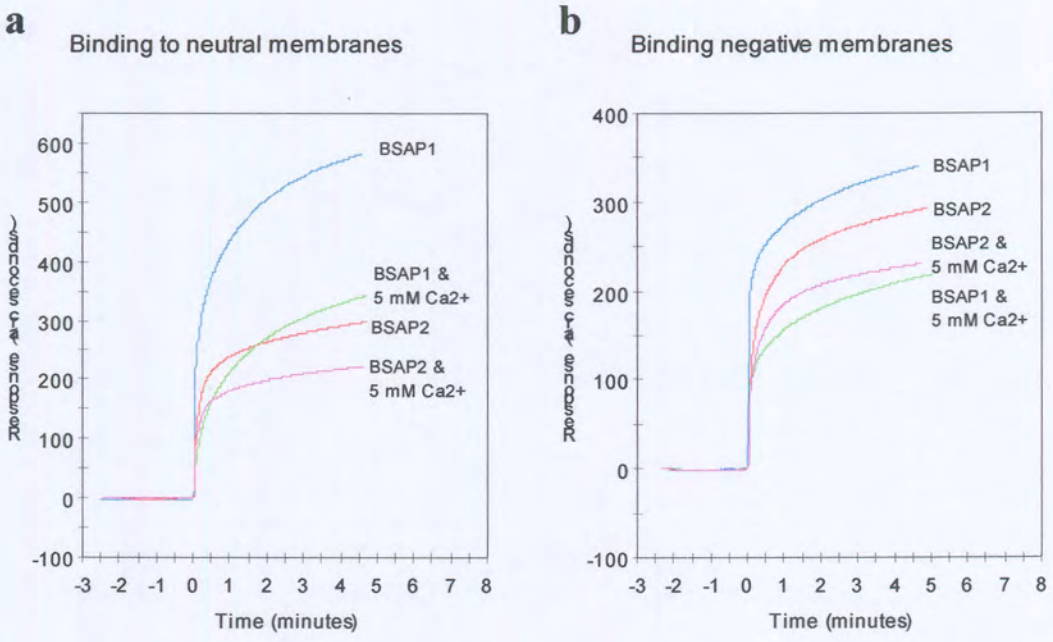


Figure 4.9: The effect of Ca^{2+} on the association of BSAP1 and BSAP2 with (**a**) neutral membranes and (**b**) negative membranes. The association was determined in the absence (blue, BSAP1; red, BSAP2) and presence (green, BSAP1; purple, BSAP2) of 5mM $CaCl_2$. Approximately 2 min of baseline data was collected at the start of each experiment. The association phase starts at 0 min. In each experiment 5µg of protein was used.

The influence of Ca^{2+} on the membrane binding of BSAP1 and BSAP2 is further compared in Figure 4.9. In Figure 4.9a there is a clear difference in BSAP1 binding when Ca^{2+} is added. In the absence of Ca^{2+} the response was about 600 arcsec, whereas in the presence of the ions the response was reduced to around 320 arcsec, a difference of 280 arcsec. The response of BSAP2 was 280 arcsec in the presence of Ca^{2+} and 200 arcsec in its absence. The difference in BSAP2's response was much smaller being only 80 arcsec. The magnitudes of the same differences on the negative membrane were 130 arcsec for BSAP1 and 60 arcsec for BSAP2. It would thus appear that BSAP1 is more severely affected by Ca^{2+} than BSAP2.

4.4 Discussion

The immobilisation of phospholipids to the IAsys cuvette was successful. The failure of the signal recorded during immobilisation to return to its baseline value or even to decrease significantly is a clear indication that liposome deposition did indeed take place (Figure 4.4 & 4.5). Furthermore, the binding to both sensor surfaces of Factor IXa, a known membrane binding protein, supports this view.

The membrane surfaces could be effectively regenerated with HCl. It was therefore possible to perform numerous binding experiments on the same surface. Both membrane surfaces appear to have remained stable for close to two months, since there were no qualitative differences in the sensograms obtained during that period. This is not surprising since coalescence generated membranes have been reported to be stable for extended periods of time (Puu, 2001).

As expected, BSA interacted poorly with both membranes and served well as negative control. Factor IXa appears to have interacted strongly and consistently with both neutral and negative membranes, both in the absence and presence of additional Ca^{2+}. Given that fIXa can bind with the membranes in the absence of additional Ca^{2+}, the protein may have contained some or all of the Ca^{2+} ions that are integral to the structure of the Gla domain, which facilitates its membrane binding. This despite the lyophilised state in which it was purchased. Similar observations have been reported for other proteins whose Ca^{2+}-dependent membrane binding ability was investigated using optical biosensors (Isawa *et al.*, 2000). It may therefore be possible that the isolated BSAPs also have such intrinsically bound Ca^{2+} ions. To investigate this possibility, experiments were carried out in the presence of 5mM EDTA (method and results not shown). The addition of EDTA to the BSAPs resulted in a stronger associative response, an observation that is in agreement with those from Figure 4.9: in the absence of Ca^{2+}, the proteins bind membranes more strongly. This implies that some Ca^{2+} may have been bound to the BSAPs before their preincubation with 5mM $CaCl_2$.

In the previous chapter it was speculated that BSAP1 and BSAP2 might have membrane-binding properties. The data collected using the optical biosensor supports such a hypothesis. Both BSAP1 and BSAP2 bind to the neutral and negative membranes. They dissociate weakly from the membranes indicating true binding. It appears that BSAP1 binds neutral and negative membranes more strongly than BSAP2. Its ability to bind negative membranes is however much poorer than its ability to bind neutral membranes. The affinity of the proteins for the membranes was not experimentally determined due to the large amounts of protein that would have to be purified.

Since BSAP1 and BSAP2 bind to membranes, it may be that they contain a hydrophobic binding site similar to that of the membrane-binding coagulation factors. The difference between the binding of BSAP1 to neutral and negative membranes, unlike BSAP2, might thus be due to such a binding site that is sensitive to membrane composition.

It was assumed that any membrane-binding properties observed for BSAP1 and BSAP2 will be dependent on Ca^{2+} ions, since they are directly or indirectly responsible for facilitating membrane binding in other $BaSO_4$-adsorbing proteins. It has been reported that the binding of prolixin-S, an anticoagulant inhibiting tenase complex formation, does so in a Ca^{2+}-dependent manner. Responses were not observed at 0.2 mM or less, but rapidly increased depending on Ca^{2+} concentration over 0.2 mM and nearly reached a plateau at 1mM (Isawa *et al.*, 2000). It was therefore assumed that the addition of 5mM $CaCl_2$, as practised by Isawa *et al.* (2000), to BSAP1 and BSAP2 would allow clear observations regarding the presumed Ca^{2+}-dependence of their membrane binding.

For neutral membranes, in the absence of additional Ca^{2+}, BSAP1s response reached about 600 arcsec and that of BSAP2 about 280 arcsec. In the presence of Ca^{2+}, the response of both proteins was lower. For negative membranes, the binding response of BSAP1 in the absence of Ca^{2+} was roughly 310 arcsec and that of BSAP2 was 270 arcsec. In the presence of Ca^{2+}, the response of both proteins, as with neutral membranes, was also lower. The binding of both proteins to both membranes was therefore poorer if

they were pre-incubated with Ca^{2+}. An observation that is inconsistent with the idea that the membrane binding of BSAP1 and BSAP2 is dependent on Ca^{2+}.

The inhibitory effect of calcium on the binding of BSAP1 and BSAP2 may indicate that this ion in some way affects their putative membrane-binding site. It is possible that both proteins bind Ca^{2+}, altering their conformation. The use of optical biosensors to infer such conformational changes in proteins is at least tentatively possible. An example is the study of pulmonary surfactant protein A (SP-A), which is similar to CP4, one of the $BaSO_4$-adsorbing proteins. Like CP4 it binds Ca^{2+}, requiring this ion for a Ca^{2+}-dependent, highly cooperative conformational change, which enables SP-A to bind liposomes. The latter property was studied using resonant mirror technology (Meyboom *et al.*, 1997).

If both proteins bind Ca^{2+}, which may alter their conformation, the nature of the membrane-binding site would change, lowering the protein's affinity for the membranes. The observed decreased affinity may therefore not entirely contradict the postulated role of calcium in BSAP1 and BSAP2 membrane binding. It does however necessitate redefining the native binding properties of the proteins. The high affinity binding to the membranes observed in the absence of additional Ca^{2+} may be an artefact of the method in which the proteins were processed prior to the experiment. The conditions used to purify the proteins are often denaturing and it is not inconceivable that ions they naturally bind were lost during $BaSO_4$ adsorption or hydrophobic-interaction chromatography, where ion exchange with the high ionic-strength eluents is possible. The conformation of the proteins in the presence of Ca^{2+} could thus represent their native state and their membrane affinity under those conditions their natural affinity for membranes. The data currently available does, however, not allow any such distinction to be made.

The difference in magnitude of the binding of BSAP1 to the neutral membrane in the absence and presence of Ca^{2+} was 280 arcsec. The same difference for BSAP2 was much smaller being only 80 arcsec. The magnitudes of the same differences for the negative membrane were 130 arcsec for BSAP1 and 60 arcsec for BSAP2. It would thus appear

that BSAP1 is more severely affected by, or naturally binds more Ca^{2+} than BSAP2. This may be due to the existence of a few more acidic amino acids in BSAP1 than in BSAP2 (see amino acid composition in Section 2.3.2).

The ability of BSAP1 and BSAP2 to adsorb to $BaSO_4$ suggested that these proteins might bind to membranes. If they are inhibitors of the extrinsic pathway and more precisely tissue factor, this may be of importance to their biological function. The proteins may need the ability to be bind to the membranes containing tissue factor in order to effect anticoagulation.

The association of BSAP1 and BSAP2 with tissue factor could also be investigated using the IAsys optical biosensor. The direct covalent coupling of tissue factor to sensor surfaces, allowing the investigation of inhibitor binding, has been described (O'Brien *et al.*, 1994; Isawa *et al.*, 2000). Alternatively, tissue factor embedded in a membrane could proof useful, in particular when trying to evaluate the influence of phospholipids on inhibition. In that case, it is essential that the orientation of proteins in the liposomes used for immobilisation is maintained in the planar, supported membrane (Jass *et al.*, 2000). The incorporation of tissue factor into supported membranes has been reported (Fan *et al.*, 1998) and should be explored in future research. Closer investigations of the possible effects of the BSAPs on tissue factor were not attempted during the current study, due to the prohibitive cost of the materials needed for such experiments.

Chapter 5

Concluding Discussion

Ticks pose a significant health risk to humans and domestic livestock due to the pathogens and toxins they can transmit. They are controlled primarily by acaricides, to which they however, develop resistance. Alternative means to control them are thus needed. The use of vaccines is a possibility, which requires the identification of potential protein vaccine candidates. The soft tick *Ornithodoros savignyi* possesses numerous potent anti-haemostatics, which are essential for tick feeding, that could be used for such a purpose. The current study focused on the purification and characterisation of two proteins from that tick that may function as anticoagulants.

Two $BaSO_4$-adsorbing proteins have been isolated to homogeneity from the crude salivary gland extract of the tick *Ornithodoros savignyi*. Both proteins selectively adsorb to commercial $BaSO_4$. The proteins co-purify during reversed-phase chromatography, but can be separated by hydrophobic-interaction chromatography. The high salt content of the collected HIC fractions necessitated desalting by reversed-phase chromatography. Since it is known that reversed-phase can lead to protein denaturation, samples to be used in the one-stage clotting assays were prepared by loading the crude salivary extract directly onto the HIC column. The column used and the conditions applied are deemed non-denaturing. Instead of reversed-phase re-chromatography, the collected fractions were desalted by dialysis.

The fact that the proteins separate on the HIC column indicates that they differ in terms of their surface hydrophobicity, since this is the property on which separation on HIC columns is based (McNay & Fernandez, 1999; Regnier, 1987). Contrary to this is the fact that these proteins have identical retention times on the reversed-phase column pointing to identical total hydrophobicities.

The nature of the adsorption of the proteins to $BaSO_4$ was investigated in the hope of optimising the purification procedure. Protein adsorption to low and high grade $BaSO_4$ was evaluated. The low-grade salt was synthesised in the laboratory, its identity being verified by energy dispersive X-ray spectrometry. The crystalline nature of this product as well as that of the commercial salt was investigated by scanning electron microscopy. This showed the laboratory synthesized $BaSO_4$ to be the low-grade and the commercial $BaSO_4$ the high-grade form of the salt. The low-grade salt should adsorb more protein than the high-grade salt due to its larger surface/gram ratio, but no such improvement was noted. Also, the size-exclusion properties of the low-grade $BaSO_4$ resulted in the co-purification of the T1, T2, and NT proteins, together with several unidentified ones. The use of high grade $BaSO_4$ to isolate BSAP1 and BSAP2 therefore seems optimal.

The homogeneity of the protein samples was assessed by means of reducing and non-reducing polyacrylamide gel electrophoresis and electro-spray mass spectrometry. The latter indicated that the mass of BSAP1 was 12.1 kDa and that of BSAP2 was 10.0 kDa. These masses are in reasonable agreement with the more exact values obtained by electro-spray mass spectrometry, which gave values of 9333.54 Da and 9173.50 Da, respectively.

The proteins seem to have a somewhat higher mobility under non-reducing conditions, indicating a more compact structure. This is most probably due to the presence of the disulphide bonds. The only slight increase in mobility however implies a minor role for these bonds in the tertiary structure of the protein. Neither protein contains any subunits, as no peptides with masses lower than that of the native proteins were observed. Molecules with roughly twice the mass of the native proteins seem to be present in electrophoresed samples of BSAP1 and BSAP2. This may indicate the existence of dimers, but most likely is the result of incomplete SDS binding.

The purified proteins differ in composition by 18 amino acid residues. The nature of these differing amino acids is not conserved, some are uncharged and polar, some non-polar and others charged. Noteworthy differences in their composition are the absence of

histidine and isoleucine in BSAP1 and the presence of one of each of these residues in BSAP2. The latter has 4 cysteine residues and the former 5, as determined by performic acid oxidation. Overall, the aspartic acid, asparagine, glutamic acid and glutamine content are high, accounting for roughly 30% of the proteins' composition. Likewise, both proteins have a high serine and glycine content. Methanesulphonic acid hydrolysis identified 2 tryptophan residues per protein.

Since a large portion of the BSAPs amino acid composition may be made up of acidic amino acids, the low isoelectric points as calculated from the amino acid composition and experimentally determined by 2D-electrophoresis are not surprising. The calculated pI of BSAP1 is 3.87 and that of BSAP2 is 4.03.

Amino acid analysis of pyridylethylated protein identified 6, possibly 7, cysteine residues in BSAP1 and 8 residues in BSAP2. The discrepancy in the number of cysteine residues as determined by performic acid oxidation and protein pyridylethylation, is probably due to the greater accuracy of the latter method. This view is supported by the fact that the overall amino acid composition of the native and the alkylated proteins is the same. The likely presence of disulphide bonds was first indicated by non-reducing electrophoresis of the purified proteins. Comparing the molar ratios of the (4-pyridylethyl) cysteine residues in the reduced and non-reduced protein samples showed that all cysteine residues, with the possible exception of one residue in BSAP1, are involved in the formation of disulphide bonds.

Each protein contains only one methionine residue. The chromatographic profiles of the cyanogen bromide cleaved proteins indicated that the methionine residues occupy different positions in the polypeptide chains of the two proteins. No amino acid sequences for either of the two BSAP1 cyanogen bromide cleavage fragments were obtained. The BSAP2 cleavage products did however yield a partial sequence (Asp/Ser-Gly-Gly-Xxx-Xxx-Ile-Leu-Gly) for what is presumably the unblocked C-terminal fragment.

The Gla-specific DBS staining of electrophoresed protein samples indicated that BSAP1 and BSAP2 do not contain γ-carboxyglutamic acid residues. This was confirmed by ESMS analysis of native and decarboxylated protein samples. Also, amino acid analysis of BSAP1 and BSAP2 did not indicate the presence of hydroxyproline or hydroxylysine. This data and the confirmed absence of Gla residues, implies that BSAP1 and BSAP2 bind to $BaSO_4$ through means other than those of the blood plasma Gla proteins or CP4. They may possibly associate with $BaSO_4$ in a manner similar to CRP, using a few amino acid residues to coordinate the binding of a Ba^{2+} ion.

The one-stage clotting assays showed that the two proteins do not inhibit the intrinsic coagulation cascade, but inhibition of the extrinsic pathway was detected. Factor VII does not seem to be the target of this inhibition. Tissue factor, being the only other unique component of the extrinsic pathway, could perhaps be the target. As discussed in Section 1.6.4, the contact phase may play only a minor role in blood coagulation, suggesting that the extrinsic pathway is of primary importance for haemostasis. Targeting the TF-fVIIa complex with an inhibitor could therefore have substantial benefits for a haematophagous organism. Some TF-fVIIa complex anti-haemostatic agents have been identified (Gordon & Allen, 1991; Stanssens *et al.*, 1996). Significant inhibition of the extrinsic pathway by crude *O. savignyi* salivary extract has been reported (Gaspar *et al.* 1995). The proteins isolated during this study may be those responsible for that inhibition.

Since both proteins seem to have similar anticoagulant activity, it may be that they are iso-forms. The protein's mass difference is relatively small (160.04 Da), they co-purify during reversed-phase chromatography, pointing to similar hydrophobic properties, and both are acidic in nature with near identical pIs. Their most striking similarity is the fact that both adsorb to $BaSO_4$, a property shared with few other proteins. Despite this evidence, the different number of disulphide bonds, as well as the different cyanogen bromide cleavage patterns, indicate the opposite.

The ability of BSAP1 and BSAP2 to adsorb to $BaSO_4$ suggested that these proteins might bind to membranes. The data collected using the optical biosensor proved that the

proteins have that ability. Both bind to neutral and negative membranes, dissociating only weakly. BSAP1 binds neutral and negative membranes more strongly than BSAP2. Its ability to bind negative membranes is however much poorer than its ability to bind neutral membranes. In contrast, BSAP2 binds both membranes equally strongly. The binding of both proteins to both membranes was poorer upon pre-incubation with Ca^{2+}.

Taken together, the above observations indicate the existence of a membrane-binding site, possibly similar to that of fIXa, which may be hydrophobic in nature. The inhibitory effect of calcium on the binding indicates that this ion affects this putative membrane-binding site either directly or indirectly, lowering the affinity of the proteins for the membranes. The interaction is therefore dependent on Ca^{2+}, although not in the manner initially envisaged. The conformation of the proteins in the presence of Ca^{2+} could represent their native state and their membrane affinity under those conditions their natural affinity for membranes. This view is supported by the fact that the proteins adsorb to $BaSO_4$, making it likely that they do associate with bications.

The difference in magnitude of binding of BSAP1 and BSAP2 to the neutral and negative membranes in the absence and presence of Ca^{2+} indicates that BSAP1 may bind more Ca^{2+} than BSAP2. This may be due to the existence of more acidic amino acids in BSAP1 than in BSAP2.

Future aims should include cloning and sequencing the genes encoding the $BaSO_4$-adsorbing proteins, their structural modelling if appropriate templates exist, and the expression of recombinant proteins. Cloning and sequencing of the BSAP's genes may aid in the elucidation of their function by screening the gene sequences against those in gene databases. Expressing recombinant proteins should greatly reduce the time and effort associated with what are often routine experiments, because there will be no need to repeatedly purify proteins from the source material. Also, the inhibition of tissue factor inferred from the clotting assays and supported by the biosensor investigations should be examined, even if only to disprove BSAP1 and BSAP2's presumed function.

One approach to improving treatment of thrombotic diseases involves the design and testing of inhibitors that block specific stages of the coagulation cascades. Inhibitors of the TF-fVII complex would facilitate greater understanding of this complex and aid in the development of potential therapeutic agents. Also, studying salivary gland proteins can contribute to the development of experimental vaccines. This study has laid the groundwork for a more detailed investigation of the proteins BSAP1 and BSAP2, which in future may find such uses.

Summary

Dissertation Title: Putative extrinsic blood coagulation pathway inhibitors from the tick *Ornithodoros savignyi*
Student: Matthias Ehebauer
Promoters: Prof. A.W.H. Neitz and Dr. A.R.M. Gaspar
Department: Biochemistry
Submitted for the degree: Magister Scientiae

Commercial (high-grade) $BaSO_4$ selectively adsorbs two proteins from crude *O. savignyi* salivary gland extracts. They co-purify during reversed-phase HPLC, but can be separated by hydrophobic-interaction chromatography. Both proteins have been characterized in terms of their molecular mass, amino acid composition and one partial internal amino acid sequence was determined. Their molecular masses were established through electro-spray mass spectrometry as 9333 Da and 9173 Da, respectively. The 9.3 kDa protein was designated BSAP1 and the 9.1 kDa protein BSAP2. Their amino acid compositions shows significant differences, in particular the presence of 6-7 and 8 cysteine residues in BSAP1 and BSAP2, respectively. It is therefore unlikely that these proteins are isoforms. All of the cysteine residues are involved in the formation of disulphide bonds, the only possible exception being one residue in BSAP1. Both proteins appear to be N-terminally blocked. An internal amino acid sequence Asp/Ser-Gly-Gly-Xxx-Xxx-Ile-Leu-Gly was obtained by sequencing a fragment of the cyanogen bromide cleaved BSAP2.

It was suspected that these proteins might exhibit anticoagulant activity. The prothrombin time (PT) and activated partial thromboplastin time (aPPT) in the presence of the presumptive inhibitors were therefore evaluated. The aPPT was not significantly prolonged. The PT however did indicate a slight delay in the clotting time. This delay is not due to inhibition of factor VII, one of only two unique coagulation factors in the extrinsic pathway. The other factor is thromboplastin, also known as tissue factor.

The nature of the protein adsorption to $BaSO_4$ was examined. From literature it is known that γ-carboxyglutamic acid-containing proteins, as well as some hydroxyproline and hydroxylysine-rich glycoproteins adsorb selectively to $BaSO_4$. The BSAPs were analysed for the presence of these modified amino acids, but all tests proved negative. The absence of Gla residues was determined using a Gla-specific stain on a polyacrylamide gel and was confirmed by performing mass spectrometry on native and decarboxylated protein samples. The absence of hydroxyproline and hydroxylysine was demonstrated by amino acid analysis.

Both BSAP1 and BSAP2 bind to neutral and negative membranes. BSAP1 binds neutral and negative membranes more strongly than BSAP2. Its affinity for negative membranes is however much lower than its affinity for neutral membranes. In contrast, BSAP2 binds both membranes equally strongly. The binding of the proteins to the membranes was significantly lowered upon pre-incubation with Ca^{2+}.

Opsomming

Titel van verhandeling: Putative extrinsic blood coagulation pathway inhibitors from the tick *Ornithodoros savignyi*
Student: Matthias Ehebauer
Promotors: Prof. A.W.H. Neitz en Dr. A.R.M. Gaspar
Departement: Biochemie
Graad waarvoor ingedien: Magister Scientiae

Kommersiële $BaSO_4$ adsorbeer selektief twee proteïene vanuit kru *O. savignyi* speekselklier-ekstrak. Hierdie proteïene suiwer gelyktydig gedurende omgekeerde-fase chromatografie, maar kan deur hidrofobiese-interaksie chromatografie geskei word. Beide proteïene is in terme van hulle molekulêre massa en aminosuursamestelling gekarakteriseer, en een gedeeltelike aminosuurvolgorde is bepaal. Die molekulêre massas is deur elektro-sproei massaspektrometrie vasgestel as 9333 Da en 9173 Da. Die 9.3 kDa proteïen word aangedui as BSAP1 en die 9.1 kDa proteïen as BSAP2. Hierdie proteïene verskil in aminosuursamestelling. Daar is 6-7 sisteïenresidue in BSAP1 en 8 in BSAP2. Dit is daarom hoogs onwaarskynlik dat hulle isoforme is. Al die sisteïenresidue is in disulfiedbindings betrokke, behalwe een residu van BSAP1. Albei proteïene se N-terminaal is geblokkeer. Die interne aminosuurvolgorde Asp/Ser-Gly-Gly-Xxx-Xxx-Ile-Leu-Gly is vasgestel deur 'n fragment van sianogeen-bromied gekloofde BSAP2 te bepaal.

Dit was vermoed dat die proteïene bloedstolling sal inhibeer. Die protrombientyd (PT) en die geaktiveerde parsiële tromboplastientyd (aPPT) is in die teenwordigheid van die vermeende inhibeerders getoets. Daar is bevind dat die aPPT nie verleng het nie, maar die PT het wel 'n verlenging van die stollingstyd gelewer. Hierdie verlenging is nie te wyte aan inhibisie van faktor VII nie. Faktor VII is een van die twee stollingsfaktore in die ekstrinsieke pad. Die ander faktor is tromboplastien, meer algemeen bekend as weefselfaktor.

Die adsorpsie van die proteïene aan $BaSO_4$ is meer deeglik ondersoek. Dit is uit die literatuur bekend dat proteïene wat γ-karboksieglutamiensuur bevat, asook glikoproteiene wat ryk is aan hidroksieprolien en hidroksielisien, selektief aan $BaSO_4$ adsorbeer. Die BSAPs is vir die teenwoordigheid van hierdie gemodifiseerde aminosure getoets. Die resultate van al die analises was negatief. Die afwesigheid van Gla-residue is deur 'n Gla-spesifieke kleurstof, wat op poliakrielamiedjelle toegepas word, vasgestel, en daarna deur massaspektrometrie van gewone en gedekarboksileerde proteïen bevestig. Die afwesigheid van hidroksieprolien en hidroksielisien is deur aminosuuranalise vasgestel.

Beide BSAP1 en BSAP2 bind aan neutrale en negatiewe membrane. BSAP1 bind sterker as BSAP2 aan neutrale en negatiewe membrane, maar die affiniteit vir negatiewe membrane is veel kleiner as die affiniteit vir neutrale membrane. BSAP2 bind, in teendeel, albei membrane ewe sterk. Die binding van die proteïene aan membrane was sterk verlaag in die teenwoordigheid van kalsium.

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