Isolation and characterisation of serine proteases and metalloproteases from the venom of African puff adders

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Abstract

The puff adder (*Bitis arietans*) is a highly venomous viperid snake responsible for many fatalities in Africa, yet despite this there have been few comprehensive analyses of its venom proteins, particularly of the proteases that play a key role in envenoming. This study set out to isolate and characterise the most abundant serine proteases and metalloproteases found in venoms of puff adders obtained from Nigeria, Tanzania and Kenya. All three contained both classes of protease, but there were clear regional differences in the types of protease and their activities. Prominent in all three venoms was an SVMP PII-a. This protease varied in degree of glycosylation between the three sources, and also in its activity. The protease isolated from Tanzanian venoms, which was non-glycosylated, proved to be highly potent, and very destructive towards laminin in particular. The SVMP PIII content of the three venoms was quite low, but in some Kenyan venoms a prominent 68 kDa SVMP PIII was found which has a novel non-covalent dimeric structure and was strongly gelatinolytic, an activity that was not observed in any of the other SVMPs identified here. The Nigerian venoms were rich in an interesting set of serine proteases (SVSPs), the main forms of which were isolated and characterised. Two distinct groups were identified: trypsin-like acidic SVSPs and chymotrypsin-like basic SVSPs, each with different activities. The acidic SVSP is responsible for the gelatinase activity observed in the Nigerian venom, which is a novel form of activity for SVSPs. This regional diversity in venom protease activities is discussed with reference to the implications it will have in the development of therapeutic interventions.

1. Introduction

The puff adder (*Bitis arietans*) is found throughout much of the African continent and western Arabia and is considered to be one of the most medically important snakes in this region, accounting for a high proportion of the 200-300,000 annual envenomings in sub-Saharan Africa (Chippaux, 2011). Despite this, there have been too few studies on the clinical effects of puff adder bites. Most notable of these is that of Warrell et al. (1975) who recorded details of 10 bites from Nigerian snakes and more recently Tianyi et al (2024) carried out a thorough case-series of puff adder bites treated at two primary

healthcare facilities in Kenya, complemented with a scoping review of published cases of individual bites. Both studies found local and systemic clinical features of puff adder bites to be diverse, though inconsistent, and included pain, swelling, necrosis, haemorrhage, coagulopathy, thrombocytopaenia, fever, and hypotension/shock.

The protein content of puff adder venoms is typical of that of other vipers (Casewell et al., 2014; Currier et al., 2010; Fasoli et al., 2010) and the three most abundant protein classes, C-type lectin-like proteins (CLPs), snake venom metalloproteinases (SVMPs) and snake venom serine proteases (SVSPs) are highly likely to be those responsible for the bulk of the venom-induced pathologies. Owing to their well-documented effects on coagulation and platelet physiology (Arlinghaus and Eble, 2012; Morita, 2005), the CLPs will be partially responsible for two of the known systemic effects of puff adder envenomating: thrombocytopenia and coagulopathy. It is likely, however, that the SVMPs and SVSPs will also play a major role in coagulopathy and are undoubtedly responsible for other commonly observed hemotoxic effects such as spontaneous haemorrhage and hypotension. A haemorrhagin was previously isolated from puff adder venom (Mebs and Pan Holzer, 1982), but this was not fully characterised. Other studies have identified EDTA-inhibited haemorrhagic components in puff adder venom, but only in two cases has an SVMP with this activity been isolated. Thus, Van der Walt et al., (1972) purified and characterised a 21.4 kDa SVMP, and Omori-Satoh Ap et al., (1995) found two distinct haemorrhagic SVMPs with molecular weights of 68 and 75 kDa. Hypotension in patients with puff adder envenoming can be an indirect consequence of haemorrhage and also of the direct action of adenosine (Graham et al., 2005) and bradykinin potentiating peptides (Kodama et al., 2015) found in puff adder venom, but protease action is implicated here also. Thus, kallikrein-like (kinin-releasing) SVSPs have been isolated from puff adder venoms with a wide range of molecular weights, from 33 to 58 kDa, most likely due to differences in N-glycan content (Megale et al., 2018; Sekoguchi et al., 1986; Nikai et al., 1993) and there is also evidence that SVMPs in puff adder venom can directly generate the vasodilator angiotensin 1-7 (Paixão-Cavalcante et al., 2015). As well as their haemotoxic effects, these proteases are likely to be responsible for initiating the processes that lead to local tissue damage following puff adder bites, including swelling, blistering, inflammation and necrosis, the combined effects of which often result in severe pain. There have been no detailed studies of the puff adder proteins that might be responsible for this, however.

The abundance and variety of SVMPs and SVSPs in *B. arietans* venom has been confirmed by proteome analysis (Casewell et al., 2014; Fasoli et al., 2010). The latter study used 2D PAGE and highlighted the complex nature of the SVSP content, which is clearly a mixture of multiple proteo- and glycoforms. Along with the various studies on individual proteins, these proteomic studies have provided some insight into the toxicity of puff adder venom, but a complete picture of the protease complement, and its regional variations, is required to better understand the diverse pathological effects of the venom and identify therapeutic targets. Here, we describe the purification of, and the structural and functional characterisation of the SVMP and SVSP complement of three regional puff adder venoms. The study was prompted by our observation of differences in content and activities of venom from Nigerian and Tanzanian snakes (Dawson et al 2024). The bulk of the work described here focusses on the proteases

in venoms from these two regions, since transcriptomes were available, along with a wide stock of individual venoms, but it was extended to include proteases from Kenyan puff adders, which initial studies suggested were different again from that of Nigerian and Tanzanian snakes.

2. Materials and methods

2.1. Venom

Venoms were extracted from five Nigerian and four Tanzanian specimens of *B. arietans* maintained within the herpetarium at the Centre for Snakebite Research and Interventions at the Liverpool School of Tropical Medicine (LSTM). Wet venoms were lyophilised for long term storage at 2 – 8 °C. The Kenyan venoms were provided by Dr. George Omondi, Kenyan Snakebite Research and Intervention Centre, Institute of Primate Research, Nairobi, Kenya.

2.2. Reagents

All reagents used were of analytical reagent grade and, unless otherwise specified, were purchased from Merck Life Science, Watford, UK or Fisher Scientific, Loughborough, UK.

2.3. Protein purification

All chromatography was carried out using either an AKTA LC system (Cytiva) or a Vanquish HPLC system (Thermo Fisher). Chromatography buffers were freshly prepared and vacuum filtered (0.1 um) immediately prior to use.

2.3.1. Size exclusion chromatography of whole venom

The initial step in all isolation procedures was a separation of whole venom on a size exclusion chromatography (SEC) column. The buffers used for the SEC separation were chosen to facilitate the transfer to the second chromatography step (cation or anion exchange) without the need for buffer exchange. Thus, for the SVMP PIIs and SVSPs, 20 mg of freeze-dried venom was resuspended in 1.5 mL ice-cold PB5.2 (50 mM sodium phosphate pH 5.2) and centrifuged at 10,000 xg for 10 mins. The supernatant was immediately loaded onto a 120 mL column of Superdex 200HR equilibrated in PB5.2. The column was operated at a flow rate of 1.0 mL/min and 2 mL fractions were collected after the void volume. Elution was monitored at 214 and 280 nm. SDS-PAGE analysis and protease assays were carried out on all protein-containing fractions to determine which were to be chosen for the second stage of isolation. For isolation of the SVMP PIII from Kenyan venoms, the SEC procedure was carried out exactly as above, but using 50 mM Tris-Cl, pH 8.5 as the separation buffer.

2.3.2. SVMP PII purification

Using general protease assays, the bulk of the EDTA-inhibited proteolytic activities were found in the middle-eluting peaks of the SEC separations associated with prominent bands on SDS-PAGE at 23-35 kDa. These SVMP-containing fractions (2-3 mg of total protein) were applied to a 1 mL HiRes Capto S column equilibrated in PB5.2. Elution was carried using a 25-column volume (CV) gradient of 0 - 0.25 M NaCl in PB5.2. The flow rate was 0.6 mL/min. The unbound material was retained and 1 mL fractions

were collected from the start of the NaCl gradient. Elution was monitored at 214 and 280 nm. Protease assays were carried out on the main peaks and SDS-PAGE was used to determine purity**.**

2.3.3. Purification of SVMP PIII from Kenyan venom

The prominent 68 kDa protein visible in some Kenyan *B. arietans* venoms was found in the first peak following the SEC separation of this venom. The main SEC fractions containing this protein, already in 50 mM Tris-Cl, pH 8.5, were applied directly to a 1 mL Mono Q column equilibrated in the same buffer. The 68 kDa protein was eluted from the column using a 20 CV gradient of 0-0.3 M NaCl in 50 mM Tris-Cl, pH 8.5. The column was operated at 0.6 mL/min and 1.0 mL fractions were collected. Elution was monitored at 214 and 280 nm.

2.3.4. Serine protease purification

For all venoms, serine protease (SVSP) activity was found in the first main peak of the SEC separation, with bands visible on SDS-PAGE in the 50-60 kDa range (reducing conditions). These were pooled and applied to a 1 mL HiRes Capto S column equilibrated in PB5.2 and operated as for SVMP PII isolation, but with a 0 – 0.7 M NaCl gradient. The basic SVSPs bound to the column under these conditions and eluted as multiple peaks in the NaCl gradient. The acidic SVSPs, which flowed unbound through the column at this pH (5.2), were retained and immediately dialysed against 50 mM Tris-Cl, pH 8.5. These were then loaded onto a 1 mL Mono Q column equilibrated in the same buffer. The acidic SVSPs were eluted from the column using a 25 CV gradient of 0-0.4 M NaCl in 50 mM Tris-Cl, pH 8.5. The column was operated at 0.6 mL/min and 0.5 mL fractions were collected. Elution was monitored at 214 and 280 nm. The final purification step on RP-HPLC was performed using a Biobasic C4 column (2.1 x 150 mm, Thermo Fisher). The flow rate was 0.2 mL/min and proteins were separated in the following gradient of acetonitrile in 0.1% trifluoroacetic acid; 0-32%/5 mins; 32-44%/30 mins; 44-70%/2 mins. Elution was monitored at 214 nm.

2.3.5. *Analytical methods*

2.3.5.1 SDS-PAGE

Samples were prepared for reducing SDS-PAGE analysis by adding sample buffer to a final concentration of 2% SDS, 5% β-mercaptoethanol and then heating at 85°C for 5 mins. Electrophoresis was performed on 4-20% acrylamide gels (BioRad TGX) using a Tris-glycine buffer system, followed by staining with Coomassie Blue R250 or visualisation using a stain-free system. Non-reducing gels were performed by omitting the heat treatment. The SVMP PIII isolated from Kenyan *B. arietans* venom required a different method of preparation for non-reducing gels in order to retain its oligomeric structure: thus, NP-40 was added to a final concentration of 0.5% before adding sample buffer containing 0.5% (final) SDS. Molecular weights of pure proteins were calculated using a calibration curve prepared from PageRuler markers (Thermo Fisher) run on the same gel (4-20% TGX, BioRad).

2.3.5.2 Analytical SEC

For analysis of the Kenyan SVMP PIII, a 24 mL Superdex 200 SEC column was set up on an AKTA LC system (Cytiva) and equilibrated in PBS (25 mM sodium phosphate, 0.15 M NaCl, pH 7.2). The column was operated at a flow rate of 0.5 mL/min and elution was monitored at 280 nm. Fifty μL of sample was loaded. The column was calibrated by running 50 μL of BioRad SEC standard under the same conditions.

2.3.6. Deglycosylation of isolated proteins

In preparation for deglycosylation, a 20 μL sample was denatured by heating for 5 mins at 85°C following the addition of 2.0 μL 1% SDS and 0.5 μL 1 M DTT. After cooling to room temperature,1.7 μL of 10% NP-40 was added and then 0.5 μL PNGase F (at the supplied concentration) was added to start the reaction. Incubation was carried out at 42°C for 3 hours or overnight at room temperature. Where deglycosylation was carried out under native conditions for gelatin zymograms, the denaturing step was omitted.

2.3.7. General protease assays

2.3.7.1 Casein degradation with SDS-PAGE analysis

Venom or protein samples were incubated in TBSC (35 mM Tris-Cl, 0.5 M NaCl, 1 mM $CaCl_2$, pH 7.4) with beta casein at a ratio of 30:1 (w/w) casein:sample protein; using a final concentration of 1.0 mg/mL casein. Incubation was carried out at 37°C for 2 hours. SDS PAGE sample buffer was added (final concentration 2% SDS, 5% β-mercaptoethanol) to stop the reaction. This was then heated at 85°C for 5 mins. The extent of casein degradation was assessed using SDS-PAGE (see 2.3.5.1). In EDTA inhibition experiments, EDTA was added to the protein sample to a final concentration of 5 mM and incubated for 15 mins prior to the addition of casein to start the reaction. Where PMSF was used as an inhibitor, this was added to a final concentration of 2 mM from a 20 mM stock solution in freshly prepared in methanol. This pre-incubation was carried out for 60 mins (room temperature) prior to addition of casein.

2.3.7.2. Insulin B digestion with RP-HPLC analysis

Venom or protein samples were incubated in TBSC with insulin B chain at a ratio of 30:1 (w/w) insulin B:sample protein, using a final concentration of 0.4 mg/mL insulin B. Where inhibitors were used, these were pre-incubated with the protein sample as in section 2.3.7.1. Incubation was carried out at 37°C for 90 mins and the reaction was stopped by the addition of trifluoracetic acid (TFA) to 1%. An aliquot containing the equivalent of 1 μg insulin B was analysed by RP-HPLC using a Biobasic C4 column (2.1 x 150 mm). The flow rate was 0.3 mL/min and the separation was carried with the following gradient of acetonitrile in 0.1% trifluoroacetic acid; 0-36%/40 mins; 36-70%/3 mins. Elution was monitored at 214 and 280 nm.

2.3.7.3. Chromogenic substrate assays

Assays using the chromogenic substrates BAEE (N-alpha-benzoyl-L-arginine ethyl ester) and BTEE (N-benzoyl-L-tyrosine ethyl ester) were carried out in TBSC with the substrate at a concentration of 0.25 mM and the protease at a final concentration of 2-5 μg/mL. The reaction was followed in quartz cuvettes at 253 nm (BAEE) or 256 nm (BTEE). Where nitroanilide esters (N-succinyl-A-A-A-p-NA, N-methoxysuccinyl-A-A-P-V p-NA, N-succinyl-A-A-P-L p-NA, N-succinyl-A-A-P-F p-NA) were used and at final concentrations, the substrates were 0.2 mM in TBSC, the proteases 2-5 μg/mL. The reaction was followed at 410 nm. In all cases specific activity was calculated as μmol product formed per minute per mg protease. The mM extinction coefficients used for this calculation were BAEE, 1.070 (253 nm); BTEE, 0.964 (256 nm) and for all the nitroanilides, 8.800 (410 nm).

2.3.7.4 Fluorogenic substrate assay

This assay, which kinetically measures the cleavage of a quenched fluorogenic substrate (ES010, R&D Biosystems) by metalloproteases was performed as previously described (Albulescu et al., 2020). Reactions were set up in a 384-well plate (Greiner) and consisted of 15 μL of pure SVMP PII isoforms at 0.05 – 0.2 mg/mL in PBS, and 75 μL of substrate [final concentration 10 μM] and were run in triplicate. Data was collected on a Clariostar (BMG Labtech) instrument at an excitation wavelength of 320 nm and emission wavelength of 405 nm at 25 °C for 1 h. The slope of the reaction between 0-2 min was calculated for each sample, the average background slope (PBS-only samples) was then subtracted, and specific activity expressed as ΔFluorescence/ time(min) /pmole protein.

2.3.8. Functional protease assays

2.3.8.1. Gelatin zymogram assay

The gels (10% acrylamide) for this assay were prepared using the method of Laemlli (1970) with the adaptations of Fling and Gregerson (1986). Gelatin was prepared by briefly heating a 20 mg/mL solution at 50°C, then adding this to the gel mix immediately prior to casting, such that the final gelatin concentration was 2 mg/mL (0.2% w/w). Where inhibitors were tested, these were pre-incubated with the protein sample as in section 2.3.7.1. Venom or protein samples were then prepared for the zymogram assay by adding standard SDS-PAGE sample buffer but with no reductant and a final SDS concentration of 1.6% (w/v). These were not heated. Following electrophoresis, the method of (Toth et al., 2012) was used to visualise gelatinase activity. Thus, the gel was rinsed, with shaking, in 2.5% (v/v) Triton X-100 for 30 mins, then washed (3 x 10 mins) with distilled water to remove the detergent. The buffer used for development was 50 mM Tris-Cl, 200 mM NaCl, 5 mM $CaCl_2$, 0.02 % (v/v) Brij 100, pH 7.8. The gel was washed for 10 mins in this buffer, then this was replaced with fresh development buffer and incubated overnight at 37°C. To visualise gelatin degradation zones, the gel was stained for 60 mins with Coomassie Blue R250 and destained until clear bands were visible against a blue background.

2.3.8.2. Digestion of basement membrane proteins

Basement membrane material (Geltrex, Thermo Fisher) was diluted with PBS to 10% of its supplied concentration (10-18 mg/mL) and stored in aliquots at -20°C. For the assay, a fresh aliquot was carefully thawed out and immediately placed on ice. Digestions were set up with basement membrane material:protein sample at 30:1 (w/w) and performed at 37°C for various time periods between 2 mins and 4 hours. Following this, SDS-PAGE sample buffer was added and the sample heated at 85°C to stop the reaction. The extent of basement membrane protein degradation was then assessed using SDS-PAGE (see section 2.3.5.1).

2.3.8.3. Digestion of plasma proteins (prothrombin and fibrinogen)

In both the prothrombin and fibrinogen degradation assays, these two substrate proteins were used in the assay at a final concentration of 1.0 mg/mL in TBSC and venom/pure protein samples were added at a ratio of 30:1 substrate:protein sample to start the reaction. Incubation was carried out at 37°C for 60 mins. Following this, SDS-PAGE sample buffer was added, the sample heated at 85°C to stop the reaction and the extent of substrate protein degradation was then assessed using SDS-PAGE (see section 2.3.5.1). An aliquot equivalent to 1.0 μg of the substrate protein was loaded per lane of the gel.

2.3.9. Trypsin digestion and MS/MS analysis.

In preparation for this, the relevant proteins were desalted on a RP-HPLC column, as above. These were then dried in a centrifugal evaporator, 20 μL H_2O was added and then re-dried. These proteins were resuspended in 8 M urea/0.1 M Tris-Cl (pH 8.5), reduced with 5 mM TCEP (tris (2-carboxyethyl) phosphine) for 20 minutes and alkylated with 50 mM 2-chloroacetamide for 15 minutes in the dark at room temperature. Samples were diluted 4-fold with 100 mM Tris-Cl (pH 8.5) and digested with trypsin at an enzyme/substrate ratio of 1:20 overnight at 37°C. The reaction was terminated by addition of formic acid (FA), and digested peptides were loaded on to Evotips and analysed directly using an Evosep One liquid chromatography system (Evosep Biosystems, Denmark) coupled with timsTOF SCP-mass spectrometer (Bruker, Germany). Peptides were separated on a 75 µm i.d. × 15 cm separation column packed with 1.9 µm C18 beads (Evosep Biosystems, Denmark) and over a predetermined 44-minute gradient. Buffer A was 0.1% FA in water and buffer B was 0.1% FA in acetonitrile. Instrument control and data acquisition were performed using Compass Hystar (version 6.0) with the timsTOF SCP operating in data-dependent acquisition mode.

Fragmentation spectra were searched against an in-house *B. arietans* venom gland derived protein sequence database sourced from Nigerian and Tanzanian specimens (see Dawson et al. 2024) using Mascot (Perkins et al., 1999). Reverse decoys and contaminants were included in the search database. Cysteine carbamidomethylation was selected as a fixed modification, oxidation of methionine was selected as a variable modification. The precursor-ion mass tolerance and fragment-ion mass tolerance were set at 10 ppm and 0.04 Da, respectively, and up to 2 missed tryptic cleavages were allowed. Mascot files were parsed into Scaffold (version 5.0.1, Proteome Software, Inc.) for validation at a protein-level false discovery rate (FDR) of < 1%.

3. Results

3.1. Comparison of the regional B. arietans venoms.

Electrophoretic analysis of Nigerian (NGA) and Tanzanian (TZA) venoms from snakes held at LSTM and Kenyan (KEN) venoms supplied by K-SRIC showed distinct differences in their protein profile, particularly the prominent bands in an otherwise clear 20 – 40 kDa region on the gel (Fig. 1). TZA venoms possessed a sharp band at 23 kDa, whereas KEN and NGA venoms had, instead, bands at 31 and 36 kDa respectively. In the higher molecular weight region of the gel, there was a lower level of the multiple protein bands between 50 and 60 kDa in TZA compared with the others. Also in this region, a very prominent protein at 68 kDa was seen in some of the KEN venoms (visible here in KEN015), including one captive-bred snake held at LSTM (not shown), which was not seen in the TZA and NGA venoms. These patterns were consistently observed in venoms from a wider selection of animals, although the intensity of the 34 kDa protein band in NGA venoms did vary greatly between individuals, some having none at all. Two individuals from each region were chosen for the analysis shown in Fig. 1 and for use in the subsequent assays of whole venoms.

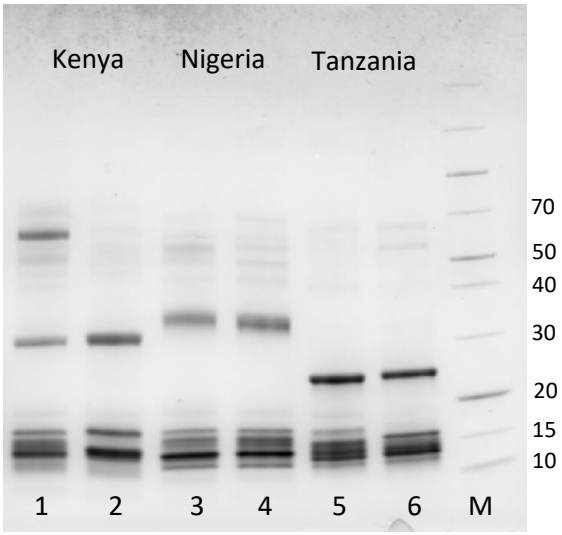


Fig. 1. SDS-PAGE analysis of the *B. arietans* venoms used in this study. The gel is 4-20% acrylamide (BioRad) and was run under reducing conditions. Eight μg of whole venom was loaded per lane and the gel and was stained using Coomassie Blue R250. The molecular weights of the markers (lane M, Thermo Page Ruler) are indicated in kDa. The venoms shown are 1, 2: KEN 015 and 042; 3, 4: NGA 011 and 016; 5, 6 TZA 005 and 006.

Suspecting that these protein differences lie in the SVMP and SVSP composition of the venoms (CLPs, the other main proteins of *B. arietans* are found in the multiple bands visible in the 12-17 kDa region), the venoms were tested in various assays aimed at determining differences in the abilities of the six chosen venoms to degrade proteins that may be of functional and pathological significance. Pronounced differences were observed in their abilities to degrade the key coagulation cascade proteins, fibrinogen and prothrombin (Figs. 2A and 2B resp.). All three regional venoms were able to quickly degrade both alpha and beta fibrinogen to some extent. The TZA venoms were particularly

destructive, however, and one of them (TZA006) even caused significant degradation of gamma fibrinogen in the short time period used here (60 min). Regional differences in the digestive power of the venoms against plasma proteins was more pronounced with prothrombin. NGA and KEN venoms had little effect on prothrombin, and this was still the case after longer incubation periods (4 hours, not shown). The venoms from both TZA snakes were highly destructive, however, and no intact prothrombin remained after a 60-minute incubation. A faint band evident at approximately 32 kDa suggests that the TZA venoms may have generated some thrombin as a result of protease action, but it was not possible to clarify this using non-reducing SDS-PAGE to determine levels of intact thrombin. Thus, this is clearly not a highly specific conversion of prothrombin to thrombin, such as can be seen with *Echis* venoms (Morita et al., 1976,Yamada et al., 1996) but rather a general degradation of prothrombin which may generate a very low level of thrombin.

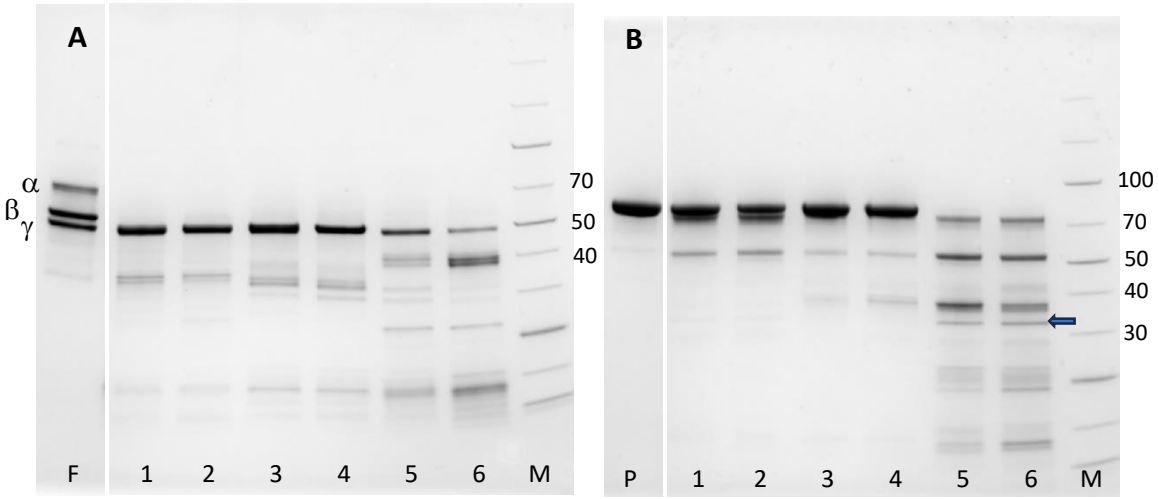


Fig. 2. Degradation of fibrinogen and prothrombin by *B. arietans* venoms. Fibrinogen (gel A) or prothrombin (gel B) were incubated at 37°C for 60 minutes with whole venoms at a 30:1 (w/w) ratio of substrate:venom. The gel was run under reducing conditions and the equivalent of 1.0 μg of substrate protein (prothrombin, fibrinogen) was loaded per lane. The gel is 4-20% acrylamide (BioRad) and was stained using Coomassie Blue R250. F is fibrinogen control (with α, β and γ bands labelled) and P is the same for prothrombin. The arrow on gel B indicates the expected position of B-thrombin. Lanes 1, 2: KEN 015 and 042; lanes 3, 4: NGA 011 and 016; lanes 5, 6 TZA 005 and 006. The molecular weights of key markers (lane M, Thermo Page Ruler) are indicated in kDa.

Two further gel-based protein degradation assays were used to determine how the venoms might act against other tissues that can be the targets of snakebite. Using a similar assay method to that used for the plasma proteins, a basement membrane protein extract (Geltrex) was incubated with the venoms (Fig. 3). Once again, the TZA venom proved to be particularly potent; in the case of TZA006, virtually no intact laminin remained after just a short incubation period (30 mins). The KEN venoms also degraded alpha laminin. In both cases the beta laminin band was degraded less than the alpha. Minor bands in the 100-150 kDa region, most likely nidogens and collagen IV (Escalante et al., 2006), disappeared after treatment with TZA and KEN venoms. In stark contrast to this, the NGA venoms (lanes 3 and 4) appeared to be wholly inactive against these basement membrane proteins.

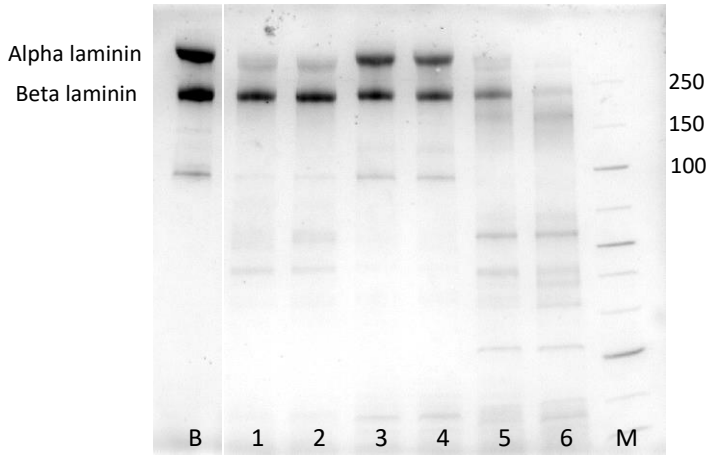


Fig. 3. Degradation of basement membrane proteins by *B. arietans* venoms. A basement membrane protein extract (Geltrex) was incubated at 37°C for 30 minutes with whole venoms at a 30:1 (w/w) ratio of substrate:venom. The gel was run under reducing conditions and the equivalent of 8 µg of substrate protein was loaded per lane. The gel is 4-20% acrylamide (BioRad) and was stained using Coomassie Blue R250. B is basement membrane protein control. Lanes 1, 2: KEN 015 and 042; lanes 3, 4: NGA 011 and 016; lanes 5, 6: TZA 005 and 006. The molecular weights of key markers (lane M, Thermo Page Ruler) are indicated in kDa.

Gelatin zymogram assays were also carried out on the venoms, and once again pronounced differences were observed between the three regional venoms (Fig. 4A). The NGA venoms contained the most gelatinase activity, with a strong band showing at around 55-60 kDa which was most active in NGA011. No such activity was observed in any TZA venoms, not just the two shown here, even at high loading levels. The result for the KEN venoms was very different, however, with a sharp band of activity at around 140 kDa. Using a higher protein loading, this band could be seen in other KEN venoms, such as KEN042 shown here, but was very prominent in KEN015 (Fig. 4A, lane 1). Because this particular assay format is able to detect the proteolytic action of specific proteins separated during the electrophoresis step, it was used to measure the effects of protease inhibitors and therefore determine the class of the protease responsible for the activity. EDTA or PMSF, inhibitors of SVMPs and SVSPs, respectively, were incubated with NGA and KEN venoms (Fig 4B). The activity in NGA was inhibited by PMSF, but not EDTA, whereas the opposite was the case in the KEN015 venom. Thus, the gelatin-degrading activity in the NGA venoms is due to a 55-60 kDa serine protease, whereas in the KEN venoms it is due to a 140 kDa metalloprotease.

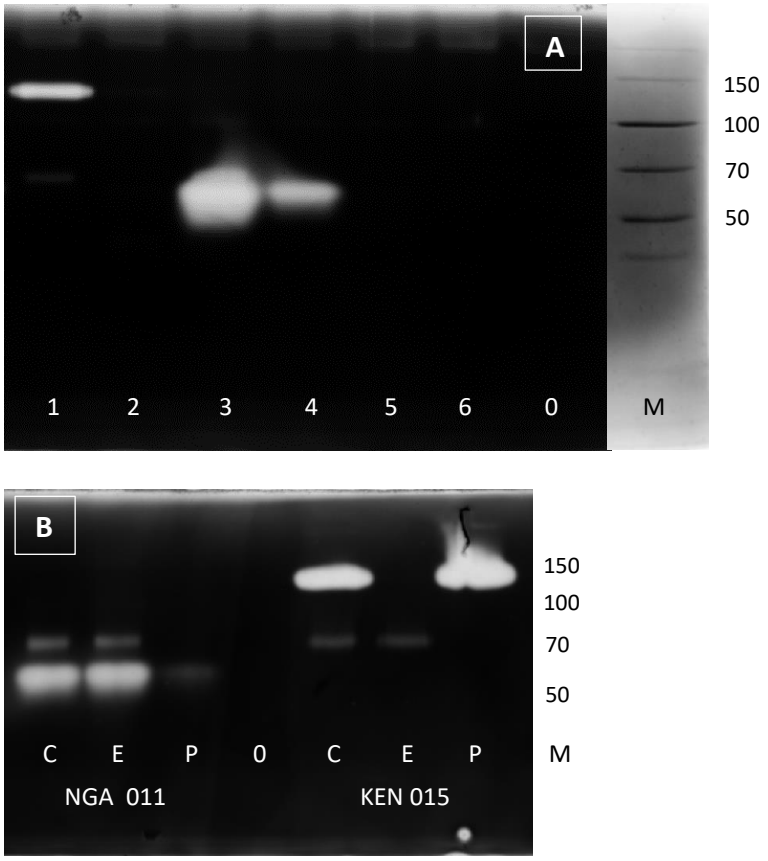


Fig. 4. Gelatinase activity (gelatin zymogram) of the *B. arietans* venoms. The zymograms were prepared with 0.2% (w/w) gelatin in a 10% acrylamide SDS-PAGE gel. The gel was run under non-reducing conditions and 2 µg of venom was loaded per lane. The zymograms were then incubated as in section 2.3.8.1 and then visualised by staining with Coomassie Blue R250. **A:** Zymogram of venoms Lanes 1, 2: KEN 015 and 042; lanes 2, 3: NGA 011 and 016; lanes 5, 6: TZA 005 and 006; lane 0: control (no venom). The molecular weights markers (lane M, Thermo Page Ruler, indicated in kDa,) were run under reducing conditions and visualised after fully destaining the gel. **B:** Zymogram of NGA011 and KEN015 venoms treated with protease inhibitors. The venoms were pre-incubated with either EDTA or PMSF as described in the main text and then run as for zymogram A. Lanes C, controls (un-inhibited venoms); E, EDTA-treated venoms; P, PMSF-treated venoms; 0, no venom; M, markers as in zymogram A

3.2. Isolation and biochemical characterisation of the SVMPs

3.2.1. Isolation of the low molecular weight SVMPs in Tanzanian, Nigerian and Kenyan venoms.

The suspected small SVMPs observed at 20-40 kDa on SDS PAGE of the various venoms were purified using SEC followed by cation exchange chromatography. A typical trace for the SEC separation of the TZA venoms can be seen in Fig. 1S. The 23 kDa band, seen in Fig 1, lanes 5 and 6, was found in the last of the 5 protein-containing peaks (1-5) at elution volume 92-95 mL. Further analysis (results not shown) revealed that peak 7 is adenosine and peak 6 contains various unidentified small molecules. The 23 kDa protein was fairly pure at this stage but was further purified using cation exchange

chromatography (Fig. 2S). The protein eluted in two peaks, the second of which contained the greater amount of the protein. SDS-PAGE analysis of the pure protein can be seen in Fig. 5, lane 4.

Fig 3S shows a typical SEC separation of NGA venom, quite different in profile to the TZA venoms. The 36 kDa NGA protein was found in the first half of the split peak 3 and so the proteins in these fractions were subject to cation exchange chromatography (Fig 4S). The 36 kDa protein was found to be pure in both of the prominent peaks eluting at 11-17 mL and the irregular peak shapes suggest it exists in multiple forms. SDS-PAGE analysis of the protein in peak 2 can be seen in Fig. 5, lane 1.

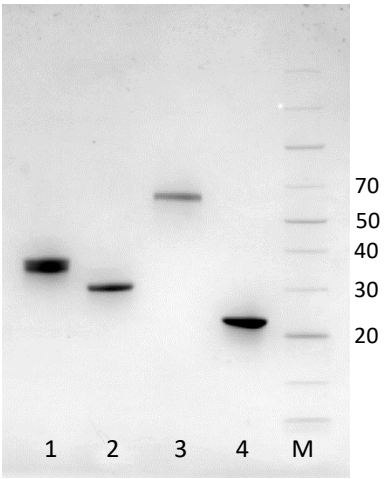


Fig. 5. SDS-PAGE analysis of the purified *B. arietans* SVMPs. The gel used was 4-20% acrylamide (BioRad) and was stained with Coomassie Blue R250. Lane 1, NGA SVMP PII; lane 2, KEN SVMP; lane 3, KEN SVMP PIII; lane 4 TZA SVMP PII; lane M, molecular weight markers (Thermo Page Ruler), indicated in kDa.

Using the casein and insulin B assays carried out in the presence or absence of EDTA, both proteins were confirmed to be SVMPs (Fig 5S and 6S). Although both the NGA and TZA SVMPs were able to fully digest insulin B chain within 90 mins, the patterns of digestion for the two are different suggesting that they have different peptide bond specificities. The NGA SVMP appears to have cleaved at just two sites, generating three products, whereas 6-7 peptides have been produced by the TZA SVMP. As will be seen in subsequent assays, the TZA SVMP is a more a potent protease than the NGA SVMP.

Both proteins were subjected to de-glycosylation with PNGase F which determined the TZA 23 kDa SVMP to be non-glycosylated (Fig. 7S). The NGA 36 kDa SVMP shifted in molecular weight by 9-10 kDa, which suggest the presence of 3 N-glycan (assuming 2.5-3 kDa per glycan), but a time-course de-glycosylation indicated only one intermediate (result not shown) and that it therefore possesses 2 N-glycans. Following de-glycosylation, the NGA SVMP had a similar molecular weight to the TZA SVMP. MS/MS analysis of the proteins purified from TZA and NGA determined that they are both SVMP PIIs. The two sequences to which these matched, found in transcriptomes from the respective snakes, are shown in Fig. 6. No disintegrin peptides were found and the two proteins match with the predicted

molecular weight of the metalloprotease domain of the SVMP PIIs. They can thus be classified as SVMP PII-a in the scheme devised by Fox and Serrano (2008). The number of predicted N-glycan sites for each protein matched to that found experimentally.

23 kDa protein from TZA venom:

TZA_Contigs 195 and 843||Toxin25512_SVMPII

SEQYRSSDPNIYFNVIVVVDNRMVNYYRGELNKITTKVHQYFNILNEMYRYFYIHPILVGIEIWSN
GDLCTVTTSASDTLHSFAEWRERDLLSRKKHDNAQLFTGTEFNGGIIGIAYVGTMCDVKRSASVVM
NYQPEIDRSVAAIMAHEMGHNLGIRHDGNQCYCGADACIMAPHISNPPPMYFSDCSWNDYNRFIST
HNPQCFRNNPSRTVIV

36 kDa protein from NGA venom:

NGA_Contigs 386 and 2346||Toxin24915_SVMPII

SEQHRSCDLNRDLKIMVVADHRLVNYYKGELEELKKKIFQVFNMLKEIFRYLYIRPILVGIEIWSN
GDLCTVTNSAYDTLRSFAEWRDRDLRQRKKHDNAQLFTGTKFNGGIIGKAYIGTICDLKLSSGVVM
NYRPEIDSAAAAIMAHEIGHNLGINHDGNQCYCYGYSCIMAAQISDPPPKYFS**NCS**WNEYNKFINT
DKAQCLC**NET**SRTVIV

Fig. 6. Amino acid sequences of the transcripts matching to the SVMPs isolated from TZA and NGA venoms. Ten μg of the pure proteins were digested with trypsin and the resulting peptide mix was analysed by LC-MS/MS (Sec. 2.3.9). Fragmentation spectra were searched against an in-house *B. arietans* (NGA and TZA) venom gland derived protein sequence database using Mascot. Predicted N-glycan sites in the NGA sequence are indicated in bold font.

Since these two SVMPs are likely to be therapeutic targets for managing envenoming by *B. arietans*, their activity was measured against a fluorogenic peptide MMP substrate routinely used in our laboratory for high-throughput drug discovery work. The TZA SVMP PII-a was found to have a much greater specific activity than the NGA form under the conditions used: 2290 +/- 211 units (ΔFluorescence/min/pmole) compared with 148 +/- 1.2 units respectively. This difference in protease activity is mirrored in the functional assays carried out on the proteins (see Sec. 3.4).

Using the same isolation protocol as that used for the NGA and TZA SVMP PII-a forms, the equivalent protein was purified from KEN venom (chromatography results not shown) which runs at 31 kDa on SDS-PAGE (Fig.5, lane 2). This was confirmed to be a metalloprotease using casein and insulin B degradation assays (Fig. 5S and 6S). The two-peptide insulin B degradation pattern suggests a narrow specificity, cleaving at just one position, one that appears to overlap with NGA SVMP PII based on the co-elution of its two peptide products with two of the three in the latter. De-glycosylation with PNGase F caused a mobility shift on SDS-PAGE (2-3 kDa) that would be expect if it possessed just one N-glycan (see Fig. 7S). No transcript information was available for the KEN *B. arietans* at the time of writing, so it was not possible to match this to a specific SVMP sequence.

3.2.2. Isolation of the high molecular weight SVMP from Kenyan venom

SEC of the KEN015 venom resulted in a large peak eluting at the start of the separation (Fig. 8S). This contained the 68 kDa protein visible on SDS-PAGE of whole venom (see Fig. 1). The fractions containing this protein were subject to anion exchange chromatography resulting in two peaks (Fig. 9S), the first of which contained the 68 kDa protein at a high degree of purity (Fig. 5, lane 3 and Fig. 7, lane 1). The second peak contained a protein with the α/β subunit pattern expected of a CLP, presumably a high molecular weight form (e.g. tetrameric) as found in other vipers (Arlinghaus and Eble, 2012). The 68 kDa protein was determined to be an SVMP using a casein assay in the presence or absence of EDTA (Fig. 6S); it is presumably an SVMP PIII because of its molecular weight. Unlike the SVMP PII-a, this SVMP PIII was unable to cleave any bonds in insulin B (Fig 5S). Because of its elution position on SEC and suspecting it to be responsible for the 140 kDa gelatinase activity in the KEN015 venom, it was expected to be a dimer (an SVMP PIII-c) but upon analysis using SDS-PAGE under non-reducing conditions (Fig. 7) the protein still ran at 68 kDa, even when using a low SDS concentration (0.5% final). If, however, the protein was treated with the non-ionic detergent NP-40 prior to the addition of a low % SDS non-reducing sample buffer, then it remained native and ran at 140 kDa (Fig. 7, lane 4). Its native molecular weight was also confirmed using analytical SEC in PBS, where it eluted with a molecular weight of 170 kDa, though a small proportion has clearly broken down into monomeric form, eluting at 70 kDa (Fig. 8).

PNGase F treatment showed the KEN SVMP PIII was likely to possess 3 N-glycans, based on the 9-10 kDa shift in molecular weight that was observed (Fig. 7S). This was confirmed by de-glycosylating under native conditions: 1- and 2-glycan intermediate bands where observed (result not shown).

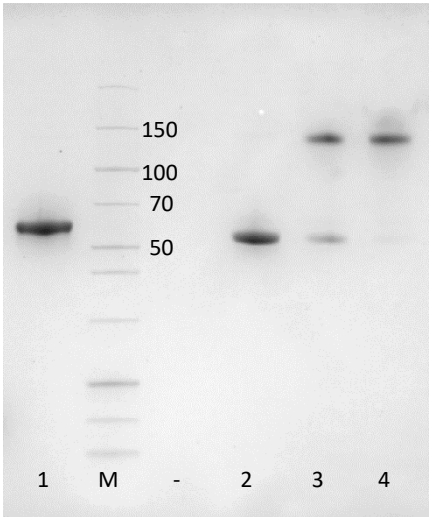


Fig 7. Effect of non-ionic detergent on the oligomeric structure of the KEN SVMP PIII. SDS-PAGE of KEN SVMP PIII run under various conditions. Lane 1, standard reducing conditions; lane M, molecular weight markers (Thermo PageRuler with molecular weights indicated in kDa), lane 2 non-reducing conditions (0.5% SDS), lane 3 as lane 2 but with 0.25% NP40 added after the SDS,

lane 4, as lane 2 but with 0.25% NP40 added prior to the SDS. The gel used was a 4-20% acrylamide (BioRad) and was stained with Coomassie Blue R250.

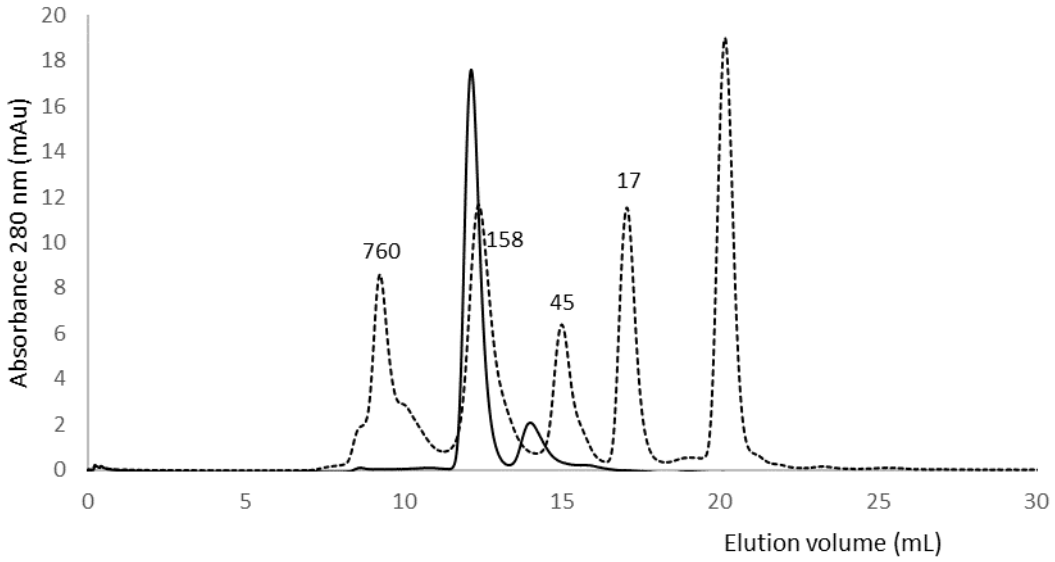
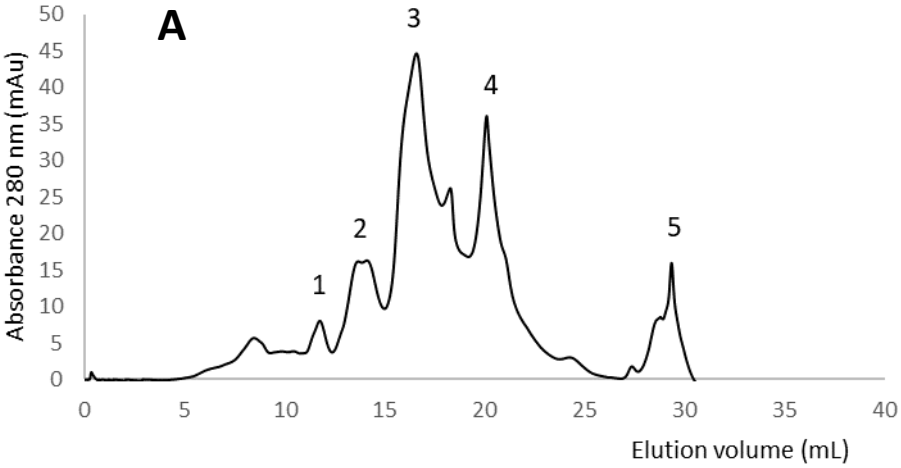


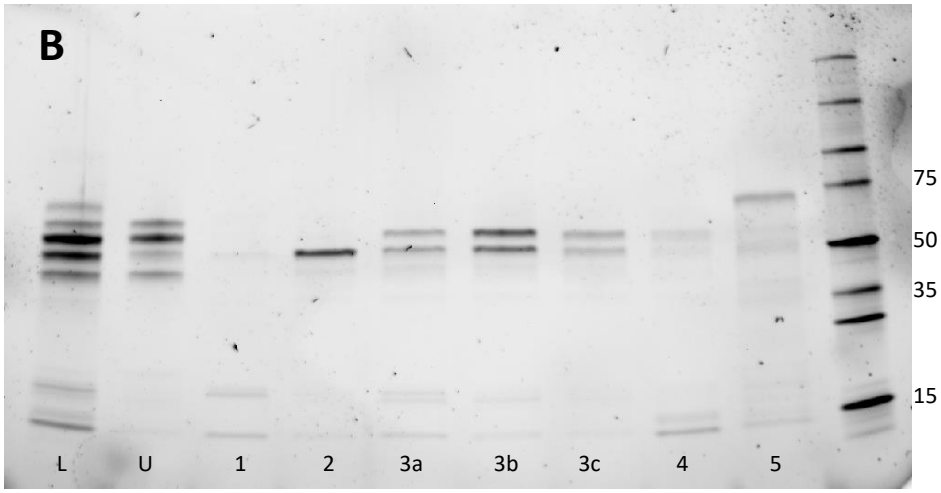
Fig. 8. Size exclusion chromatography analysis of KEN SVMP PIII. Analysis was carried out using a 24 mL Superdex 200 SEC column operating at 0.5 mL/min in 25 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Elution was monitored at 280 nm. Fifty µL (20 µg) of sample was loaded. Solid line, pure KEN SVMP PIII (peak 1 in Fig. 9S); dashed line, SEC standards (BioRad: ferritin, IgG, ovalbumin, myoglobin, cyanocobalamin) with native molecular weights (kDa) of the proteins indicated above the respective peak.

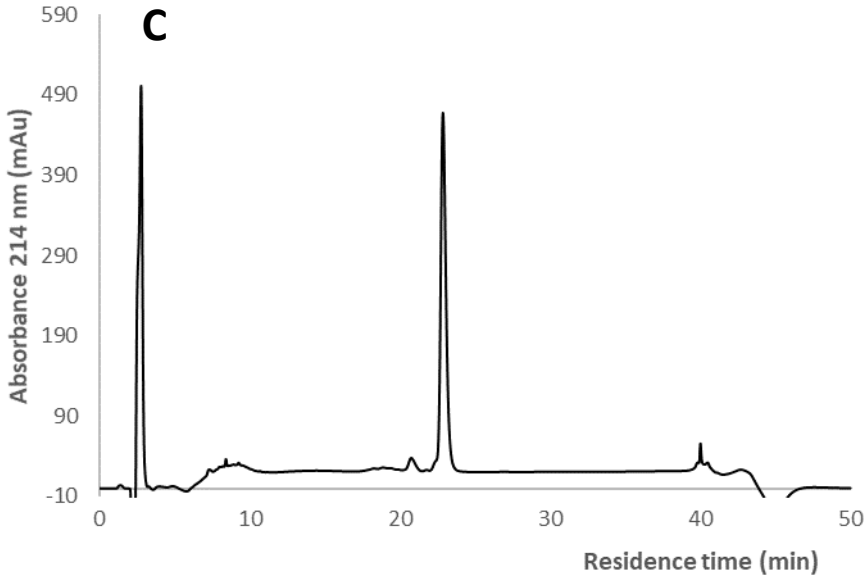
3.3. Isolation and biochemical characterisation of the serine proteases (SVSPs)

In NGA and TZA venoms, the second peak eluting from SEC (Figs. 1S and 3S, peak 2) contained multiple proteins in the 50-65 kDa size range and was rich in protease activity. Neither EDTA or PMSF could fully inhibit this activity, indicating that there are both SVSPs and SVMPs in this peak. Of the two key venoms, TZA and NGA, the latter had the greater number of proteins in this peak (this is also evident in the 40-65 kDa range on SDS-PAGE, see Fig. 1) as well as in the protease activity found here. The SVSP gelatinase activity observed in the whole NGA venom (Fig. 4) was also found here, so this venom was used to develop isolation methods for the individual proteases in the 50-65 kDa size range. The material from this peak was applied to a cation exchange chromatography column (see Sec. 2.3.4). This resulted in a large amount of material passing through unbound as well as material which bound and then eluted in the NaCl gradient in multiple peaks (Fig. 9A). Peaks 2 and 3 were found to contain serine protease activity, inhibited by PMSF but not EDTA, using both casein and insulin B degradation assays (Fig. 10S and 11S for the results for peak 3). Peak 2 contained a single 52 kDa protein and peak 3 contained proteins at 52 and 56 kDa (Fig. 9B). RP-HPLC was used to separate the individual SVSPs and, following removal of the solvent and resuspension in PBS, their protease activity was found to have been retained. This RP-HPLC step enabled a full separation of the two proteins in

cation exchange chromatography peak 3 (Fig. 9D, peak 1 is 56 kDa; peak 2 is 52 kDa). These two proteins and that from peak 2 will be referred to henceforth as basic SVSPs.







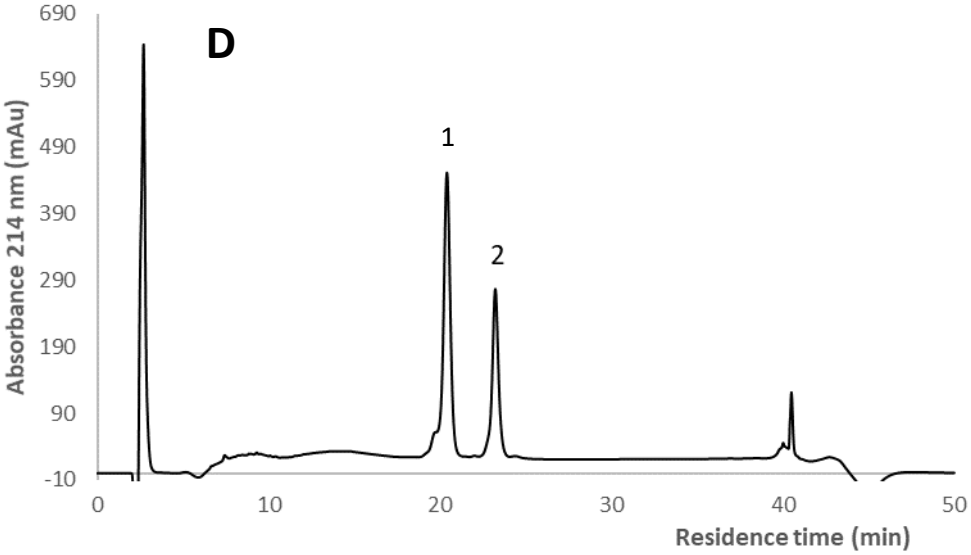
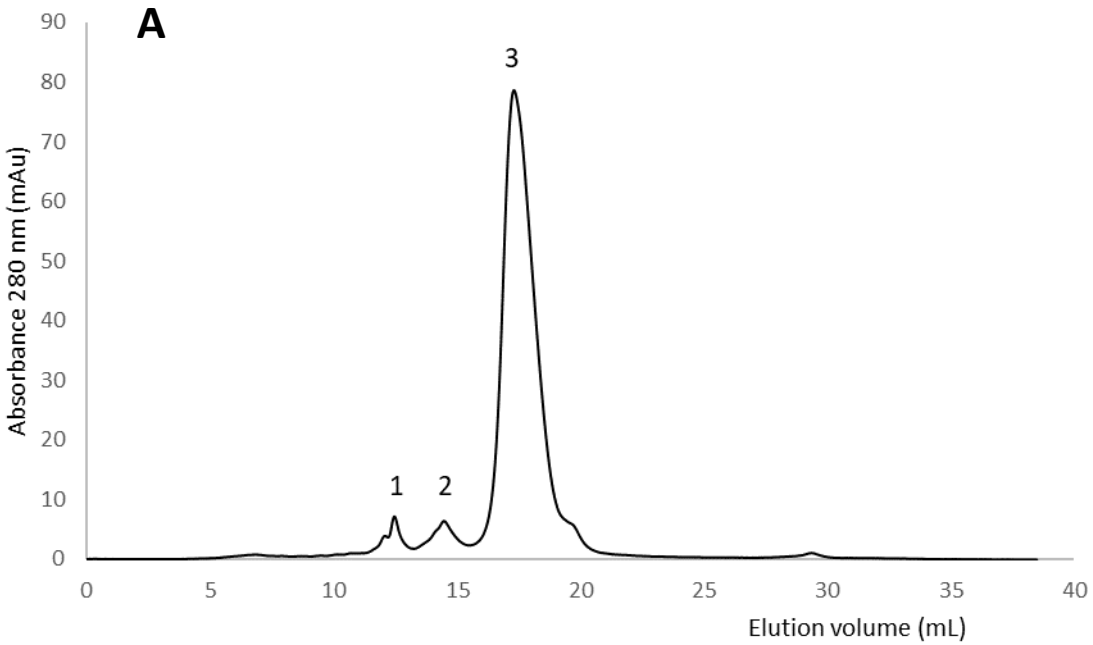


Fig. 9. Purification of the basic serine proteases from NGA SEC peak 2. **A:** The proteins in peak 2 from SEC (Fig. 3S) were applied to a 1 mL HiRes Capto S column equilibrated in 50 mM sodium phosphate pH 5.2. Elution was carried using a gradient of 0 - 0.7 M NaCl and monitored at 280 nm. The proteins were loaded onto the column independently of the program used to perform the chromatography, so the peak of unbound protein used for the subsequent anion exchange chromatography (Fig. 10) is not visible here**. B:** SDS-PAGE of the main fractions from the cation exchange run. L, load; U, unbound proteins; 1-5 fractions from the respective chromatography peaks (peak 3 was run as three fractions a-c); M, markers (Promega Broad Range), molecular weights of key markers are indicated in kDa. The gel was 4-20% acrylamide, stain-free (BioRad). **C** and **D:** C4 RP-HPLC (see Sec. 2.3.4) of the main proteins from cation exchange chromatography, C, peak 2; D, peak 3b.

The proteins that did not bind to the cation exchange column (see lane U, Fig. 9B) also possessed SVSP activity. These were dialysed against 50 mM Tris-Cl, pH 8.5 and subjected to anion exchange chromatography. The bulk of the bound protein eluted in one main peak (peak 3, Fig. 10) which contained strong bands at 58 and 62 kDa. An earlier-eluting peak (1) contained a band at 43 kDa. Using a casein assay, the proteins in peak 3 were shown to contain only serine protease activity, inhibited by PMSF but not EDTA (Fig. 10S). They were not as active towards casein as were the basic SVSPs and, in stark contrast to the latter, the acidic SVSPs were unable to cleave any of the peptide bonds of insulin B (Fig. 11S). The proteins in peaks 2 and 3 were subject to RP-HPLC (Fig. 10C) and resulted in a good separation of the two proteins in cation exchange chromatography peak 3 (Fig. 10C, peak 1 is 60 kDa; peak 2 is 56 kDa). As in the case of the basic SVSPs, both retained their protease activity following this final isolation step.


A
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Elution volume (mL)
1
2
3
0
10
20
30
40
50
60
70
80
90
0
5
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25
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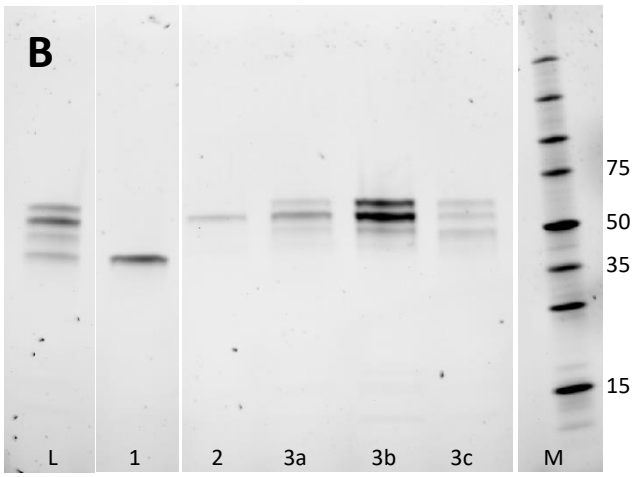
B
75
50
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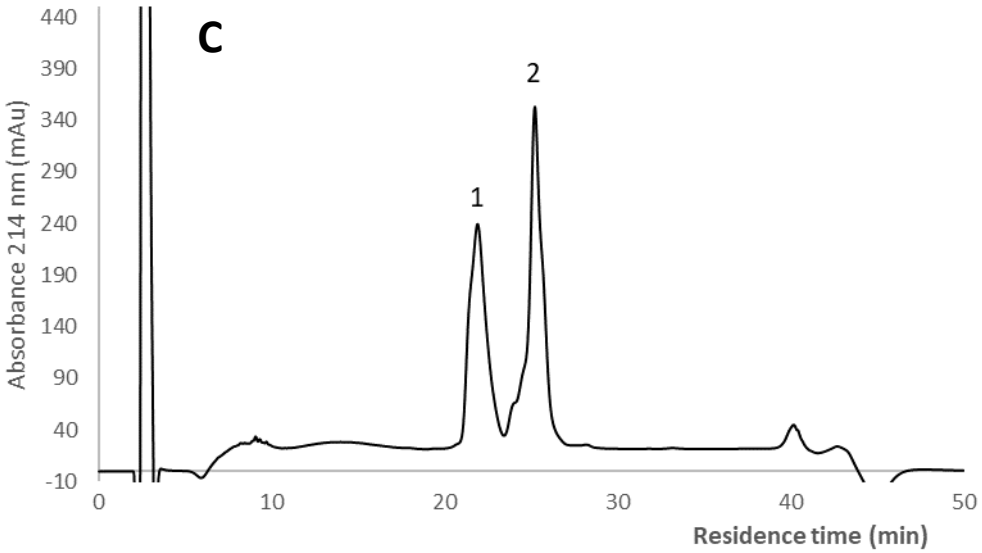
C
Absorbance 214 nm (mAu)
Residence time (min)
1
2
440
390
340
290
240
190
140
90
40
-10
0
10
20
30
40
50

Fig. 10. Purification of the acidic serine proteases from NGA SEC peak 2. **A**: The unbound material from the cation exchange step (Fig. 9) was dialysed against 50 mM Tris-Cl, pH 8.5 and loaded onto a 1 mL Mono Q column equilibrated in the same buffer. The acidic SVSPs were eluted from the column using a gradient of 0-0.4 M NaCl and elution was monitored at 280 nm. **B**: SDS-PAGE of the main fractions from the cation exchange run. L, load; 1-3, fractions from the relevant chromatography peaks (peak 3 run as three fractions a-c); M, markers (Promega Broad Range), molecular weights of key markers are indicated in kDa. The gel was 4-20% acrylamide, stain-free (BioRad). **C:** C4 RP-HPLC (see Sec. 2.3.4) of the main protein peak (3b) from cation exchange chromatography.

PNGase F treatment was used to determine the number of N-glycans on the basic and acidic SVSPs (Fig. 12S). The proteins in the main ion exchange peaks were used for this analysis. In both cases, glycan cleavage was incomplete, but as a consequence allowed visualisation of the intermediates with the full range of N-glycans. For both proteins, the lowest molecular weight band was around 24 kDa, a size that would be predicted from the sequence (see Discussion). From there upwards (increasing molecular weight) and compiling the results from all 4 lanes on the gel, bands can be seen in 2-3 kDa steps which would account for the protein chain plus 1 (just above the PNGase F band at 33 kDa), 2, 3, 4 (alongside the 50 kDa marker), 5 and 6 N-glycans. This would suggest that the two bands in the basic SVSP peak have 4 (the 52 kDa form) and 5 (the 56 kDa form) N-glycans, and that of acidic SVSPs, 5 (the 56 kDa form) and 6 (the 60 kDa form) N-glycans.

A set of chromogenic esterase substrates were used to determine the substrate site (P_I) specificities of the purified serine proteases (Table 1). The acidic SVSPs showed activity against just the R-containing BAEE substrate, confirming them to be trypsin-like SVSPs. The basic SVSPs had no activity against BAEE, nor towards the BTEE substrate traditionally used to determine chymotrypsin-like SVSP activity. Using a set of nitroanilide esters with V, L, A and F in the key P_I site, the basic SVSPs showed a small but definite activity towards that containing F (N-succinyl-A-A-P-F-pNA).

Table. 1. Determination of the primary specificity of NGA SVSPs

	Specific activity (µmol product formed/min/mg SVSP)					
	Ethyl esters		**Nitroanilide esters**			
Enzyme	***BAEE***	***BTEE***	***A***	***L***	***V***	***F***
Trypsin	36.5	ND	ND	ND	ND	0
Chymotrypsin	ND	27.3	0	1.85	0	12.2
Elastase	ND	ND	2.50	8.56	1.83	0
Acidic SVSP	7.13	0	0	0	0	0
Basic SVSP	0	0	0	0	0	0.031

Each result is the average of three determinations. The basic SVSP used was a pooled fraction of the 52 and 56 kDa RP-HPLC peaks (see Fig. 9D). The acidic SVSP used was from anion exchange peak 3b (see Fig. 10)

3.4. Activity of the isolated proteases in the functional assays.

3.4.1. Fibrinogen and prothrombin degradation.

The assays performed with whole venom (section 3.1) were repeated with the various purified proteases to determine which are responsible for the observed degradation and to explain the regional variations between the venoms in this respect. The NGA basic and acidic SVSPs used for this analysis were proteins in the main ion exchange peaks (Figs. 9A and 10A).

All of the isolated proteases were able to digest fibrinogen to some degree (Fig. 11A). The alpha band disappeared in all cases and the beta was degraded by the acidic SVSP and all SVMPs. The SVMP PII-a from TZA was the most proteolytic (lane A5). The degradation pattern for the acidic SVSP (lane A1) differs from that of the basic (lane A2), the latter not able to digest beta-fibrinogen. In an equivalent experiment with prothrombin, only the SVMP PIIs showed any proteolytic activity (Fig. 11B). Once again, the TZA SVMP PII-a (lane B5) has proved to be particularly potent, with no intact prothrombin remaining at the end of the 2-hour time period, and, as in the case with whole venom, little evidence of the production of intact thrombin. The result for the putative KEN SVMP PII is not shown here but gave very similar result to that of the NGA SVMP PII-a (lane B4).

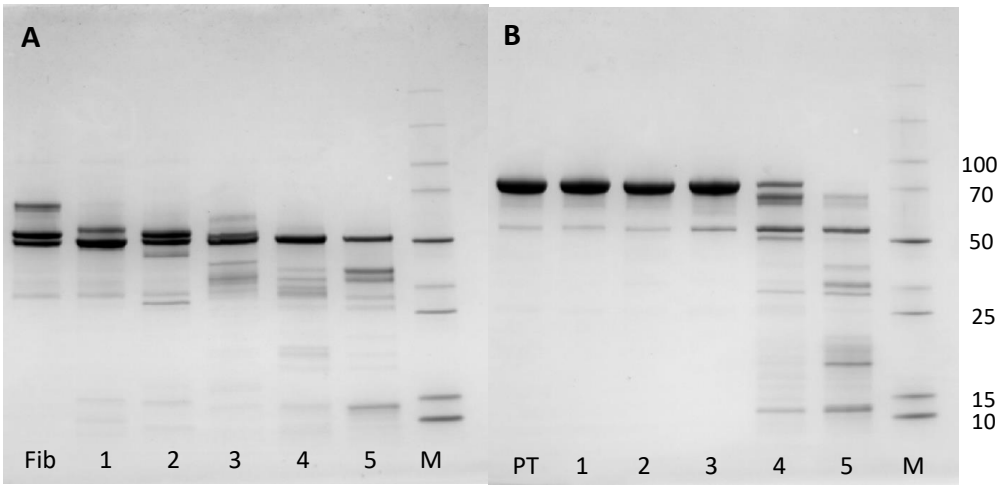

Fig. 11. Degradation of fibrinogen and prothrombin by purified *B. arietans* proteases. Fibrinogen (gel A) or prothrombin (gel B) were incubated at 37°C for 120 minutes with the proteases at a 30:1 (w/w) ratio of substrate:protease. The gel was run under reducing conditions and the equivalent of 1.0 μg of substrate protein (prothrombin, fibrinogen) was loaded per lane. The gel is 4-20% acrylamide (BioRad) and was stained using Coomassie Blue R250. F is fibrinogen control and P is the same for prothrombin (lane 1 on both gels). Lanes: Fib and PT, controls, no enzyme; lane 1, NGA acidic SVSP; lane 2, NGA basic SVSP, lane 3: KEN SVMP PIII; lane 4, NGA SVMP PII; lane 5 TZA SVMP PII. The molecular weights of key markers (lane M, Promega Broad Range) are indicated in kDa.

3.4.2. Basement membrane protein degradation

As was the case for prothrombin, the serine proteases had no significant effect on basement membrane proteins (Fig. 12A, lanes 1 and 2). The KEN SVMP PIII (lane 3) appears to have degraded the nidogens but has not the laminins. All SVMP PIIs degraded the proteins to some extent, with the

non-glycosylated SVMP PII-a from TZA being the most effective (lane 6), fully degrading the laminins. Of the two glycosylated SVMP PIIs, that from the KEN venom (lane 4) was a lot more degradative than the NGA protein (lane 5). The TZA and NGA SVMP PIIs were tested for their speed of action in a time-course experiment (Fig. 12B): significant levels of digestion by the TZA SVMP PII-a were seen against laminin and nidogen within 5 mins of incubation, whereas the NGA SVMP PII had little effect, even within 30 mins.

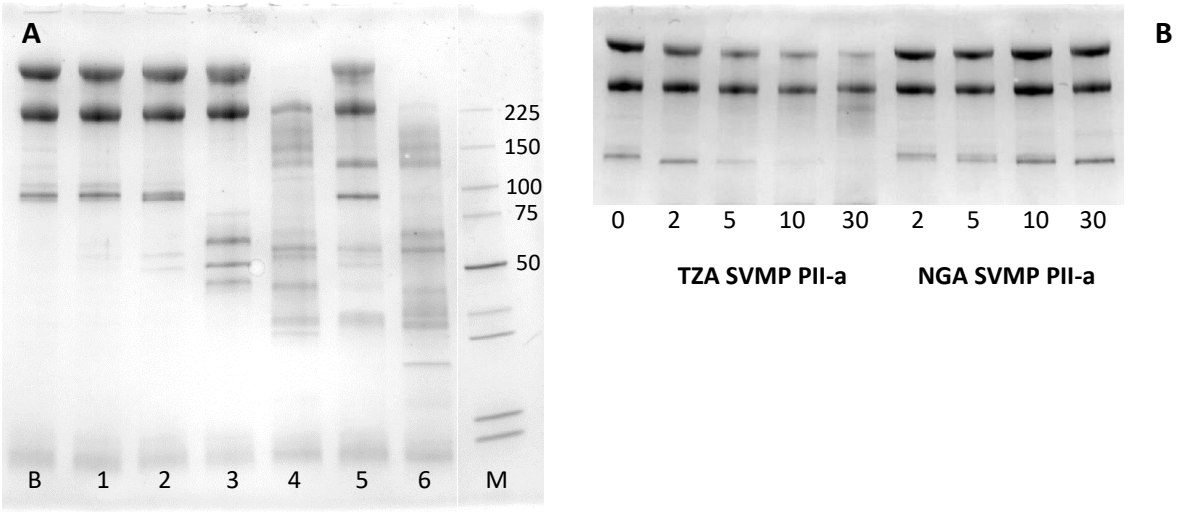

Fig. 12. Degradation of basement membrane proteins by proteases isolated from *B. arietans* venoms. A basement membrane protein extract (Geltrex) was incubated at 37°C for 30 minutes with the purified proteases at a 30:1 (w/w) ratio of substrate:protease. The gel was run under reducing conditions and the equivalent of 8 μg of substrate protein was loaded per lane. The gel is 4-20% acrylamide (BioRad) and was stained using Coomassie Blue R250. **A:** Activity of the individual proteases: Lane B, basement membrane protein control; lane 1, NGA acidic serine protease; lane 2, NGA basic serine protease, 3: KEN SVMP PIII; lane 4, KEN 31 kDa SVMP; lane 5, NGA SVMP PII; lane 6, TZA SVMP PII. The molecular weights of key markers (lane M, Promega Broad Range) are indicated in kDa. **B:** Time course of degrading activity of the TZA and NGA SVMP PIIs; reaction was stopped after 2-, 5-, 10- and 30-mins incubation; lane 0, basement membrane protein control.

3.4.3. Gelatinase activity

The studies using whole venom in gelatin zymogram assays showed that the gelatinase activity in the NGA venoms was due to the action of serine proteases (see Fig. 4). Using the purified NGA SVSPs in the same assay it became clear that this activity resides solely with the acidic SVSPs (Fig. 13A). The activity of the latter was not inhibited by EDTA but was so by PMSF. The basic SVSPs demonstrated no gelatinase activity. Both RP-HPLC purified 56 and 60 kDa acidic SVSPs were active (Fig. 13B) with gelatin-degrading activity observed at their respective molecular weights.

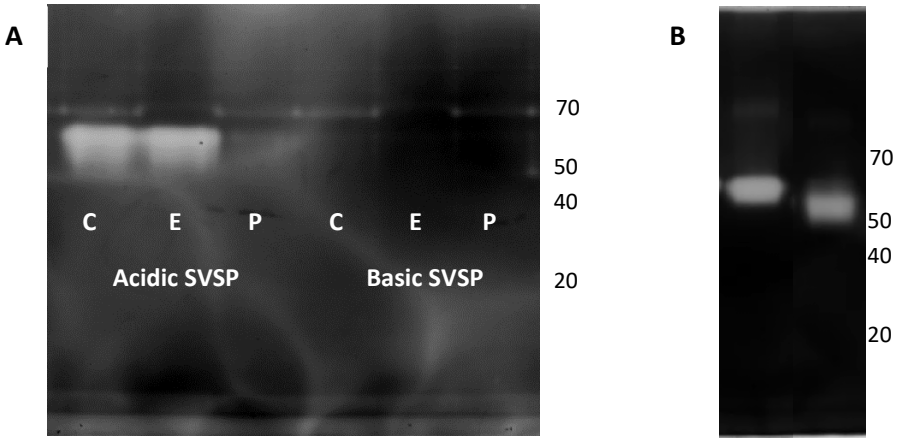

Fig. 13. Gelatinase activity (gelatin zymograms) of the serine proteases from NGA *B. arietans* venoms. The zymograms were prepared with 0.2% (w/w) gelatin in a 10% acrylamide SDS-PAGE gel, which was run under non-reducing conditions. The zymograms were then incubated as in section 2.3.8.1 and then visualised by staining with Coomassie Blue R250. **A**: purified acidic and basic proteases (0.1 μg) with inhibitors. C, control (no inhibitor), E, 10 mM EDTA; P, 2 mM PMSF. **B**. Protein (0.5 μg) from peak 1 and 2 of RP-HPLC of acidic SVSPs (Fig. 10C). Molecular weight markers (Thermo Page Ruler), indicated in kDa on the right of each zymogram.

Following its isolation, the SVMP PIII from the KEN015 venom was shown to be responsible for the gelatinase activity seen in the whole venom (Fig. 14). It is only active in its dimer form: in lane 1 the clear band at 140 kDa is due to the active dimer, but that which has broken down to a monomer under these conditions, visible as a Coomassie-stained band at around 60 kDa, is not active. Carrying out de-glycosylation under native conditions, it was found that the glycans are not essential for the gelatinase activity of the SVMP PIII, but the protein is only active in its dimeric form. Thus, when run after prior treatment with NP-40 (Fig 14, lane 2), all of the SVMP PIII remained in the 140 kDa dimeric form and is fully active. Lane 3 shows the shift in molecular weight (Ca. 10 kDa) due to removal of the N-glycans by PNGase F, but the dimer form of the de-glycosylated protein is still able to degrade gelatin. Despite the presence of NP40, some of the de-glycosylated SVMP PIII has broken down to inactive monomer (stained band at around 45 kDa) following de-glycosylation, however, suggesting that the N-glycans may play some role in maintaining structural stability and/or solubility.

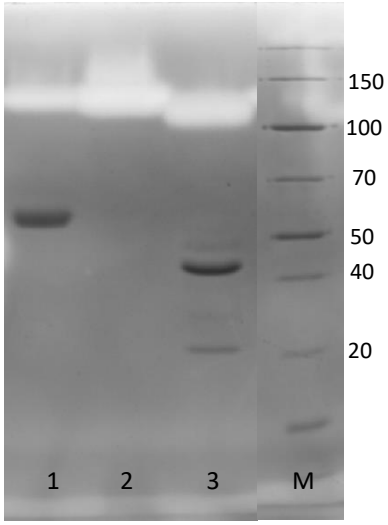

Fig 14. Gelatinase activity of native and de-glycosylated KEN SVMPIII. The zymogram was prepared with 0.2% (w/w) gelatin in a 10% acrylamide SDS-PAGE gel. The gel was run under non-reducing conditions. The zymograms were then incubated as in section 2.3.8.1 and then visualised by staining with Coomassie Blue R250. This zymogram was destained for sufficient time to visualise the Coomassie-stained SVMP PIII bands. Lane 1, untreated SVMP PIII; lane 2 (control) and 3 (+ PNGase F), deglycosylation in the presence of 0.4% NP-40; lane M, molecular weight markers. Both sets of markers are Thermo PageRuler with molecular weights indicated in kDa.

4. Discussion

Our study on puff adder venoms has revealed a diverse and interesting set of proteases, including some structures and functions that are novel for snake venoms. In all three regional venoms studied here the major SVMP was a processed form of SVMP PII containing just the metalloprotease domain, classed as SVMPII-a (Fox and Serrano 2008). Although the full protein (metalloprotease + disintegrin) and disintegrin domains have often been isolated and structured, particularly from *Bothrops* and *Trimeresurus* spp., SVMP PII-a forms have not been identified in many other venoms (Olaoba et al., 2020). The most studied SVMP PII-a has probably been atrolysin E of *C. atrox* (Hite et al., 1992) which has been shown to degrade ECM components (Baramova et al., 1989). Processing to the SVMP PII-a form should mean that the liberated disintegrin domains will be present in puff adder venoms. These will be 9-12 kDa in size and are likely to be found in the mix of proteins, predominantly CLPs, in the 10-17 kDa region on SDS-PAGE of whole venom (see Fig. 1). Disintegrin derived peptides from an SVMP PII sequence have been identified in *B. arietans* venom using LC-MS/MS (Currier et al., 2010).

Two of the SVMP PII-a proteases identified here were shown experimentally to be glycosylated; the NGA form has two N-glycans (these sites were also identified in the primary structure, see Fig. 6) and the KEN form has 1 N-glycan. With the availability of three forms of the same protein having different degrees of glycosylation (none on the TZA form, one on the Kenyan form and two on the Nigerian) it should be possible to assess whether these N-glycans are important for their activity. Thus, of the three forms, the KEN SVMP PII possessed the narrowest specificity, cleaving insulin B at just one position whereas the TZA SVMP PII-a demonstrated a broader specificity towards insulin B, cleaving at 4 or 5

sites. On the other hand, although the KEN and NGA SVMP PIIs were similar in their action against fibrinogen and prothrombin, the 1-glycan KEN SVMP had greater ability to degrade laminins, almost as effectively as the TZA SVMP PII-a. So, there appears to be no correlation between N-glycan content and activity, quantitatively and qualitatively, and as is the case with many glycans found throughout the eukaryotes, they may simply help to maintain solubility of the protein in the extracellular environment. It is more likely that the pronounced differences in activity reside in the primary structure, particularly at and around the active site. Interestingly, analysis of the venom gland transcriptomes showed regional differences in the tripeptide SVMP inhibitor (SVMPi) expressed by the puff adders. The NGA venom has pELW (the presence of this in the venom has been confirmed in our laboratory) in the relevant transcript, whereas the TZA venom transcript predicts an SVMPi with the sequence pEVW. This suggests that there are structural differences at the active site of the two SVMP PIIs.

Of most interest amongst these SVMP PIIs is the very potent TZA SVMP PII-a. In an earlier study from our laboratory, TZA puff adder venoms were shown to possess a greater measurable SVMP activity than the NGA venoms (assayed by cleavage of the GL bond in the fluorogenic MMP substrate ES010, Dawson et al. 2024). The assays carried out in this study using the same measurement on the purified proteins points to the differences in the specific activity of the TZA and NGA SVMP PII-a forms being responsible for this. The TZA SVMP PII-a is a very active protease with a broad specificity, acting strongly and rapidly against virtually all the proteins used as substrates in this study. The only exception to this was the gelatin substrate of the zymogram assay, against which the SVMP PII-a and the TZA venom as a whole had no effect. Based on its identical molecular weight and two-peak elution pattern on cation exchange chromatography (Fig. 2S), this is almost certainly the same protease ('Protease A') as that isolated from puff adder venom (most likely South African) by Van Der Walt and Joubert (1971, 1972) and there are good matches between the sequence of TZA SVMP PII-a and those of tryptic peptides from Protease A sequenced by this group in a subsequent paper (Strydom et al., 1986). These studies also showed that it acts with a broad specificity. What is especially notable about the TZA SVMP PII-a was the speed with which it acted against the proteins in the basement membrane extract (Fig. 12B), clearly causing laminin degradation within minutes of exposure. One of the doubts over using this assay to determine potential haemorrhagic abilities of proteases are concerns on the duration of the assays and their clinical relevance, with the proteases showing their effects after several hours in this in vitro assay (Escalante et al., 2006), much longer than would happen in a clinical setting. That is not the case for this SVMP.

Although they are not as visible on SDS-PAGE as they are in other viper venoms such as *Echis* spp., SVMP PIIIs at around 60-65 kDa were isolated from TZA and NGA venoms (results not shown). These were found to be present only in small quantities, however, and were therefore considered not to be of major clinical relevance. An SVMP PIII was found in the KEN venoms, however, that was particularly prominent in the venom of one animal, highly visible on SDS-PAGE of crude venom (Fig. 1, lane 1). Because of its abundance in the venom, this SVMP PIII was easily isolated and proved to be quite an interesting protein, appearing to have a narrower specificity than other viper SVMP PIIIs. It was not active at all against insulin B, a commonly used assay for venom SVMPs (Yamakawa et al., 1995).

Although it showed some ability to degrade fibrinogen, as all venom proteases seem to do, it showed little activity against basement membrane proteins and none against prothrombin. However, the purified protein did turn out to be responsible for the strong gelatin-degrading activity of the whole venom. A high molecular weight (60-70 kDa) gelatinase activity was observed in just 1 of 12 NGA *B. arietans* tested venoms in an earlier study (Currier et al., 2010) and this correlated with a pronounced ~65 kDa band seen after western blotting with an SVMP antibody. This is likely to be due to a SVMP PIII similar or identical to that found here in the KEN venom. Paixão-Cavalcante et al., (2015) observed gelatinase activity in a mix of puff adder venoms from Guinea, São Tomé, Angola and Mozambique, but at a different molecular weight of around 100 kDa. This variability of its presence in puff adder venoms suggests that this SVMP PIII gelatinase is a protein that is only expressed under certain circumstances or possibly that there can be variations in the puff adder venom protease complement even within the same region.

This 68 kDa Kenyan SVMP PIII also turned out to possess a novel oligomeric structure. Analytical SEC and non-reducing PAGE in the presence of NP40 showed that it exists naturally as a 140 kDa dimer, but not through disulphide bonds, as would be the case it if were an SVMP of the PIII-c class. Gelatinase activity was observed only when the protein was in this dimeric form and not when it had broken down to the monomer. Each monomer was also found to possess 3 N-glycans which were shown to be unnecessary for its gelatinase activity but appeared to be important in maintaining solubility. This is a novel form of SVMP PIII, not presently accounted for in the SVMP classification system (Fox and Serrano, 2008). It may be that this form of SVMP PIII is peculiar to some *B. arietans*, but it is possible that this form does exist in other SVMP PIII-rich vipers and because of the ease with which it can revert to monomer form during analysis, it may not have been characterised as a dimer. In many respects, this KEN SVMP PIII compares with the 68 and 75 kDa *B. arietans* SVMPs (BHRa and BHRb) characterised by (Omori-Satoh Ap et al., 1995; Yamakawa et al., 1995). These were shown to be able to degrade gelatins from a variety of collagens and it is tempting to suggest that they are all regional variants of the same protein (it is not clear which regional puff adder venom was the basis for this work).

All the venoms studied here possessed serine protease activity, but there were far greater quantities found in the NGA venoms. This is evident in the faint but multiple bands at 50-65 kDa on SDS-PAGE (see Fig.1): more are visible in the NGA venoms than the TZA. Because these are spread out across a large size range, due to their existence as multiple forms, their quantity is easy to underestimate, but if somehow compacted together in one band on a gel, these would be at least as prominent as the SVMP PII-a band. During isolation, these SVSPs separated into two groups: basic and acidic forms. Despite the apparent similarity in molecular weight and degree of glycosylation, the basic and acidic SVSPs are distinct proteins with different enzymatic activities. Using chromogenic substrates, the acidic SVSPs were shown to be trypsin like, but the basic SVSPs were not and showed a preference for F at the P_1 site. The study of Dawson et al (2024) showed that NGA puff adder venoms have a greater serine protease activity than that of TZA venoms, but this was assayed using a chromogenic substrate for trypsin-like SVSPs which would not have detected activity of the basic SVSPs, thus the full SVSP

activity of the venom would have been underestimated. Both forms of SVSP were able to degrade casein, though poorly so in the case of the acidic SVSPs. Neither had any effect on the basement membrane proteins, nor on prothrombin. Therefore neither forms are prothrombin activators, an established role for some venom SVSPs found in Australian snakes (Kini, 2006). There was some difference in their effect on fibrinogen cleavage. The acidic SVSPs cleaved both alpha and beta fibrinogen in a pattern that could be described as thrombin-like, as might be expected for a trypsin-like SVSP, whereas the basic SVSPs demonstrated only alpha-fibrinogenase activity.

The most pronounced differences in the activities of the two forms of SVSPs were observed in their actions against insulin B chain and gelatin. The basic forms cleaved insulin B at multiple sites (though the degradation pattern was quite different from that of the various puff adder SVMPs), but the trypsin-like acidic forms had no proteolytic activity against this substrate, despite the presence of both an R and a K residue in the peptide chain. In contrast to this, the acidic SVSPs were found to be responsible for the strong gelatinase activity observed in NGA venoms, whereas the basic SVSPs possessed no such activity. This is a previously unknown activity for isolated snake venom serine proteases, which have been shown to act only on plasma proteins (see Serrano, 2013). There is evidence in the literature for such activity, however; a PMSF-inhibited 36 kDa gelatinase was found on zymograms of *Bitis nasicornis* whole venom (Paixão-Cavalcante et al., 2015). This SVSP gelatinase activity thus appears to be the key difference between the action of NGA and TZA puff adder venoms. Such activity, at around 50-60 kDa, was observed in 11 of 12 NGA *B. arietans* tested venoms in an earlier study (Currier et al., 2010), but was reasonably assumed to be due to the action of an SVMP, since such activity would be unexpected for a snake venom serine protease based on the knowledge at the time. Despite there being different classes of protein, it is interesting that both the KEN SVMP PIII and acidic SVSP gelatinases described here are unable to degrade insulin B and, except for fibrinogen, had no activity towards the other protein substrates used here: this points to the gelatinase activity being a specific role of these proteins. It is interesting to note that we have isolated a gelatinase SVMP PIII from *Echis* venom which is also inactive towards insulin B, in contrast to all other SVMP PIIIs isolated from the same venom (manuscript in preparation). This suggests that gelatinase activity in venom proteases is quite specific, with primary specificity to amino acids at both P_1 and P'_1. Because of the possible functional and clinical relevance of the ability of a venom protease to degrade gelatin (predominantly denatured fibrillar collagen I) these SVSP and SVMP gelatinases warrant further studies beyond that of a simple scientific interest.

The availability of a full set of transcriptome data for both snakes allowed us to match the biochemical characteristics of the purified SVSPs to predicted primary structures. An analysis of the NGA puff adder transcriptome found it to contain 23 different SVSP sequences (contrasting with only 6 found in that of TZA). Within this set there is a broad range of predicted isoelectric points, ranging from 5.1 to 8.7 (not including contribution from sialic acids of the N-glycans) but a full analysis of the sequences indicated the presence of two distinct groups of acidic and basic proteases. The numbers of predicted N-glycans also matched the experimentally determined values: all the basic forms are predicted to have 4 or 5 N-glycans and the acidic forms 5 or 6 N-glycans, except for one form with just 3 predicted N-glycan sites.

The latter may be the 43 kDa protein in Fig. 10B, lane 1. A critical difference can be found at their respective substrate-binding sites, however. Figure 14 shows the relevant sections of the six NGA *B. arietans* SVSP transcripts predicted to be the most abundantly expressed in the venom gland.

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Hum trypsin      CVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWG-DGCAQKNKPGVYTKVYNY
Acidic SVSP_7    CAGVLEGGKDTCIADSGGPLICNGEFHGIVAWGRHPCAQPHLPAFYTKVFDY
Acidic SVSP_2    CAGVLEGGKDTCTGDSGGPLICNGEFHGIVSWGGHPCAQPQWLAFYTKVFDY
Acidic SVSP_5    CAGVLEGGXDTCIGDSGGPLICNGEFHGIVSWGDHPCAQPHLLAFYTKVFDY
Basic SVSP_1     CAGDRRRRIGSCKGDSGGPLICNGQIHGIVSWGRNPCAYSFSPGMYTKVFDY
Basic SVSP_4     CAGDRRRRIGSCKGDSGGPLICNGQIHGIVSWSRDPCAYSFSPGMYTKVFDY
Basic SVSP_3     CAGDRRRRIGSCKGDSGGPLICNGQIQGIVSWGRDPCAYSFSPGMYTKVFDY
M. lebetina AF   CAGVPRRRIGSCLGDSGGPLICNGQIQGIASWGSDPCVNHGAPGVYTKVFDY
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Fig. 14. Sequence alignment of the substrate-binding region of the 6 most abundant SVSP transcripts from NGA puff adder. The top 6 most abundant transcripts were selected and subject to a CLUSTAL multiple sequence alignment by MUSCLE 3.8 (Larkin et al., 2007). Also included for comparison are sequences from human trypsin-1 (P07477) and *M. lebetina* alpha-fibrinogenase MLAF (Q8JH85 VSPA_MACLB). In bold is the S_1 residue (D189 or G189) plus the 5 residues immediately N-terminal to this. The active site S195 is shown in bold/italics. Shown in bold/underlined are the three key substrate pocket residues 189, 216 and 226 (DGG in trypsin; DGA in the acidic SVSPs; GGG or GSG in basic SVSPs and GGG in MLAF). All residues are numbered using the chymotrypsin system. The predicted pI for the three acidic SVSPs shown is 5.1-6.1 and for the basic SVSPs, 7.1-8.8.

The transcriptome data for the NGA acidic SVSPs support the experimental findings (Table 1) in that they are trypsin-like serine proteases, with an aspartate (D) at the bottom of the substrate pocket (S_1 binding site, residue 189 using chymotrypsin numbering). They also have sequences immediately N-terminal to the substrate-binding site (VLEGGKDT or ILEGGIDS) similar to those of other well-documented venom serine proteases, such as batroxobin (Itoh et al., 1987). Thus, in most respects, these *B. arietans* acidic SVSPs are typical snake venom serine proteases (Serrano, 2013), though none have so far been shown to have the gelatinase activity of the NGA acidic SVSPs isolated here. At the two other positions that form the substrate pocket, 216 and 226, the acidic SVSPs have G and A residues respectively. The A at position 226 in lieu of the smaller G residue found here in trypsin and thrombin might restrict access of large residues in the P_I position of the substrate, but despite this it appears to cleave both alpha and beta fibrinogen in a similar manner to thrombin (Fig. 11A). It is quite rare for an SVSP to cleave both alpha and beta fibrinogens (Kini, 2006). It is difficult, however, to explain the inability of the acidic NGA SVSP to cleave at the K and R residues in insulin B. This may be a consequence of the amino acids either side of these residues in this peptide: for example, inability to cleave the K of insulin B may be due to steric effects of the proline residue immediately N-terminal of this K. This is presumably not an issue for the R in the small artificial substrate BAEE which it was able to hydrolyse at around 20% of the rate of trypsin (Table 1). It is not clear which residues in gelatin (collagen I) would be the target of the NGA acidic SVSP, but collagen I is rich in R and K residues.

In contrast to the acidic SVSPs, the basic forms have a G in lieu of the D at position 189 and a distinct arginine-rich sequence (DRRRRIG) on the N-terminal side of the substrate-binding site. This is partly responsible for their basic pI and is found in very few snake species: a BLAST search found just four such proteins: rhinocerase 4 and 5 (Vaiyapuri et al., 2011), alpha-fibrinogenase ML-AF (Siigur, 2003) and an *E. ocellatus* serine protease (Submitted to GenBank: ADE45139.1). Notably, like *B. arietans*, these all are African or Middle Eastern Viperinae. The well-studied SVSP Ancrod (*Calloselasma rhodostoma*) has an arginine-rich sequence (DLRGRRD) in this region and although the D at S_1 makes it a trypsin-like SVSP, like the NGA basic SVSPs it cleaves alpha-fibrinogen only and not beta. The basic NGA SVSP also has G at both 216 and 226 positions, which, along with the G at S_1, would form a large substrate binding pocket and hence it is not surprising that it has a preference for F at P_1 (Table 1). This is similar to the action of chymotrypsin which has a S189G216G226 substrate pocket and also acts strongly towards F-containing substrates. This NGA basic SVSP is able to cleave insulin B at multiple sites (Fig. 11S), more than might be expected for a peptide substrate with three F residues, two of which are adjacent, so it must be able to cleave at other amino acids, presumably those of a larger size like F. Many SVSPs have a G189G216G226 substrate pocket (Vaiyapuri et al., 2012), but the only one that has been fully characterised is the alpha fibrinogenase ML-AF of *M. lebetina* (Siigur, 2003) and this protein bears many similarities to the NGA basic SVSP. As well as the GGG, ML-AF also has the arginine-rich sequence (PRRRRIG), a highly basic isoelectric point and cleaves insulin B at three positions including the FF and FY peptide bonds but also at YL (Mahar et al., 1987). Both proteins are also alpha fibrinogenases and this may be their main role in the venom, thus contributing to any coagulopathic effects of the venom.

Although these are initial biochemical studies, we can begin to relate the regional differences in the protease activities to clinical features. The differences in gelatinolytic activity of the NGA and the rapid laminin-degrading activity of the TZA venoms may translate to differences in the extent of tissue damage from local envenoming. In the NGA venoms, the gelatinase activity of the acidic SVSPs combined with the action of the SVMP PII-a may contribute to tissue damage at the bite site, ultimately leading to dermonecrosis. Because of their potential ability to degrade fibrillar collagens at the dermal-epidermal junctions, the gelatinolytic acidic SVSPs could also contribute to blister formation at and around the bite site (Gutiérrez et al., 2018; Jiménez et al., 2008). This feature may also be true for those KEN venoms which possess the gelatinolytic SVMP PIII. In contrast, the potent laminin-degrading SVMP PII-a in the TZA venoms suggests a possible role in the haemorrhagic action of these venoms. Some clinical studies have reported patients developing subcutaneous haemorrhage after cases of puff adder envenoming, but the region of origin of the snakes were not specified (Wakasugi et al., 2021) (Husain et al., 2023). At this stage, much of this is speculative and confirmation of this would require that these proteases are subject to further in vitro and in vivo studies; in the case of the possible haemorrhagic role of the TZA SVMP PII-a, the exemplar would be the extensive characterisation that has been carried out on the haemorrhagic *Bothrops* SVMPs (Gutiérrez et al., 2016a, 2016b). Whatever its precise target(s), the work presented here shows that the potent TZA SVMP PII-a surely plays a critical role of the in the action of venoms from these puff adders, as well as from any other regions in which the presence of this protein can be identified.

For all three venoms studied here, there is evidence that the proteases underlie the mechanisms of coagulopathy, as is the case in other vipers. All possess proteases that degrade fibrinogen and all do so quite differently from each other. The acidic SVSP of the NGA venom appears to act like thrombin towards fibrinogen and this along with the general fibrinogen-degrading activities of the basic SVSPs and SVMP PII-a will result in depletion of fibrinogen, suggesting that an anticoagulant effect may predominate. The ability of the TZA venom to completely degrade prothrombin via the action of the SVMP PII-a also suggests a complex effect on the coagulation pathway, with the production of small amounts of thrombin. The poor yield of thrombin would be unlikely to cause consumptive coagulopathy as is seen in *Echis* spp., but which is not an observed feature of puff adder envenomation.

The possible role of the SVMPs and SVSPs in producing the hypotension that is commonly seen in puff adder bite victims has not been considered here, but these and the other venom components suspected of causing this clinical manifestation (adenosine, BPPs and VEGF, see Péterfi et al., 2019for review) will be the subject of a further study. The SVMPs isolated here may play a role in the production of vasodilatory angiotensin 1-7, as suggested by the study of Paixão-Cavalcante et al., (2015), and amongst the SVSP fractions are likely to be kallikrein like (kinin-releasing) enzymes found by others. Thus, within the acidic SVSP transcripts, our sequences matched with kinin-releasing *B. arietans* SVSPs that have previously been characterised. The N-terminal sequence that Nikai et al. (1993) found for their 58 kDa acidic SVSP matches very well with those found in our transcriptome, and the tryptic peptide sequences from Kn-Ba obtained by Megale et al. (2018) matched exactly with the NGA acidic SVSP_7 (Fig. 14). Kn-Ba is only 33 kDa in size, but the sequence of SVSP-7 predicts just 3 N-glycans (as opposed to the 5 or 6 predicted for the other 22 transcripts), so it would be smaller than the main group of SVSPs. This may even be the small acidic SVSP in lane 1 of Fig. 10B.

In summary, the results presented here clearly reveals the regional variations in the protease complement of puff adder venoms. Some variation was evident even within a single region, particularly the presence of the Kenyan SVMP PIII gelatinase. Whilst carrying out this work we also briefly looked at other puff adder venoms held at LSTM. Both the 21 kDa SVMP, which when isolated was identical in action to the TZA SVMP PII-a, and acidic SVSPs with gelatinase activity like those of the NGA venom were isolated from a Ghanaian venom sample. On SDS-PAGE a South African venom contained a strong band at the same size as the TZA SVMP PII-a (most likely that isolated by Van Der Walt and Joubert, 1971, see above) and Guinean venom contained a distinct 34 kDa protein that is most likely a glycosylated SVMP PII-a similar to that found in or KEN and NGA venoms. It is perhaps not surprising, therefore, that such a variety of clinical manifestations are observed following puff adder envenomation (Frank). This diversity in protease activity means that the well-documented issues with developing targeted therapeutics will be a particular problem for puff adder bites. The work presented here is a good starting point to address this issue, however; providing methods for the isolation of the proteases to be used in the further studies outlined above, to be used as immunogens for generation of toxin-specific antibodies and as the basis for experimental testing of small molecule therapeutics.

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