

**Isolation and characterisation of insulin-releasing compounds from *Pseudechis australis* and *Pseudechis butleri* venom**

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## **Abstract**

Crude venom from two elapid snakes *Pseudechis australis* and *Pseudechis butleri* was fractionated by gel filtration chromatography and selected fractions screened for *in vitro* insulin-releasing activity using clonal pancreatic BRIN-BD11 cells. Following acute 20-min incubation at 5.6 mM glucose, 9 fractions exhibited significant ( $P < 0.001$ ) insulin-releasing activity. Structural characterisation of active fractions was achieved primarily using MALDI-TOF MS and N-terminal Edman degradation sequencing. The partial N-terminal sequences are reported for a total of 7 venom components. Their homology to existing sequences as determined using BLAST searching uncovered the main insulin-releasing families as being phospholipases A<sub>2</sub> and short  $\alpha$ -neurotoxins. A number of sequences are reported for the first time from *Pseudechis butleri* venom which is much less studied than the related *Pseudechis australis*.

*Keywords:* *Pseudechis australis*, *Pseudechis butleri*, elapid, venom, insulin secretion, diabetes

## **1.0 Introduction**

Reptiles and their venom products have been used traditionally for many years in the treatment of human disease and ailments. Snake fat has been used as both an anti-rheumatic ointment and as relief for sore throats whilst dried snake skin has been claimed to act as a cough suppressant, a diuretic for kidney stones and to have anti-rhinitic properties (Quave *et al.* 2010). Snake meat is classed as a medicinal food; fried and eaten to purify the blood, with leftover cooking used oil as an anti-scabies treatment, or being applied to disinfect and heal wounds (Quave *et al.* 2010). The composition of snake venom components has been

extensively reviewed (Tu, 1996). Several factors have been shown to be influential in determining composition of snake venom through differences at the genus level (Vonk *et al.* 2011). They contain a mixture of both enzymatic and non-enzymatic proteins (Walker *et al.* 2013). As reviewed by Escoubas and colleagues (2008), the main structural families present in snake venom include serine proteinases, CRISP (cysteine-rich secretory proteins), L-amino acid oxidases, C-type lectin-like proteins, disintegrins, metalloproteinases and phospholipases A2.

Recent figures estimate that expenditure for diabetes care and associated complications generate a considerable social and economic burden (Seuring *et al.* 2015). The quest for new biomolecules that may be useful for diabetes therapy has opened up the exploration of natural products and in particular animal venoms. Indeed, the best known example of a venom-derived treatment for type 2 diabetes is the incretin mimetic exendin-4 (Byetta) which was isolated from the Gila monster *Heloderma suspectum* (King 2011). Exendin-4 is a 39-amino acid peptide which has a 53% sequence identity to glucagon-like peptide-1 (GLP-1) and displays bioactivities similar to GLP-1 but with a protracted biological half-life (Lund *et al.* 2014).

There are several examples of snake venom constituents and crude snake venom suggestive of insulin-releasing properties. A decrease in glucose concentrations was detected following injection of crude venom from *Crotalus durissus terrificus* rattlesnake (Hernández Cruz *et al.* 2008). Cardiotoxin (CTX-1), a peptide isolated from the venom of the *Naja kaouthia* snake, has been described by Nguyen and co-workers (2012) as a potent insulin secretagogue when tested in INS-1E cells with no cytotoxicity. Various *Crotalus* species have also been examined, with a dose-dependent increase in insulin secretion reported by Toyama and

colleagues (2005) for the crotamine isoform Cro 2 from the *Crotalus durissus cascavella*. Indeed, research from our laboratory has shown that venom from the vipers *Crotalus adamanteus*, *Crotalus vegrandis* and *Bitis nasicornis* contain insulin-releasing compounds such as serine proteinases, phospholipases A<sub>2</sub> (PLA<sub>2</sub>) and disintegrins (Moore *et al.* 2015). Supported by our previous work and existing literature on the ability of various snake venom components to stimulate insulin-release, two Elapidae snakes, *Pseudechis butleri* and *Pseudechis australis*, were chosen for study. A number of venom chromatographic fractions, corresponding to phospholipase A<sub>2</sub> and  $\alpha$ -neurotoxins, exhibited a significant elevation in insulin-release when examined in BRIN-BD11 pancreatic beta-cells.

## **2.0 Materials and methods**

### *2.1 Materials*

*Pseudechis australis* and *Pseudechis butleri* venoms were obtained from Venom Supplies, Pty Ltd. (Tanunda, South Australia). HPLC-grade water (VWR International, Geldenaaksebaan, Leuven, Belgium) and ammonium acetate (Fisher Scientific, UK) were used to prepare LC mobile phase. RPMI-1640 tissue culture medium, foetal calf serum, penicillin and streptomycin were obtained from Gibco (Paisley, Strathclyde, UK). Sinapinic acid and  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) were obtained from Fluka, Sigma-Aldrich, (Dorset UK) and used as matrices for MALDI-TOF MS analysis. Reagents for N-terminal Edman degradation sequencing were obtained from Perkin-Elmer Biosystems (Warrington, UK). All other chemicals were of analytical grade or better.

## 2.2 *Crude venom fractionation*

Crude snake venom was fractionated by gel filtration chromatography on a Spectra System HPLC (Thermo Separation Products, Riviera Beach, FL, USA) using Superdex 75 10/300 GL column (10 x 300 mm GE Healthcare Life Sciences, Buckinghamshire, UK). Venoms (15 mg/mL in 0.1 M ammonium acetate) were centrifuged (10,000 rpm for 5 min) and supernatant (200 µL) chromatographed at ambient temperature using isocratic mobile phase (100% 0.1 M ammonium acetate) at flow rate of 0.7 mL/min. LC effluent was monitored at 214 and 280 nm and fractions collected every min using automatic fraction collector (Pharmacia LKB Frac-100, Uppsala, Sweden) over 90-min run time and fractions capped and stored at 4°C prior to further analysis.

## 2.3 *MALDI-TOF MS and N-terminal Edman degradation sequencing*

Guided by LC-UV response for each venom fractionation, individual test fractions were analysed using PerSeptive Biosystems Voyager-DE Biospectrometer MALDI-TOF mass spectrometer. Internal mass calibration instrument was performed prior to sample analysis using Peptide Calibration Mix 2 as described previously (Moore *et al.* 2015). Test sample (1.5 µL) was pipetted onto one pre-defined well of a 100-well stainless steel plate and allowed to dry at room temperature. A 10 mg/mL solution of appropriate matrix (CHCA or sinapinic acid) was prepared in 50% acetonitrile: 50% HPLC water with 0.1% TFA. The plate was inserted into the MALDI-TOF and all measurements collected in linear positive ionisation mode using minimum of 50 laser shots. For CHCA matrix, accelerating voltage was maintained at 20,000 V with grid voltage and guide wire voltages set at 94% and 0.05%, respectively. For sinapinic acid matrix, accelerating voltage was changed to 25,000 V and grid voltage and guide wire voltage set to 92% and 0.3%, respectively. Nitrogen laser (337 nm) was directed toward the densest area of the sample/matrix spot, the laser intensity

adjusted to obtain optimal spectral response and mass/charge ratio plotted against relative abundance. For Edman sequencing, sample was immobilised onto BioBrene Plus filter (Applied Biosystems) and partial amino acid sequence of a number of fractions performed using Perkin Elmer Applied Biosystems 491 Protein Sequencer using standard protocols (Moore *et al.* 2015).

#### 2.4 *In vitro* insulin release studies

Test fractions for acute insulin-release assay were selected based primarily on MALDI-TOF MS results. An aliquot of each LC fraction (500  $\mu$ L) and blank (500  $\mu$ L 0.1 M ammonium acetate) were placed in the freezer overnight at  $-70^{\circ}\text{C}$  overnight and subsequently freeze-dried using Heto PowerDry PL6000 freeze drier (Thermo Electron Corporation, Waltham, MA, USA). BRIN-BD11 cells (see McClenaghan *et al.* 1996 for origin and characteristics of BRIN-BD11 cells) were cultured in sterile tissue culture flasks (Orange Scientific, Braine-l'Alleud, Belgium) using RPMI-1640 tissue culture medium containing 11.1 mM glucose, 10% v/v foetal calf serum, and 1% (v/v) antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin). Cells were incubated in a LEEC incubator (LEEC, Nottingham, UK) at  $37^{\circ}\text{C}$  in atmosphere of 5%  $\text{CO}_2$ . For acute insulin-release studies BRIN-BD11 cells were seeded into 24-well plates and experiments performed as described previously (Moore *et al.* 2015). Samples for testing (500  $\mu$ L) were reconstituted and insulin-release performed at  $37^{\circ}\text{C}$  for 20 min using Krebs Ringer Bicarbonate Buffer (KRBB) supplemented with 5.6 mM glucose (500  $\mu$ L). We chose to test selected compounds from snake venom at 5.6 mM glucose as this represents a physiological non-stimulatory concentration to test for novel insulinotropic activity (McClenaghan *et al.* 1996). Following incubation, insulin released into the supernatant was determined by radioimmunoassay (Flatt and Bailey, 1981). Basal (unstimulated) levels of insulin release were set to 100% (control).

## 2.5 Statistical Analysis

Insulin-release data are expressed as mean  $\pm$  SEM and results analysed using Student's unpaired *t*-test.  $P < 0.05$  was deemed to be statistically significant.

## 3.0 Results

### 3.1 Crude venom fractionation

Figures 1a and 1b illustrate the UV profile for *Pseudechis australis* and *Pseudechis butleri*, respectively. Chromatograms for both *Pseudechis* species are identical with exception of one additional peak with *Pseudechis butleri* eluting at 21.11 min on the shoulder of the main peak at approximately 19 min. In line with the principles of size-exclusion chromatography, MALDI-TOF data for the individual venom fractions generally indicates a decrease in molecular weight of eluted components with increasing retention time. No further purification was performed on any of the LC fractions and consequently the individual fractions are likely to contain more than one component. MALDI-TOF MS analysis was performed on all of the fractions tested for insulin-release using both CHCA and sinapinic acid matrices. Further characterisation of the fractions comprised a review of the experimental MALDI-TOF masses with those deposited in the Uniprot database and N-terminal degradation sequencing of suitable fractions.

### 3.2 *Pseudechis australis* in vitro insulin-release and structural characterisation

Four *Pseudechis australis* fractions were selected for insulin-release testing and all exhibited significant insulin-releasing activity (1.6 to 1.9-fold;  $P < 0.001$ ) compared to 5.6 mM glucose alone (Figure 2). MALDI-TOF mass spectrum for *Pseudechis australis* fraction 17 collected with sinapinic acid matrix showed singly charged species as the base peak at  $m/z$  27704.21

with the doubly charged species also evident at  $m/z$  13854.33. Closer inspection also revealed a further two peaks of lower intensity at  $m/z$  27555.01 and  $m/z$  27859.60 either side of the base peak. When subjected to N-terminal degradation sequencing the first 30 residues was determined as: NLYQFYEMIECANFGTISGLAYAGYYCYCG. A BLAST search indicated this sequence to have 90% homology with residues 28 to 57 of the phospholipase A2 enzyme *Pseudechis cf. australis* HI-2009 (GenBank: BAJ07178.1; Table 1).

Edman degradation sequencing for fraction 19 indicated similarity to phospholipase A2 family. With at least three main components of masses of approximately 13 kDa, partial Edman sequence determined NLIQFGWMIQCANKGSRPSLDYADYGCYCGWG forms a core stretch in each of these. As shown in Table 1 this experimental sequence shares a 97% homology with each of the following: phospholipase A2 enzyme *Pseudechis cf. australis* HI-2009 (GenBank: BAJ07180.1); Pa-11 precursor from the *Pseudechis australis* (GenBank: AAZ22660.1); basic phospholipase A2 PA-11 (UniProtKB/Swiss-Prot: P04056.1); and basic phospholipase A2 PA-12C (UniProtKB/Swiss-Prot: P20256.1).

MALDI-TOF spectrum for fraction 21 showed two main peaks at  $m/z$  13191.41 and  $m/z$  13114.77 with the former peak also present in fraction 22 at approximately 82% intensity. Based solely on observed experimental masses, these are most probably PLA<sub>2</sub> type compounds. The base peak in fraction 22 with  $m/z$  6711.52 is not comparable to any of the UniProt masses listed for *Pseudechis australis*. However, a similar sized peak of  $m/z$  6745.08 discussed later with the results for *Pseudechis butleri* fraction 22 was found to belong to the snake three-finger toxin family. Further sequencing would be required to confirm if this is indeed the correct family assignment. Structural characterisation by Edman was not attempted as the fraction contained a mixture of peptides.

### 3.3 *Pseudechis butleri* in vitro insulin-release and structural characterisation

A significantly elevated insulin-release (1.3 to 2.3-fold;  $P < 0.001$ ) was observed for *Pseudechis butleri* test fractions 14, 18, 19, 21 and 22 (Figure 2). Fraction 14 coincided with the first peak eluted peak on the UV profile for the *Pseudechis butleri* fractionation, but the complexity and presence of high molecular weight components at ~23.5 kDa and 53 kDa restricted further structural characterisation. These masses were not comparable with any of the masses published in UniProt database for *Pseudechis butleri*. Although complex with two main peaks at  $m/z$  13085.87 and 13246.16, fraction 18 was subjected to N-terminal degradation sequencing with a partial sequence established. The first sequence was determined as: NLIQFGNMIQCANKGQYPELAYADY which shares 84% homology with residues 1 to 25 of the basic phospholipases A2 PA-11 (UniProtKB/Swiss-Prot: P04056.1), PA-12C (UniProtKB/Swiss-Prot: P20256.1) and PA-12A (UniProtKB/Swiss-Prot: P20255.1). Furthermore, it also shares 84% homology with residues 28 to 52 of the Pa-11 precursor *Pseudechis australis* (GenBank: AAZ22660.1) and phospholipase A2 enzyme *Pseudechis cf. australis* HI-2009 (GenBank: BAJ07180.1). The mass of this second component at ca. 13 kDa would imply that it belongs to the PLA<sub>2</sub> family, yet as shown in Table 2, a BLAST search restricted to the *Pseudechis* species shows only a 69% homology with residues 28 to 43 of the phospholipase A2 enzyme *Pseudechis cf. australis* HI-2009 (GenBank: BAJ07178.1). Widening the search parameters indicated 56% homology to a PLA<sub>2</sub> protein from the Indian elapid *Bungaris fasciatus*. Further purification for this fraction would be required to allow the second component to be sequenced on its own.

The MALDI-TOF spectra of fractions 19 and 21 showed some commonality with a main cluster of peaks present at approximately 13 kDa. Although both fractions 19 and 21

promoted significant insulin-release ( $P < 0.001$ ), fraction 21 elicited greater insulin-release which has a notable peak of  $m/z$  6733.41 at 70% intensity. Fraction 19 was not sequenced but the partial sequences for two components of fraction 21 were established by Edman degradation sequencing. As shown in Table 2, a BLAST search of the first sequence NILQFRKMIQCANKGSRAAWHYLDYG showed 100% homology with the N-terminal sequences deposited for the basic phospholipases A2 PA-13 (UniProtKB/Swiss-Prot: P04057.1) and PA-15 (UniProtKB/Swiss-Prot: P20257.1) from *Pseudechis australis*. It also showed 100% homology with residues 28 to 53 of both the *Pseudechis australis* Pa-13 precursor (GenBank: AAZ22665.1) and phospholipase A2 enzyme *Pseudechis cf. australis* HI-2009 (GenBank: BAJ07181.1). The second sequence identified for fraction 21 was: NTCENCTSSEPLTITKCCGGDSSCCKC. Results of the BLAST search indicate a shared homology of 56% with short neurotoxin 1 from *Pseudechis porphyriacus* (UniProtKB/Swiss-Prot: A8HDJ4.1) and 58% homology with another short neurotoxin 1 from *Naja mossambica* (UniProtKB/Swiss-Prot: P01431.1). It had been assumed that the two sequences for fraction 21 would have corresponded to the two most intense peaks at  $m/z$  13185.43 and  $m/z$  13111.23 and both been PLA<sub>2</sub> type compounds. However, results for the Edman sequencing indicate one PLA<sub>2</sub> and one short neurotoxin. This would suggest that the residues sequenced for PLA<sub>2</sub> are core to both of the main peaks at ~13 kDa with the sequence for the short neurotoxin corresponding to the peak of  $m/z$  6733.41 at 70% intensity.

For *Pseudechis butleri* fraction 22, the small peak detected on the UV profile eluting on the shoulder of the main peak at 21.11 min is manifest as the base peak at  $m/z$  6745.08 on the MALDI-TOF MS spectrum. Similar to fraction 21, the presence of a PLA<sub>2</sub> type compound of ~13 kDa and a lower mass compound of ~6.7 kDa in fraction 22 appears to have a positive influence on promoting insulin-release. The following sequence information was obtained for

*Pseudechis butleri* fraction 22 when subjected to Edman degradation sequencing: MTCANQQSIQPKTTTINAGGESSCYKKTW (Table 2). This sequence shows 90% homology with the 83 amino acid short neurotoxin 1 isolated from *Pseudechis australis* (UniProtKB/Swiss-Prot: P25497.2) which belongs to the snake three-finger toxin family.

#### **4.0 Discussion**

The insulin-releasing activity of snake venom components fractionated from *Pseudechis australis* and *Pseudechis butleri* crude venoms was examined using clonal BRIN-BD11 beta-cells. Due to the relatively higher mass range of snake venom constituents, gel filtration chromatography is frequently used for their fractionation (Graham *et al.* 2008; Blacklow *et al.* 2010). From UV elution profiles presented for each of the snake venoms there is a comparable profile for the two *Pseudechis* venoms investigated. Despite differing run conditions there is also similarity between chromatograms presented in this study and a size-exclusion profile for the related species *Pseudechis papuanus* as reported by Graham and colleagues (2008). These authors claimed that size-exclusion chromatography offered a robust means of correctly classifying a snake as belonging to either the viper or elapid families based on the observed profile and our findings corroborate this. Nine fractions were found to exhibit significantly elevated insulinotropic activity. Structural characterisation of the active fractions either by Edman degradation sequencing or by comparison of the experimental MALDI-TOF derived masses with published masses revealed snake toxins of varying molecular weight, from a range of different toxin families. In fact, this study is the first to report the presence of PLA<sub>2</sub> and short neurotoxin proteins in the lesser-studied *Pseudechis butleri* venom.

The phospholipase A<sub>2</sub> (PLA<sub>2</sub> EC 3.1.1.4 phosphatidylcholine 2-acylhydrolyase) family of snake toxins predominate the fractionations of *Pseudechis australis* and *Pseudechis butleri* venom. All of the venom test fractions showing evidence of PLA<sub>2</sub> type compounds exhibit a significant insulin-releasing activity. This finding is in keeping with previous work on elapid venom, in particular from *Naja mossambica mossambica* (Juhl *et al.* 2003) and *Micropechis ikaheka* (Gao *et al.* 2001) which contain insulinotropic PLA<sub>2</sub> proteins. Venom from *Crotalus vipera* has also been studied where the presence of PLA<sub>2</sub> compounds had a significant effect on insulin secretion (Moore *et al.* 2015; Yamamoto *et al.* 1983; Nogueira *et al.* 2005). The mechanism behind the insulinotropic activity of phospholipase molecules is thought to involve hydrolysis of membrane phospholipids initiating release of arachidonic acid. This is converted to lipoxygenase products which in turn results in elevated cytosolic Ca<sup>2+</sup> and insulin secretion (Persaud *et al.* 2007).

Takasaki and colleagues (1990) reported the amino acid sequences of eight phospholipases isolated from *Pseudechis australis* and define the following hydrophilic residues Arg-43, Lys-46, Asp-50, Glu-54, Lys-58, Asp-90 and Glu-94 as being responsible for presynaptic toxicity. For those sequenced PLA<sub>2</sub>s in *Pseudechis butleri* fraction 18 and *Pseudechis australis* fraction 19 which show homology with Pa-11, a phospholipase A<sub>2</sub> that is reported by Nishida and co-workers (1985) as being toxic, there is a possibility of non-specific toxic effects on beta-cells in this study. Following the same line of reasoning, *Pseudechis butleri* fraction 21 was shown to be comparable to the non-toxic Pa-13 and therefore might be expected to be free from adverse effects (Nishida *et al.* 1985). However, further studies using pure preparations are required to elucidate exact molecular mechanisms of action together with potential toxic effects.

As reviewed by Barber and co-workers (2013), snake venom  $\alpha$ -neurotoxins are known as postsynaptic neurotoxins which bind to the skeletal nicotinic acetylcholine receptor and block neuromuscular transmission. With ability to cause paralysis, their presence in snake venom is key to efficient prey acquisition. Short chain  $\alpha$ -neurotoxins belonging to the three-finger toxin family were identified in the elapid snake venoms of this study. On the three occasions where short  $\alpha$ -neurotoxins were either inferred from the MALDI-TOF mass (*Pseudechis australis* fraction 22) or confirmed through Edman sequencing (*Pseudechis butleri* fractions 21 and 22), there is also evidence of PLA<sub>2</sub> type compounds co-eluting in the same fraction. Each of these fractions enhanced insulin-release, but given the known insulin releasing properties of phospholipases A<sub>2</sub>, it is difficult to assess the individual contribution of the short  $\alpha$ -neurotoxins to insulintropic activity. As mentioned previously there was also a PLA<sub>2</sub> entity present in this fraction which was deemed to be comparable to the non-toxic Pa-13.

The current study and previous work from our group (Moore *et al.* 2015) have identified the potential of elapid and viper venoms as a source of molecules with insulin-releasing properties. Although further detailed studies are required to evaluate any possible therapeutic ability, the small scale proteomic analysis of the individual snake venoms examined in this research, combined with biological activity testing has made a significant contribution to the rapidly emerging field of venomics.

#### **Compliance with Ethical Standards:**

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Conflict of Interest: Authors Moore, Gault and McClean received funding from Nicobrand Ltd, Coleraine, N. Ireland in respect of a PhD studentship for SWMM. They declare no conflict of interest.

Author P.R. Flatt declares that he has no conflict of interest.

Author V. Bhat declares that he has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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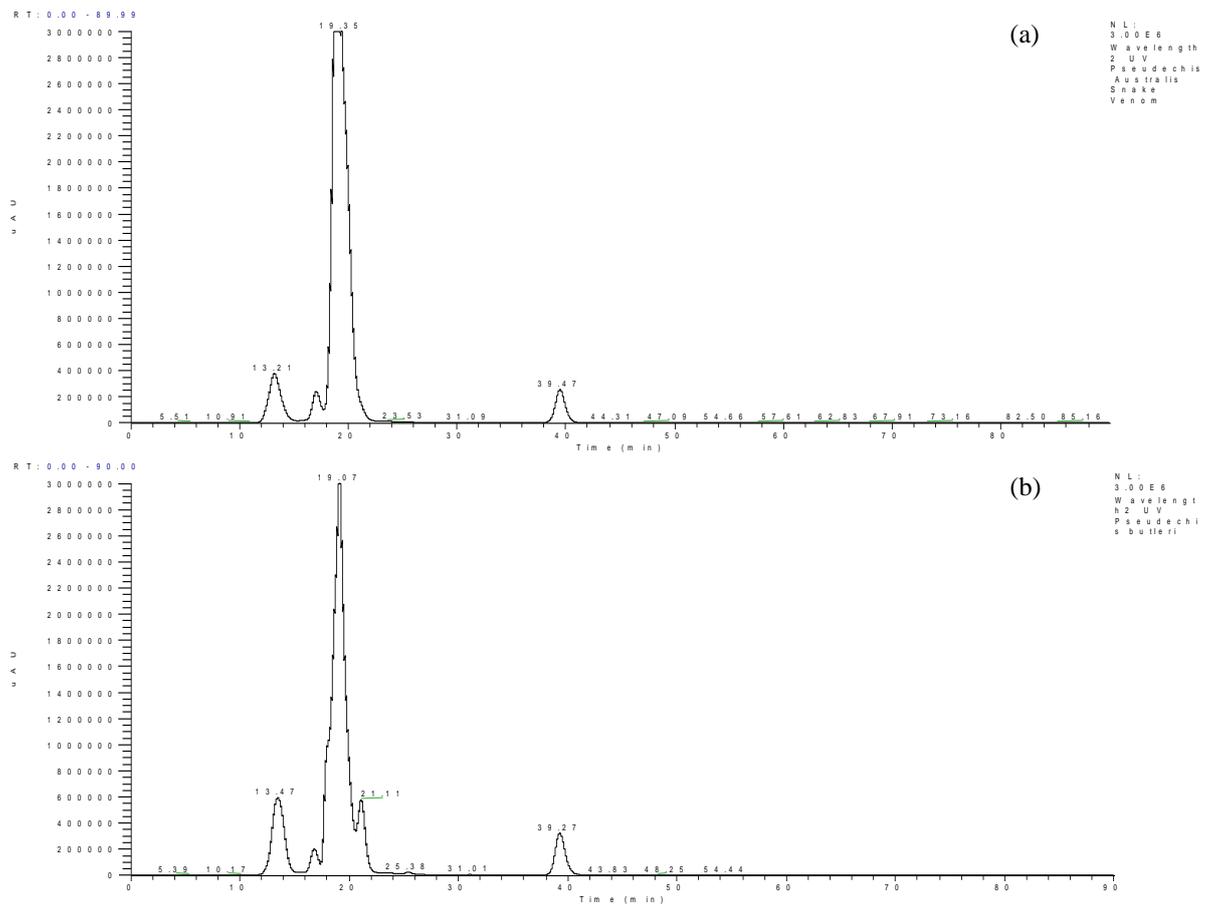
## Tables and Figures

**Table 1** Edman degradation sequencing results for *Pseudechis australis* fractions aligned against homologous sequences identified using BLAST searching.

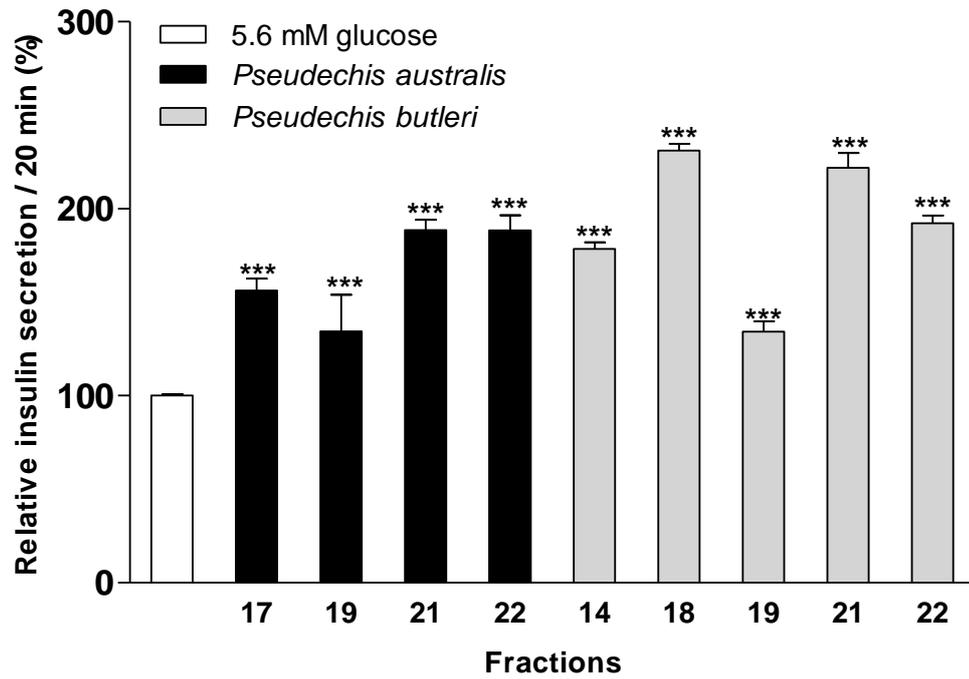
	Sequence (Residue numbers in bold text)	Homology	Database matches
17	<b>1</b> NLYQFYEMIECANFGTISGLAYAGYYCYCG <b>30</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>28</b> NLYQFKEMIECANKGTISGLAYAGYGCYCG <b>57</b>	90%	Phospholipase A2 enzyme [ <i>Pseudechis cf. australis</i> HI-2009] (GenBank: BAJ07178.1)
19	<b>1</b> NLIQFGWMIQCANKGSRPSLDYADYGCYCGWG <b>32</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>28</b> NLIQFGNMIQCANKGSRPSLDYADYGCYCGWG <b>59</b>	97%	Phospholipase A2 enzyme <i>Pseudechis cf. australis</i> HI-2009 (GenBank: BAJ07180.1);
	<b>28</b> NLIQFGNMIQCANKGSRPSLDYADYGCYCGWG <b>59</b>	97%	Pa-11 precursor from the <i>Pseudechis australis</i> (GenBank: AAZ22660.1);
	<b>1</b> NLIQFGNMIQCANKGSRPSLDYADYGCYCGWG <b>32</b>	97%	Basic phospholipase A2 PA-11 <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P04056.1)
	<b>1</b> NLIQFGNMIQCANKGSRPSLDYADYGCYCGWG <b>32</b>	97%	Basic phospholipase A2 PA-12C <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P20256.1),

**Table 2** Edman degradation sequencing results for *Pseudechis butleri* fractions aligned against homologous sequences identified using the BLAST search.

	Sequence (Residue numbers in bold text)	Homology	Database matches
18	<b>1</b> NLIQFGNMIQCANKGQYPELAYADY <b>25</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>1</b> NLIQFGNMIQCANKGSRPSLDYADY <b>25</b>	84%	Basic phospholipase A2 PA-11 <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P04056.1)
	<b>1</b> NLIQFGNMIQCANKGSRPSLDYADY <b>25</b>	84%	Basic phospholipase A2 PA-12C <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P20256.1)
	<b>1</b> NLIQFGNMIQCANKGSRPSLDYADY <b>25</b>	84%	Basic phospholipase A2 PA-12A <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P20255.1)
	<b>28</b> NLIQFGNMIQCANKGSRPSLDYADY <b>52</b>	84%	Pa-11 precursor <i>Pseudechis australis</i> (GenBank: AAZ22660.1)
	<b>28</b> NLIQFGNMIQCANKGSRPSLDYADY <b>52</b>	84%	Phospholipase A2 enzyme <i>Pseudechis cf. australis</i> HI-2009 (GenBank: BAJ07180.1)
	<b>1</b> DLYEFLEMIECAAKVTFPLRDYLT <b>25</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>28</b> NLYQFKEMIECANKGT <b>43</b>	69%	Phospholipase A2 enzyme <i>Pseudechis cf. australis</i> HI-2009 (GenBank: BAJ07178.1)
18	DLLQFNEMIECTIPGSFPLLDYMDY <b>42</b>	56%	Acidic phospholipase A2 Kbf-grIB <i>Bungarus fasciatus</i> (Genbank: DQ508411.1)
21	<b>1</b> NILQFRKMIQCANKGSRAAWHYLDYG <b>26</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>1</b> NILQFRKMIQCANKGSRAAWHYLDYG <b>26</b>	100%	Basic phospholipase A2 PA-13 <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P04057.1)
	<b>1</b> NILQFRKMIQCANKGSRAAWHYLDYG <b>26</b>	100%	Basic phospholipase A2 PA-15 <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P20257.1)
	<b>28</b> NILQFRKMIQCANKGSRAAWHYLDYG <b>53</b>	100%	Pa-13 precursor <i>Pseudechis australis</i> (GenBank: AAZ22665.1)
	<b>28</b> NILQFRKMIQCANKGSRAAWHYLDYG <b>53</b>	100%	Phospholipase A2 enzyme <i>Pseudechis cf. australis</i> HI-2009 (GenBank: BAJ07181.1)
	<b>1</b> NTCENCT SSEPLTITKCCGGDSSCKC <b>27</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>23</b> TC--CNQOSSQPKTTTTCAGGESSCYK <b>47</b>	56%	Short neurotoxin 1 <i>Pseudechis porphyriacus</i> (UniProtKB/Swiss-Prot: A8HDJ4.1)
	<b>8</b> SSEPPTTTRCSGGETNCYK <b>26</b>	58%	Short neurotoxin 1 <i>Naja mossambica</i> (UniProtKB/Swiss-Prot: P01431.1)
22	<b>1</b> MTCANQOSIQPKTTTTINAGGESSCYKKTW <b>29</b>	N/A	Determined by N-terminal Edman degradation sequencing
	<b>22</b> MTCCNQOSSQPKTTTTICAGGESSCYKKTW <b>50</b>	90%	Short neurotoxin 1 <i>Pseudechis australis</i> (UniProtKB/Swiss-Prot: P25497.2)



**Figure 1** HPLC fractionation of (a) *Pseudechis australis* and (b) *Pseudechis butleri* crude venom using a Superdex 75 10/300 GL column (10 x 300 mm) and an isocratic mobile phase of 0.1 M ammonium acetate at 0.7 mL/min over 90 min with UV detection at 280 nm.



**Figure 2** Effects of selected HPLC fractions of *Pseudechis australis* and *Pseudechis butleri* crude venom on insulin secretion from BRIN-BD11 cells in the presence of 5.6 mM glucose. Values represent means  $\pm$  SEM. \*\*\*P<0.001 compared with 5.6 mM glucose control (n=5).