Chapter 2: Characterization of savignygrin, a platelet aggregation inhibitor from the soft tick, *Ornithodoros savignyi* *

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2.1.1 Introduction: Integrins

Integrins are a family of adhesion receptors that propitiate cell-cell and cell-matrix interactions. Numerous physiological processes like hemostasis (receptors involved in platelet interaction with damaged vessel walls and platelet aggregation), fertilization (adhesion of sperm and oocyte), neuron-neuron interaction and inflammation (accumulation of leukocytes at affected sites) are mediated by integrins (Clemetson, 1998). The functional receptor is expressed as a transmembrane hetero-dimer assembled from various combinations of the 17α and 8β sub-units identified to date, forming the more than 20 current family members (Longhurst and Jennings, 1998; Plow *et al.* 2000). Integrins have different ligand specificities ($\alpha_2\beta_1$-collagen, $\alpha_5\beta_1$-fibronectin, $\alpha_6\beta_1$-laminin, $\alpha_v\beta_3$-vitronectin and $\alpha_{IIb}\beta_3$-fibrinogen), the latter which can also recognize fibronectin, vitronectin and von Willebrands factor. Most ligands recognized by integrins possess the recognition motif Arg-Gly-Asp (RGD) (Ruoslahti and Pierschbacher, 1987). Some ligands may also contain other sequences recognized by integrins, like the dodecapeptide sequence HHLGGAKQAGDV contained in the γ-chain of fibrinogen that binds to $\alpha_{IIb}\beta_3$ (Andrieux *et al.* 1989).

2.1.2 $\alpha_{IIb}\beta_3$ as model integrin

$\alpha_{IIb}\beta_3$ (GPIIbIIIa) is the most thoroughly characterized integrin and is the major integrin of platelets and the only adhesion receptor capable of mediating platelet aggregation by binding of fibrinogen or von Willebrands factor (Calvete, 1994; Calvete, 1995; Shattil *et al.* 1997; Plow *et al.* 2000; Plow *et al.* 2001). On resting platelets $\alpha_{IIb}\beta_3$ exists in an inactive conformation that binds irreversibly to the γ-chain C-terminal dodecapeptide (HHLGGAKQAGDV) of immobilized fibrinogen (Calvete, 1995). The unactivated form also has a ligand-binding site accessible to small molecules that contain RGD, KGD, RYD or OrnGD motifs, that are presented on mobile recognition loops protruding 14-17Å from the protein core (Calvete, 1995). The ligand-binding site can

also be reached by RGD-peptides that extend 11-32Å from the surface of polyacronitrile beads (Beer, Springer and Kohler, 1992). These results suggest that the binding-pocket in unactivated $\alpha_{IIb}\beta_3$ may resemble a narrow cavity buried 10-20Å below the protein surface (Calvete, 1995). Upon platelet activation by various agonists, $\alpha_{IIb}\beta_3$ undergoes a conformational change that allows binding of macromolecules containing the RGD-motif (Fig. 2.1). The ligand-binding site is probably discontinous and is formed by both α_{IIb} and β_3 subunits. It is speculated that there could be a divalent cation (Ca^{2+} ?) binding site inside the ligand-binding site that interacts with ligands containing aspartic acid residues, such as contained in the RGD motif (Calvete, 1994; Plow *et al.* 2000). A ternary complex between RGD ligand and the receptor-bound divalent cation may be formed, until stabilization of the RGD-integrin interaction, whereupon the divalent cation is displaced (D'Souza *et al.* 1994). Investigations into the secondary structure of the RGD motif using GRGDSP peptides predict that it occurs as a highly ordered and unusual structure that exhibits a double β-bend nature, which could provide the necessary conformational restraints to bind to cell-surface adhesion receptors (Reed *et al.* 1988).

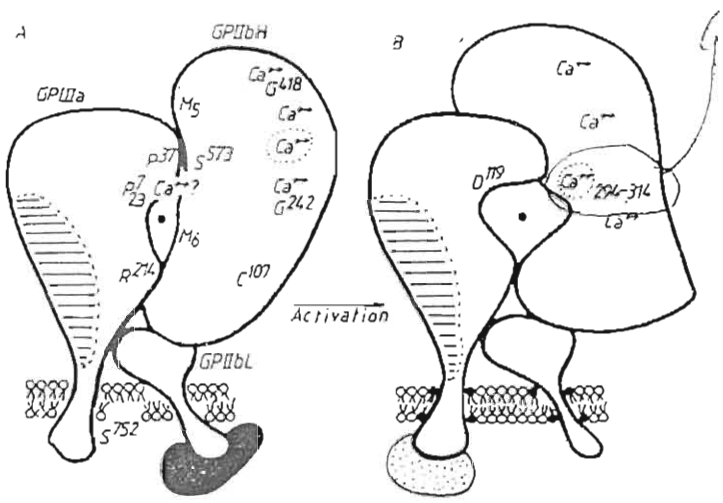


Fig. 2.1: Activation dependent conformational change of $\alpha_{IIb}\beta_3$. The buried ligand-binding site of $\alpha_{IIb}\beta_3$ in resting platelets (A), are exposed after platelet activation (B). The ligand-binding site as well as the putative cation-binding site is indicated by the dot. The circled Ca^{++} site around 294-314 indicates the binding site for the γ-chain fibrinogen C-terminal peptide. Adapted from Calvete (1994, 1995).

2.1.3 Snake venom disintegrins as platelet aggregation inhibitors

Inhibitors specific for integrins are useful for the study of integrin function and for the development of pharmaceutical compounds. The disintegrins identified in snake venom are the most extensively characterized $\alpha_{IIb}\beta_3$ antagonists. Their function is the maintenance of hemorrhage through inhibition of platelet aggregation, mediated by interaction with $\alpha_{IIb}\beta_3$, thereby preventing fibrinogen binding (Huang, 1998). Disintegrins are a family of low molecular weight (5400-9000 Da) proteins that contain the RGD motif, except for barbourin (Scarborough *et al.* 1991). Most disintegrins inhibit platelet aggregation with IC_{50} values 3000-30 000 times lower (nM range) than the tetra peptide RGDS (μM range) (Gould *et al.* 1990). The three dimensional structures (Fig. 2.2) of the *Viperidae* (viper) snake venom disintegrins echistatin, kistrin, flavoridin, dendroaspin (mambin) from *Elapidae* (mamba) and the leech derived decorsin show no classical secondary structure (Adler *et al.* 1991; Saudek, Atkinson and Pelton, 1991; Senn and Klauss, 1993; Krezel *et al.* 1994; Sutcliffe *et al.* 1994), but rather a dense core, consisting of β-turns kept intact by disulphide bonds, with a protruding hairpin-loop region that presents the RGD motif (Huang, 1998).

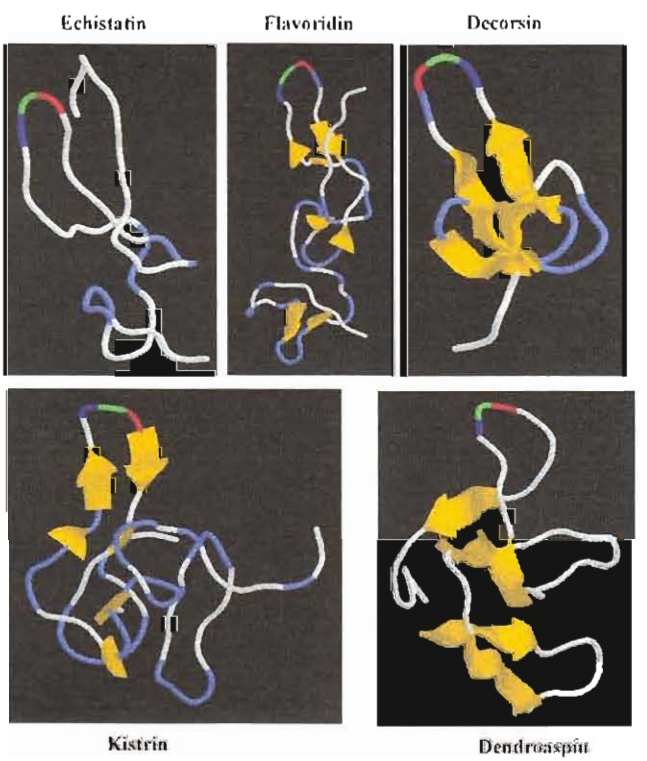


Fig. 2.2: Three-dimensional structures of $\alpha_{IIb}\beta_3$ antagonists. Antagonists from the *Viperidea* (echistatin, kistrin, flavoridin) and *Elapidae* (dendroaspin) snake venoms and leech-derived decorsin. The RGD sequence is colored in blue (Arg), green (Gly) and red (Asp), respectively.

The spatial configuration of the RGD motif at the end of the loop and the flanking sequences have been found to be important contributors to specificity for different integrins (Gould *et al.* 1990; Scarborough *et al.* 1993). The structural differences between the different snake venom inhibitors emphasize that their inhibitory activities are solely determined by the appropriate presentation of the RGD sequence (McDowell *et al.* 1992).

Disintegrins are classified as a protein family based on alignment of sequences by conserved cysteines (Huang, 1998). Only a certain set of proteins from the *Viperidiae* snake family fall in this rigid classification scheme (Fig. 2.3). *Elapidae* snake venom-derived (mambin), leech-derived (decorsin and ornatin) and tick-derived (variabilin and disagregin) inhibitors do not fall into this aligned family (Huang, 1998; Wang *et al.* 1996; Karczewski, Endris and Connolly, 1994). It can be expected that more diversification of this classification scheme will follow as more non-snake derived inhibitors are identified.

Fig. 2.3: Amino acid sequences of different $\alpha_{IIb}\beta_3$ antagonists. Disintegrins from the *Viperidae* family are similar, while dendroaspin from *Elapidae* snake venom have a totally different sequence and conformation. Leech-derived decorsin and ornatin shows similarity, while both tick-derived inhibitors (disagregin and variabilin) are unique. RGD sequences are boxed in grey and cysteines are shown in bold. Adapted from Huang, 1998.

2.1.4 Tick-derived $\alpha_{IIb}\beta_3$ antagonists

No homology exists between the tick-derived $\alpha_{IIb}\beta_3$ antagonists. Variabilin, from the hard tick, *Dermacentor variables*, contains a RGD motif, not flanked by cysteine residues, while disagregin from the soft tick *Ornithodoros moubata*, contains no RGD sequence (Wang *et al.* 1996). Disagregin does however, inhibit the binding of the RGD containing peptide echistatin, which suggests that its binding site could be in close proximity to the RGD-binding site on $\alpha_{IIb}\beta_3$. In addition, the γ-fibrinogen sequence HHLGGAKQAGDV competes with its binding to soluble $\alpha_{IIb}\beta_3$, indicating an inhibition mechanism distinct from the disintegrins as well as possible multiple binding sites (Karczewski, Endris and Connolly, 1994; Karczewski and Connolly, 1997). It has not been determined whether the RED sequence of disagregin, might play a role in its mechanism of action. Closely related homologues could thus provide important information on its mechanism of action if conserved regions necessary for function can be identified. With this in mind, a homologue (designated savignygrin) from the tick, *O. savignyi* was purified.

2.2 Materials and methods

2.2.1 Chemicals used

All materials were of analytical grade and deionized water was used in all experiments. Tris(hydroxymethyl) aminomethane (Tris), NaCl, ascorbic acid, ethylene diamine tetra-acetic acid (EDTA), $HgCl_2$, sodium azide, methanol, acetic acid, glycine, ammonium persulphate and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were obtained from Merck, Darmstadt, Germany. $CaCl_2$, $MgCl_2$, ethylene-bis(oxyethylene nitrilo) tetra-acetic acid (EGTA) was from BDH Chemicals Ltd. Poole, England. ADP-di(monocyclohexylammonium) salt, diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), fibrinogen, p-nitrophenol and tris(hydroxymethyl) aminomethane (tricine) were obtained from Sigma Chemical Co., USA). Human $\alpha_{IIb}\beta_3$, α-thrombin, activated fXa and plasmin were obtained from Enzyme Research Laboratories, Inc. (South Bend, OR, USA). Microtitre plates were obtained from Bibby Sterilin, UK. Low molecular weight marker proteins were purchased from Pharmacia, USA and acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS) from BDH Laboratory Supplies LTD., England. Coomassie Brilliant Blue was obtained from Bio-Rad Laboratories, USA and the Protein Assay Kit from Pierce, USA. Primers used were synthesized by GIBCO BRL

Custom Primers (Life Technologies) or Integrated DNA Technologies and resuspended at 100 pmole/μl in 20% acetonitrile and stored at –20 °C. Final concentrations were confirmed by spectrophotometric measurement at 260 nm. Isopropyl β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (X-gal) and RNAse were obtained from Roche Diagnostics. Yeast extract and tryptone were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England).

2.2.2 Collection of ticks and preparation of salivary gland extract (SGE)

Ticks were collected from the North Western Province area of South Africa by sifting of sand. The salivary glands were obtained by dissection (Mans *et al.* 1998a). Female ticks were embedded in molten wax with their dorsal parts visible. The integument was then removed by lateral dissection of the cuticle with a #11 scalpel under a 0.9% NaCl solution using a binocular stereomicroscope (10x magnification). Salivary glands were removed with fine forceps, frozen in liquid nitrogen and stored at -70 °C. A stock solution of salivary gland extract was prepared by resuspension of 12 salivary glands in 500 μl buffer solution (20 mM Tris-HCl, 0.15M NaCl pH 7.6 at 25 °C). Resuspended glands were sonified with a Branson sonifier cell disrupter B-30 (Branson Sonic Power Co.) for 3x6 pulses at 30% duty cycle and an output control of 3, while keeping the solution on ice. Extracts were centrifuged in a microfuge (10 000xg for 5 min) and the supernatant used for further studies.

2.2.3 Measuring platelet aggregation using an aggregometer

Platelet aggregation studies using an aggregometer were performed as described (Mans *et al.* 1998a) to monitor inhibition of platelet aggregation induced by ADP (10 μM), collagen (40 μg, Diagnostica Stago), thrombin receptor activating peptide (TRAP, 50 μM, Sigma) and epinephrine (10 μM, Dioagnostica Stago). Aggregation of platelets was monitored using the photometric method of Born and Cross (1963). Fresh blood was collected in Vacutainer tubes containing a citrated buffer solution (0.109 M), from human donors using no substances known to inhibit platelet aggregation, such as alcohol, garlic and aspirin for at least two weeks prior to donation. Platelet rich plasma (PRP) was prepared by centrifugation (170xg for 10 min) and plasma was collected in plastic tubes. Platelet poor plasma was prepared by further centrifugation of the remaining blood from the platelet rich plasma at 2700xg for 15 min. Platelet rich plasma

was diluted to a final count of 300 x 10^9 l^{-1} using a Coulter counter. Savignygrins (20 μl at indicated concentrations) or 20 μl saline solution as control were pre-incubated with 400 μl of the diluted platelet rich plasma for 5 minutes to monitor for spontaneous aggregation. Platelet poor plasma (PPP) was set at 100% transmission and PRP at 0% transmission. Aggregation was then induced by adding 20 μl of different agonists to the PRP.

2.2.4 Disaggregation of aggregated platelets by savignygrin

It has been shown that apyrase can disaggregate aggregated platelets (Mans *et al.* 2000). To investigate whether the savignygrins could have a disaggregation effect on aggregated platelets, platelets were aggregated with 10 μM ADP for 4 minutes before their addition (41 μM final concentration). Platelets were allowed to disaggregate for a further 4 minutes. As controls, platelets were also incubated with savignygrin without addition of ADP or before addition of ADP. Platelets were also analyzed with or without the addition of ADP in the absence of savignygrin. All preparations were incubated for 8 minutes. Further analyses were conducted using electronmicroscopy (Mans *et al.* 2000).

2.2.5 Electron microscopical analysis of platelet disaggregation by savignygrin

Platelets were collected by centrifugation in 0.1% glutaraldehyde (0.075M phosphate buffer pH 7.5) before fixing in 1% glutaraldehyde (phosphate buffer) for 2 hours. Platelets were secondary fixed in OsO_4 before sequential dehydration in ethanol. For scanning electron microscopy (SEM), platelets were critical point dried and gold sputter coated before viewing in a JEOL 840 SEM (WD 16 mm, 5 keV, 4000X magnification). For transmission electron microscopy (TEM), platelets were infiltrated with Quetol resin and 100 nm thick sections were prepared using a microtome. Sections were contrasted with lead citrate and uranyl acetate before viewing with a Philips 301 TEM.

2.2.6 Platelet aggregation assay using a micro-plate method

For monitoring of anti-platelet activity during purification and also for IC_{50} determination of ADP-induced platelet aggregation, a microplate assay was used (Bednar *et al.* 1995). PRP was prepared as described and 100 μl was used per

microplate well. Savignygrins or saline (10 μl of indicated concentrations) were added and left to incubate for 3 minutes before addition of 10 μl ADP (10 μM final concentration). Aggregation was allowed to proceed for 10 minutes, before absorbance was measured at 620 nm in a microplate reader (Titertek). Platelet suspensions were stirred with a Titertek® microplate shaker (Flow Laboratories) set at a speed of 5 during the expreriment.

2.2.7 Inhibition of binding between monoclonal antibody P2 (α-CD41) and platelets by savignygrin

Monoclonal antibody P2 has been shown to interact specifically with α_{IIb} (CD41) of the intact $\alpha_{IIb}\beta_3$ complex (McGregor *et al.* 1983). Inhibition of P2 binding to platelets was assayed using flow cytometry (Liu *et al.* 1994). Platelet poor plasma (PPP) was prepared and used to dilute platelet rich plasma (PRP) to a count of $300x10^9$ platelets/liter. PRP (20 μl), savignygrin (20 μl, at various concentrations) and 150 μl Tyrode buffer (0.4 mM NaH_2PO_4, 0.4 mM NaCl, 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH7.4, 3.5 mg/ml BSA) were incubated for 30 minutes at room temperature in the presence or absence of ADP (20 μM final concentration). P2-FITC conjugated mAb (10 μl, Immunotech, Coulter) was added and incubated for 30 minutes at room temperature. The unfixed platelet solution (120000 platelets/10μl) was diluted to 500 μl with Tyrode buffer before analysis with an Epics2 flow cytometer (Coulter Electronics, Inc.) by measurement of 10000 events. Fluorescent measurement was gated to count intact, non-aggregated platelets (3-5 μm diameter). Three separate experiments were used to determine the mean fluorescence.

2.2.8 Inhibition of $\alpha_{IIb}\beta_3$ binding to immobilized fibrinogen by savignygrin

The binding of $\alpha_{IIb}\beta_3$ to fibrinogen was performed as described (Seymour *et al.* 1990). ELISA plates were coated overnight with 100 μl fibrinogen (10μg/ml in 20 mM Tris-HCl, pH 8.5) before blocking with 100 μl block buffer (20 mM Tris-HCl, pH 7.4, 0.12 M NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, 0.5% BSA) for 1 hour. Wells were washed 3X with 100 μl block buffer before 50 μl of savignygrin (at various concentrations) were added to each well followed immediately by 50 μl of purified $\alpha_{IIb}\beta_3$ (40 μg/ml, Enzyme Research Laboratories). ELISA plates were incubated for 2 hours before washing wells

with block buffer (3X) and incubating wells for 1 hour with 100 µl P2-FITC (50X dilution from stock), followed by washing 3X with block buffer. Block buffer (100 µl) was added to wells and fluorescence measured using a Fluoroscan Ascent FL (Thermo Labsystems) with excitation at 485 nm and emission at 438 nm. Background was subtracted using wells with no $\alpha_{IIb}\beta_3$.

2.2.9 Inhibition of osteosarcoma cell adhesion by savignygrin

The specificity of the savignygrins for integrins was investigated using the osteosarcoma cell line MG-63, known to express the integrin $\alpha_v\beta_3$ which binds to vitronectin and fibrinogen (Stuiver, Ruggerri and Smith, 1996). Adhesion studies were perfomed as described (Wang *et al.* 1996). Cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum (FCS). Cells at 60% confluence were trypsinized (0.25% trypsin, 3 mm EDTA in phosphate buffered saline) at 37°C, 4 min, before neutralization with DMEM/FCS. Cells were pelleted at 120xg (5 min), resuspended in DMEM/FCS and were incubated at 37°C (30 min) before centrifugation (120xg, 5 min). Cells were then suspended in serum free DMEM at $2x10^5$ cells/ml and incubated with various concentrations of savignygrin for 30 min at 37°C. This mixture (100 µl) was added to ELISA plate wells pre-coated overnight with 1µg/ml vitronectin (Sigma Co., USA) or fibrinogen (Sigma Co., USA) and incubated for 2 hours at 37 °C. Wells were washed 3X with wash buffer (20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, 0.12M NaCl) before staining with 0.1% bromophenol blue in 1% acetic acid for 1 hour. Wells were then washed 3X with 1% acetic acid before air drying. Bromophenol blue was eluted with 100 µl buffer (50 mM Tris-HCl, pH 9.2) before absorbance was measured at 595 nm.

2.2.10 Inhibition of platelet adhesion to fibrinogen

Adhesion of platelets to fibrinogen was performed as described previously (Keller *et al.* 1992). The same coating procedures for fibrinogen (section 2.2.9) were followed, but 100 µl PRP ($2-3X10^8$ platelets/ml) was added to wells. Savignygrin (10 µl at various concentrations) was added and platelets were incubated for 2 hours at room temperature. Wells were washed with block buffer before lysis with reaction buffer (20

mM citrate, pH 5.5, 0.1% Triton X-100, 10 mM p-nitrophenol). The reaction was allowed to proceed at room temperature and absorbance was measured at 340 nm in a microplate reader (Titertek) every hour, until an adequate reading was obtained.

2.2.11 Temperature stability of savignygrin

Savignygrin (14 μg protein in 300 μl 20 mM Tris, 0.15M NaCl, pH 7.4) was incubated at 94 °C for different time periods and immediately placed on ice for 10 minutes before centrifugation (14000xg for 15 minutes at room temperature), followed by measurement of platelet aggregation inhibitory activity. Activity was measured relative to untreated inhibitor.

2.2.12 High performance liquid chromatography (HPLC)

Beckman instrumentation was used for all HPLC work, which consists of two pumps (module 110B), an analog interface for control of solvent delivery from the pumps (module 406), a UV detector (module 166) and a Beckman 340 organiser with injector. This hardware was controlled using Beckman System Gold software (1987).

2.2.13 Size exclusion HPLC (SEHPLC)

Salivary gland extract (40 salivary glands, ~1600 μg protein) were prepared, filtered through a 0.22 μm filter (Millex GV4, Millipore Corporation, USA) and applied to a size exclusion column ($G2000SW_{XL}$, 7.8mm x 30 cm, TosoHaas, USA). Isocratic conditions (20 mM TrisHCl, 0.15 M NaCl pH 7.6, flow speed: 1 ml/min, A_{280}) were used during SEHPLC. Prior to application onto the size exclusion column, buffers were filtered through 0.22 μm membranes (Millipore Corporation, USA). The column was calibrated using molecular mass markers and a physiological saline buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.6) for elution. Molecular mass markers used were aldolase (158 kDa, 0.1 mg/ml), bovine serum albumin (BSA) (67 kDa, 0.1 mg/ml), ovalbumin (43 kDa, 0.1 mg/ml), chymotrypsin (25 kDa, 0.05 mg/ml) and ribonuclease (13.5 kDa, 0.1 mg/ml). Markers were obtained from Pharmacia. SGE was applied to the column and 1 ml fractions were collected and assayed for platelet aggregation inhibitory activity.

2.2.14 Anion exchange HPLC (AEHPLC)

Fractions from SEHPLC were dialyzed against water (Slide-A-Lyzer cassettes, 10kDa molecular weight cutoff, Pierce) for two hours prior to application onto an anion exchange column (DEAE-5PW, 7.5 mm x 7.5 cm, TosoHaas). Proteins were fractionated using a gradient system (flowspeed: 1ml/min, A_{280}) with buffer A (20 mM Tris-HCl, pH 7.6) and buffer B (20 mM Tris-HCl, 1 M NaCl, pH 7.6) as set out in Table 3.3. Proteins with known iso-electric points were used as standard markers. These were, apo-transferrin (pI~5.9, 1mg/ml), β-lactoglobulin (pI~5.14, 1 mg/ml) and chicken egg ovalbumin (pI~4.6, 1 mg/ml) obtained from Sigma, USA. Fractions (1 ml) were collected and assayed for enzyme activity.

Table 2.1: Flow conditions used during AEHPLC.

Time (min.)	Flow (ml/min.)	% Buffer B	Duration (min.)
0	1	0	5
5	1	60	21
26	1	100	10
36	1	0	0.5
36.5	1	0	10
46.5	END		

2.2.15 Reversed phase HPLC (RPHPLC)

Fractions from AEHPLC were desalted and fractionated using RPHPLC (Jupiter 5μ C5 300 Å, 4.6 mm x 25 cm, Phenomenex, USA, flowspeed: 1ml/min, A_{280} or A_{230}). Elution was achieved with a gradient of buffer A (0.1% TFA, 0.1% acetonitrile) and buffer B (0.1% TFA, 60% acetonitrile) from 0-100% over 60 minutes. Peaks collected were dried in a vacuum concentrator (Bachoffer), rechromatographed on AEHPLC and desalted with RPHPLC.

Table 2.2: Flow conditions used during RPHPLC.

Time (min.)	Flow (ml/min.)	% Buffer B	Duration (min.)
0	1	0	5
5	1	100	60
60	1	100	10
70	1	0	0.5
70.5	1	0	10
80.5	END		

2.2.16 Quantitation of proteins

Total protein of SGE was determined with the method of Bradford (1976), using the Pierce Coomassie Protein Assay kit (Pierce, USA). BSA was used as standard reference protein. Standard protein or sample (150 μl) were pipetted into a microtitre plate well and 150 μl of the Coomassie Protein Assay Reagent added. The microplate was shaken for 10 minutes before reading the absorbance at 620 nm with a SLT 340 ATC scanner (SLT Labinstruments). Determinations were performed in triplicate. Purified proteins were quantitated using amino acid analysis.

2.2.17 Amino acid analysis

Amino acid analysis was performed according to the PICO-TAG method (Bidlingmeyer, Steven and Tarvin, 1984). Protein was dried in pyrolized hydrolysis tubes (PICO.TAG system) and placed in a larger glass vessel that contained 200 μl of 6M HCl, 7% thioglycolic acid solution and flushed with N_2 and evacuated prior to incubation at 110 °C for 24 hours. After hydrolysis the amino acids were extracted three times from the membranes using 50 μl of 30% methanol, 0.1 M HCl while drying the extracts each time under vacuum. Derivatization of the amino acids was performed by adding 10 μl of a 2:2:1 mixture of methanol: water: triethylamine which was then dried again under vacuum before adding 20 μl of a mixture of 7:1:1:1 methanol: water: triethylamine: phenylisothiocyanate (PITC). This was left at room temperature for 20 minutes before the unreacted PITC was removed under continuous vacuum for 1 hour. Standard PITC-amino acid mixture (Waters) or samples were solubilized in 200 μl 10% Na_2HPO_4, 5% acetonitrile buffer and the pH was adjusted to 7.4 with 10% H_3PO_4. Samples were filtered through a 0.45 μm membrane before 20 μl of standard or sample was injected onto the column. A reversed phased column (PICO-TAG, 3.9 mm x 150 mm,Waters) was used for the separation of the amino acids with 0.14 M sodium acetate, pH 5.7 for Buffer A and 60% acetonitrile for Buffer B. Gradient elution was used as set out in Table 3.4 and elution was monitored at 254 nm. The results were analyzed using Beckman System Gold software.

Table 2.3: HPLC gradient conditions for amino acid analysis.

Time (min.)	Flow (ml/min.)	% Buffer B	Duration (min.)
0	1	10	10
10	1	51	0.5
10.5	1	100	2.2
12.7	1.5	100	0.5
13.2	1.5	10	7.5
20.7	END		

2.2.18 Total cysteine determination by performic acid oxidation

Performic acid was prepared by adding H_2O_2 (10 µl) to formic acid (90 µl) and incubating the mixture at room temperature for 1 hour with regular vortexing. Protein samples (pyrolized tubes) and performic acid were then incubated on ice (30 min). Performic acid (20 µl) was added to each sample, mixed thoroughly and incubated for 16 hours at 4 °C. Octanol (20 µl) was added, followed by addition of HBr (7.5 µl), a vortex step and incubation on ice (30 min). The samples were evaporated under nitrogen gas and normal amino acid analysis procedures described above were then followed.

2.2.19 Alkylation of cysteines with 4-vinyl pyridine (4-VP)

Inhibitor (250 pmole) was denatured with 8M guanidinium chloride (GdCl, 250 µl) for 2 hours in the presence or absence of 4% DTT, before addition of 2 µl 4-VP (Sigma, St. Louis, MO, USA). Mixtures were incubated for 2 hours and alkylated protein was desalted using RPHPLC and vacuum dried before amino acid analysis, activity measurements and N-terminal sequence determination.

2.2.20 Tryptophan determination by methanesulfonic acid (MSA) hydrolysis

MSA (20 µl, 4M containing 0.2% w/v tryptamine HCl) was added to protein in pyrolyzed hydrolysis tubes. The tubes were added to a larger vessel containing water (100 µl) and evacuated. Hydrolysis was performed at 110 °C (24 hours). After hydrolysis the vial was cooled to room temperature and the MSA neutralized with 4 M KOH (22 µl), before drying under vacuum. Amino acids were extracted, derivatized and identified as described in section 2.2.13.

2.2.21 N-terminal sequence analysis

N-terminal sequence analysis of ~1 nmole alkylated protein was performed in a gas phase amino acid sequencer (Hewick *et al.* 1981) modified as described (Brandt *et al.* 1984), at the sequence facility of Prof. W.F. Brandt (Department of Biochemistry, University of Cape Town).

2.2.22 Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified proteins were analyzed using a tricine SDS-PAGE system (Schägger and von Jagow, 1987) that is suitable for resolution of proteins in the range of 1-100 kDa. A 16.5%T, 3%C separating gel (1 M Tris-HCl, 0.1% SDS, pH 8.45) and a 4%T, 3%C stacking gel (0.75 M Tris-HCl, 0.075% SDS, pH 8.45) were prepared from acrylamide (48% acrylamide/ 1.5% N', N'-methylene bisacrylamide) and electrophoresis buffer (3M Tris-HCl, pH 8.45, 0.3%SDS) stock solutions. These solutions were degassed for 30 minutes and polymerized by addition of 50 μl of 10% ammonium persulphate and 5 μl of TEMED.

Protein was diluted 1:4 in reducing buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS (w/v), 0.1% glycerol (v/v), 0.05% β-mercaptoethanol (v/v) and 0.025% bromophenol blue (w/v) and boiled at 94 °C for 4 minutes. Low molecular mass and peptide mass markers were used for mass determination. The low molecular mass markers were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14.5 kDa). Peptide mass markers are fragments from a cyanogen bromide digestion of myoglobin to give a ladder of masses as indicated in results. Electrophoresis was carried out with anodal buffer (0.2M Tris-HCl, pH 8.9) and cathodal buffer (0.1M Tris-HCl, 0.1M Tricine, 0.1% SDS, pH~8.2 with no adjusting of pH) using a Biometra electrophoresis system (Biometra, GmbH) with an initial voltage of 60 V for 45 minutes and then a voltage of 100 V until the bromophenol blue front reached the bottom of the gel.

2.2.23 Staining of SDS-PAGE gels

Savignygrins eluted from the gel under non-reducing conditions and normal methanol (40%), acetic acid (10%) fixation. Gels were thus fixed by washing steps in several

fixatives (Phoeling and Neuhoff, 1981). Solution A (30% methanol, 3.4% sulphosalicylic acid di-hydrate, 5.7% trichloroacteic acid): 15 min, solution B (50% methanol, 0.12% acetic acid): 30 min, solution C (5% methanol): 2 X 15 min, solution D (10% glutardialdehyde): 30 min, solution C: 3 X 30 min. Proteins were visualized by staining in 0.1% Coomassie Brilliant Blue (40% methanol, 10% acetic acid) and were destained in an excess of destaining solution (50% methanol, 10% acetic acid).

2.2.24 Tricine SDS-PAGE in the presence of urea

Unfolding of inhibitors in the presence of urea was investigated by including 8 M urea in the tricine separating gel, or by using a gradient of 0-8 M urea perpendicular to the direction of electrophoresis (Goldenberg and Creighton, 1984).

2.2.25 Electrospray mass spectrometry (ESMS)

Molecular masses of the native or alkylated inhibitors were determined using electrospray mass spectrometry (ESMS) by Dr. M.J. van der Merwe (Department of Biochemistry, University of Stellenbosch). A VG Micromass Quattro triple quadropole mass spectrometer equipped with an electrospray ionisation source was used (Micromass, UK). Capillary voltage (3.5 kV) was applied in the positive mode with source temperature 80 °C, cone voltage 70 V and skimmer lens at 5 V. Proteins (~300 pmoles) were dissolved in 50% acetonitrile/0.1% formic acid (100 µl) and injected (10 µl) into a stream of 50% acetonitrile at flow speed of 10 µl/min, supplied from a LKB/Pharmacia 2249 gradient pump. Data was aquired in the continuum mode, scanning from m/z 500-2000 at 100 amu/second. Horse heart myoglobin was used to calibrate the instrument before analysis.

2.2.26 Peptide mapping using matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS)

Peptide mapping was performed at the mass spectrometry unit (Department of Biochemistry University of Cape Town). Protein (10 µg) was digested with trypsin (1:10) for 4 hours at 37 °C in digestion buffer (100 mM sodium carbonate pH 8.2, 2mM $CaCl_2$). Digested protein (5 pmole) was crystallized in an equal volume of α-cyano-4-hydroxycinnamic acid matrix before analysis with a DE-PRO MALDI-TOF (Perseptive

Biosystems, USA) mass spectrometer with a laser intensity of 1200, accelerating voltage (20000), grid voltage (91-92%) and guide wire voltage (0.1%) using a linear flight path. The spectrometer was calibrated using a calibration mixture of external standards and forty to sixty scans were averaged.

2.2.27 Origins of (+) and (-) forms: alleles or gene duplicates

The possibility that the (+)/(-) forms might be alleles was considered. To test this hypothesis, SGE from twenty individuals were heat treated (60°C, 10 minutes), before separation with RPHPLC. Savignygrin fractions were analyzed using non-reducing tricine SDS-PAGE.

2.2.28 Total RNA purification

Whole nymphae (0.2g) were ground up in the presence of liquid nitrogen, while salivary glands (120 glands) from female *O. savignyi* ticks were dissected as described previously and immediately transferred to 750 µl of TRI-REAGENT® (Molecular Research Center, INC). Total RNA was isolated according to the manufacturer's instructions. The RNA was dissolved in FORMAzol® (Molecular Research Center, INC) and stored at –20 °C. The purity of the RNA was determined by measurement of the 260/280 nm ratio which gave values of 1.24-1.29 for salivary gland preparations and 1.53-1.65 for mouse lung preparations. RNA quality was assessed by electrophoresis on a 1% agarose gel prepared in 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, 18 % formaldehyde (Sigma) and DEPC treated ddH_2O. RNA samples were prepared in 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, 20% formaldehyde, deionized formamide (Gibco, BRL) in DEPC treated, double distilled deionized water. Electrophoresis was conducted at 70 V for 30 minutes. RNA was quantified by spectrophotometric measurement at 260 nm, which gave a final concentration of 80 µg/ 20 µl for salivary glands, 106 µg/ 20µl for nymph and 93 µg/ 20 µl for mouse lung.

2.2.29 cDNA synthesis from total RNA

Single-stranded cDNA, was prepared using Superscript™ II (Life Technologies) and the anchor-dT primer (Table 2.4) (Joubert *et al.* 1998). Total RNA (0.5 µl – 2 µg) in 7.5µl DEPC treated H_2O was denatured at 70 °C for 3 minutes and snap cooled on ice. Five pmoles anchor-dT primer (1 µl), 3 µl DTT (0.1 M), 200 units of Superscript™ II

(1μl), 4μl 5X first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3) was added and incubated at 42 °C for 60 minutes before inactivation at 70 °C (2min). Single-stranded cDNA was stored at –20 °C until used. Double stranded full-length cDNA was prepared with the Marathon cDNA amplification kit (ClonTech) according to the manufacturers instructions. Total RNA (4 μg) was used for synthesis and first strand cDNA was synthesized with the anchor primer (10 pmole) described above.

2.2.30 Rapid amplification of 3' cDNA ends (3'RACE)

To obtain the coding gene and 3' untranslated region (3' UTR) a degenerate primer (GrinA) was designed from the first seven amino-acids (YQPECLE) obtained by Edman degradation, using the program OLIGO Version 4.0 (National Biosciences, Hamel, USA) (Rychlick and Rhoades, 1989). Single-stranded cDNA (0.5 μl of the previously described single strand cDNA stock), GrinA (100 pmole- 1 μl), anchor primer (5 pmole – 1 μl), 5μl 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 4 μl $MgCl_2$ (2 mM final concentration) and 4 μl dNTP's (200 μM final concentration) were adjusted to 40 μl with H_2O. cDNA was denatured at 94 °C (3 minutes) and then cooled to 80 °C after which 0.5 μl TaKaRa Taq™ (5U/μl, TAKARA Biotechnology) diluted in 10 μl H_2O was added. Amplification consisted of 34 cycles of DNA denaturation (94 °C, 30s), annealing (55 °C, 30s) and extension (72 °C, 2 minutes), followed by a final extension (72 °C, 7 minutes). All amplification procedures were conducted in a Gene Amp® PCR System 9700 (Perkin Elmer Applied Biosystems).

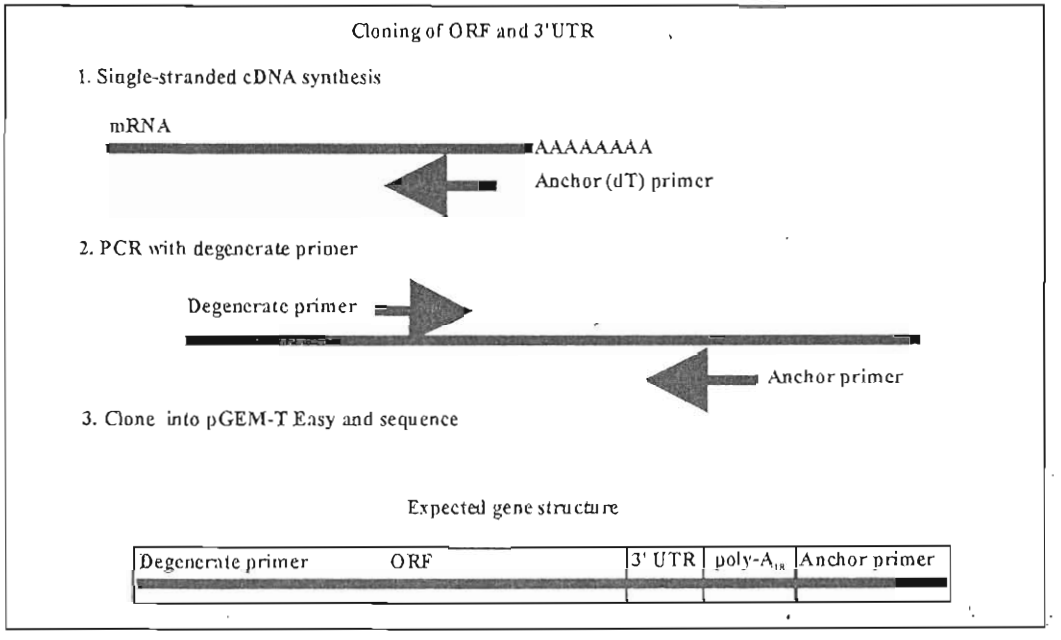


Fig. 2.4: Cloning strategy to obtain ORF and 3'UTR. (1) Single standed cDNA was synthesized with a anchor (dT) primer. (2) A degenerate primer and a anchor primer was used to amplify the product of interest. (3) The product was cloned into the pGEM T-Easy vector and sequenced. Indicated is the expected gene structure obtained with this method.

2.2.31 Rapid amplification of 5' cDNA ends (5'RACE)

To obtain the 5' UTR and signal peptide sequence a gene specific primer (GrinA1RB: ACT ATT TCC GTT CTG AAG) complementary to the coding sequence of the last six amino acids of savignygrin (KKACGNA) was designed. Double-stranded full-length cDNA (1 μl of a 50X dilution of cDNA stock solution), GrinA1RB (5 pmoles), and AP2 (5 pmoles, Clontech Marathon kit) were used for amplification. All other amplification conditions were identical to that of the 3' RACE.

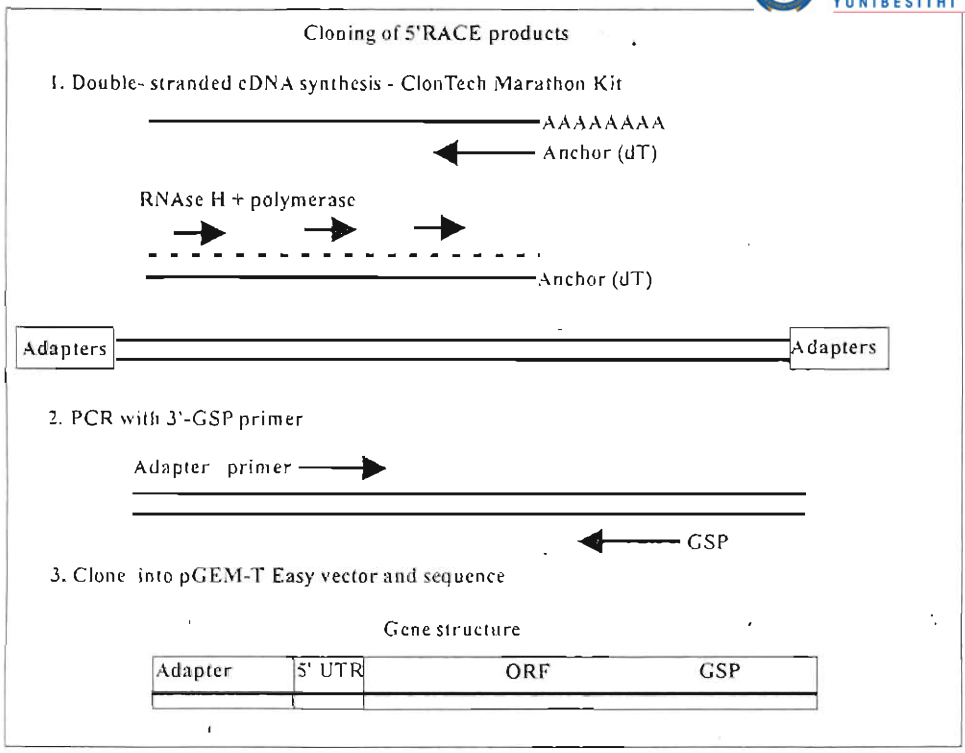


Fig. 2.5: Cloning strategy to obtain 5'UTR and ORF. (1) Double-standed cDNA was synthesized by first-strand cDNA synthesis from total RNA with an anchor (dT) primer, followed by second-strand cDNA synthesis with RNAse H and polymerase. Adapter primers were then ligated to the ends of the double-stranded cDNA. (2) A primer specific for the 3'end of the ORF (GSP) and a adapter primer were used to amplify the product of interest. (3) The product was cloned into the pGEM T-Easy vector and sequenced. Indicated is the expected gene structure obtained with this method.

2.2.32 Cloning of low-molecular mass savignygrin inhibitor

During the characterization of savignygrin two isoforms were observed. ESMS indicated that one had a slightly higher molecular mass (6966 Da, designated the high mass inhibitor) than the other (6808 Da, designated the low mass inhibitor). 3' RACE with the degenerate primer produced the high mass inhibitor only. A single colony obtained from the 5' RACE gave a sequence that differered at a single nucleotide (R52G). To determine whether this might be the low mass form a primer (GrinAB: ACT ATT TCC GTT CTG AAG) was designed with the single nucleotide differerence at the 3' end and 3' RACE was performed as described. A product was obtained that showed both R52G and N60G differences with the high mass inhibitor sequence. To confirm this difference a GSP (LMMprime: TGT ACC TCT CCT TGA AC) was designed in the 3'UTR where differences were observed with the high mass inhibitor sequence. 5'

RACE was performed with the GSP to obtain the full-lenght low mass inhibitor sequence.

2.2.33 Analysis of PCR products

Amplified products were analyzed by electrophoresis (68 Volts ~ 1.5 hours) using a 2% analytical grade agarose (Promega, Wisconsin, USA) gel with TAE (0.04 M Tris-acetate, 0.001 M EDTA) as running buffer in a minigel apparatus (Biometra, GmbH).

2.2.34 Quantitation of DNA

DNA was quantitated by visual comparison to known concentrations of 100 bp ladder markers (Promega, Wisconsin, USA).

2.2.35 Elution of RACE products from agarose gels

Amplified products were extracted from agarose gels with the silica elution method (Boyle and Lew, 1995). RACE products were excised from gels and gel pieces were dissolved in 1 ml NaI (0.9g/ml) at 55 °C. DNA was adsorbed on 5μl silica (100 mg/ml) (Sigma, USA) by incubation for 30 minutes on ice. Silica was washed 2X with wash buffer (10 mM Tris-HCl, 50 mM NaCl, 2.5 mM EDTA, pH 7.5 and 50% v/v ethanol) by centrifugation (30s in a microfuge). DNA was eluted with 10 μl 1mM Tris-HCl, pH 8.0 at 60 °C (5 minutes). Yield was determined by comparison with known concentrations of 100 bp ladder electrophoresis markers (Promega, Wisconsin, USA).

2.2.36 Purification of PCR products

For certain applications PCR products were purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). PCR product (100 μl) was added to 500 μl binding buffer (3M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), pH 6.6) before addition to High Pure filter tubes. Tubes were centrifuged for 1 minute at 12000xg and the flow through discarded. Filters were washed with 500μl wash buffer (2 mM Tris-HCl, 20 mM NaCl, 20% ethanol (v/v), pH 7.5) by centrifugation (12000xg, 1 minute), followed by another wash with 200μl wash buffer. PCR product was eluted with 100μl elution buffer (1 mM Tris-HCl, pH 8.5).

2.2.37 A/T cloning of RACE products into pGEM T-Easy

DNA product (3:1 insert to vector ratio) was ligated into the pGEM®-T Easy Vector (1µl ~ 50 ng) using A/T cloning at 4 °C (16-48 hours) with 1µl T4 ligase and 5µl 2X buffer of the pGEM®-T Easy Vector System I (Promega, Wisconsin, USA). T4 ligase was inactivated at 70 °C prior to transformation of competent cells. The products of at least three PCR reactions were cloned and at least 3 different clones of each PCR product cloned were sequenced from both up-and down stream ends.

2.2.38 Preparation of competent cells

Competent SURE (Stratagene, La Jolla, CA, USA) *E. coli* cells were prepared using the calcium/manganese-based method (Hanahan *et al.* 1991). Bacteria were grown overnight on M9 minimal medium agar (0.05 M Na_2HPO_4-$2H_2O$, 0.02 M KH_2PO_4, 8 mM NaCl, 0.02 M NH_4Cl, 2 mM $MgSO_4$, 0.01M D-glucose, 0.1 mM $CaCl_2$, 1 mM thiamine hydrochloride, 1.5% agar (w/v), pH 7.4). Colonies were plated onto LB plates (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5, 1.5% (w/v) agar with 12.5 µg/ml tetracycline) and grown overnight at 30 °C. Several colonies were suspended in SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0) by vortexing and inoculated into 50 ml SOB medium. Cells were grown at 30 °C with shaking (250xg) until OD_{600}~0.3 after which the cells were pelleted by centrifugation (10000xg, 15 min at 4 °C). The supernatant was discarded and the pellet suspended in 16.6 ml CCMB 80 medium (80 mM $CaCl_2$-2H2O, 20 mM $MnCl_2$-4H2O, 10 mM $MgCl_2$-$6H_2O$, 10 mM K-acetate, 10% glycerol, pH 6.4) and incubated on ice for 20 min. Cells were pelleted by centrifugation (10000xg, 10 min at 4 °C) and the supernatant discarded. The pellet was suspended in 4.1 ml CCMB 80 medium, aliquoted and stored at –70 °C.

2.2.39 Transformation of SURE *E. coli* cells

Transformation of competent cells was performed according to the heat-shock method (Sambrook *et al.* 1989). Transformation of cells with ligated plasmid was performed by preincubation of 100 µl cells (OD_{600} ~ 0.3) with plasmid at 4 °C (30 minutes), heat shock at 42 °C (60s) and incubation at 4 °C (3 minutes) before growing cells in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 50 mM D-

glucose, pH 7.1) for 1 hour before plating 100 µl of transformed cells onto 1.5% agar that contained 50 µg/ml ampicillin, 40 µl/plate X-gal (20 mg/ml DMSO) (Roche) and 4 µl/plate IPTG (400 mM) (Roche Diagnostics). Postitive colonies were selected using blue-white selection with conventional minipreps of plasmids and restriction enzyme digestion (Eco RI) of plasmid to identify inserts with the correct size.

2.2.40 Screening for recombinant clones

Blue/white selection is based on the fact that the β-galactamase gene is disrupted during insertion of foreign DNA into the multiple cloning site of the pGEM T-Easy vector. Vector that ligated without foreign DNA produces β-galactamase under induction of IPTG and hydrolyses X-galactose which then forms a blue colored product that yields blue colonies. When the gene for β-galactamase is disrupted, no hydrolysis of X-galactose occurs, which yields white colonies. White colonies were inoculated into 3 ml LB broth (10 µg/ml ampicillin) and grown for 16 hours at 30 °C.

2.2.41 Miniprep of plasmids

Cells from positive colonies (1 ml culture) were collected by centrifugation (12000xg, 1 min) in a microfuge and resuspended in 100 µl miniprep solution 1 (25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, pH 8.0). Miniprep solution 2 (150 µl, 0.2M NaOH, 1% SDS) were added and the solution were mixed by gentle inversion before incubation on ice (5 min) to lyse the cells. Ice cold miniprep solution 3 (250 µl, 3M potassium acetate, pH 4.2) was added and the solution was mixed by gentle inversion and incubated on ice (5 min) before centrifugation (12000xg, 5 min) in a microfuge to precipitate chromosomal DNA. Absolute ethanol (1 ml) was added to the supernatant and incubated at room temperature (10 min) before centrifugation (12000xg, 15 min) in a microfuge to precipitate plasmids. The supernatant was discarded and the pelleted plasmid was washed with 70% ethanol (1 ml) and again centrifuged. The supernatant was discarded and the pellet dried using the Bachoffer vacuum concentrator (5 min). Pellets were dissolved in 30 µl water (20 µg RNAse) and 8 µl were digested with 1 µl EcoRI (10U) in digestion buffer (90mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5) for 2 hours before agarose gel analysis. Of those recombinant plasmids that showed

the correct insert size, the remaining 2 ml of culture was used for high pure plasmid purification for sequencing.

2.2.42 High pure plasmid isolation

Plasmids were purified for sequencing with the high pure plasmid purification kit (Roche Diagnostics). Cells (2 ml culture) were collected with centrifugation (12000xg, 1 min) in a microfuge and suspended in 250 µl suspension buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0 at 25 °C, 0.1mg/ml RNAse). Cells were lyzed with 250 µl lysis buffer (0.2M NaOH, 1%SDS) by gentle inversion and incubation at room temperature (5 min) before addition of 350 µl binding buffer (4 M guanidine hydrochloride, 0.5M potassium acetate, pH 4.2). The solution was mixed by gentle inversion and incubated on ice (5 min) to precipitate chromosomal DNA. Chromosomal DNA was removed by centrifugation (12000xg, 10 min) and the supernatant added to a High Pure filter tube, which has a glass fibre filter for specific binding of plasmid DNA in the presence of chaotropic salts. The solution was centrifuged (12000xg, 1 min) and the flow through discarded before addition of 700 µl wash buffer II (2 mM Tris-HCl, 20 mM NaCl, 80% ethanol, pH 7.5) and recentrifugation (12000xg, 1 min). After another dry centrifugation (12000g, 1 min) to remove all ethanol, the High Pure filter tube was transferred to a new collection tube and plasmid was eluted with 100 µl of elution buffer (1 mM Tris-HCl, pH 8.5).

2.2.43 Sequencing of recombinant pGEM T-Easy plasmids

Sequencing was performed using the Big Dye Sequencing Kit (Perkin Elmer, Foster City) on an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, California, USA) according to the manufacturers instructions using 2 µl ready reaction mixture, 3 µl 5X buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 1 µl primer (3.2 pmoles of upstream T7 or downstream SP6 promoter primers respectively), plasmid (150-250 ng) and water to a final volume of 20 µl. Cycle sequencing was performed using 25 cycles of denaturation (96 °C, 10s), annealing (50 °C, 5s) and extension (60 °C, 4 min). Big Dye products were precipitated with 64% ethanol (final volume 100 µl) for 30 minutes and pelleted by centrifugation (12000xg, 25 minutes). Pellets were washed with 70% ethanol and dried on a vacuum concentrator (Bachoffer) for 7

minutes. The pellet was suspended in 3 μl loading dye (5:1 ratio of deionized formamide: 25 mM EDTA, pH 8.0 and blue dextran, 30 mg/ml). Samples were denatured at 95 °C (2 min), snapcooled on ice and analyzed on a 36 cm gel according to the ABI Prism 377 Genetic Analyser User's Manual. At least 3 different clones were sequenced for each fragment and each clone was sequenced with both primers. Sequences obtained were analyzed using the Staden package. DNA and deduced protein sequences were analyzed using BLAST (Altschul *et al.* 1990) and alignments were performed with ClustalX (Jeanmougin *et al.* 1998). Protein molecular mass, amino acid composition and theoretical tryptic digests of the deduced sequence were determined with the PAWS program (http://www.proteometrics.com/software/paws.htm).

2.2.44 Primers used during study

Primers used for the cloning and sequencing of the various proteins described in this study are indicated in Table 2.4.

Table 2.4: Primers used in study

Name	Primer sequence	Chapter
General		
Anchor-dT	GCT ATC ATT ACC ACA ACA CTC T_{18}VN	2, 3, 7
Anchor	GCT ATC ATT ACC ACA ACA CTC	2,3, 7
AP2	AAC TCA CTA TAG GGC TCG AGC GGC	2, 3, 7
T7Seq	TAA TAC GAC TCA CTA TAG GG	2, 3, 7
SP6Seq	ATT TAG GTG ACA CTA TAG	2, 3, 7
Savignygrin		
GrinA	TAY CAR CCN GAR TGY YTI G	2
GrinA1RB	GGA TCC TCA CGC ATT TCC GCA TGC CTT CTT	2
GrinAB	ACT ATT TCC GCT CTG AAG	2
GrinA1F	CAT ATG TAC CAA CCC GAG TGC CTG GAG	2
LMMprime	TGT ACC TCT CCT TGA AC	2
Savignin		
ThrombA	YTN AAY GTI MGI TGY AAY AA	3
Thromb C1	CTC GAG TTC CAT TGA AAC GCC ACA	3
TSGP's		
TOC	TTY CCI ACI GAY GCN TA	7
TOE	TTY CCI ACI GAR GCN TA	7
C1T2	CTA GCA GTC CTT GTC TT	7
NTC1	GTT CCA ACA TCC ACA TG	7
TOKS1	GCN AAY GAY GTI TGG AAY GT	7
C1T1	CTA CGG AAC TCT GCA GCC TT	7
20kD	GGI CCI GAY GGI TGY GT	7
20kDC1	GTG TAG GGG ATG GGG CCA	7

2.3 Results

2.3.1 Purification of the savignygrins

Inhibition of ADP-induced platelet aggregation was used as a measure of activity during purification. Size exclusion chromatography was used as a first step in the purification procedure (Fig. 2.6a). Inhibition of platelet aggregation was observed across the whole protein spectrum and could be ascribed to the presence of apyrase activity in the high molecular mass region (500-20 kDa) as described previously (Mans *et al.* 1998b). Heat inactivation of apyrase shifted the inhibition pattern to the low molecular mass region (<20kDa). Fractionation of the size exclusion fractions on the anion exchange column indicated inhibitory activity over a broad range (10-13 min) at an approximate iso-electric point range of 5-6 (Fig. 2.6b). Reversed phase chromatography separated the inhibitory activity into two distinct peaks designated A and B (Fig. 2.6c). ESMS analysis showed that both peaks contained a high (6966 Da) and low (6808 Da) molecular mass species (results not shown). Separation of these species were attempted with rechromatography using AEHPLC (Fig 2.6d). Both peaks A and B separated into two peaks, indicating two species that differ in charge with approximate iso-electric points of 5.9 and 5.5, respectively. These peaks were designated A+, A-, B+ and B- on the basis of charge (as observed on AEHPLC) and hydrophobicity (as observed on RPHPLC). These different species were then desalted using RPHPLC (Fig. 2.6e and 2.6f). The yields obtained for three different separations were 45±15 μg, 37±9μg, 27±9μg and 27±9μg for the different forms (A+, A-, B+ and B-), respectively. This corresponds to approximately 1-3% of the total soluble salivary gland protein for each isoform.

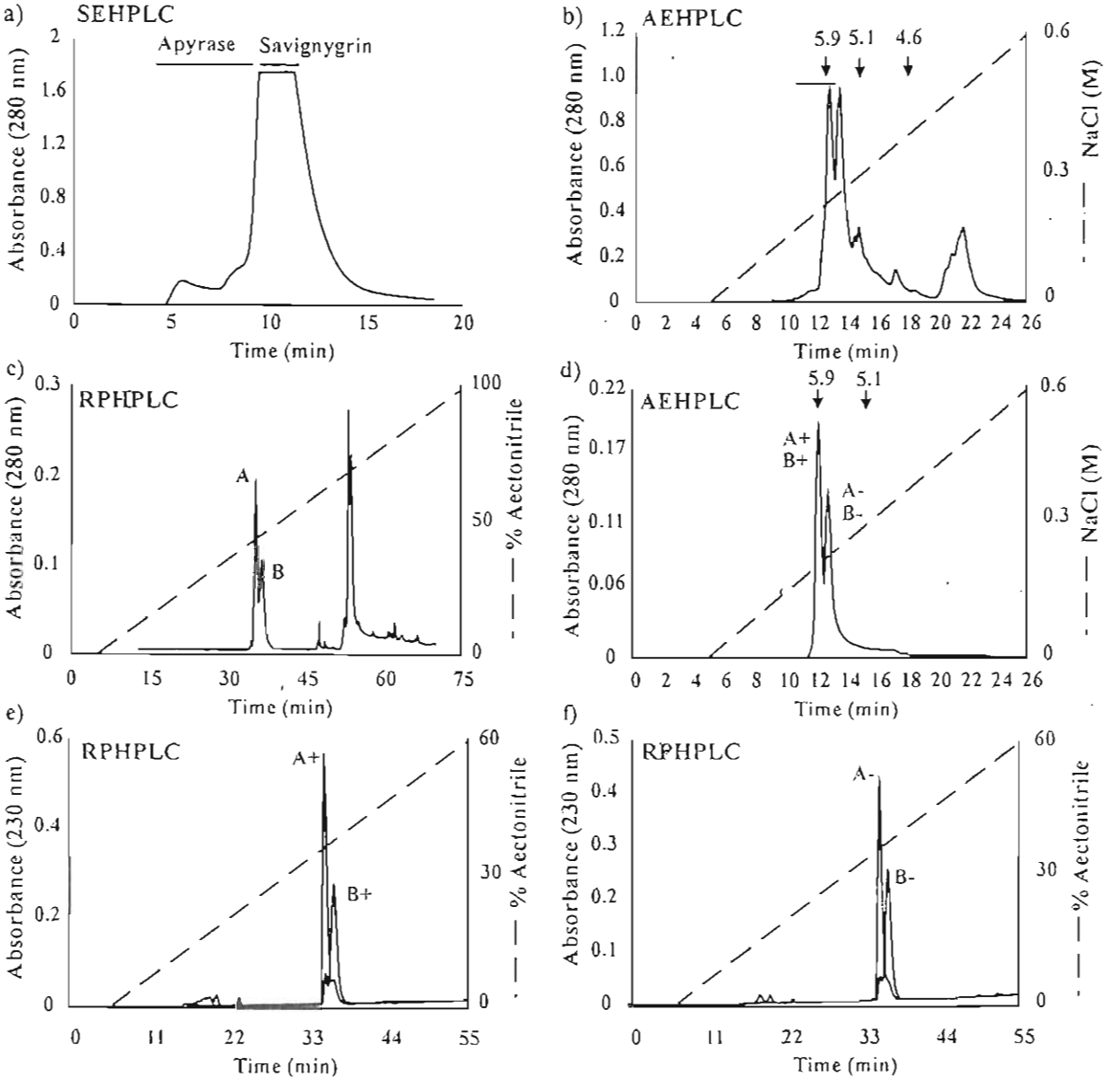


Fig. 2.6: Purification of savignygrin. (a) SEHPLC of tick salivary gland extract. Lines indicate apyrase activity and fractions pooled for AEHPLC (savignygrin). (b) AEHPLC of the fractions obtained after SEHPLC. The line indicates the region of platelet aggregation inhibitory activity and also those fractions pooled for RPHPLC. Relative iso-electric points are indicated with arrows. (c) RPHPLC of fractions with inhibitory activity obtained from AEHPLC. Inhibitory activity was observed in two peaks assigned A and B. (d) Rechromatography of fractions A and B on AEHPLC. The chromatograms of A and B are superimposed and peaks collected are indicated as A+, A-, B+ and B-. (e) Rechromatography of fractions A+ and B+ with RPHPLC. Chromatograms are superimposed. (f) Rechromatography of fractions A- and B- with RPHPLC. Chromatograms are superimposed.

2.3.2 Tricine SDS-PAGE analysis of the savignygrins

Tricine SDS-PAGE analysis was used to determine the purity of the inhibitors and to investigate their behaviour under reducing as well as non-reducing conditions (Fig. 2.7a). Reducing conditions indicated that the isoforms were pure with a relative Mr of 6.3 kDa for all four. Non-reducing conditions showed a reduction in electrophoretic mobilities for the different inhibitors. The A+ form migrated at ~15 kDa and the B+ form at ~12 kDa. The minus forms had surprisingly an even lower mobility with A- at ~32 kDa and B- at ~30 kDa. This indicates that disulphide bonds may play an important role in the maintenance of conformation and that the different inhibitors have distinct conformations. Lack of unfolding and reduced SDS binding could account for the lower mobilities. Electrophoresis in the presence of urea was thus investigated (Fig. 2.7b). In the presence of 8M urea the reduced inhibitors had a slight decrease (~2kDa) in electrophoretic mobility, indicating that reduced forms still bind SDS in the presence of urea. The non-reduced (+) forms however, showed a significant decrease in mobility.

This indicated unfolding by urea (increasing the hydrodynamic volume). By using a urea gradient (0-8M) perpendicular to the direction of electrophoresis the unfolding kinetics of a protein can be observed (Goldenberg and Creighton, 1984). Unfolding kinetics suggest that the (-) forms are already in an unfolded form due to the little change in mobility across the gradient, while the plus forms are unfolded to a greater extent (Fig. 2.7c). These results suggested that the (-) forms are less stable than the (+) forms and correlates with the finding that the (-) forms were less stable than the (+) forms when their temperature stability was measured (Fig. 2.7d). Although, all four inhibitors were extremely stable (retaining >50% activity after 3 hours of boiling at 94 °C), the (-) forms were slightly less stable and probably unfold more easily under the extreme conditions of boiling in the presence of SDS as was performed for electrophoretic analysis. There were no marked inflection points for the inhibitors that would indicate a rapid transition from a folded to unfolded state. There is rather a gradual decrease in electrophoretic mobility with increased urea concentration, indicating slow unfolding of a very stable conformation (Goldenberg and Creighton, 1984). This is most possibly due to a dense core structure kept intact by disulphide bonds, which is inaccessible to SDS. These

proteins would hence have a lower SDS:protein ratio and migrate to a greater extent on intrinsic charge.

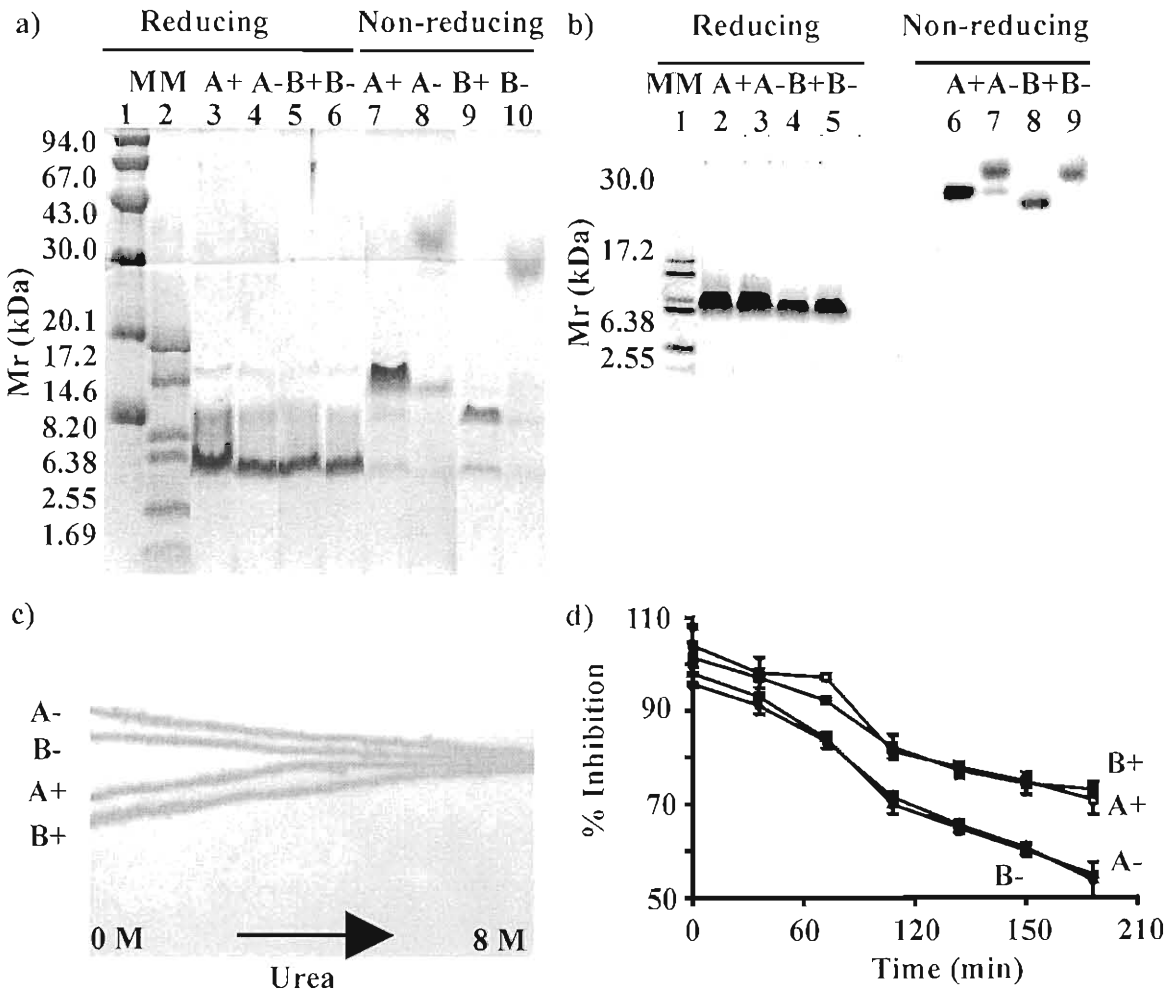


Fig. 2.7: Electrophoretic analysis of the savignygrins. (a) Tricine SDS-PAGE analysis of the savignygrins. Lane 1 indicates low molecular mass markers. Lane 2 indicates peptide molecular mass markers. Lane 3 to 6 shows electrophoresis under reducing conditions for A+, A-, B+ and B-, respectively. Lane 7-10 shows electrophoresis under non-reducing conditions for A+, A-, B+ and B-, respectively. (b) Tricine SDS-PAGE in the presence of 8M urea. Lane 1 shows peptide molecular mass markers. Lane 2-5 indicate inhibitors (A+, A-, B+ and B-, respectively) under reducing conditions. Lanes 6-9 show inhibitors (A+, A-, B+ and B-, respectively) under non-reducing conditions. (c) Tricine SDS-PAGE analysis under non-reducing conditions, with a 0-8 M urea gradient perpendicular to the direction of electrophoresis. (d) Temperature stability of the savignygrins. Inhibitors were incubated at 94 °C for the indicated time periods before activity was determined.

2.3.3 Electrospray mass spectrometry of the savignygrins

ESMS of the different forms shows that the (+) forms have the same molecular mass (6966 Da), while the (-) forms also have the same mass (6808Da), but are 158 Da smaller (Fig. 2.8). These masses correlate well with those obtained by SDS-PAGE under reducing conditions. The spectra show the M^{5+} to M^{8+} ion species for all four forms. The maximum number of positive charges correspond well with amino acid analysis and sequence data which indicate nine lysine and arginine residues (Fig. 2.10).

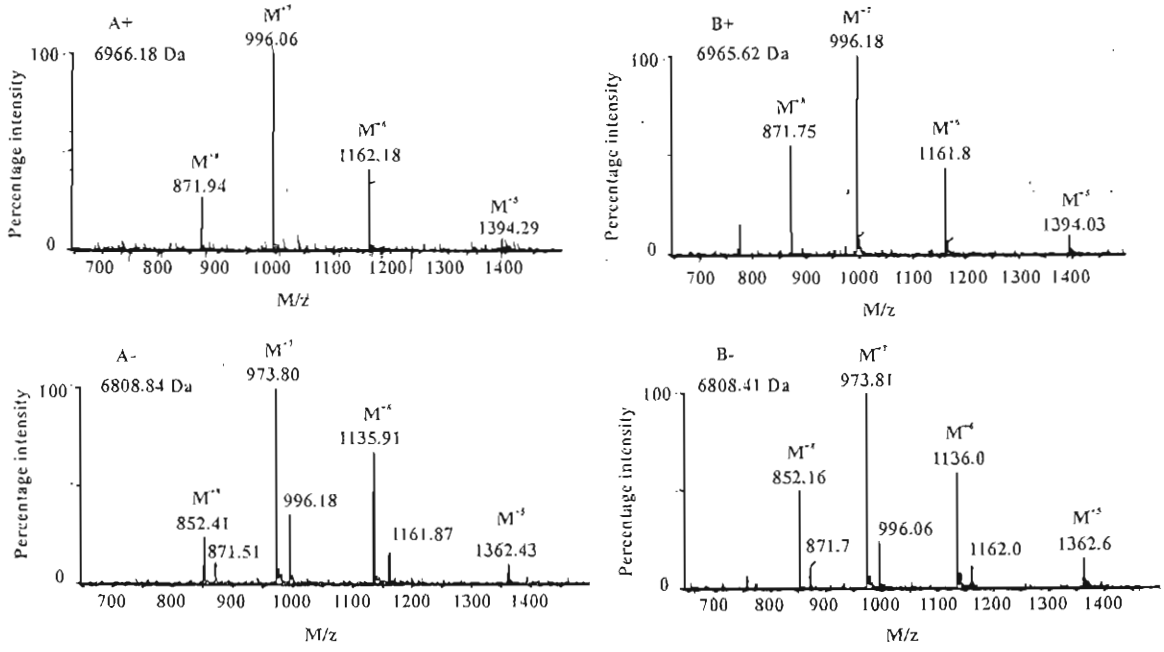


Fig. 2.8: Electrospray mass spectrometry analysis of the savignygrins. The M^{+5}, M^{+6}, M^{+7} and M^{+8} protonated species of each inhibitor is shown. Molecular masses obtained after deconvolusion of the respective spectra are included in each figure.

2.3.4 Peptide mass fingerprinting of the savignygrins

Peptide maps of the different inhibitors obtained by trypsin digestion and analyzed by MALDI-TOF-MS, was essentially the same (Fig. 2.9). These results indicate that the amino acid sequences are similar. No difference could be observed that could account for the discrepancy between the (+) and (-) forms.

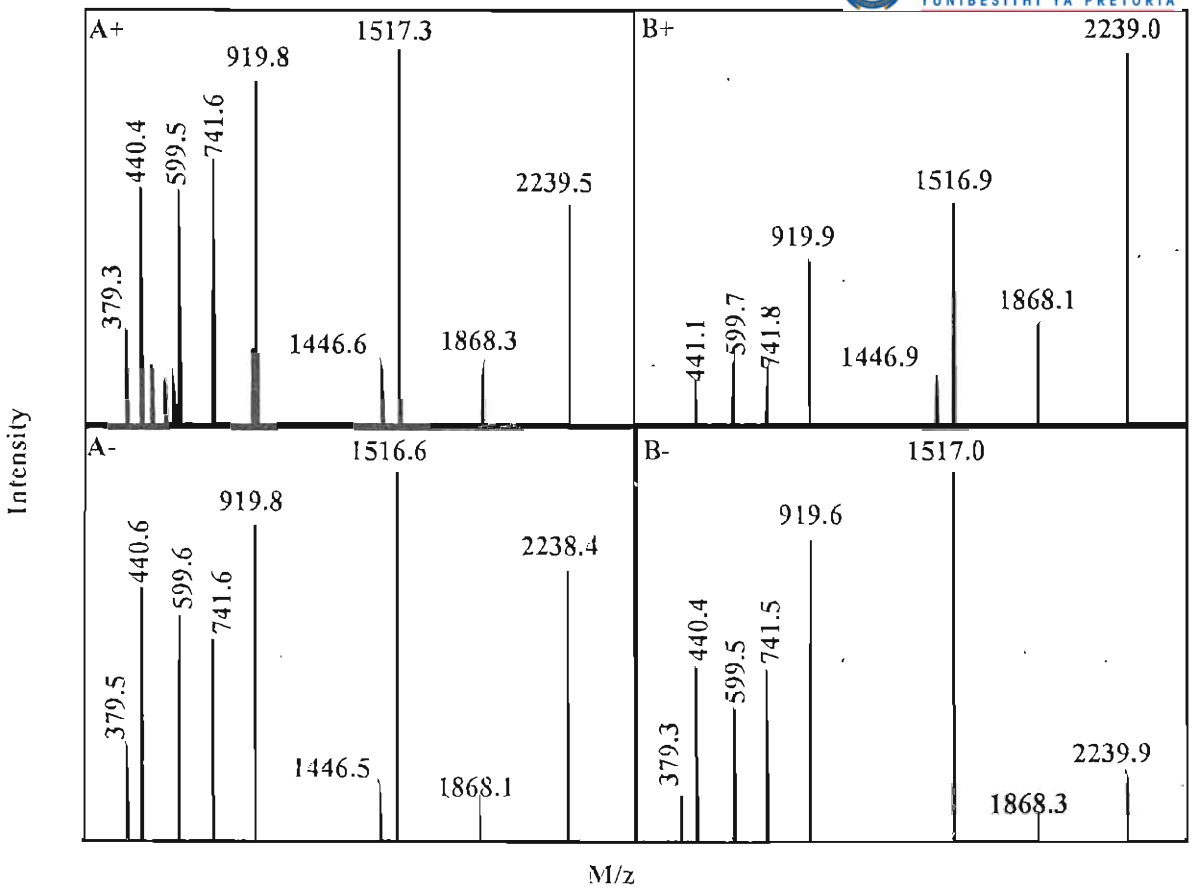


Fig. 2.9: Peptide molecular mass fingerprint analysis of the savignygrins. MALDI-TOF-MS spectra of tryptic digests of the A+, B+, A- and B- forms. Masses obtained for the fragments are indicated.

2.3.5 Amino acid analysis of the savignygrins

Amino acid analysis indicated that the overall compositions of the different inhibitors are similar (Fig. 2.10). No isoleucine or valine was detected, not even after hydrolysis up to 72 hours (results not shown). From these data the percentage of hydrophobic amino acids (using Ala, Leu and Phe) are approximately 13%. Performic acid oxidation, alkylation of cysteines with 4-vinylpyridine and ESMS of the alkylated forms (results not shown) all confirmed that 6 cysteines are present.

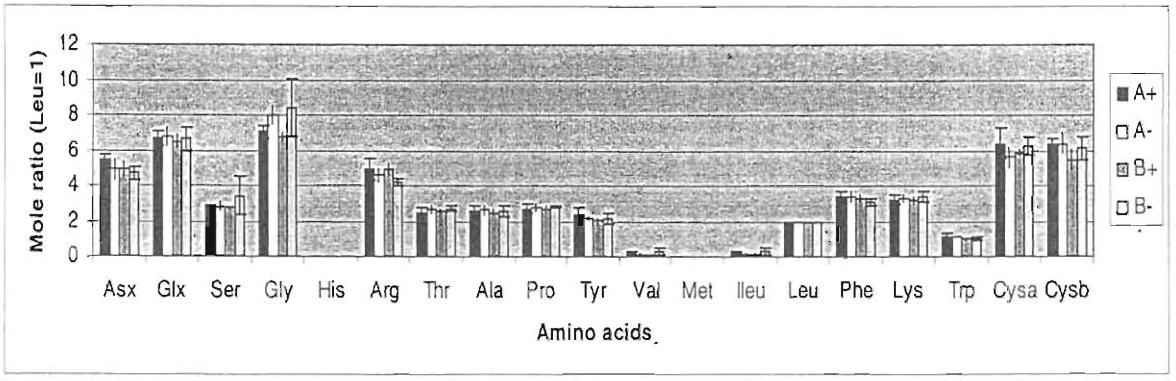


Fig. 2.10: Amino acid analysis of savignygrins. Indicated are mole ratios of amino acids relative to Leu for the different inhibitors. Cysa indicates performic acid oxidation and cysb alkylation with 4-vinylpyridine. SD is for three individual determinations.

2.3.6 Determination of disulphide content of the savignygrins

By using 4-vinylpyridine in the presence or absence of DTT, the existence of any free sulphydryl groups can be indicated. No alkylated cysteines were detected in the absence of DTT implying that all the cysteines are involved in disulphide bonds (Fig. 2.11).

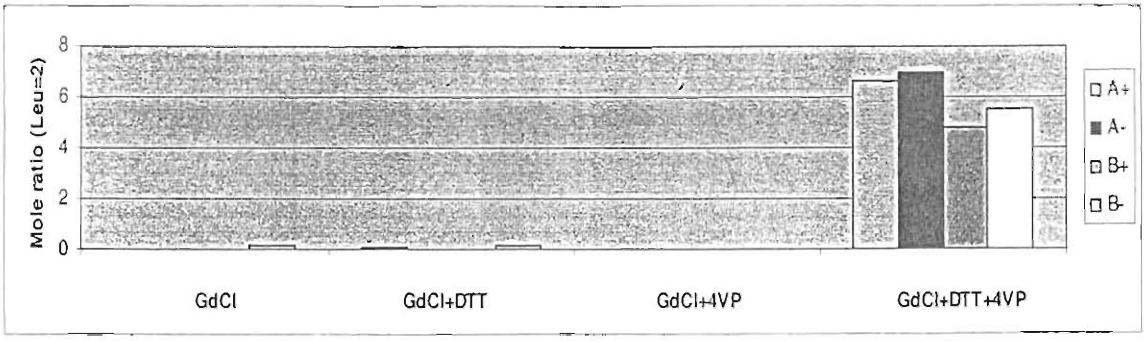


Fig. 2.11: Determination of the disulphide content of the savignygrins. Savignygrins were alkylated in the presence or absence of a reducing agent (DTT). Indicated are results obtained from amino acid analysis of savignygrins that were denatured (GdCl), denatured and reduced (GdCl+DTT), denatured and alkylated (GdCl+4VP) and denatured, reduced and alkylated (GdCl+DTT+4VP).

2.3.7 Disulphide bonds and their relevance for activity

Alkylation with 4-VP in the presence of DTT, or reduction with DTT alone, abolished inhibitory activity completely (Fig. 2.12), indicating that disulphide bonds are essential for the maintenance of activity.

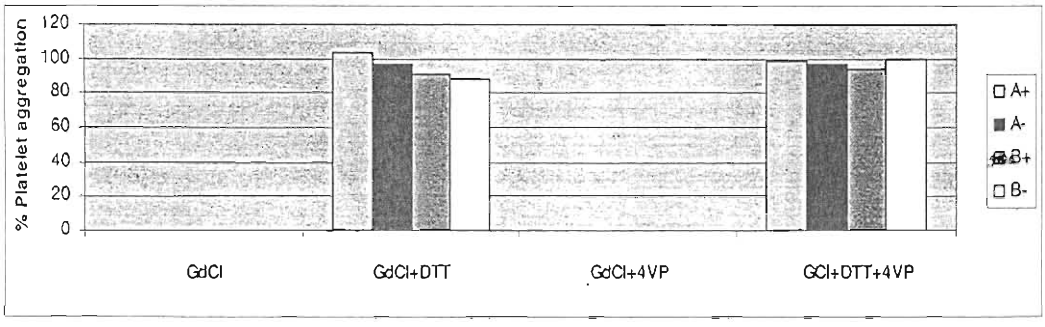


Fig. 2.12: The effect of disulphide bonds on activity of the savignygrins. Activity of savignygrins that were denatured (GdCl), denatured and reduced (GdCl+DTT), denatured and alkylated (GdCl+4VP) and denatured, reduced and alkylated (GdCl+DTT+4VP). A final concentration of 300 nM for the savignygrins was used for activity measurements. All values were calculated with respect to a control sample that was not treated in any way.

2.3.8 Biological activity of the savignygrins

All four inhibitors inhibited platelet aggregation induced by ADP, collagen, TRAP and epinephrine while platelets aggregated with ADP were also disaggregated (Fig. 2.13a). An IC_{50} of approximately 130 nM was determined for all four inhibitors (Fig. 2.13b). This is well in the range of the disintegrin inhibitors and close to that of disagregin (104 nM) (Gould *et al.* 1990; Karczewski, Endris and Connolly, 1994). A decrease in transmittance observed with added inhibitor indicates activation of platelets and concomitant shape change to the spherical form (Fig. 2.12a). This suggests post-activation inhibition at the level of the platelet integrin $\alpha_{IIb}\beta_3$. To investigate this further, platelets treated with savignygrin were analyzed using electron microscopy.

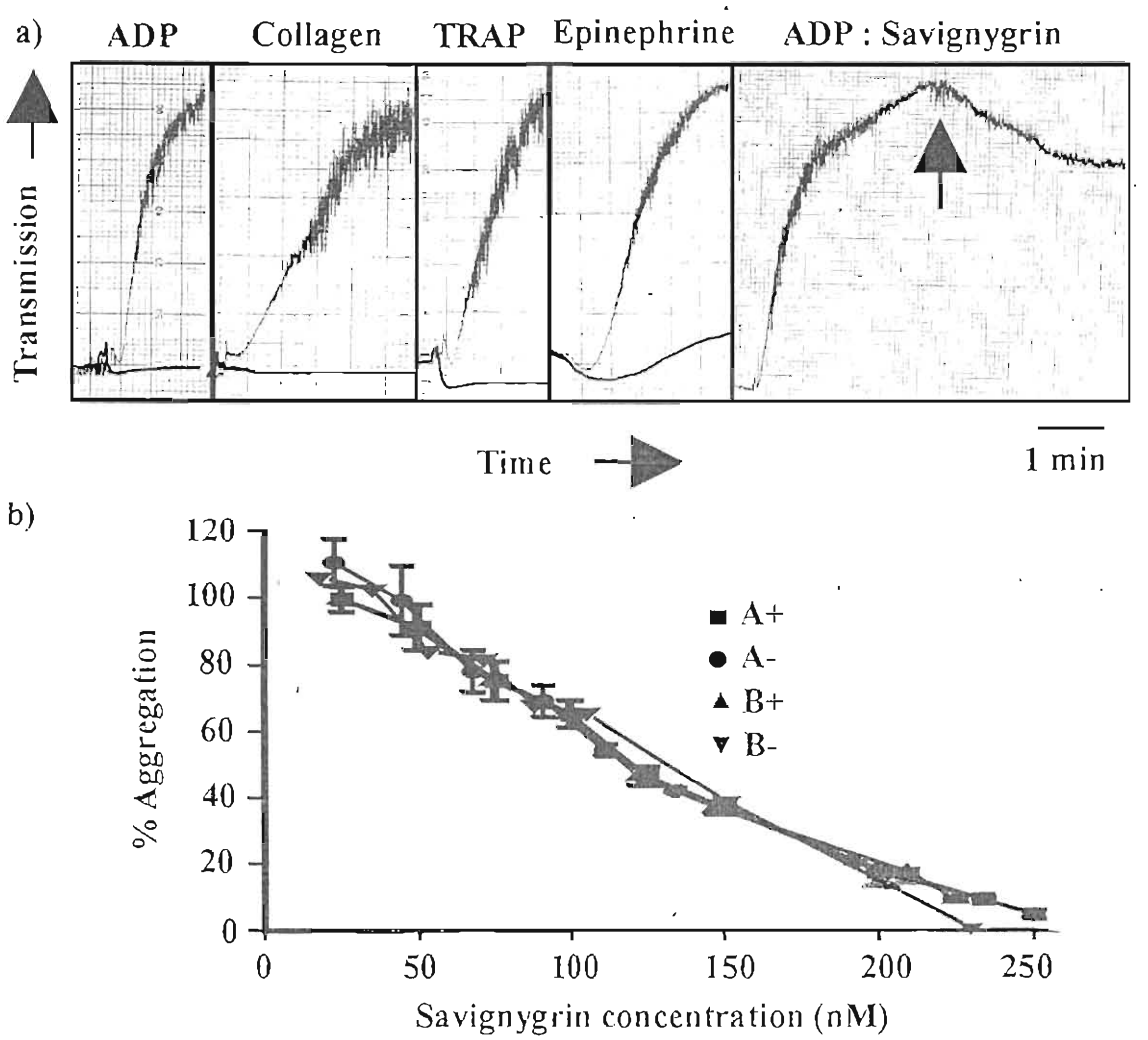


Fig. 2.13: Inhibition of platelet aggregation induced by various agonists by savignygrin. ADP (10 μM), collagen (2 μg), TRAP (50 μM) and epinephrine (10 μM) were used to induce platelet aggregation. Platelet rich plasma was incubated with savignygrin (300 nM final concentration, black tracings). Controls were incubated with saline (grey tracings). Platelet aggregation is indicated by an increase in transmission. Similar tracings were observed for all the different isoforms.

2.3.9 The influence of savignygrin on platelet shape change

Platelets used as negative control show a discoid (resting) shape with their granules intact (2.14a). Platelets aggregated with ADP show a fused state and secretion of granules (Fig. 2.14b). Platelets incubated with savignygrin, without ADP-induced activation, retain the discoid form associated with non-activated platelets and retain their granules (Fig. 2.14c). Platelets incubated with savignygrin and ADP possess an activated, spherical form and

retain their granules (Fig. 2.14d). Platelets aggregated with ADP and disaggregated with savignygrin possess the same shape as for those incubated with savignygrin and ADP but show a higher degree of dilatory open canaliculary system and show signs of degranulation (Fig. 2.14e). This is in contrast to results previously obtained for apyrase, which showed that removal of ADP leads to disaggregation but also signal transduction that manifests as reversible shape change (Mans *et al.* 2000). Platelets rather exhibited a similar shape as was found for platelets disaggregated with plasmin, a fibrino-(geno)-lytic enzyme, that severed the fibrinogen link between platelets, but had no signal transducing effect on platelets. These results are concurrent with targeting by savignygrin of the fibrinogen receptor that allows activation and degranulation of platelets, but inhibits fibrinogen binding, with no further effect on platelet signal transduction as observed for apyrase.

2.3.10 Targeting of $\alpha_{IIb}\beta_3$ by savignygrin

To test the possibility that $\alpha_{IIb}\beta_3$ is targeted, inhibition of binding of αCD41 (P2-FITC) to platelets and purified $\alpha_{IIb}\beta_3$ to immobilized fibrinogen was tested. Binding of P2-FITC to platelets was inhibited in a concentration dependent manner, both in the presence and absence of ADP (IC_{50}~15 μM) (Fig. 2.15a). Inhibition in the absence of ADP, indicate that the savignygrins can bind to resting $\alpha_{IIb}\beta_3$. Inhibition in the presence of ADP is much less than in the absence, suggesting that more competition exists between the antibody and the inhibitor. Furthermore, adhesion of $\alpha_{IIb}\beta_3$ to fibrinogen was inhibited with an IC_{50} ~3 nM (Fig. 2.15b). This is comparable with that of variabilin (9nM) (Wang *et al.* 1996) and decorsin (1.5nM) (Seymour *et al.* 1990). Taken together these results indicate the targeting of $\alpha_{IIb}\beta_3$ by the savignygrins.

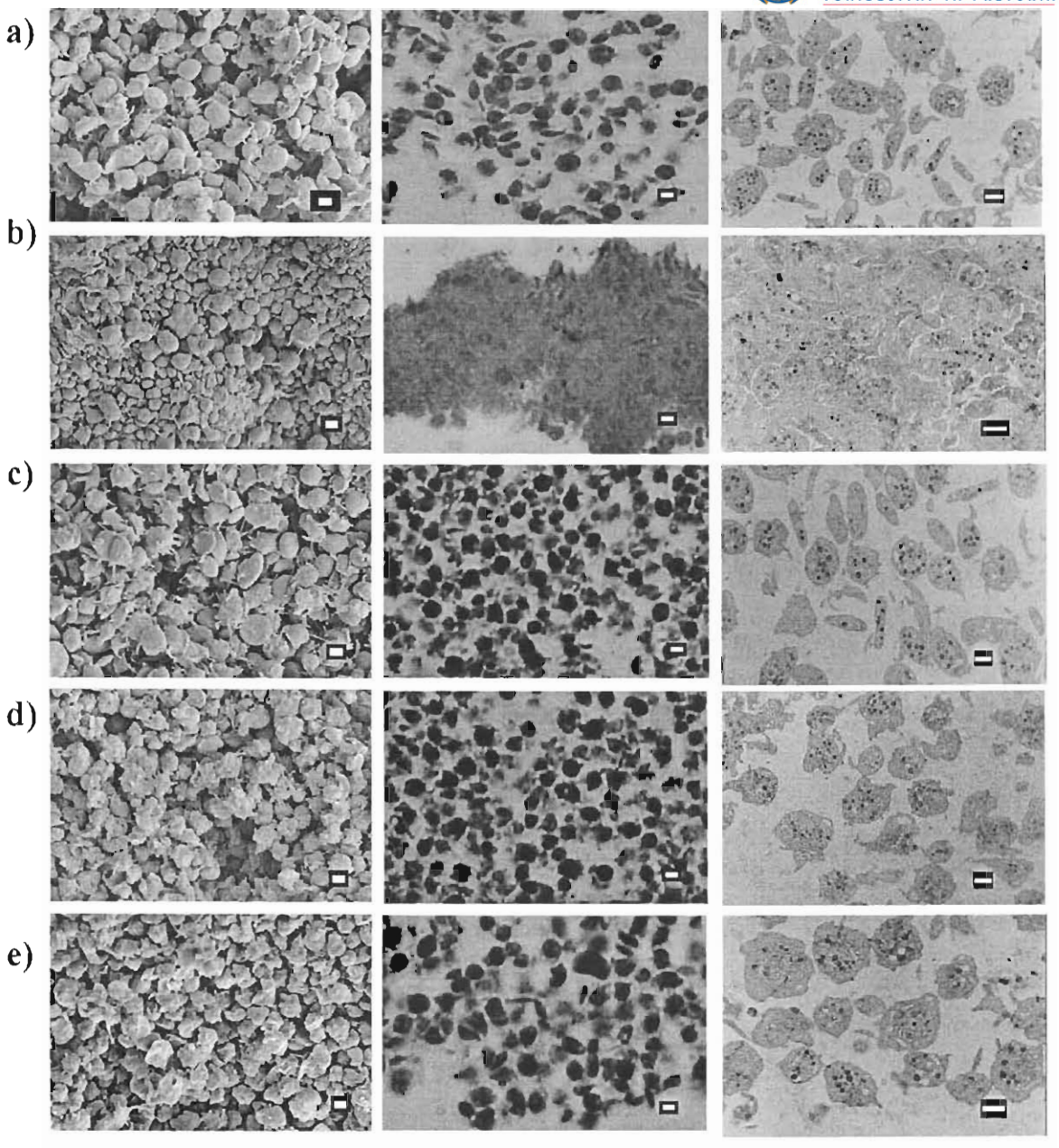


Fig. 2.14: The disaggregation effect of savignygrin on aggregated platelets. (a) Platelets without addition of any compounds. (b) Platelets aggregated with ADP (10 μM final concentration). (c) Platelets incubated with savignygrin. (d) Platelets incubated with savignygrin before addition of ADP (10 μM final concentration). (e) Platelets aggregated with ADP for 4 minutes and disaggregated with savignygrin. Scale bar = 1μm.

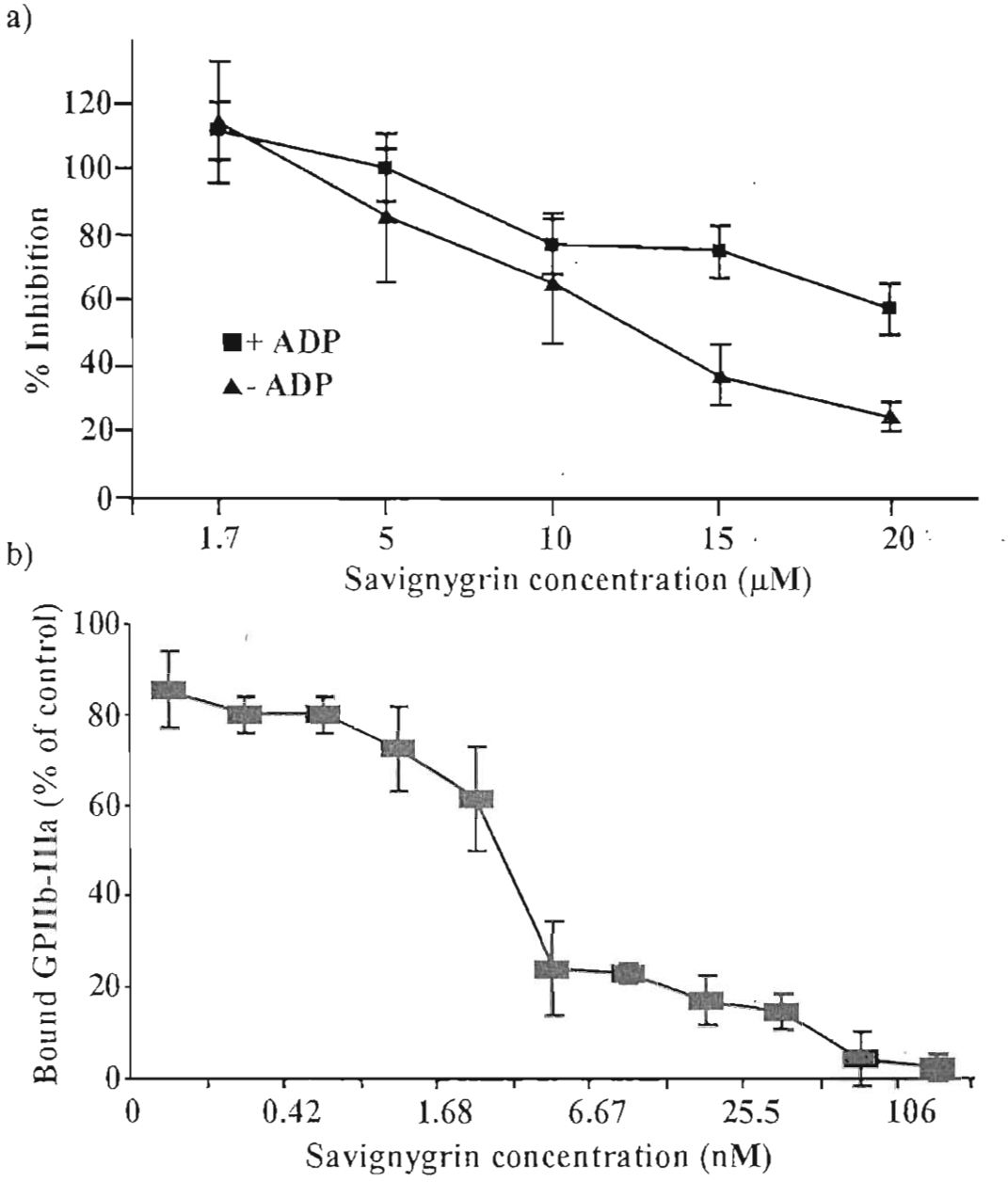


Fig. 2.15: Targeting of $\alpha_{IIb}\beta_3$ by savignygrin. (a) Inhibition of αCD41-FITC binding to platelets. Platelets were incubated with savignygrin, at various concentrations in the presence or absence of ADP, before incubation with αCD41-FITC. SD is for triplicate values. (b) Inhibition of the binding of purified $\alpha_{IIb}\beta_3$ to immobilized fibrinogen. $\alpha_{IIb}\beta_3$ was incubated with various concentrations of savignygrin before addition to fibrinogen. Values indicated are SD for triplicate values.

2.3.11 Integrin specificity of savignygrin

The integrin $\alpha_v\beta_3$ recognizes most ligands, such as vitronectin and fibrinogen that bind to $\alpha_{IIb}\beta_3$ (Plow *et al.* 2000). No inhibition of osteosarcoma cell adhesion to vitronectin or fibrinogen by savignygrin was observed, not even at a concentration of 10μM, although adhesion of platelets to fibrinogen was inhibited at concentrations of 20 nM (5% of control) (Fig. 2.16). This suggests that savignygrin is specific for $\alpha_{IIb}\beta_3$.

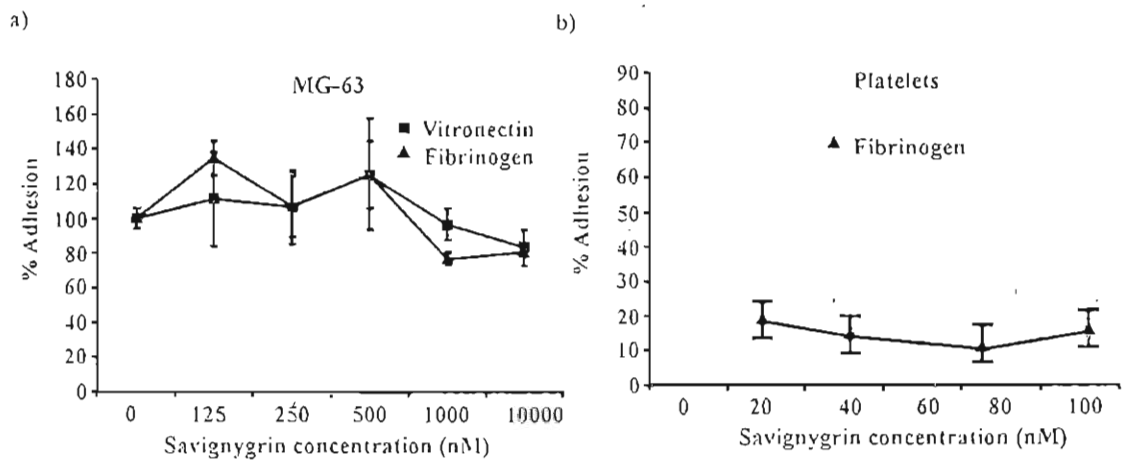


Fig. 2.16: Integrin specificity of the savignygrins. a) Adhesion of osteosarcoma cells to vitronectin and fibrinogen in the presence of savignygrin. b) Adhesion of platelets to fibrinogen in the presence of savignygrin. SD are for values in triplicate.

2.3.12 N-terminal amino acid sequence determination of the savignygrins

N-terminal amino acid sequence determination of the inhibitors shows that they all have the same sequence (results not shown). The yields for the A+ form are indicated and lie on the linear curve expected for the cycles determined (Fig. 2.17). Alignment with disagregin from *O. moubata* (Karczewski, Endris and Connolly, 1994) showed, that the cysteines were conserved although the N-terminal sequences show little similarity. A RGD motif in the sequence of savignygrin corresponds to the RED motif in the sequence of disagregin. Significant is that the sequence around the RGD motif is homologous to that of disagregin with the only difference being an aspartic acid residue at position 17 instead of a glutamic acid. This suggests that the sequence around the RGD motif is important for function, with negatively charged residues being prominent.

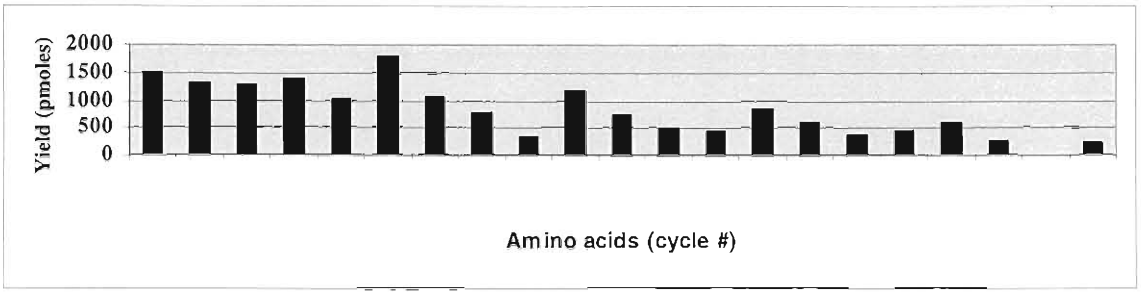


Fig. 2.17: N-terminal sequence analysis of savignygrin. Sequencing yields for different cycles of A+ are indicated. Similar results were obtained for the A-, B+ and B- isoforms.

2.3.13 Isolation of total RNA

Mouse lung total RNA showed the characteristic 28S, 18S and 5.8S rRNA bands. Total RNA from tick salivary glands and nymphs only showed the 18S rRNA band and a second band with a much lower mobility (Fig. 2.18). This is unusual as the 28S rRNA intensity is normally double that of the 18S rRNA (Farrell, 1993). There was however, a fragment with a much lower mobility that may represent unprocessed 28S-5.8S rRNA. Similar results have been obtained when *Xenopus* oocytes are depleted of U8 sno RNA, which is essential for 5.8S and 28S rRNA maturation (Peculis, 1997).

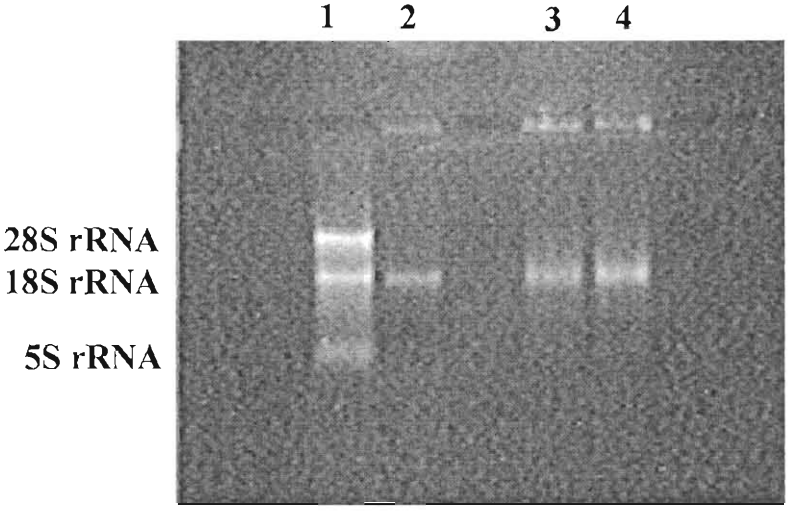


Fig. 2.18: Electrophoretic analysis of total RNA. Lane 1 indicates control RNA isolated from mouse lung. Lane 2 indicates isolations of total RNA from nymphae, and lanes 3 and 4 from tick salivary gland extracts.

2.3.14 Cloning and sequencing of savignygrin

PCR under optimized conditions using the degenerate primer designed from the N-terminal sequence of savignygrin showed a product of 300 bp while 5' RACE of the 5' UTR indicated a slightly smaller product (Fig. 2.19). Using the GrinAB primer a product of 170 bp was amplified for the 3'RACE, while a product of 400 bp was amplified for the 5'RACE using the GrinLMM primer. Sequencing showed that the 3' RACE (291 bp) and 5' RACE (324 bp) fragments corresponded well with the results obtained with PCR for the (+) form (Fig 2.20).

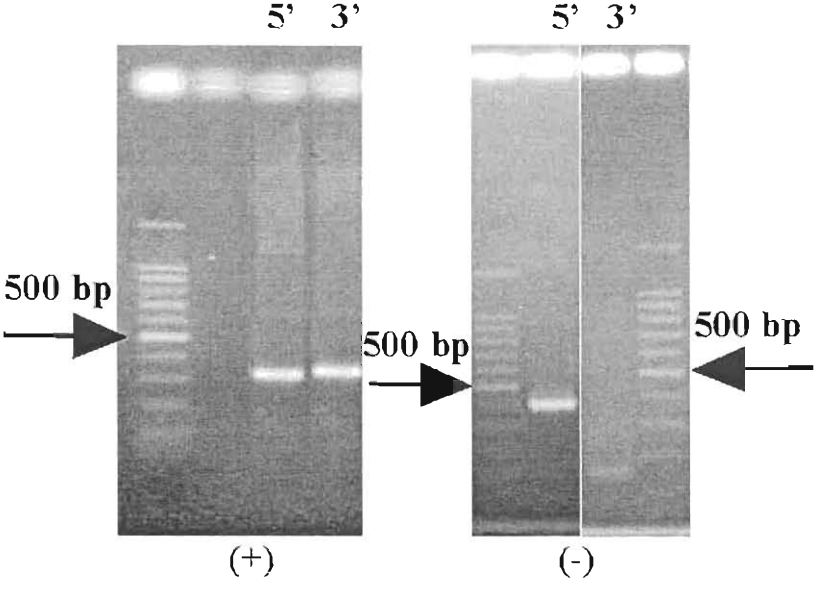


Fig. 2.19: RACE of the high (+) and low (-) mass forms of savignygrin. The 500 bp marker is indicated with an arrow. 5'RACE and 3'RACE products are indicated for the (+) and (-) forms, respectively.

2.3.15 Analysis of the recombinant amino acid sequence of savignygrin

The cDNA sequences obtained for both (+) and (-) forms showed all primers used during PCR and the poly-A tail used during cDNA synthesis. The cDNAs also contained the stop codon (TGA) and the poly-adenylation signal AATAAA. The translated amino acid sequences correlated with an immature protein of 80 amino acids, while the mature chain consisted of 61 amino acids, with the first 21 amino acids corresponding to that obtained with Edman degradation (Fig. 2.20). It is well known that threonine residues give low yields during Edman degradation, which explains the threonine at position 20 that corresponds with the gap in the Edman derived sequence. Analysis of the immature protein using SignalP (Nielsen *et al.* 1997), predicted the presence of a signal peptide (19

amino acids) and the correct cleavage site. There are relatively few differences between the (+/-) forms at sequence level. The only gap present is in the 5'UTR of the (-) form and most of the differences occur at isolated positions in the 3'UTR. Two non-synonymous substitutions occur at positions R52G (R73G immature protein) and N60G (N81G immature protein) for the (+/-) forms, respectively and one synonymous substitution at position K56 (K77 immature protein).

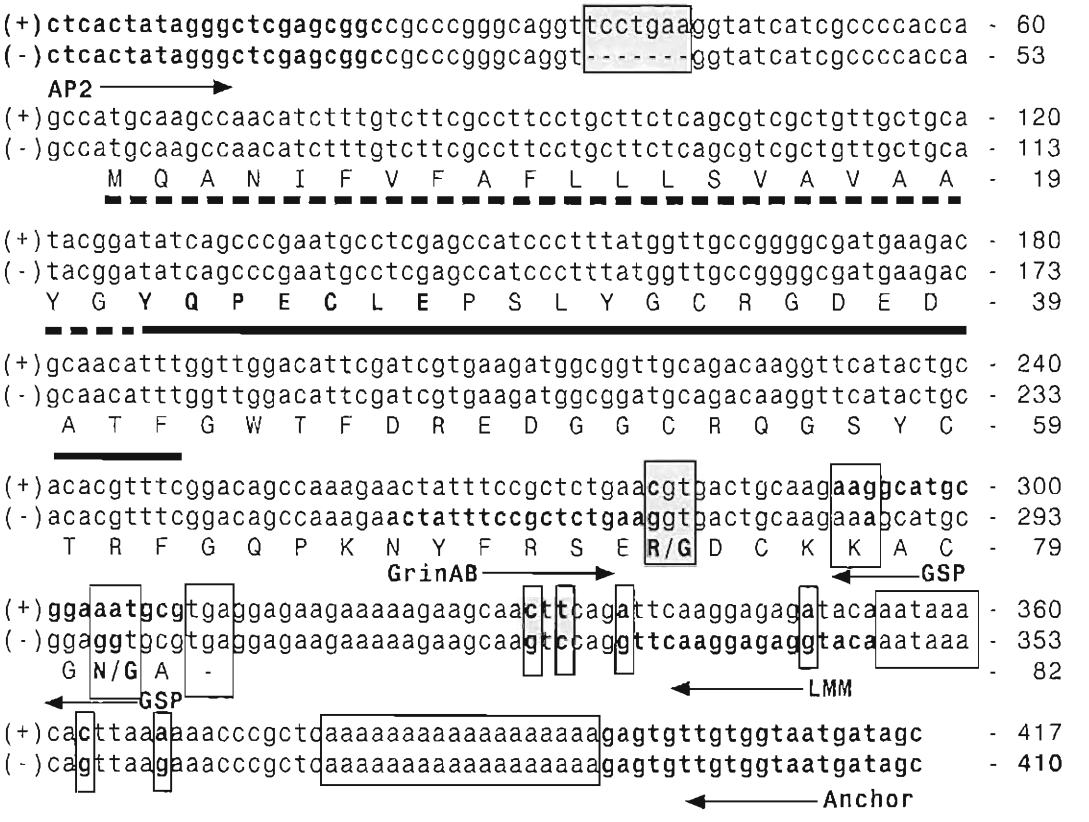
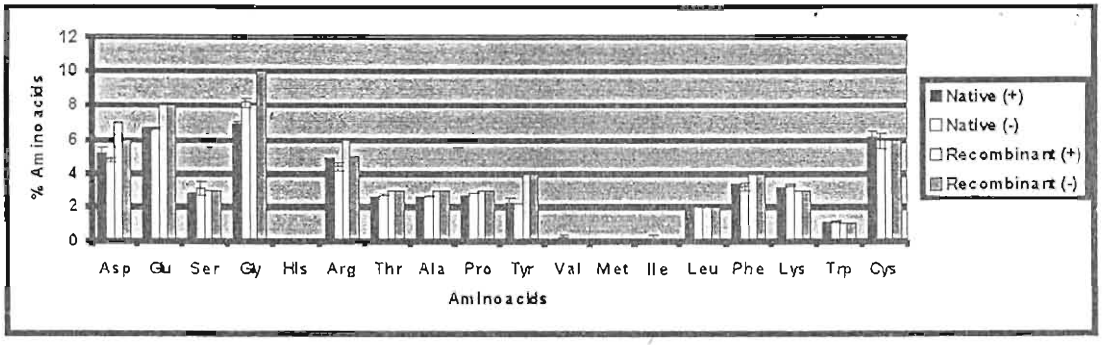


Fig. 2.20: Full-length cDNA sequence for savignygrin. The 5' adapter, 3' gene specific and 3' anchor primer are shown in bold. The stop codon (TGA), the poly-adenylation signal (AATAAA) and the poly-A tail are boxed. The N-terminal sequence obtained with Edman degradation are underlined while the N-terminal sequence used for degenerate primer design is shown in bold. The signal peptide is underlined with a broken line.

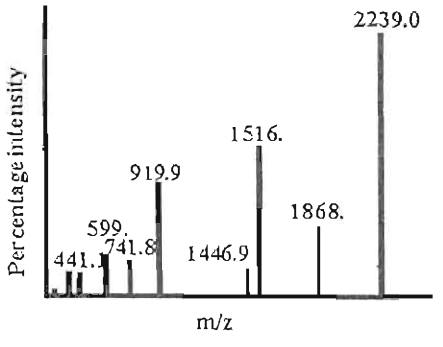
2.3.16 Comparison of data obtained from the deduced amino acid sequence and data from native savignygrin

The theoretical peptide masses obtained for a tryptic digest of the deduced amino acid sequence correlate well with the empirical data obtained for the native inhibitors and show that the peptides obtained were distributed across the whole sequence. The calculated masses of the deduced amino acid sequences also correspond with the masses of the (+)/(-) forms obtained with ESMS. Amino acid compositions obtained for the native inhibitors and the composition calculated from the deduced amino acid sequence obtained after cloning correlate closely (Fig. 2.21). Taken together, these data suggest that the correct sequences for the savignygrins were obtained.

a)



b)



c)

Cleavage product		Molecular mass (Da) Experimental	Deduced
YQPECLEPSLYGCR (1-14)		1868.1	1866.1
GDEDATFGWTFDR (15-27)		1516.6	1516.5
EDGGCR (28-33)		741.6	738.9
QGSYCTR (34-40)		919.6	918.0
NYFR (46-49)		599.6	598.7
GDEDATFGWTFDRED GGCR (15-33)		2238.4	2238.3
Molecular mass of protein	(+)	6966.1	6965.6
	(-)	6808.8	6809.4

Fig. 2.21: Comparison of native savignygrin and the deduced amino acid sequence. (a) A comparison of the amino acid composition obtained for the native (+)/(-) forms and the composition calculated from the deduced amino acid sequences obtained after cloning and sequencing. (b) A representative peptide mass fingerprint of all four iso-forms, obtained for the savignygrins. (c) A comparison of the peptide masses obtained experimentally for the native savignygrins and those calculated for the deduced amino acid sequence. Total protein molecular mass obtained for the (+)/(-) forms by ESMS and a calculated molecular mass are also indicated.

2.3.17 Comparison of savignygrin with disagregin

BLAST analysis of the protein sequences of savignygrin indicated significant homology (E-value: $4x10^{-13}$) to the platelet aggregation inhibitor disagregin, from the related soft tick, *O. moubata*. Alignment using the Dayhoff PAM250 matrix gave an identity of 44% and similarity of 72% (Fig. 2.22).

```
Savignygrin (+): 1-YQPECLEPSLYGCRGDEDATFGWTFDREDGGCRQGSYCTRFGQPKNYFRSERDCKKACGNA - 61
Savignygrin (-): 1-YQPECLEPSLYGCRGDEDATFGWTFDREDGGCRQGSYCTRFGQPKNYFRSEGDCKKACGGA - 61
Disagregin     : 1-SDDKCQGRPMYGCREDDDSVFGWTYDSNHGQCWKGSYCKHRRQPSNYFASQQECRNTCGA- - 60
SSPro prediction:  CCCCCCCCC********CC*EEEEE*CCCCCEEEEEECCCCCCCCCC***HHHHHHH*CC
```

Fig. 2.22: Alignment of savignygrin with disagregin. Identity (66%) is boxed in black, while similar amino acids are boxed in grey. Secondary structure prediction using the SSPro server is indicated with C: random coil, E: extended β-sheet, H: α-helix and *: no consensus secondary structure. GenBank accession codes are for savignygrin (+) (AF452885), savignygrin (-) (AF452886) and disagregin (A54369).

2.3.18 Origins of (+)/(-) forms: alleles or gene duplication?

The origins of protein isoforms are important when the evolutionary history of a protein is considered. Isoforms could be a conformational artefact inherent in the primary structure of the protein itself, as was observed for the A/B forms. Proteins can also be post-translationally modified to various extents, which can confer different chemical and physical properties to isoforms. The (+)/(-) forms have been shown to differ on the gene level. As such, these isoforms might be either different alleles or might be gene duplications. To investigate these different possibilities, savignygrins from twenty individuals were prepared by RPHPLC and analyzed with tricine SDS-PAGE. The analysis indicated that all four isoforms were present in all twenty individuals (Fig. 2.23). It is known that *O. savignyi* is diploid with the sex chromosomes being XX and XY for females and males, respectively (Howell, 1966a). It could further be assumed that the collected ticks are representative of a population that mated randomly. For a diploid random population, the Castle-Hardy-Weinberg Law states that the gene frequencies can be expressed as: $(p+q)^2 = p^2 + 2pq + q^2$, where p and q are the frequencies of the homozygous alleles (AA, aa) and pq the heterozygous alleles (Aa). The frequencies of the alleles vary between 0 and 1 so that the sum always equals 1. Under these assumptions, the maximum heterozygosity attainable is at the point where p=q so that the

frequency of 2pq can never exceed 0.5 (Spiess, 1989). The probability of picking 40 alleles (20 ticks) at random and finding all heterozygous is ~1 in a trillion (0.5^{40}), seems very unlikely. Another possibility could have been the localization of the savignygrins on the sex chromosomes, so that males would always be heterozygous. This was excluded by analysis of females only. The other possibility is positive overdominance, which is found when the heterozygote has a higher mean fitness than either homozygote (Parsons and Bodmer, 1961). In the present case this implies that both homozygotes are lethal as they are not present at all in the analyzed population. The close similarity in sequence and biological activity between the high and low mass forms would make this scenario extremely unlikely. The savignygrins are most of the time stored away until feeding and probably do not interact at all with important cellular processes within the tick. From these considerations, the possibility that the savignygrins are different alleles is very unlikely. The more likely scenario is that of a recent gene duplication event that would ensure representation of the savignygrins in all ticks tested.

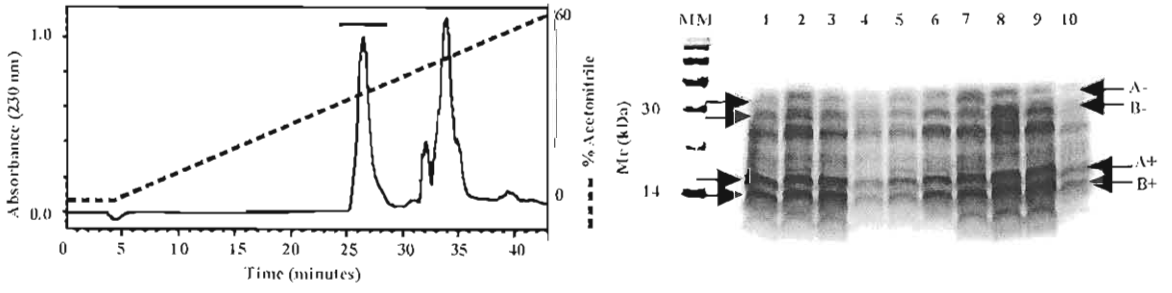


Fig. 2.23: Analysis of savignygrin isoforms in twenty individuals. A representative chromatogram of the SGE from one tick fractionated with RPHPLC is indicated on the left. The horizontal line indicates fractions analyzed. Non-reducing tricine SDS-PAGE analysis of savignygrin preparations from 10 ticks is indicated on the right. Arrows indicate the savignygrin isoforms. Results for the other ten ticks were similar.

2.4 Discussion

2.4.1 Savignygrin (+)/(-) isoforms: conformational and genetic origins

Amino acid composition, peptide mass fingerprinting, ESMS and N-terminal sequence analysis indicate that the 4 isoforms purified for savignygrin, have similar amino acid sequences. The translated amino acid sequences confirmed the near identical sequences of the high (+) and low (-) mass forms and account for the ESMS derived mass difference observed, as well as the separation of these forms during AEHPLC. Careful analysis of the native and recombinant amino acid compositions show that the (-) forms have lower aspartic acid and arginine values, but a higher glycine value, with respect to the (+) forms. This corresponds with the sequence data. It has been shown that the (+)/(-) forms must be gene duplicates. Interestingly, no isoforms were observed for disagregin and no difference could be observed in the electrophoretic mobilities under reduced and non-reduced conditions (Karczewski, Endris and Connolly, 1994). This could indicate that the (+)/(-) gene duplication is a fairly recent event that occurred after the divergence of these two tick species from a common ancestor.

The presence of the A/B conformational isoforms are more problematic, as no sequences were obtained that could explain the differences. It is not surprising to find separation of a single protein into two peaks during RPHPLC and has been observed for a number of proteins. Such two-peak separations are generally observed for proteins that are stable under reversed-phase chromatography conditions (low pH and high concentrations of organic mobile phase), so that native as well as unfolded forms are present (Kunitani and Johnson, 1986). The high stability of the savignygrins correlates well with this data. It is thus proposed that the A/B forms are not sequential isoforms, but rather an artefact of the purification procedure.

2.4.2 Conformational and stability differences observed between the (+)/(-) forms

Electrophoretic analysis and temperature stability studies indicated that the (-) forms are less stable than the (+) forms. The R52G difference between the high and low mass forms occurs in a region predicted to be a α-helix. Destabilization of this α-helix by G52 could lead to the lower stability observed for the low mass form. Glycine has been shown to be

one of the most helix-destabilizing residues (Chakrabartty, Schellman and Baldwin, 1991 and references therein). This destabilizing effect is described as being due to greater flexibility of the glycine peptide backbone, which thermodynamically prefers a random coil stucture instead of the α-helical structure. Glycine also lacks the enthalpic interaction between Cβ and the α-helix backbone that favours the helix. G52 as such probably does not destabilize the helix under native conditions, but under conditions of high temperature and SDS concentration as used during electrophoresis, leads to destabilization of the structure and the observed shift in electrophoretic mobility. The N60G difference between the high and low mass forms occurs outside any secondary stucture elements, right at the end of the protein sequence and would not be expected to have significant effects on the protein structure.

2.4.3 Electrophoretic behaviour of the savignygrins

All isoforms showed a decrease in electrophoretic mobility under non-reducing conditions, suggesting a compact structure that inhibits SDS binding. This is a phenomenon observed for proteins with intra-chain disulphide bonds (Pitt-Rivers and Impiombato, 1968). The same unusual electrophoretic behaviour was observed for echistatin under non-reducing conditions, while an unusual rapid migration was observed for the S-alkylated protein (Gan *et al.* 1988). The low hydrophobicity (13% hydrophobic amino acids) of the savignygrins could also influence electrophoretic behaviour, as it was observed that peptides with a low hydrophobicity behave unusually during electrophoresis, presumably due to reduced SDS binding (Hayashi and Nagai, 1980). A compact structure stabilized by disulphide bonds could explain the high temperature stabilities and why these inhibitors are not fixed by normal methanol:acetic acid fixation procedures after electrophoresis under non-reducing conditions. High temperature stabilities have also been observed for proteins with low hydrophobicity (Russouw *et al.* 1995).

2.4.4 Biological activity of savignygrins

The biological activity of the savignygrins was not affected by sequence or conformation differences, as indicated by similar IC_{50} values (~130 nM) for all four isoforms. This

could be accounted for in part by the RGD motif that is present in all four isoforms. Activation of platelets pre-incubated with savignygrin was indicated by a decrease in transmittance during inhibition of platelet aggregation induced by various agonists. Electron microscopic analysis of platelets incubated with savignygrin before activation with ADP confirmed the discoid to spherical shape change associated with activation. Maintenance of a spherical shape during disaggregation of aggregated platelets by savignygrin indicated that no signal transduction events occur except for a dissociation of fibrinogen from $\alpha_{IIb}\beta_3$. This suggests post-activation inhibition by the savignygrins and implicates the common denominator of platelet aggregation, the integrin $\alpha_{IIb}\beta_3$. Inhibition of the binding of αCD41-FITC in the presence or absence of ADP strongly suggests that savignygrins bind to resting as well as activated $\alpha_{IIb}\beta_3$, corresponding with the above hypothesis. This is furthermore supported by the inhibition of binding of purified $\alpha_{IIb}\beta_3$ to fibrinogen. The discrimination observed for savignygrin between $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ was also found for disagregin (Karczewski, Endris and Connolly, 1994).

2.4.5 The RGD motif of the savignygrins

It has been shown that disagregin inhibits the binding of echistatin (which contains the RGD motif) to platelets, suggesting interaction with the RGD binding site of $\alpha_{IIb}\beta_3$ (Karczewski, Endris and Connolly, 1994). This is strongly supported by the presence of an RGD motif in the sequence of savignygrin. Although disagregin has a RED motif and peptide studies showed that the peptide REDV does not inhibit platelet aggregation, a restricted conformation induced by the three dimensional structure of disagregin could account for inhibitory activity (Chen *et al.* 1991). This is supported by the dependence of savignygrin on intact disulphide bonds for the maintenance of an active but restricted biological conformation. The sequence adjacent to the RGD motif of savignygrin (YGCRGDEDA) is similar to that of disagregin (YGCREDDDS), which suggests that the sequence around the RGD motif could have an important effect on its function. Of particular interest is the fact that the two downstream residues are negatively charged. It is possible that these residues interact with the Ca^{2+} binding site localized inside the $\alpha_{IIb}\beta_3$ ligand-binding locus speculated to interact with the negatively charged aspartic acid of the RGD motif (Calvete, 1994). It was also shown that disagregin can inhibit the

binding of the dodecapeptide sequence of γ-fibrinogen to $\alpha_{IIb}\beta_3$ (Karczewski and Connolly, 1997). There may thus be other binding sites for $\alpha_{IIb}\beta_3$, apart from the RGD motif in the savignygrins.

Secondary structure elements are normally conserved in proteins with the same structural folds. This is exemplified by the conserved areas observed for the predicted β-sheet and α-helix elements in the alignment of disagregin and savignygrin. Of interest is the fact that the region surrounding the RGD/RED motif is the largest conserved stretch between disagregin and savignygrin. However, no consensus secondary structure is predicted in this region. As residues are normally conserved either for structural or functional purposes, this suggests that this region is important for activity.

2.4.6 Secretion of savignygrin during feeding

To have any biological significance during tick feeding, bioactive components have to be secreted (Law, Ribeiro and Wells, 1992). Secretory proteins are targeted to the endoplasmic reticulum via a hydrophobic signal peptide in their N-terminus, from where they are transported to the Golgi-network and finally secreted by either constitutive or regulated secretion in secretory granules. Extracellular proteins in general are disulphide rich and disulphide bonds are absent in intracellular proteins due to the reducing environment inside the cell (Gierasch, 1989; von Heijne, 1990; Fahey, Hunt and Windham, 1977). The presence of a signal peptide in the full-length sequences savignygrin and the fact that all cysteines are involved in disulphide bonds, indicate that the savignygrins are targeted to the secretory pathway. No evidence that suggests secretion has been described for variabilin (Wang *et al.* 1996) or disagregin (Karczewski, Endris and Connolly, 1994).

2.4.7 Independent adaptation of hard and soft ticks to a blood feeding environment

Variabilin, a 40 amino acid platelet aggregation inhibitor from the hard tick, *D. variabilis* contains a RGD motif in the last third of its sequence (Wang *et al.* 1996). There is no amino acid sequence similarity between variabilin and savignygrin and the RGD position is completely different. This suggests that platelet aggregation inhibitors with RGD-like

motifs have evolved after the divergence of hard and soft ticks. This implies that the main tick families have adapted to their blood feeding environments independently.

2.5 Summary

The relationship between the different isoforms of savignygrin is summarized in Fig. 2.24.

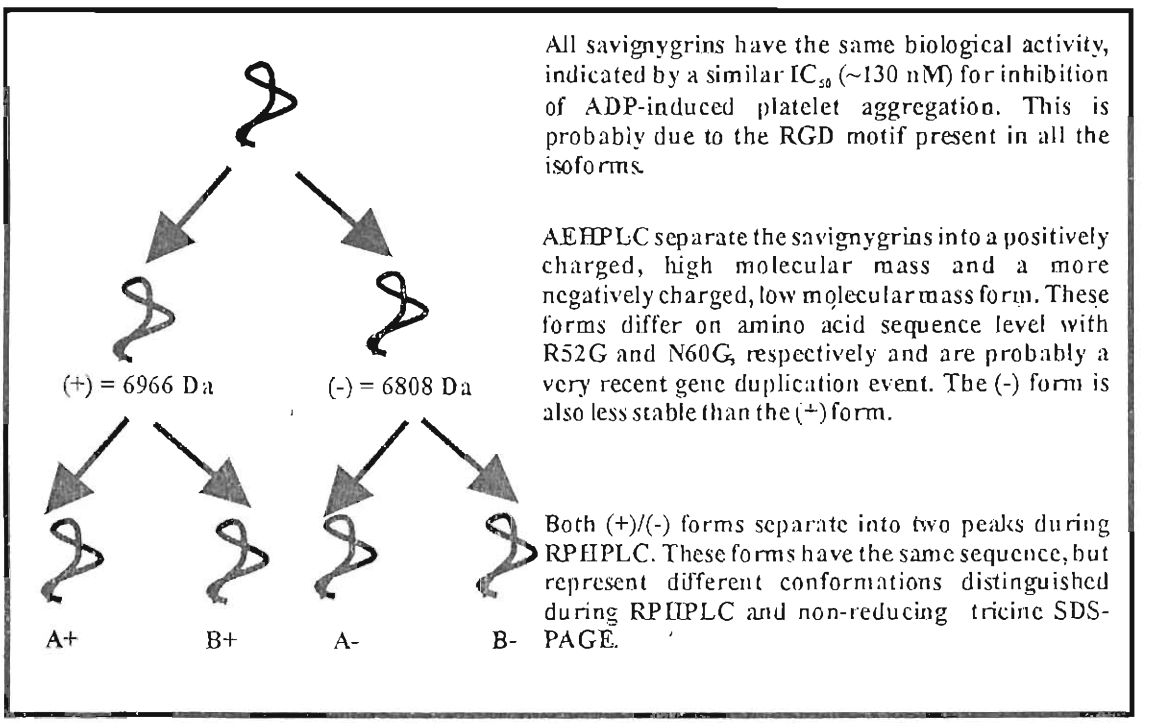


Fig. 2.24: The relationship between the different isoforms of savignygrin.

During the characterization of savignygrin, it became apparent that the platelet agregation inhibitors were related to the BPTI-like coagulation inhibitors of soft ticks. This relationship and its implications for the structure and function of the platelet aggregation inhibitors will be treated in Chapter 4. The amino acid sequences for the fXa inhibitors of *O. savignyi* and *O. moubata* were known, as well as the sequence for ornithodorin, the thrombin inhibitor from the tick, *O. moubata*. The amino acid sequence for savignin, the thrombin inhibitor from the tick *O. savignyi* was not known at this stage and for the sake of a complete phylogenetic analysis, savignin was also cloned and sequenced. Chapter 3 deals with the cloning, sequencing and molecular modeling of savignin and will serve as an introduction to the coagulation cascade inhibitors found in soft ticks.