ELSEVIER

Toxicon xx (2003) xxx–xxx

TOXICON

www.elsevier.com/locate/toxicon

The purification and partial characterisation of two novel metalloproteinases from the venom of the West African carpet viper, *Echis ocellatus*

J.-M. Howes[a], M.C. Wilkinson[b], R.D.G. Theakston[a], G.D. Laing[a,*]

[a]*Venom Research Unit, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK*
[b]*School of Biological Sciences, Biosciences Building, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK*

Received 20 February 2003; accepted 5 April 2003

Abstract

Separation of previously uncharacterised *Echis ocellatus* venom by phenyl-Superose FPLC (Fast Liquid Protein Chromatography) yielded eight protein fractions. Three of these displayed high proteolytic activity when assayed by in vivo and in vitro assays (including enzyme linked immunosorbant assay), and were further separated using Superdex 75 and Mono-Q FPLC. This resulted in the purification of a non-haemorrhagic 24 kDa metalloproteinase (EoVMP1, p*I* 7.0), and a haemorrhagic 56 kDa metalloproteinase (EoVMP2, p*I* 5.5). Following tryptic digest, short amino acid sequences of EoVMP1 and EoVMP2 were obtained using Edman degradation. Both sequences displayed homology when aligned with existing snake venom metalloproteinases (SVMPs). The strong homology observed among previously well-characterised SVMPs suggests that principles governing the interaction of substrates and inhibitors are likely to be similar for EoVMP1, EoVMP2 and all members of the reprolysin family.

Keywords: Venom; Metalloproteinase; Gelatinase; Collagenase; Enzyme linked immunosorbant assay; Haemorrhage

1. Introduction

The *Echis* genus is responsible for more snakebite deaths worldwide than any other venomous snake (Warrell et al., 1977). The saw-scaled viper (*Echis ocellatus*) is the most abundant venomous snake in savannah Nigeria (Trape et al., 2001) and a source of major health concerns (Pugh and Theakston, 1987; Pugh and Theakston, 1980; Pugh et al., 1979; Warrell and Arnett, 1976). At some times of the year, victims can occupy 74% of regional hospital beds (Revault, 1996). Snakebite by *Echis* vipers results in two clinical scenarios: (i) local venom effects which include swelling at the bite site, blistering, necrosis and local bleeding and (ii) life-threatening systemic haemorrhage and incoaguable blood induced through entry of venom into the circulation (Pugh and Theakston, 1987; Bjarnason and Tu, 1978; Gutiérrez and Rucavado, 2000). Symptoms of local and systemic haemorrhage symptoms are induced primarily by zinc-containing venom metalloproteinases (VMPs; Kamiguti et al., 1998), which selectively cleave key peptide bonds of basement membrane components (Sanchez et al., 1991; Rodrigues et al., 2000; Aragon-Ortiz and Gubensek, 1987), effecting its interactions with endothelial cells (Gutiérrez and Rucavado, 2000). Metalloproteinases have been identified in the venoms of other members of the *Echis* species such as Carinactivase (Yamanda et al., 1996), and Ecarin (Kornalik and Blomback, 1975). However, despite its clinical significance, the venom of *E. ocellatus* is poorly characterised with information lacking on important components, including the metalloproteinases. This paper describes the isolation of two metalloproteinases by FPLC (Fast Liquid Protein Chromatography), assessment of their

* Corresponding author. Tel.: +44-151-7053164; fax: +44-151-7053371.
E-mail address: gavin.laing@liv.ac.uk (G.D. Laing).

0041-0101/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0041-0101(03)00096-5

biological activities using in vivo and in vitro methods and sequence information.

At present, the only effective treatment for systemic envenoming by *E. ocellatus* is specific antivenom (Meyer et al., 1997), which currently is scarce and relatively expensive (Theakston and Warrell, 2000; Laing et al., 1995). Although effective against systemic envenoming, antivenom is generally ineffective for alleviating local haemorrhage or necrosis (Gutiérrez et al., 1998) unless it is administered very soon after the bite, as indicated by work in experimental animals (Iddon et al., 1987).

The information obtained from further characterisation and inhibition studies on venom metalloproteinases from this and other venoms will be used to guide the identification of potential targets for existing drugs or specific inhibitors for possible eventual use in snakebite therapy.

2. Materials and methods

2.1. Venom sample preparation

Pooled venom was obtained from 200 specimens of *E. ocellatus* maintained at the Liverpool School of Tropical Medicine. The specimens were wild-caught in the area of Kaltungo and Zamko, central Nigeria. Venom was extracted manually, lyophilised, and stored at 4 °C in dark glass bottles before use.

2.2. Venom separation

2.2.1. Phenyl-Superose FPLC

One hundred milligrams of lyophilised whole venom were dissolved using bath sonication in 2 ml filtered, degassed double-distilled water. After centrifugation at 5000 × g for 10 min at 18 °C, the supernatant was mixed with 2 ml start buffer (20 mM Tris-HCl, 1.0 mM $CaCl_2$, 2.0 M $(NH_4)_2SO_4$, pH 7.4) and applied to a phenyl-Superose HR 10/10 column (Amersham Biosciences, Bucks., UK) equilibrated with start buffer. Elution was performed by a decreasing salt gradient and monitored at 280 nm. The flow rate was 0.5 ml/min and 2.5 ml fractions were collected.

2.2.2. Superdex 75 FPLC

Phenyl-Superose peaks displaying optimum biological activity were applied to a Superdex 75 column (Amersham Biosciences, Bucks., UK) equilibrated with 0.05M PBS, 1 mM $CaCl_2$, pH 7.4 and eluted under the same conditions.

2.2.3. Mono-Q FPLC

Superdex 75 fraction 1 was applied to a Mono-Q HR 5/5 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, 1.0 mM $CaCl_2$, pH 6.8. Elution based on anion exchange was carried out with an increasing gradient of 1 M NaCl.

2.3. SDS-page

Venom fractions were run under reducing conditions on 4–12% gradient and 12% SDS-PAGE gels (Laemmli, 1970).

2.4. Assessment of the haemorrhagic activity of venom fractions

2.4.1. Mouse intradermal assay

Fractions 1–8 from phenyl-Superose separation were dialysed against 20 mM Tris-HCl, pH 7.4 at 4 °C overnight. Following dialysis, 10 μg of protein in a total volume of 50 μl of each fraction was injected into the shaved intradermal dorsal region of male CD-1 strain mice (18–20 g) under light isofluorane/oxygen anaesthesia. Three mice were used for each dose and sterile saline was injected as a negative control. After 24 h, the animals were killed, the dorsal skin removed, and the diameter of the haemorrhagic lesion measured on the inner surface of the skin in two directions at right angles using callipers and background illumination (Theakston and Reid, 1983). The mean diameter of the haemorrhagic lesion was calculated for each fraction. The experiment was repeated.

2.5. Degradation ELISA (Enzyme linked immunosorbant assay)

Venom fractions 1–8 from the phenyl-Superose separation at a concentration of 0.1 mg/ml and collagenase as a positive control (2 units/ml) were added to 96-well ELISA Plate (Nunc A/S, Denmark) coated with 50 μl of biotinylated collagen IV (origin human placenta 0.5 μg/ml; Fluka, Dorset, UK) or biotinylated gelatin (Bovine type B, Sigma, UK) according to the method of Koritsas and Atkinson, 1995. Briefly, pre-coated wells were incubated with 50 μl of PBS for 15 min. The PBS was then removed, 50 μl of venom fraction added, and the plate incubated at 37 °C for 30 min. The degradation reaction was stopped by washing five times with PBS-Tween. The wells were then incubated with 50 μl of streptavidin-alkaline phosphatase conjugate (1:2500 dilution in water) for a further 15 min, and washed as above. After the addition of chromogenic substrate (SIGMA FAST™ *p*-nitrophenyl phosphate tablets), the absorbances of the well contents were read at 405 nm. The degradation of gelatin and collagen IV was determined with reference to the OD max value.

2.6. Amino acid sequencing

Samples of venom fractions (EoVMP1 and EoVMP2) in PBS were submitted to Edman degradation following in-gel digestion, which was carried out using an adaptation of the method of Rosenfeld et al, (1992). Gel slices from SDS-PAGE were cut in half, placed in an Eppendorf tube and washed (2 × 30 min) with 50% acetonitrile, 0.2 M

ammonium bicarbonate pH 8.9 and then freeze-dried for 1 h. The slices were re-swollen in re-hydration buffer (RHB) (0.2 M ammonium bicarbonate pH 7.8, 0.02% Tween 20) with 2 M urea and containing a quantity of trypsin equivalent to 10% (w/w) of target protein. This buffer was added to the slices in 10–20 μl aliquots, allowing each gel slice to take up all the buffer before adding the next aliquot. When completely re-swollen, the slices were incubated at 37 °C overnight. At the end of the incubation period, excess RHB/2 M urea was removed to a second Eppendorf tube and peptides were extracted from the gel slices with two lots of 60% acetonitrile, 0.1% Trifluoroacetic acid (TFA). These washes were pooled with the excess buffer, concentrated by centrifugal evaporation and applied to a PE-Biosystems PepMap C18 RP-HPLC column (100 × 2.1 mm) equilibrated in 0.08% TFA. Peptides were separated with a 95 min gradient of 0–64% acetonitrile in 0.08% TFA. Elution was monitored at 214 nm. Peptides were subjected to N-terminal sequencing by Edman degradation using an Applied Biosystems model 471A Protein Sequenator. The resulting amino acid sequence was subjected to protein-protein BLAST (Basic Local Alignment Search Tool).

3. Results

One hundred milligrams of whole *E. ocellatus* venom, separated on the basis of hydrophobicity on a phenyl-Superose FPLC column, resulted in eight fractions (numbered 1–8 in order of elution). The protein content of fractions 1–8 were analysed by SDS-PAGE (Fig. 1) and their biological activities determined in vitro by degradation ELISA using biotinylated gelatin and collagen IV substrates. Gelatin degradation ELISA has been used previously to detect the proteolytic activity in snake venoms (Bee et al., 2001). A fairly uniform level of gelatin degradation was demonstrated (data not shown). Collagen IV-ase activity increased significantly ($P < 0.0001$, Student's t-test) in fractions 6–8 compared with fractions 1–5 (Fig. 2). An increase in collagenase activity is indicative of the presence of venom metalloproteinases due to their ability to degrade type IV collagen and various gelatin substrates (Selistre-de-Araujo et al., 2000; Soto et al., 1998; Rodrigues et al., 2000; Gomis-Rüth et al., 1993). The relationship between collagen IV degradation and haemorrhagic activity was corroborated by studies on the eight phenyl-Superose FPLC fractions. Fractions 6–8 possessed significantly more haemorrhagic activity ($P < 0.01$, Student's t-test) when compared with fractions 1–5 (Fig. 2). SDS-PAGE of phenyl-Superose fractions 6–8 revealed that all three fractions contained a 56 kDa protein band absent in fractions 1–5 (Fig. 1). Fractions 6–8 were therefore pooled and subjected to gel filtration on a Superdex 75 FPLC column. Application of the Superdex 75 void volume onto an anion exchange mono-Q FPLC column resulted in a cleaner, four-peak profile (Fig. 3) with two purified proteins, EoVMP1, (24 kDa, p*I* 7.0) and EoVMP2, (56 kDa, p*I* 5.5) when analysed by SDS-PAGE (Fig. 3).

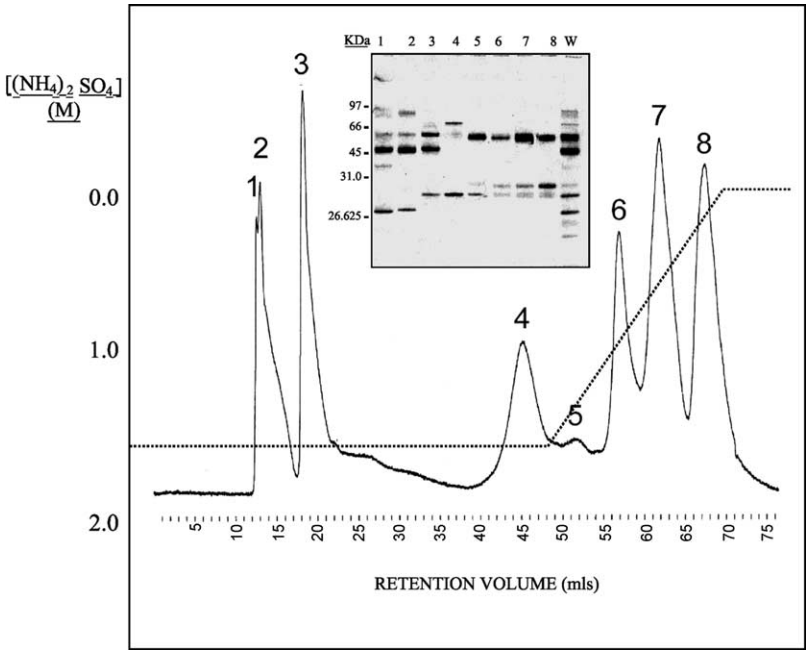

Fig. 1. Elution profile of crude *E. ocellatus* venom on phenyl-Superose FPLC. Crude *E. ocellatus* venom eluted from a phenyl-Superose FPLC column in eight peaks. Reduced SDS-PAGE of phenyl-Superose fractions 1–8; W, whole *E. ocellatus* venom. 4–12% bis-tris (…) elution gradient.

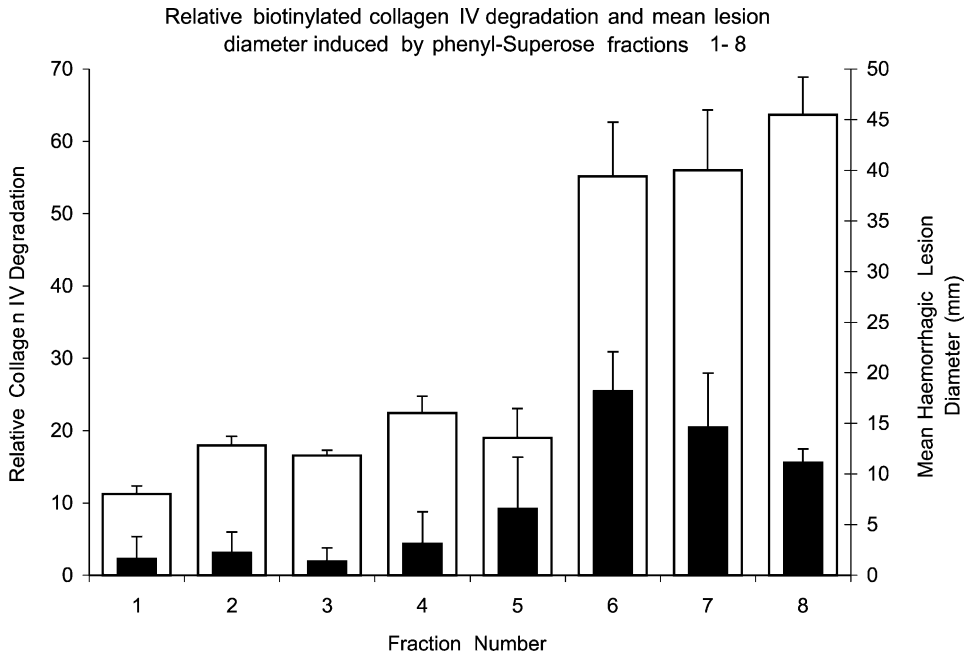

Fig. 2. Haemorrhagic lesion induction and relative substrate degradation induced by *E. ocellatus* venom fractions. Diameter of haemorrhagic lesion induced by phenyl-Superose fractions 1–8 in male CD-1 mice (■); Biotinylated collagen IV (□) substrate degradation by phenyl-Superose fractions 1–8. Results were expressed as the mean ± SD ($n = 6$).

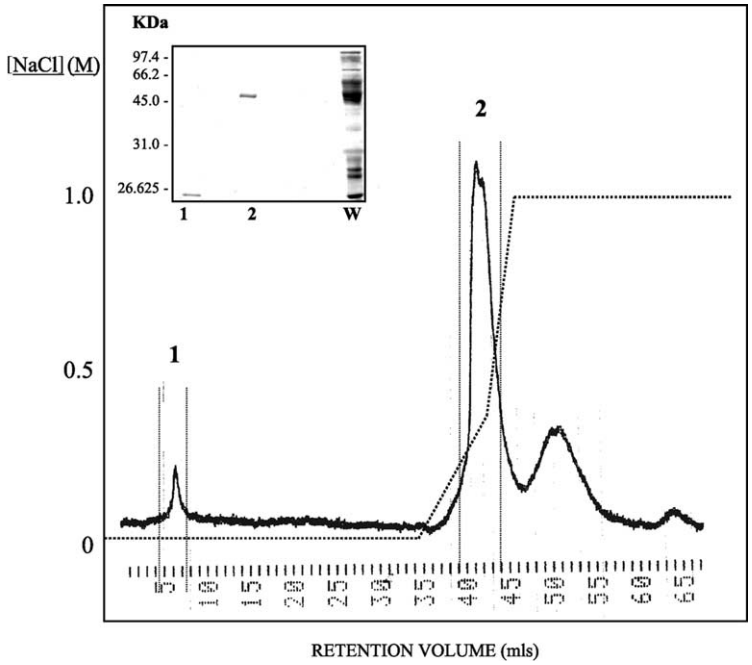

Fig. 3. Mono-Q FPLC of *E. ocellatus* venom fractions. Superdex 75 void volume eluted from mono-Q FPLC in four peaks. 4–12% bis-tris gel (reduced) of pure proteins eluted from the mono-Q column. 1, EoVMP1; 2, EoVMP2; W, whole *E. ocellatus* venom; (…) elution gradient.

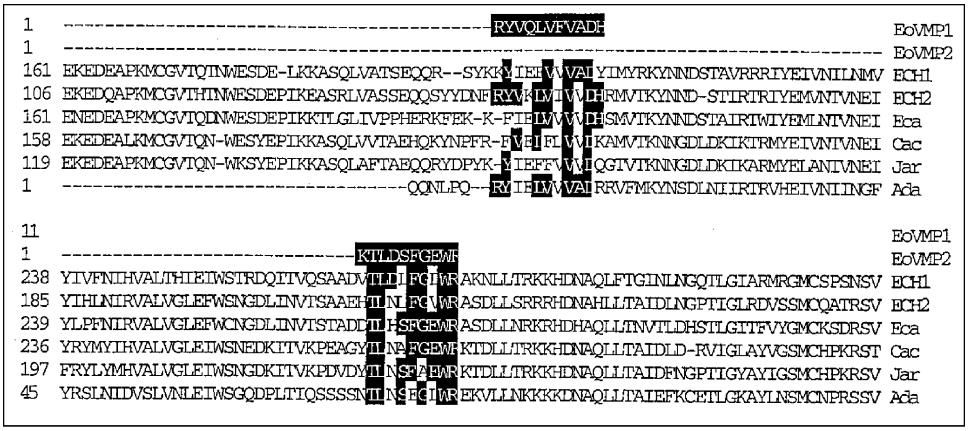

Fig. 4. Alignment of EoVMP1 and EoVMP2 BLAST sequences with existing snake venom MPs. (a) EoVMP1 and EoVMP2 AA sequence alignment with the metalloproteinase region of existing snake venom SVMPs: ECHI and ECHII (SVMPs from *Echis pyramidum leakeyi* venom (Paine et al. (1994); Eca (SVMP from *Echis carinatus* venom (Kornalik and Blomback, 1975); Cac (SVMP from *Crotalus atrox* venom (Zhou et al., 1995) Jara (SVMP from *Bothrops jararaca* venom (Paine et al., 1992); Ada (adamalysin a non-haemorrhagic short-chain metalloproteinase from *Crotalus adamanteus* venom (Gomis-Rüth et al., 1993).

No haemorrhagic lesions were induced by intradermal injection of EoVMP1, but EoVMP2 was intensely haemorrhagic, producing a mean lesion diameter of 9.8 ± 3.99 mm at a dose of 10 μg per mouse. EoVMP1 and EoVMP2 were subjected to in-gel digestion and Edman degradation for identification. The sequence data obtained following tryptic digest showed homology when aligned with existing venom metalloroteinases (Fig. 4) and confirmed EoVMP1 and EoVMP2 as snake venom metalloproteinases (SVMPs).

4. Discussion

Two metalloproteinase enzymes, EoVMP1, a non-haemorrhagic 24 kDa short-chain metalloproteinase (p*I* 7.0), and EoVMP2, a haemorrhagic 56 kDa long-chain metalloproteinase (p*I* 5.5) were isolated in pure form from the venom of *E. ocellatus.* They join the large number of zinc metalloproteinases of varying molecular weights and biological functions that have previously been isolated from viperid and crotalid venoms (Jia et al., 1996; Leonardi et al., 2001; Rodrigues et al., 2001; Wu et al., 2001). Although EoVMP1 and EoVMP2 differ in biological function, they display high sequence homology to other SVMPs (reprolysins); EoVMP1 had 73% homology with ECH II (a metalloproteinase from *Echis pyramidum leakeyi* venom (Paine et al., 1994), and EoVMP2 showed 80% homology with Ecarin (a metalloproteinase from *Echis carinatus* venom (Kornalik and Blomback, 1975).

SVMPs are primarily responsible for the haemorrhagic effects characteristic of viperine and crotaline snake envenoming. Sequential and structural analysis of members of the reprolysin family have resulted in the division of SVMPs into four classes, P-I to P-IV (Hite et al., 1994; Gutiérrez and Rucavado, 2000). The P-I class of SVMP includes proteins of 20–30 kDa containing only the metalloproteinase domain. Sub-class P-1A proteins display high haemorrhagic activity, while those in class P-1B display little or no activity and include 'Neuwidase' from *Bothrops neuwiedi* venom (Rodrigues et al., 2000) and 'Adamalysin II' from *Crotalus adamanteus* venom (Gomis-Rüth et al., 1998). Our results suggest that EoVMP1 belongs to class PI-B. The P-1B SVMPs can also display fibrinogenase activity with specificity for the Aα chain and they lack arginine esterase activity. The class P-II SVMPs contain both metalloproteinase and disintegrin domains, while P-III SVMPs have an additional cysteine-rich domain and include Jararhagin (Paine et al., 1992). EoVMP2 is almost certainly a P-III enzyme as judged by intense haemorrhagic activity, size and p*I* value. The class P-IV SVMPs have an additional lectin-like polypeptide linked to metalloproteinase-containing polypeptide chain (Jia et al., 1996; Rodrigues et al., 2000; Gutiérrez and Rucavado, 2000).

SVMPs are secreted as zymogens in the venom gland. Subsequent processing via the 'cysteine switch' mechanism converts the zymogen to the active proteolytic form (Grams et al., 1993). Proteolytic activities are commonly associated with haemorrhagic factors (Soto et al., 1998) and can be utilised to aid the identification of SVMPs such as EoVMP2. Haemorrhagic activity is primarily due to the selective enzymatic action of metalloproteinases on capillary basement membrane components such as collagen IV, laminin, and fibronectin (Baramova et al., 1989), thereby affecting the interactions between basement membrane and endothelial cells. Some metalloproteinases may induce haemorrhage by directly affecting capillary blood vessels (Gutiérrez and Rucavado, 2000; Rucavado et al., 2002).

Reprolysins such as EoVMP1 and EoVMP2 share some degree of structural similarity to other zinc MP sub-families, including matrix MPs (MMPs), bacterial serralysins and astacins, primarily in the zinc-binding region (Zhang et al., 1994). Matrix metalloproteinases (MMPs) contribute to the degradation of matrix components including collagen and proteoglycans in degenerative diseases such as arthritis, cancer periodonitis and osteoporosis (Mattei et al., 2002). Recent advances in protease inhibition have led to the development of synthetic MMP inhibitors and phase III clinical trials have been undertaken to test 'broad range' inhibitors of metalloproteinases including Bay-12 9566, Batimastat, Marimastat and AG3340 (Heath and Grochow, 2000). Escalante et al. (2000) reported the ability of Batimastat to neutralise local tissue damage induced by BaP1, a haemorrhagic metalloproteinase from the venom of *Bothrops asper*. Future work will assess the effectiveness of a range of synthetic peptide inhibitors against venom metalloproteinase activity in vitro and in vivo.

In conclusion, two metalloproteinases have been purified from the venom of *E. ocellatus*: EoVMP1 is a low weight non-haemorrhagic class P-IB SVMP, and EoVMP2 is a high weight class P-III SVMP. The partial amino acid sequences for both proteins show high homology with other members of the reprolysin family. Further characterisation and inhibition studies of EoVMP1, EoVMP2 and other SVMPs may lead to the development of a parallel treatment alongside antivenom for venom-induced local necrosis and tissue damage that is not completely dependent on venom specificity.

Acknowledgements

We want to thank the Gunter Charitable Trust for support of Joanna-Marie Howes, the Nigerian Ministry of Health for the collection of snakes, and P.D. Rowley for help with extraction of venom.

References

Aragon-Ortiz, F., Gubensek, F., 1987. Characterisation of a metalloproteinase from Bothrops asper (terciopelo) snake venom. Toxicon 25 (7), 759–766.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B., Fox, J.W., 1989. Degradation of extracellular matrix proteins by haemorrhagic metalloproteinases. Archives of Biochemistry and Biophysics 15, 63–71.

Bee, A., Theakston, R.D.G., Harrison, R.A., Carter, S.D., 2001. Novel in vitro assays for assessing the haemorrhagic activity of snake venoms and for demonstration of venom metalloproteinase inhibitors. Toxicon 39, 1429–1434.

Bjarnason, J.B., Tu, T., 1978. Hemorrhagic toxins from the western diamondback rattlesnake (*Crotalus atrox*) venom. Isolation and characterisation of five toxins and the role of zinc in haemorrhagic toxin e. Biochemistry 17 (16), 3395–3404.

Escalante, T., Franceschi, A., Rucavado, A., Gutierrrez, J.M., 2000. Effectiveness of batimastat, a synthetic inhibitor of matrix metalloproteinases, in neutralising local tissue damage induced by BaP1, a haemorrhagic metalloproteinase from the venom of the snake Bothrops asper. Biochemical Pharmacology 15, 269–274.

Gomis-Rüth, F.-X., Kress, L.F., Bode, W., 1993. First structure of a snake venom metalloproteinase: a prototype for matrix metalloproteinases/collagenases. EMBO Journal 12 (11), 4151–4157.

Gomis-Rüth, F.-X., Meyer, E.F., Kress, L.F., Politi, V., 1998. Structures of Adamalysin II with peptidic inhibitors. Implications for the design of tumour necrosis factor α convertase inhibitors. Protein Science 7, 283–292.

Grams, F., Huber, R., Kress, L.F., Moroder, L., Bode, W., 1993. Activation of snake venom metalloproteinases by a cysteine switch-like mechanism. FEBS 1, 76–80.

Gutiérrez, J.M., Rucavado, A., 2000. Snake venom metalloproteinases: their role in the pathenogenesis of local tissue damage. Biochimie 82, 841–850.

Gutiérrez, M.J., Le?n, G.R., Lomonte, B., Rucavado, A., Chaves, F., 1998. Neutralization of local tissue damage induced by *Bothrops asper* (Terciopelo) snake venom. Toxicon 36 (11), 1529–1538.

Heath, E.I., Grochow, L.B., 2000. Clinical potential of matrix metalloproetinase inhibitors in cancer therapy. Drugs 59 (5), 1043–1065.

Hite, L.A., Jia, L.G., Bjarnason, J.B., Fox, J.W., 1994. cDNA sequences for four snake venom metalloproteinases: structure, classification, and their relationship to mammalian reproductive proteins. Archives of Biochemistry and Biophysics 301 (1), 1–329.

Iddon, D., Theakston, R.D.G., Ownby, C.L., 1987. A study of the pathogenesis of local skin necrosis induced by *Naja nigricolis* (spitting cobra) venom using simple histological staining techniques. Toxicon 25 (6), 665–672.

Jia, L.-G., Shimokawa, K.-I., Bjarnason, J.B., Fox, J.W., 1996. Snake venom metalloproteinases: structure, function and relationship to the Adams family of proteins. Toxicon 11 (12), 1269–1276.

Kamiguti, A.S., Zuzel, M., Theakston, R.D.G., 1998. Snake venom metalloproteinases and disintegrins: interactions with cells. Brazilian Journal of Medical and Biological Research 31, 853–862.

Koritsas, V.M., Atkinson, H.J., 1995. An assay for detecting nanogram levels of proteolytic enzymes. Analytical Biochemistry 227, 22–26.

Kornalik, F., Blomback, B., 1975. Prothrombin activation induced by Ecarin- a prothrombin converting enzyme from *Echis carinatus* venom. Thrombosis Research 6 (1), 57–63.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Laing, G.D., Lee, L., Smith, D.C., Landon, J., Theakston, R.D.G., 1995. Experimental assessment of a new, low-cost antivenom for treatment of carpet viper (*Echis ocellatus*) envenoming. Toxicon 33, 307–313.

Leonardi, A., Gubenšek, F., Križaj, I., 2001. Purification and characterisation of two haemorrhagic metalloproteinases from the venom of the long-nosed viper, *Vipera ammodytes ammodytes*. Toxicon 40, 55–62.

Mattei, M., Carnieri, E., Politi, V., D'Alessio, S.D., Sella, A., Cassol, M., Robeva, A., Colizzi, V., Sumerska, T., 2002. Inhibition of contact hypersensitivity reaction to picryl chloride: effect of small molecular weight peptidomimetric compounds possessing inhibitory activity against metalloproteinases. International Immunopharmacology 2, 699–710.

Meyer, W.P., Habib, A.G., Onayade, A.A., Yakubu, A., Smith, C., Nasidi, A., Daudu, I.J., Warrell, D.A., Theakston, R.D.G., 1997. First clinical experiences with a new ovine FAB *Echis ocellatus* snake bite antivenom in Nigeria: Randomised comparative trial with Institute Pasteur serum (ISPER) Africa antivenom. American Journal of Tropical Medicine and Hygiene 56 (3), 291–300.

Paine, M.J.I., Desmond, H.P., Theakston, R.D.G., Crampton, J.M., 1992. Purification, cloning, and molecular characterisation of a high molecular weight hemorrhagic metalloproteinase, jararhagin, from *Bothrops jararaca* venom. Insights into the disintegrin gene family. Journal of Biological Chemistry 15 (32), 22869–22876.

Paine, M.J.I., Moura-da-Silva, A.M., Theakston, R.D.G., Crampton, J.M., 1994. Cloning of metalloproteinase genes in the carpet viper (*Echis pyramidum leakeyi*). Further members of the metalloproteinase/disintegrin gene family. European Journal of Biochemistry 224, 483–488.

Pugh, R.N.H., Theakston, R.D.G., 1980. Incidence and mortality on snake bite in savannah Nigeria. The Lancet 29 (2), 1181–1183.

Pugh, R.N.H., Theakston, R.D.G., 1987. A clinical study of viper bite poisoning. Annals of Tropical Medicine and Parasitology 81 (2), 135–149.

Pugh, R.N.H., Bourdillon, C.C., Theakston, R.D.G., Reid, H.A., 1979. Bites by the carpet viper in the Niger Valley. The Lancet 22 (2), 625–627.

Revault, P., 1996. Ecology of *Echis ocellatus* and peri-urban bites in Ouagadougou. Toxicon 34 (2), 114.

Rodrigues, V.M., Soares, A.M., Guerra-Sa, R., Rodrigues, V., Fontes, M.R., Giglio, J.R., 2000. Structural and functional characterisation of neuwiedase, a nonhaemorrhagic fibrin(o-gen)olytic metalloproteinase from Bothrops neuwiedi snake venom. Archives of Biochemistry and Biophysics 15, 213–224.

Rodrigues, V.M., Soares, A.M., Andrião-Escarso, S.H., Franceschi, A.M., Rucavado, A., Gutiérrez, J.M., Giglio, J.R., 2001. Pathological alterations induced by Neuwidase, a metalloproteinase isolated from *Bothrops neuwiedi* snake venom. Biochimie 83, 471–479.

Rosenfeld, J., Capdeville, J., Guillemot, J.-C., Ferrara, P., 1992. In-gel digestion of proteins for internal sequence analysis after one or two-dimensional gel electrophoresis. Anals of Biochemistry 203, 173–179.

Rucavado, A., Escalante, T., Teixeira, C.F., Fernandes, C.M., Diaz, C., Gutiérrez, J.M., 2002. Incrememts in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake *Bothrops asper*. Mediators of Inflammation 11 (2), 121–128.

Sanchez, E.F., Magalhes, A., Mandelbaum, F.R., Diniz, C.R., 1991. Purification and characterisation of the haemorrhagic factor from the venom of the bushmaster snake (*Lachesis muta muta*). Biochimica et Biophysica Acta 6, 347–356.

Selistre-de-Araujo, H.S., de Souza, E.L., Beltramini, L.M., Ownby, C.L., Souza, D.H.F., 2000. Expression, refolding and activity of a recombinant nonhaemorrhagic snake venom metalloproteinase. Protein Expression and Purification 19, 41–47.

Soto, J.G., Perez, J.C., Minton, S.A., 1998. Proteolytic, haemorrhagic and haemolytic activities of snake venoms. Toxicon 26 (9), 875–882.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterisation of snake venoms. Bulletin of the WHO 61, 949–956.

Theakston, R.D.G., Warrell, D.A., 2000. Crisis in antivenom supply for Africa. The Lancet 356, 2104.

Trape, J.F., Pison, G., Guyavarch, E., Mane, Y., 2001. High mortality from snakebite in south-eastern Senegal. Transactions of The Royal Society of Tropical Medicine and Hygiene 95, 420–423.

Warrell, D.A., Arnett, C., 1976. The importance of bites by the saw-scaled or carpet viper (*Echis carinatus*): Epidemiological studies in Nigeria and a review of world literature. Acta Tropica 63, 308–341.

Warrell, D.A., Davidson, N.McD., Greenwood, B.M., Ormerod, L.D., Pope, H.M., Watkins, B.J., Prentice, C.R.M., 1977. Poisoning by bites of the saw-scaled or carpet viper (*Echis carinatus*) in Nigeria. Quarterly Journal of Medicine 181, 33–62.

Wu, W.B., Chang, S.C., Liau, M.-Y., Huang, T.-F., 2001. Purification, molecular cloning and mechanism of action of graminelysin I, a snake-venom-derived metalloproteinase that induces apoptosis of human endothelial cells. Biochemical Journal 357, 719–728.

Yamanda, D., Sekiya, F., Morita, T., 1996. Isolation and characterisation of carinactivase, a novel prothrombin activator in *Echis carinatus* venom with a unique catalytic mechanism. Journal of Biological Chemistry 271, 5200–5207.

Zhang, B., Botos, I., Gomis-Rüth, F.-X., Doll, R., Blood, C., Njoroge, F.G., Fox, J.W., Bode, W., Meyer, E.F., 1994. Structural interaction of natural and synthetic inhibitors with the venom metalloproteinase, atrolysin C (from d). Proceedings of the National Academy of Science, USA 91, 8447–8451.

Zhou, Q., Smith, J.B., Grossman, M.H. 1995. Molecular cloning and expression of catrocollastatin, a snake venom protein from crotalus atrox (western diamondback rattlesnake) which inhibits platelet adhesion to collagen. Biochem Journal 307, 411–417.