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Isolation and characterisation of acanmyotoxin-2 and acanmyotoxin-3, myotoxins from the venom of the death adder *Acanthophis* sp. Seram

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Abstract

Death adder (genus *Acanthophis*) venoms display neurotoxic activity but were thought to be devoid of myotoxic components. Studies from our laboratory have shown that some species (i.e. *Acanthophis rugosus* and *Acanthophis* sp. Seram) posses venom with myotoxic activity [Wickramaratna JC, Fry BG, Aguilar M, Kini RM, Hodgson WC. Isolation and pharmacological characterisation of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*A. rugosus*). Br J Pharmacol 2003;138:333–342; Wickramaratna JC, Fry BG, Hodgson WC. Species-dependent variations in the in vitro myotoxicity of death adder (*Acanthophis*) venoms. Toxicol Sci 2003;74:352–360]. The present study describes the isolation and characterisation of two myotoxins (acanmyotoxin-2 and acanmyotoxin-3) from *A*. sp. Seram venom. Venom was fractionated into approximately 12 major peaks using reverse phase high performance liquid chromatography. Two components caused concentration (0.1–1 μM) dependent inhibition of direct (2 ms, 0.1 Hz, supramaximal V) twitches and an increase in baseline tension in the chick biventer cervicis nerve-muscle. Histological examination of the muscle confirmed damage. PLA_2 activity was detected in both acanmyotoxin-2 (390.2 ± 19.7 μmol/(min mg); $n = 4$) and acanmyotoxin-3 (14.2 ± 7.7 μmol/(min mg); $n = 4$). In comparison, *A*. sp. Seram whole venom had a specific activity of 461.3 ± 90.4 μmol/(min mg) ($n = 3$). Mass spectrometry analysis indicated acanmyotoxin-2 had a mass of 13,082 Da and acanmyotoxin-2 13,896 Da. Acanmyotoxin-2 and acanmyotoxin-3 accounted for approximately 7 and 4% of total venom composition, respectively. N-terminal sequencing of the first 30 amino acids of each toxin indicated they shared some sequence homology with known myotoxins. In conclusion, clinicians should be aware that symptoms of envenoming by some species of death adder may include signs of myotoxicity as well as neurotoxicity. Future studies will investigate the efficacy of the current antivenom treatment against the myotoxic components of *A*. sp. Seram venom.

Keywords: Death adder; *Acanthophis*; Acanmyotoxin; Myotoxin; Chick biventer cervicis nerve-muscle; Phospholipase A_2; Venom

1. Introduction

A number of species of death adder (genus *Acanthophis*) are found throughout Australia and South East Asia. They are the largest ranging of the Australasian elapids, inhabiting most parts of mainland Australia and also inhabit surrounding areas, including Papua New Guinea, Irian Jaya and the Indonesian Islands (e.g. Seram). Although death adders belong to the Elapidae family, they resemble members of the Viperidae family, with large rectangular heads and thick muscular bodies ending in a thin tail. Previous studies in our laboratory have shown that venom of the common death adder (*Acanthophis antarcticus*) has neurotoxic activity with negligible myotoxic activity [1]. Given that the venom of *A. antarcticus* (including several regional variants) has been the most intensively studied [1,2], it was assumed that the venom of all members of the genus were predominantly neurotoxic. It is only recently that a number of the major species and regional variants have been investigated by liquid chromatography/mass spectrometry [3]. This study revealed that there is great diversity in death adder venoms. Based on recent studies in our laboratory [4,5] and clinical reports from Papua New Guinea [6], it is clear that the venoms of several death adders posses myotoxic activity. Wickramaratna et al. [5]

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compared the in vitro myotoxic effects of 10 *Acanthophis* venoms and showed that at least three had marked myotoxicity with *Acanthophis* sp. Seram and *A. rugosus* venoms being particularly myotoxic. A myotoxin (i.e. acanmyotoxin-1) from *A. rugosus*, the Irian Jayan death adder, was subsequently isolated [4]. This is the first myotoxin isolated from a death adder venom. Although a neurotoxin from *A.* sp. Seram has been isolated and characterised [7], no myotoxins have yet been isolated from this venom.

The aim of this study was to isolate and characterise myotoxins from *A.* sp. Seram venom. It is important to understand the composition and mechanism of action of this venom so that envenomation can be recognised and appropriate treatments can be administered to victims of snake bite.

2. Materials and methods

2.1. Fractionation of venom

Freeze dried venom was dissolved in MilliQ water and fractionated by RP-HPLC using a Shimadzu system (LC-10ATVP pump and SPD-10AVP detector). Phenomenex Jupiter semi-preparative (250 mm × 10 mm, 5 μm, C18 300 A) and Phenomenex Jupiter analytical (150 mm × 2 mm, 5 μm, C18 300 A) columns were equilibrated with 0.1% trifluoroacetic acid (TFA) (solvent A). Solvent A was then mixed with a varying gradient of 90% acetonitrile in 0.09% TFA solution (solvent B). The concentrations of solvent B were 0–20% for 0–5 min (4% gradient), 20–60% for 5–45 min (1% gradient) and 60–80% for 45–50 min (4% gradient). The semi-preparative column was used with a flow rate of 2 ml/min, while the analytical column was used with a flow rate of 0.2 ml/min. The elutant was monitored at 280 and 214 nm.

2.2. Mass spectrometry analysis

Analysis was carried out on a Micromass ZMD Electrospray mass Spectrometer (Micromass UK Ltd., Manchester, UK) under the following conditions; 3.0 kV capillary, 30, 60 or 90 V cones, in positive ion mode. Nitrogen gas was used as a curtain gas with a flow rate of 3.3 l/min. Samples were injected by direct infusion at 8 μl/min. Data processing were performed using MassLynx Version 3.5 (Micromass UK Ltd.).

2.3. Determination of PLA_2 activity

PLA_2 activity of the isolated components and whole venom was determined using a secretory PLA_2 colourmetric assay kit (Cayman Chemical, USA). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as a substrate. Free thiols generated upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5′-dithiobis(2-nitrobenzoic acid)). Colour changes were monitored using a CER-ES900C microplate reader (Bio Tek Instruments, USA) at 405 nm, sampling every min for a 5-min period. PLA_2 activity was expressed as phosphatidylcholine hydrolysed μmol/min/mg enzyme.

2.4. Amino acid sequence determination

The pure peptides were N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems Procise pulsed-liquid-phased peptide sequencer fitted with a 140C microgradient LC and 785A programmable absorbance detector (Applied Biosystems, CA, USA).

2.5. Chick biventer cervicis nerve-muscle preparation

Male chicks aged between 4 and 10 days were killed with CO_2 and both biventer cervicis nerve-muscles removed. The muscles were mounted under 1 g resting tension in 5 ml organ baths containing physiological salt solution (NaCl, 118.4 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; glucose, 11.1 mM). The solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34 °C. Direct twitches were established by electrical stimulation (0.1 Hz, 2 ms, supramaximal voltage) using a Grass S88 stimulator with an electrode placed around the body of the muscle. The preparation was equilibrated for 30 min, after which d-tubocurarine (d-TC; 10 μM) was added to the bath. This blocks indirect (i.e. nerve-mediated) twitches ensuring that the twitches are due only to direct muscle stimulation. The preparation was left for a further 30 min for equilibration or until a steady, consistent twitch height was obtained. Toxins or venom fractions were then added and left in contact with the tissue for a period of 3 h. A contracture of the skeletal muscle and a diminished twitch height were indicative of myotoxicity [8].

2.6. Morphological studies

On completion of the in vitro experiments, the tissues were placed in Tissue Tek, frozen with liquid nitrogen and stored at −80 °C until required. The tissues were cut into transverse sections (14 μm) using a Leica CM1800 cryostat and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in distilled water, stained with eosin and haematoxylin and examined under a light microscope (Olympus BX 51, Olympus Co., Japan). Slides were photographed using an Olympus C-4040ZOOM (Olympus Optical Co., Japan) digital camera.

2.7. Chemicals and drugs

The following drugs and chemicals were used: trifluoroacetic acid (Auspep, Melbourne, Australia), acetonitrile

(Merck, Darmstadt, Germany), d-tubocurarine, eosin, Mayer's Haemalum (Sigma Chemical Co., St. Louis, USA), *A*. sp. Seram venom (Venom Supplies Pty Ltd., Tanunda, Australia). Isolated toxins were reconstituted in filtered MilliQ water for in vitro experiments and for RP-HPLC.

2.8. Analysis of results and statistics

For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System. Twitch height was expressed as a percentage of the original twitch height prior to the administration of venom fractions. Change in baseline tension was measured as an increase/decrease of the original baseline prior to the administration of venom fractions. Statistical difference was determined by a one-way analysis of variance (ANOVA) on the change in twitch height or baseline tension at the 180 min time point. All ANOVAs were followed by a Dunnett's Multiple Comparison Test against the vehicle control. Statistical significance was indicated when $P < 0.05$.

3. Results

3.1. Isolation and purification of myotoxins from A. sp. Seram venom

Two myotoxins were identified and isolated from *A*. sp. Seram venom by successive reverse phase high performance liquid chromatography (RP-HPLC) separations. The initial fractionation of *A*. sp. Seram venom using the analytical column produced approximately 12 major peaks (Fig. 1a). Preliminary studies in the chick biventer cervicis nerve-muscle preparation showed that peaks 10 and 11 exhibited myotoxic activity. Peaks 10 and 11 were then collected from multiple RP-HPLC runs using the semi-preparative column. The samples were freeze dried and then reconstituted in MilliQ water. To test the purity, and determine the homogeneity and location of the isolated toxins, venom and the two toxins were run on the same conditions using the analytical column (Fig. 1b and c). If any contaminants were detected then the sample was run through the analytical column and then put through the freeze drying process again. Peak 10 eluted at 29.55 min and accounted for approximately 7% of total venom composition, while peak 11 eluted at 32.90 min and accounted for approximately 4% of total venom composition.

Peak 10 is subsequently referred to as acanmyotoxin-2 and Peak 11 referred to as acanmyotoxin-3.

3.2. Purity and molecular mass determination

The homogeneity and molecular mass of both toxins were determined by electrospray mass spectrometry. The purified samples of acanmyotoxin-2 and acanmyotoxin-3 displayed consistent peaks at several charged states and could be reconstituted into single masses of 13,082 Da (Fig. 2a) and 13,896 Da, respectively (Fig. 2b).

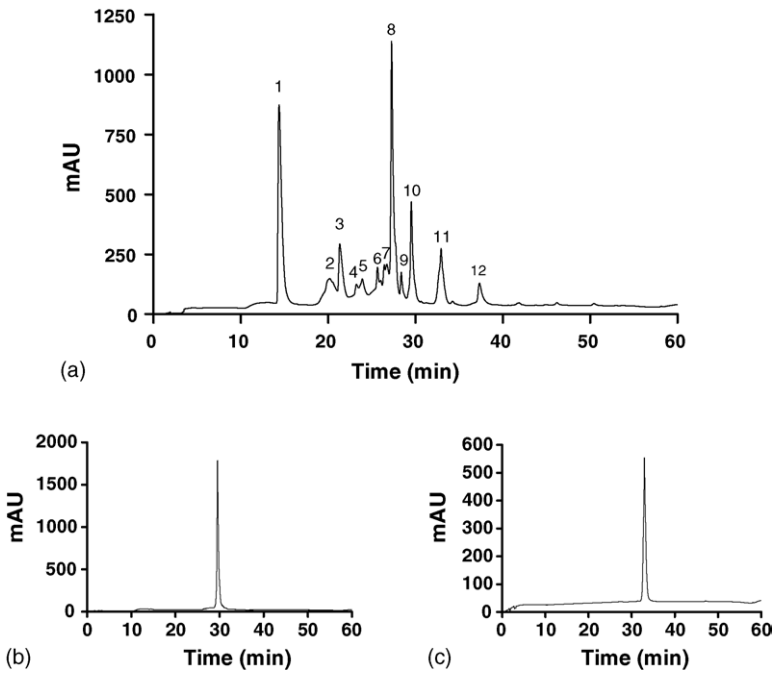

Fig. 1. RP-HPLC chromatograph of (a) *A*. sp. Seram venom with numbered peaks, (b) acanmyotoxin-2 and (c) acanmyotoxin-3 run with the same conditions on the Jupiter analytical column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0–20% for 0–5 min, 20–60% for 5–45 min and 60–80% for 45–50 min. Flow rate of 0.2 ml/min.

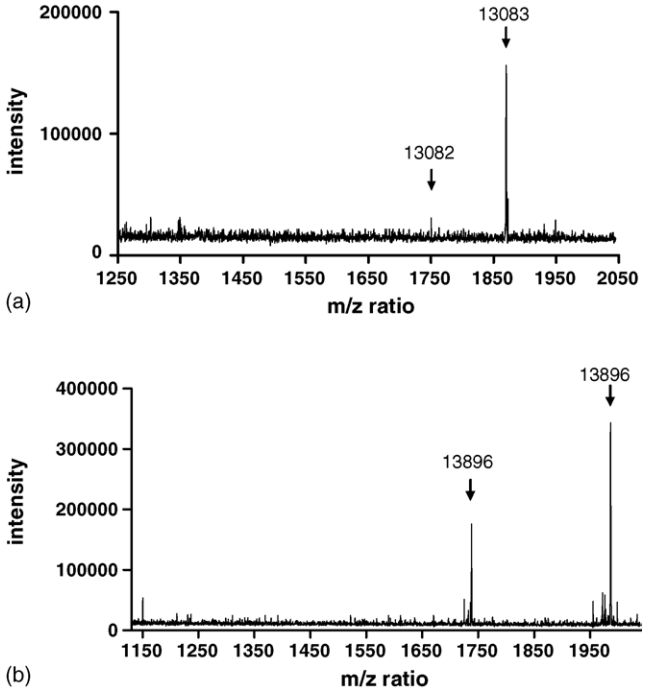

Fig. 2. Electrospray mass spectrometry of (a) acanmyotoxin-2 and (b) acanmyotoxin-3. The spectra shows a series of multiple-charged ions, arrows indicate charges found consistently at different voltages.

3.3. Phospholipase A_2 activity

PLA_2 activity was detected in both acanmyotoxin-2 and acanmyotoxin-3. The specific activity for acanmyotoxin-2 was 390.2 ± 19.7 μmol/min/mg ($n = 4$) and acanmyotoxin-3 was 14.2 ± 7.7 μmol/min/mg ($n = 4$). *A.* sp. Seram venom had a specific activity of 461.3 ± 90.4 μmol/min/mg ($n = 3$). The positive control, bee venom PLA_2, had a specific activity of 637.0 ± 172.1 μmol/min/mg ($n = 3$).

3.4. N-terminal amino acid sequence

The first 30 amino acids were identified for both acanmyotoxin-2 and acanmyotoxin-3 (Table 1). The sequence was compared with other protein sequences at the National Centre for Biotechnology Information (NCBI) database using the BLAST service. Acanmyotoxin-2 shares 53% sequence identity with acanthin II, isolated from *A. antarcticus* venom [9] and 50% sequence identity with pseudexin A, isolated from the red-bellied black snake (*Pseudechis porphyriacus*) [10]. Acanmyotoxin-3 shares 38% sequence identity with taipoxin-α, a toxin isolated from the Australian taipan (*Oxyuranus scutellatus scutellatus*) [11].

3.5. Chick biventer cervicis nerve-muscle preparation

Acanmyotoxin-2 (0.1 and 1.0 μM) and acanmyotoxin-3 (0.1 and 1.0 μM) caused significant and concentration-dependent decreases in twitch height compared to vehicle ($n = 4$; one-way ANOVA, $P < 0.05$; Fig. 3a and b). Acanmyotoxin-2 caused a significantly greater decrease than acanmyotoxin-3 at 1.0 μM. Both toxins caused a significant increase in baseline tension at the higher concentration (1.0 μM) compared to vehicle ($n = 4$; one-way ANOVA, $P < 0.05$; Fig. 4a and b). The lower concentration (0.1 μM) had no significant effect on baseline tension.

3.6. Morphological studies

Light microscopy studies of tissues exposed to acanmyotoxin-2 and acanmyotoxin-3 indicated morphological changes. Acanmyotoxin-2 (1 μM) caused the most muscle damage with obvious tissue breakdown and vacuolisation (Fig. 5b) compared to the tissue exposed to vehicle (Fig. 5a). Acanmyotoxin-3 (1 μM) (Fig. 5c) showed simi-

Table 1
N-terminal sequence of PLA_2 components isolated from other death adder species and other elapid snake venoms with myotoxic activity

Species	PLA_2 component	N-terminal sequence		
A. antarcticus	Acanthin I[a]	DLFQFGGMIG	CANKGARSWL	SYVNYGCYCG
A. antarcticus	Acanthin II[a]	NLYQFGGMIQ	CANKGARSWL	SYVNYGCYCG
A. antarcticus	Acanthoxin A1[b]	NLYQFGGMIQ	CANKGARSWL	SYVNYGCYCG
A. praelongus	Acanthoxin B[c]	DLFQFGFMIQ	CANKGSRPVF	
A. pyrrhus	Acanthoxin C[c]	NLFQFGGMIG	CANKGTRSWL	SYVNYGCYCG
Pseudechis australis	Pa-1G[d]	NLIQFGNMIQ	CANKGSRPTR	HYMDYGCYC
Oxyuranus scutellatus scutellatus	Taipoxin α chain[e]	NLLQFGFMIR	CANRRSRPVW	HYMDYGCYCG
Pseudechis porphyriacus	Pseudexin A[f]	NLYQFKNMIQ	CANKGSRSWL	DYVNYGCYCG
A. rugosus	Acanmyotoxin-1[g]	NLLQIGIMKR	CANKRRRPVF	HYRDYGCYC
A. sp. Seram	Acanmyotoxin-2	NLYQFGGMIG	CANKGTRSWL	SYVNYGCYCG
A. sp. Seram	Acanmyotoxin-3	NLLQFAFMIR	QANKRRRPVI	PYEEYGLYYM

[a] Chow et al. [9].
[b] van der Weyden et al. [18].
[c] van der Weyden et al. [19].
[d] Takasaki et al. [24].
[e] Lind and Eaker [11].
[f] Schmidt and Middlebrook [10].
[g] Wickramaratna et al. [4].

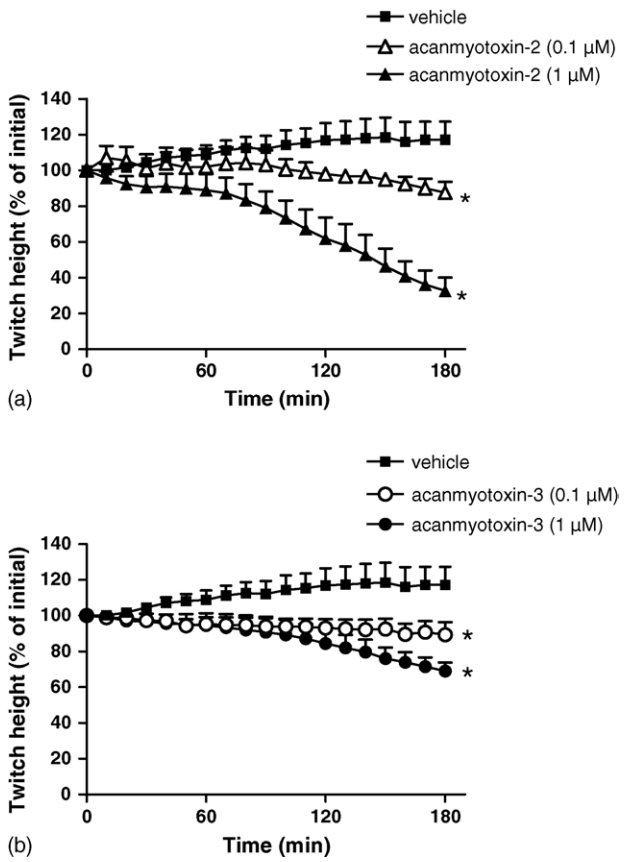

Fig. 3. The effect of (a) acanmyotoxin-2 (0.1 and 1 μM, $n = 4$) and (b) acanmyotoxin-3 (0.1 and 1 μM, $n = 4$) on direct twitches (0.1 Hz, 2 ms, supramaximal voltage) in the CBCNM preparation. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

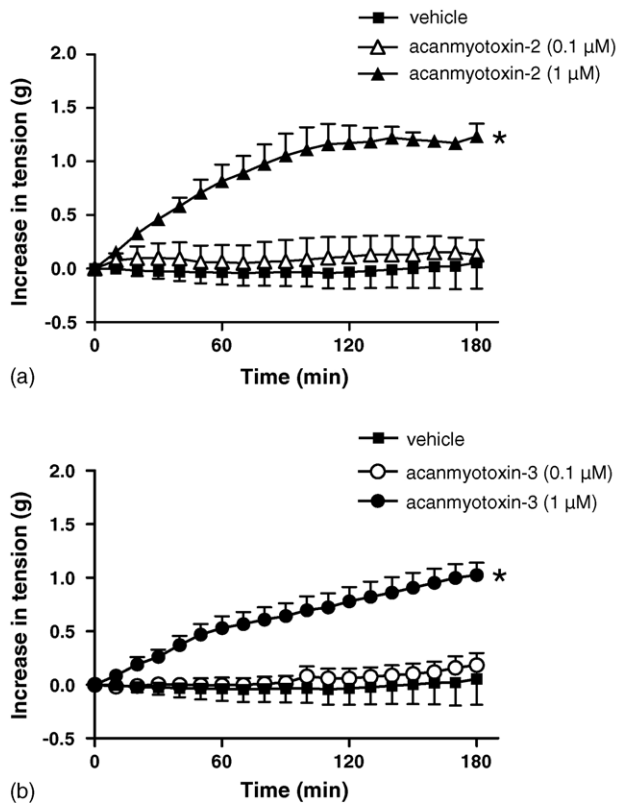

Fig. 4. The effect of (a) acanmyotoxin-2 (0.1 and 1.0 μM, $n = 4$) and (b) acanmyotoxin-3 (0.1 and 1.0 μM, $n = 4$) on baseline tension in the CBCNM preparation. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

lar damage but not to the extent of acanmyotoxin-2. There was some indication of early muscle damage caused by both toxins at lower concentrations (0.1 μM; data not shown). These results are consistent with the concentration-dependent effects observed on direct twitches in the CBCNM preparation.

4. Discussion

It has recently been reported that some species of death adder venom have myotoxic activity [5,6], an activity that was originally thought to be absent from this genus. Clinical reports from Papua New Guinea indicated signs of myotoxic activity in the venom of a local species [6]. Consequently, venom from *A. rugosus* was studied and a myotoxin isolated (i.e. acanmyotoxin-1), the first from a death adder [4]. A further study compared the myotoxic effects of 10 death adder venoms [5]. The venom from *A.* sp. Seram showed the highest myotoxic activity and is the focus of the current study.

After fractionation of *A.* sp. Seram venom and screening of the pooled fractions for myotoxic activity, two myotoxins were isolated as single peaks by successive RP-HPLC. Using electrospray mass spectrometry the molecular mass for both toxins was determined (i.e. acanmyotoxin-2, 13,082 Da and acanmyotoxin-3, 13,896 Da). It has been documented that snake venom PLA_2 components have masses in the range of 12–14 kDa [12–14]. Acanmyotoxin-3 is similar in molecular weight to that of acanmyotoxin-1 (13,811 Da), the toxin isolated from *A. rugosus* [4]. Previous LCMS analysis has indicated that *A.* sp. Seram venom contains a toxin with a molecular weight of 13,898 Da and this was classified as a PLA_2 due to its molecular weight and N-terminal sequence [3]. However, PLA_2 activity had not been confirmed and no characterisation has been performed. What the current study does show is an apparent match and confirmation of this toxin in *A.* sp. Seram venom.

The N-terminal sequences showed similarities with other elapid toxins. Acanmyotoxin-2 shares 53% sequence identity with acanthin II, a potent PLA_2 inhibitor of platelet aggregation from the venom of *A. antarcticus* [9]. Acanmyotoxin-3 shares 38% sequence identity with taipoxin-α chain, isolated from the Australian coastal taipan (*Oxyuranus scutellatus scutellatus*) [11]. Taipoxin has been shown to act as a potent presynaptic neurotoxin but also exhibits myolytic and necrotic activity [15–17]. Some sequence similarities can also be seen with toxins previously isolated from *A. antarcticus* [18,19].

In the present study, PLA_2 activity was determined and confirmed for both of the isolated fractions. The activity of

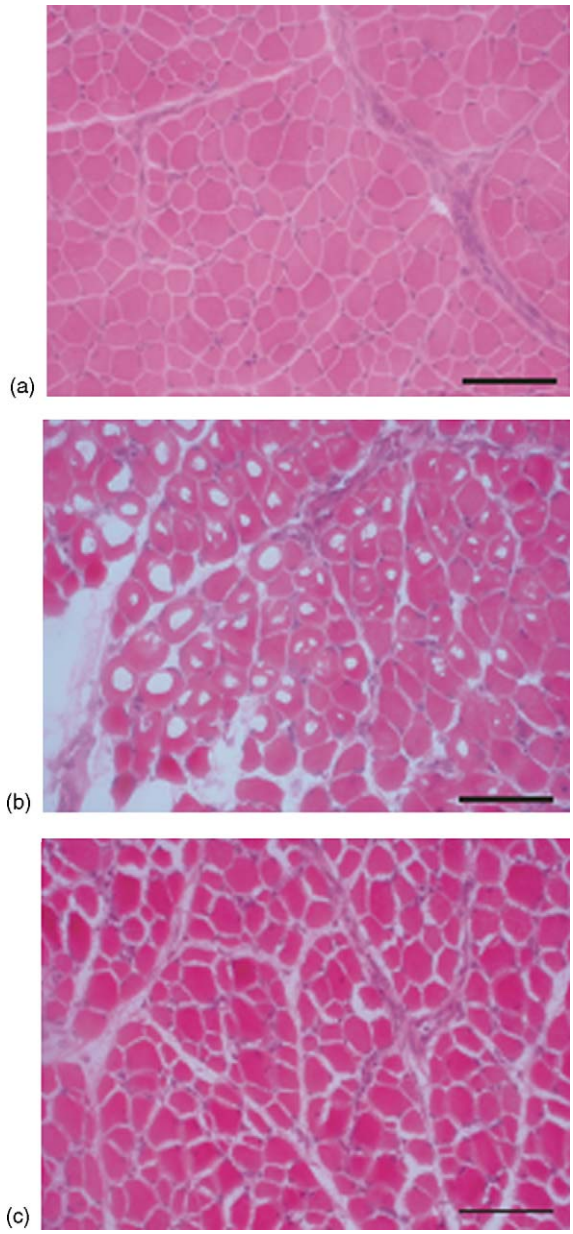

Fig. 5. Transverse sections of CBCNM preparations exposed to (a) vehicle (50 μl 0.1% BSA) for 3 h, (b) acanmyotoxin-2 (1 μM) and (c) acanmyotoxin-3 (1 μM) for 3 h. Scale bar = 100 μm.

acanmyotoxin-2 was similar to that of acanmyotoxin-1 from *A. rugosus* venom [4]. The specific PLA_2 activity of acanmyotxin-3 was much lower than that of acanmyotoxin-2. This lower level of enzymatic activity correlates with the lesser amount of damage caused by acanmyotoxin-3 at the tested concentrations. It is possible that acanmyotoxin-3 may be a Lys-49 PLA_2 myotoxin and acanmyotoxin-2 a Asp-49 PLA_2 myotoxin. Lys-49 PLA_2s do not exhibit catalytic activity and have a lysine substitution for asperate at position 49 of the amino acid sequence. This substitution results in an inability of the protein to bind to calcium, and hence has no catalytic activity [20]. It has been shown that Lys-49 PLA_2s still cause marked inflammatory reactions [21], suggesting that catalytic activity is not the sole mechanism of action for myotoxic PLA_2s. The current study has identified the first 30 amino acids for both acanmyotoxin-2 and acanmyotoxin-3, further sequencing is required to discover the identity of the amino acid at position 49. Future experimentation could also include the use of catalytic inhibitors such as *p*-bromophenacyl bromide (pBPB), to investigate if acanmyotoxin-3 does in fact act by non-catalytic mechanisms and also investigate the activity of acanmyotoxin-2 with its high level of catalytic activity being inactivated. pBPB has been used commonly as a catalytic inhibitor for the study of myotoxic PLA_2s [4,20,21]. It is possible that there are PLA_2 components in the venom that are yet to be identified. In the present study, only the venom fractions that exhibited in vitro myotoxic activity were further examined for PLA_2 activity. PLA_2 activity is commonly associated with myotoxic activity [4,5,22–24], but is not specific to venom myotoxins. Components such as presynaptic neurotoxins [25,26] and toxins that effect blood coagulation [27] also have PLA_2 activity.

Acanmyotoxin-2 and acanmyotoxin-3 were both examined for myotoxicity in vitro using the directly stimulated CBCNM preparation. The results indicated a concentration-dependent myotoxic action for both toxins. In addition, light microscopy studies were carried out to identify morphological changes within the tissues. Both acanmyotoxin-2 and acanmyotoxin-3 produced obvious changes in the skeletal muscle compared to that of the control tissues. The combination of these results suggests that both acanmyotoxin-2 and acanmyotoxin-3 are myotoxic. Acanmyotoxin-2 was seen to exhibit greater myotoxic action than acanmyotoxin-3. *A.* sp. Seram venom has previously been examined in vitro, in our laboratory, using the CBCNM preparation [5]. In this study, the venom showed a more rapid decrease in twitch height than that observed to either acanmyotoxin-2 or acanmyotoxin-3. This effect is likely to be due to the synergistic action of the different myotoxins found in the whole venom producing a greater level of tissue breakdown than either alone.

Death adder envenoming in Australia is not very common. However, there is a much greater incidence on some of Indonesia's islands and Papua New Guinea [28,29]. The prescribed treatment for systemic death adder envenoming is CSL death adder antivenom [30] which is raised against *A. antarcticus* venom, i.e. a non-myotoxic venom [1,31]. Myotoxicity can cause serious morbidity if not monitored and treated correctly. Therefore, clinicians need to be aware that the symptoms of envenoming by some species may include signs of myotoxicity as well as neurotoxicity.

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