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Cloning and activity of a novel α -latrotoxin from red-back spider venom[#]

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The DNA sequence of α -latrotoxin-Lh1a (α -LTX-Lh1a) reported in this paper has been deposited in the EMBL Nucleotide Sequence Database, under Accession Number FR851877, and the ArachnoServer Spider Toxin Database (ID#1290). The deduced amino acid sequence of α -LTX-Lh1a has also been deposited in the ArachnoServer Spider Toxin Database.

ABSTRACT

The venom of the European black widow spider *Latrodectus tredecimguttatus* (Theridiidae) contains several high molecular mass (110–140 kDa) neurotoxins that induce neurotransmitter exocytosis. These include a vertebrate-specific α -latrotoxin (α -LTX-Lt1a) responsible for the clinical symptoms of latrodectism and numerous insect-specific latroinsectoxins (LITs). In contrast, little is known about the expression of these toxins in other *Latrodectus* species despite the fact that envenomation by these spiders induces a similar clinical syndrome. Here we report highly conserved α -LTX, α -LIT and δ -LIT sequence tags in *L. mactans*, *L. hesperus* and *L. hasselti* venoms using tandem mass spectrometry, following bioassay-guided separation of venoms by liquid chromatography. Despite this sequence similarity, we show that the anti- α -LTX monoclonal antibody 4C4.1, raised against α -LTX-Lt1a, fails to neutralize the neurotoxicity of all other *Latrodectus* venoms tested in an isolated chick biventer cervicis nerve-muscle bioassay. This suggests that there are important structural differences between α -LTXs in theridiid spider venoms. We therefore cloned and sequenced the α -LTX from the Australian red-back spider *L. hasselti* (α -LTX-Lh1a). The deduced amino acid sequence of the mature α -LTX-Lh1a comprises 1180 residues (~132 kDa) with ~93% sequence identity with α -LTX-Lt1a. α -LTX-Lh1a is composed of an N-terminal domain and a central region containing 22 ankyrin-like repeats. The presence of two furin cleavage sites, conserved with α -LTX-Lt1a, indicates that α -LTX-Lh1a is derived from the proteolytic cleavage of an N-terminal signal peptide and C-terminal propeptide region. However, we show that α -LTX-Lh1a has key substitutions in the 4C4.1 epitope that explains the lack of binding of the monoclonal antibody.

Abbreviations: α -LTX, α -latrotoxin; BSA, bovine serum albumin; 4C4.1, murine monoclonal antibody raised against α -latrotoxin from *L. tredecimguttatus*; CBCNM, chick biventer cervicis nerve-muscle; CNS, central nervous system; ESI-Q-TOF, electrospray ionization quadrupole time-of-flight; FPLC, fast performance liquid chromatography; gDNA, genomic DNA; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; α -LCT, α -latrocrustatoxin; LD₅₀, median lethal dose; LIT, latroinsectotoxin; LMWP, low molecular weight protein (also known as latrodectin); MS-MS, tandem mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

1. INTRODUCTION

The most clinically significant group of spiders worldwide are the widow or comb-footed spiders, belonging to the genus *Latrodectus* (Araneae: Araneomorphae: Theridiidae). Widow spider envenomation manifests clinically as latrodectism, resulting in pain either at the bite site or radiating proximally to the whole of the bitten limb, local and regional diaphoresis, and less commonly piloerection and muscle fasciculation. Symptoms may become severe and generalized and include hypertension, tachycardia, nausea, vomiting, and abdomen, back or chest pain. Although death is uncommon, patients may be left with symptoms of envenomation for days or weeks if left untreated [1, 2].

The toxin responsible for the clinical symptoms of latrodectism is the vertebrate-specific α -latrotoxin (α -LTX) which has been isolated from the venom of the European widow spider, *L. tredecimguttatus* [3]. α -LTX is a large ~130 kDa hydrophilic protein that elicits massive spontaneous neurotransmitter release from neuronal and neuroendocrine nerve terminals [4-6]. Under non-reducing conditions, α -LTX exists as a homodimer [7] with each monomer composed of four domains: a signal peptide (domain I), a conserved N-terminal domain with two hydrophobic segments that has no substantial homology to known proteins (domain II), a domain composed of 20 ankyrin repeats (or 22 if two imperfect repeats are included; domain III), and a C-terminal propeptide domain (domain IV) [8]. α -LTX is synthesized as a larger preprotoxin of ~157 kDa where domains I and IV are believed to be removed by proteolytic cleavage during venom maturation by the endopeptidase furin, leaving a mature 130 kDa toxin composed of domains II and III [8-11]. Using electron cryo-microscopy, the 3D structure of the mature α -LTX monomer was found to contain three regions: the wing (majority of domain II), the body (one quarter of domain II and first 15–16 ankyrin repeats) and the head (ca. 4.5 C-terminal ankyrin repeats) [7].

L. tredecimguttatus venom also contains five insect-specific toxins known as latroinsectotoxins (LIT) α , β , γ , δ and ϵ [12], and one crustacean-specific protein, α -latrocrustatoxin (α -LCT) [13-15]. Of these, an additional three latrotoxins have been cloned and fully sequenced: α -LCT [16], α -LIT [9] and δ -LIT [10] with molecular masses of 110–140 kDa. In addition, two acidic low molecular weight proteins (LMWPs or latrodectins; ~7.9 kDa) have been found to co-purify with α -LTX [17-19]. α -LTX-associated LMWPs are structurally related to crustacean hyperglycemic hormones and are non-toxic [17, 18, 20]. Nevertheless they appear to augment the neurotoxicity of latrotoxins [12, 20], probably by increasing their affinity for the membrane target [21] and reducing vertebrate phyla-specificity such that α -LTX becomes active in insects [20].

Currently, α -LTX-induced neurotransmitter exocytosis is believed to involve an initial step of α -LTX binding to extracellular cell surface membrane proteins. Three structurally-unrelated cell adhesion receptor classes have been identified to mediate this increase in neurotransmitter release by

α -LTX: (i) neurexin 1 α , a neuronal protein with a single transmembrane domain [22, 23], (ii) latrophilin 1 (CL1 or lectomedin) otherwise known as calcium-independent receptor of α -LTX (CIRL; [24, 25]; a member of the CL family of G-protein-coupled receptors [26, 27]), and more recently (iii) receptor-like protein tyrosine phosphatase σ [28]. It is believed that these receptors serve as sites to target α -LTX to the appropriate location on the cell surface, such as the active zone of nerve terminals at the neuromuscular junction [29]. Once bound, α -LTX oligomerization occurs allowing the formation of amphipathic cyclical tetramers, followed by membrane insertion and non-selective cation channel formation [7]. This pore formation results in an osmotic-mediated increase in vesicular exocytosis and non-vesicular leakage of neurotransmitters. In parallel, vesicle exocytosis ensues as a result of Ca^{2+} influx through the toxin-induced pore, and receptor-mediated vesicle exocytosis. Interestingly, the anti- α -LTX monoclonal antibody 4C4.1 blocks neurotransmitter release by preventing tetramerization of *L. tredecimguttatus* α -LTX and also inhibits the ionophore activity of α -LTX once inserted into cell membranes [30, 31]. The epitope of the monoclonal antibody 4C4.1 is located in a hinge region between the body and head of α -LTX, thus highlighting the importance of this region in pore formation and toxicity.

While extensive investigations have been carried out on toxins from *L. tredecimguttatus* venom, remarkably little research has been undertaken on the composition of venoms from other *Latrodectus* species, including the clinically significant Australian red-back spider *L. hasselti*. Nevertheless, the venoms of all *Latrodectus* species are believed to contain similar α -LTX-like proteins since envenomation by any widow spider results in a similar clinical syndrome. Moreover, widow-spider antivenoms, produced using the venoms of specific *Latrodectus* species, reverse the effects of envenomation from other *Latrodectus* and *Steatoda* spiders (another member of the family Theridiidae) both in experimental animal models and in clinical settings [32-35]. The presence of a 130 kDa band in the venom of many theridiid spiders, detected by immunoblotting with red-back spider antivenom, suggests the presence of α -LTX-like or antigenically similar proteins in the venoms of all *Latrodectus* species as well as the related theridiid spider *Steatoda grossa* [32]. It has therefore been assumed, but not proven, that α -LTX is present in the venom of all widow spiders. The aim of the present study was therefore to determine whether the clinical symptoms of envenomation by other *Latrodectus* species and *S. grossa* can be explained by the presence of α -LTX molecules related to those found in *L. tredecimguttatus* venom.

2. MATERIALS AND METHODS

2.1 Sources of venoms, toxins and antibodies

Purified α -LTX, derived from *L. tredecimguttatus*, was obtained from Alomone Laboratories (Jerusalem, Israel), reconstituted in 50% v/v glycerol and stored at -20°C . Female *L. mactans* (Southern black widow spider), *L. hesperus* (Western black widow spider) and *L. lugubris* (Ili River

Valley widow spider, Kazakhstan) venoms were obtained as lyophilized milked venom, extracted by electrostimulation of the venom apparatus, from Spiderpharm. Female *L. tredecimguttatus* venom was purchased as lyophilized venom gland extract from Latoxan (Rosans, France). Whilst originally described as a separate theridiid spider species, *L. lugubris* may simply represent a geographic variant of *L. tredecimguttatus* as Platnick [36] no longer recognizes this species as distinct from *L. tredecimguttatus*. All venoms were reconstituted in water and stored at -20°C .

Female *L. hasselti* (Australian red-back spider) venom was purchased from Venom Supplies Pty. Ltd. (Tanunda SA, Australia), as lyophilized whole venom glands or obtained from venom glands freshly dissected from spiders donated to the Neurotoxin Research Group. Spiders were anaesthetized using CO_2 . Venom glands were removed through an incision on the dorsum of the cephalothorax and placed in 400–600 μl of water at 4°C . The glands were then crushed in a glass/glass Dounce vessel and the homogenate centrifuged for 30 min at 10,000 g at 4°C . The resulting supernatant was lyophilized and stored at -80°C until required. *S. grossa* (Brown cupboard spider) venom was extracted from venom sacs dissected from spiders raised in the Neurotoxin Research Group. Spiders were originally obtained by field collection from Gore Hill, Sydney, Australia and were formally identified as *S. grossa* by Dr Robert J. Raven of the Queensland Museum (South Brisbane QLD, Australia). Venom was extracted in an identical fashion to that of *L. hasselti*.

A rabbit-derived polyclonal antibody to α -LTX raised against the venom of *L. tredecimguttatus* was purchased from Alomone Laboratories. A murine-derived monoclonal antibody to α -LTX from the venom of *L. tredecimguttatus* (4C4.1) was produced according to the methods of Pescatori and Grasso [30], and supplied as lyophilized hybridoma supernatant by Dr Alfonso Grasso. This was reconstituted at a concentration of 5 $\mu\text{g}/\text{ml}$.

2.2 Isolated vertebrate bioassays

Vertebrate toxicity of whole theridiid spider venoms, or venom fractions, were bioassayed using the isolated chick biventer cervicis nerve-muscle (CBCNM) preparation as described previously [37]. All animal experimentation was approved by the Animal Care & Ethics Committee of the University of Technology, Sydney (RNSH/UTS 0011-036A). In antibody neutralization studies, *Latrodectus* spp. and *S. grossa* venom samples were pre-treated with 1 ml of reconstituted monoclonal antibody 4C4.1 solution and allowed to equilibrate with venom for 5 min at 37°C prior to application to the nerve-muscle preparation.

2.3 Gel electrophoresis and Western blotting

Western blotting was performed following initial sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [32]. Purified *Latrodectus* spp. venom fractions were transblotted for subsequent protein sequencing by Edman degradation. The PVDF

membrane was incubated with 0.025% Coomassie blue R250 in 40% methanol for 30 min immediately after transblotting and destained with 50% methanol. Bands of interest were excised and sent for Edman sequencing (Australian Proteomic Analysis Facility, Macquarie University, Sydney, Australia) on an Applied Biosystems/Perkin Elmer Procise 492 cLC protein sequencer. Secondly, transblotted *Latrodectus* spp. and *S. grossa* venom proteins were incubated with polyclonal rabbit-derived anti- α -LTX antibody (1:100 dilution) or murine-derived anti- α -LTX monoclonal antibody 4C4.1 [30] as primary antibodies using previously described methods [32]. The blot was then incubated with specific secondary antibodies for 1 h. For the polyclonal anti- α -LTX antibody a 1:10,000 dilution of alkaline phosphate conjugated goat anti-rabbit IgG (Sigma-Aldrich) was used. For the anti- α -LTX monoclonal antibody 4C4.1, a 1:2,000 dilution of alkaline phosphate conjugated goat anti-mouse IgG (Sigma-Aldrich) was employed.

2.4 Toxin purification and peptide sequencing

One milligram samples of *Latrodectus* spp. and *S. grossa* venoms were initially purified by size-exclusion fast performance liquid chromatography (FPLC) using a Superdex[®] 200 HR 10/30 FPLC column (1 x 30 cm, Pharmacia-Biotech, Uppsala, Sweden) using a buffer of 20 mM Tris/NaCl, pH 7.6 and a flow rate of 0.5 or 1 ml/min. β -Galactosidase (118 kDa) was used as an indicator of the retention time as its mass is similar to α -LTX. Protein fractions were monitored at 215 and 280 nm using a Pharmacia LKB variable wavelength monitor model 2141 (Pharmacia-Biotech). Venom fractions were collected manually and stored at 4°C. Further fractionation of the high molecular mass fractions was performed using anion-exchange FPLC within one day.

Anion-exchange FPLC was performed using a Mono Q[®] HR 5/5 FPLC column (Pharmacia-Biotech). Protein fractions were eluted using 20 mM Tris/NaCl, pH 7.6 as the mobile phase, employing a linear NaCl gradient from 20 to 400–600 mM over 30–35 min at a flow rate of 1 ml/min. Desalting of anion-exchange FPLC fractions was performed using either a HiTrap[®] Desalting Column (Pharmacia-Biotech) or using reverse-phase high pressure liquid chromatography (RP-HPLC; see below). After desalting, fractions were lyophilized and stored at –80°C.

Further separation, prior to Edman sequencing and MS-MS, was performed using an analytical C18 RP-HPLC column (25 cm x 4.6 mm, 5 μ m pore size; Vydac, Hesperia, CA, USA). The column was equilibrated with 95% buffer A consisting of 0.1% v/v trifluoroacetic acid (TFA) in water and 5% buffer B (0.085% v/v TFA in acetonitrile). Elution of venom components was achieved using a linear gradient of 5–50% buffer B over 30 min followed by a linear gradient of 50–70% buffer B over 35 min at flow rate of 1 ml/min. Purified toxins were collected, lyophilized, and stored at –80°C until required. Toxin quantification was performed using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Absorbances were read at 560 nm on a Titertek Multiscan microplate reader (Eflab, Finland).

2.5 Acute Insect Toxicity Testing

For analysis of insecticidal activity, anion-exchange FPLC fractions were dissolved in insect saline with 0.1% BSA and injected into house crickets (*Acheta domesticus*) as previously described [38]. Groups of 3–5 crickets received intrathoracic injections at a dose of 1 µg/g. A control group of three crickets were also injected with the same volume of 0.1% BSA in insect saline. Insects were monitored for 96 h following injection for signs of toxicity, and the percentage knockdown and lethality was determined. Knockdown was defined as the inability to maintain an upright posture, with intermittent or continuous twitches of appendages.

2.6 α -LTX fragment sequencing by tandem mass spectrometry

Purified protein samples were analyzed using electrospray ionization time-of-flight tandem mass spectrometry (ESI-Q-TOF MS). Briefly, samples were purified after fractionation by RP-HPLC using SDS-PAGE gel electrophoresis on 15% Tris-glycine gels as described above. Gels were stained with 0.025% Coomassie Blue R-250 in 40% methanol for 20 to 30 min and destained with 50% methanol. Protein bands of interest were excised from the gel, dried, and exposed to a 16 h tryptic digestion at 37°C prior to peptide analysis. The resulting peptides were purified using a ZipTip® (Millipore, Bedford, MA, USA) to concentrate and desalt the samples. The samples were then analyzed by tandem mass spectrometry (MS-MS) using a Micromass Q-TOF MS equipped with a nanospray source (Micromass, Manchester, England). Samples were dissolved in 5 µl 50% acetonitrile and 0.5% formic acid. Data were manually acquired in the positive mode using borosilicate capillaries with a source temperature of 80°C. A potential of 850 V was applied to the nanoflow tip. Data were acquired over the m/z range 400–1,800 to select peptides for MS-MS analysis. After peptides were selected, the MS was switched to MS-MS mode and the data collected over the m/z range 50–2,000 with variable collision energy settings. Peptides were subsequently compared with the known amino acid sequences of α -LTX, α -latroinsectotoxin, and δ -latroinsectotoxin using NCBI-Blast protein-protein comparison which uses the protein sequences derived from the venom of *L. tredecimguttatus* for sequence homology [8, 10, 39]. Raw data were processed using the MAXENT algorithm included in the MASSLYNX program.

2.7 Genomic DNA extraction from *L. hasselti*

Two *L. hasselti* spiders were immobilized by freezing at –20°C for 5 min and their cephalothoraces dissected. Cell lysis was performed by firstly crushing the cephalothoraces in 500 µl of 4 M guanidium isothiocyanate (GITC) (Sigma-Aldrich) in 100 mM Tris (pH 7.0) with 0.1% β -mercaptoethanol. Fifty microlitres of 3 M sodium acetate (pH 5) and 500 µl of phenol (saturated solution, pH 6.6) were then added sequentially to extract the proteins. Subsequent to this, 100 µl of chloroform/isoamyl alcohol (49:1) was added to extract the cellular proteins. The solution was centrifuged at 10,000 g for 10 min to separate the aqueous and organic layers. The upper layer and genomic DNA (gDNA) interface were carefully pipetted from the protein layer and placed in

separate tube where DNA precipitation was performed. To the suspension was added two volumes of 100% ethanol at room temperature. The precipitate was subsequently centrifuged at 10,000 *g* for 15 min resulting in the formation of a DNA pellet. The supernatant was decanted from the DNA pellet and the pellet resuspended in 100 μ l dH₂O.

gDNA used for the amplification of the segment which comprises bases 3320-3830 in the nucleotide sequence, was obtained from dissected legs that were ground with a chilled, sterile mortar and pestle in liquid nitrogen. After pulverization, the tissue (~100 mg) was added to 400 μ l of low salt buffer as previously described [40]. Before amplification by the Polymerase Chain Reaction (PCR), quality and quantity of all extracted DNA was assessed by measuring the OD_{260/280} and by running a 1.1% agarose gel, stained with SyberSafe[®].

2.8 PCR and Sequencing

Non-degenerate paired forward and reverse DNA primers were designed from the previously determined DNA sequence of *L. tredecimguttatus* α -LTX [8] and peptide sequences derived from MS-MS sequencing. Primer pairs were selected at approximately 400–600 bp distances and Primerfinder (<http://eatworms.swmed.edu/~tim/primerfinder/>) was utilized to reduce the likelihood of primer dimers or hairpins. Primers were supplied by Sigma GenoSys, Australia.

Red-Taq DNA Polymerase (Sigma) was used to amplify all segments of the α -LTX from *L. hasselti* by PCR, except for the segment at position 3320–3830 bp which was amplified using Platinum[®] Taq (Invitrogen). Each 50 μ l reaction contained 250–300 ng of gDNA template, 1X Sigma PCR buffer (or 1X PCR reaction buffer and 1 mM MgCl₂), 0.2 μ M dNTPs, 0.6 μ M of each forward and reverse primer, 1 U Taq Red-Taq polymerase (or 1U Platinum Taq polymerase). Cycling conditions were: 1 min at 94°C followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final cycle of 72°C for 10 min.

The PCR products were purified using the QIAGEN QIAquick PCR purification kit and sent directly for sequencing whereas clean PCR products from the segment 3320-3830bp were cloned into the pCR II[®] vector using the TA dual Cloning Kit (Invitrogen). The resulting clones were grown overnight in LB media with ampicillin. Plasmids were extracted using the QIAprep Spin Miniprep kit. Sequencing was performed at the Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC) where samples were prepared and mixed with 70 to 100 pmol of respective forward or reverse primers. In later experiments sequencing was performed at the Australian Genome Research Facility (AGRF - Brisbane node) in a reaction containing 3 μ l of Plasmid DNA with M13 primers. Sequences were aligned and analyzed using MACVECTOR (v11.0.4) or GENEIOUS PRO software (v4.8.3)

Given the similar, but non-identical, sequences of the α -LTX peptides determined in this work

when compared to α -LTXs from *L. tredecimguttatus*, it is proposed that a new nomenclature system be employed to describe the α -LTX homologs from other *Latrodectus* venoms. Accordingly, we used a nomenclature based on that recently described for venom peptides [41] and which has already been adopted by ArachnoServer [42, 43] and UniProtKB [44]. Hence, α -latrotoxin from *L. tredecimguttatus* is renamed α -latrotoxin-Lt1a (α -LTX-Lt1a) and α -latrotoxins from the venom of *L. hasselti*, *L. hesperus* and *L. mactans* are named α -LTX-Lh1a, α -LTX-Lhe1a and α -LTX-Lm1a, respectively, in order to clearly distinguish them from α -LTX-Lt1a.

3. RESULTS

3.1. *In vitro* monoclonal antibody neutralization studies

The venoms from *L. tredecimguttatus*, *L. lugubris*, *L. hesperus* and *L. hasselti* (Fig. 1, top panels) all produced a characteristic rapid muscle contracture and loss of twitch tension when added to the isolated and indirectly-stimulated chick biventer cervicis nerve-muscle (CBCNM) preparation. This is consistent with the known ability of α -LTX to increase neurotransmitter release in this preparation [32, 45]. The murine-derived monoclonal antibody 4C4.1, raised against α -LTX-Lt1a from *L. tredecimguttatus*, was then assayed for its ability to inhibit the *in vitro* effects of these *Latrodectus* venoms. The responses of *L. tredecimguttatus* venom on twitch tension and contracture were inhibited by 4C4.1 in a dose-dependent fashion. Following a 5 min incubation with 1 ml of 4C4.1 (5 μ g/ml) at 37°C, *L. tredecimguttatus* venom failed to alter twitch tension (Fig. 1Ab). Incubation with reduced amounts of 4C4.1 (<2.5 μ g/ml) resulted in the production of a moderate contracture and slow, but eventual, loss of twitch tension (data not shown). In contrast, incubation with 1 mL of 4C4.1 failed to prevent the typical, rapid contracture associated with diminishing twitch tension associated with *L. lugubris*, *L. hesperus*, and *L. hasselti* venoms (Fig. 1Bb, Cb and Db). These observations suggest that either the concentration of antibody was insufficient to completely block the neurotoxic effects of these latter venoms or more likely that the epitope that binds 4C4.1 may not be present in the α -LTX-like molecules found in other *Latrodectus* venoms.

3.2 Venom electrophoresis and Western blotting

SDS-PAGE revealed comparable high molecular mass proteins for all *Latrodectus* spp. venoms with a protein band around 130 kDa, consistent with the presence of α -LTX (Fig. 2A). Subsequent Western blotting with polyclonal anti- α -LTX-Lt1a antibody revealed that this 130-kDa band present in all venoms has antigenic similarity to α -LTX-Lt1a from *L. tredecimguttatus* (Fig. 2B). In support, Western blotting with the monoclonal antibody 4C4.1 showed binding with α -LTX-Lt1a derived from *L. tredecimguttatus* venom as well as the 130-kDa band found in *L. tredecimguttatus* (Fig. 2C). Interestingly, however, no binding was observed with any protein bands found in *L. hasselti*, *L. mactans*, *L. hesperus*, or with the α -LTX-like protein previously described in

S. grossa venom [46], (Fig. 2C) or with *L. lugubris* (data not shown). These results are consistent with the inability of 4C4.1 to neutralize the neurotoxicity of *L. hasselti*, *L. hesperus*, and *L. lugubris* venoms. This indicates that the epitope responsible for binding 4C4.1 is either not present, or is modified, in the α -LTX molecules found in other *Latrodectus* venoms and suggests that the protein structure of these toxins may vary from the known structure of α -LTX-Lt1a [47].

3.3 Size-exclusion FPLC

Bioassay-guided fractionation of *Latrodectus* and *S. grossa* venoms was undertaken to provide proteins for N-terminal sequencing. Initial separation of venoms was undertaken using size exclusion FPLC employing isocratic elution with 20 mM Tris/NaCl (pH 7.6). These conditions are similar to that used previously for the separation of α -LTX-Lt1a from *L. tredecimguttatus* venom [48]. The chromatograms resulting from fractionation of the venoms of *L. tredecimguttatus*, *L. lugubris*, *L. hasselti*, and *S. grossa* all contained a high molecular mass fraction (~200 kDa) eluting at 8–12 min (Fig. 3A-D), a fraction containing intermediate masses (120–130 kDa) eluting at 12–18 min, and a number of lower molecular mass proteins eluting at longer retention times (Fig. 3H). In contrast, the venoms from *L. mactans* and *L. hesperus* were almost completely devoid of the high molecular mass fraction (Fig. 3E-F) and had reduced expression of lower molecular mass proteins (Fig. 3H); these venoms were instead dominated by the intermediate-mass fraction (shaded black in each of the chromatograms in Fig. 3). The early-eluting high-mass fraction may represent contaminant proteins extracted from the venom gland itself, although this fraction is present in the venom from *L. lugubris* milked by electrostimulation. The intermediate-mass fraction had a similar retention time to β -galactosidase (118 kDa; Fig. 3G), suggesting that it likely contains α -LTX-like molecules. Consistent with this hypothesis, these fractions exhibited an increased muscle contracture and decreased twitch tension similar to crude venom extracts when bioassayed using the CBCNM preparation (data not shown). Interestingly, the 120–130 kDa size-exclusion fraction from *S. grossa* venom exhibited much less activity in the nerve-muscle bioassay with minimal increases in muscle contracture seen in the absence of any changes in twitch tension. This is consistent with the observations made earlier in comparisons of the bioactivity of crude venom from this species with that of *Latrodectus spp.* venoms [46].

Initial attempts at using C18 RP-HPLC fractionation employing 0.1% TFA in acetonitrile resulted in loss of activity of resultant venom fractions bioassayed using the CBCNM preparation. This was most likely due to the acidic/denaturing environment resulting in neutralization of toxin activity. Therefore all 120–130 kDa venom fractions were subsequently purified by anion exchange FPLC.

3.4 Anion Exchange FPLC

The intermediate-mass FPLC fractions exhibiting neurotoxic actions in the CBCNM bioassay were further separated by anion-exchange FPLC using a NaCl gradient. All *Latrodectus spp.* and

S. grossa venoms exhibited a large number of fractions that sequentially eluted with increasing NaCl concentration (Fig. 4A-F). *Latrodectus* venoms all contained a vertebrate-toxic fraction as determined by the CBCNM bioassay that resulted in a typical increase in resting muscle tension and loss of twitch tension (Fig. 4G-J) comparable to that observed with crude venom extract (Fig. 1Aa-Da). This was invariably found in the final peak eluting at a NaCl concentration of 360–370 mM NaCl for *L. tredecimguttatus* (f6) and *L. lugubris* (f6), consistent with that previously seen for *L. tredecimguttatus* [11], and 440–460 mM for *L. mactans* (f6), *L. hesperus* (f6) and *L. hasselti* (f7) (black peaks in Fig. 4A-E). SDS-PAGE of these anion-exchange fractions revealed that they contained at least one high-molecular mass protein band (120–130 kDa) with some also containing 70-kDa contaminants (Fig. 5A-C). An attenuated response in the nerve-muscle preparation was also seen with the fraction immediately preceding these final fractions suggesting that there was some degree of cross-contamination of these venom fractions with the α -LTX-like protein found in the final fraction of each venom (data not shown). Furthermore, acute insect toxicity was observed in the *L. hasselti* (f6), *L. mactans* (f5), *L. hesperus* (f5), and *L. lugubris* (f5) anion-exchange fractions (white peaks in Fig. 4B-E) preceding the vertebrate-toxic fraction (black peaks in Fig. 4A-E). In the insect bioassays, 30–100% knockdown was observed at 24 h postinjection, with 100% lethality at 96 h. The insect toxicity of these fractions suggests that the high-molecular weight bands seen on SDS-PAGE may contain latroinsectotoxin homologs.

Attempts to isolate the vertebrate-active fraction of *S. grossa* venom using a similar method to that employed for the *Latrodectus* venoms were unsuccessful. Although the high molecular weight size exclusion fractions of the venom showed limited activity, the various anion-exchange fractions purified from the size-exclusion fractions failed to elicit a response in the CBCNM preparation. This suggests that the *S. grossa* anion-exchange venom fractions may have become inactivated during the fractionation process. Hence, further attempts at isolation and characterization of *S. grossa* toxins was not attempted.

3.5 N-terminal sequencing of α -LTX-associated low molecular weight proteins

N-terminal sequencing via Edman degradation was attempted using the vertebrate-active final peak of all the *Latrodectus* spp. venoms fractionated by anion-exchange FPLC. Initial attempts to sequence α -LTX from the vertebrate-active anion-exchange FPLC peak of *L. tredecimguttatus* (f6) venom proved unsuccessful, with only a partial sequence of a co-eluting low-molecular weight protein (LMWP, also named latroductin) associated with α -LTX [17-19, 21, 47] being identified (see Supplementary Fig. S1). Similarly, a partial N-terminal sequence of LMWP was identified from the corresponding vertebrate-active fraction (f6) of *L. lugubris* venom (Supplementary Fig. S1). This showed 100% identity with the reported sequence of LMWP from *L. tredecimguttatus*, providing further support for the contention that *L. lugubris* is not a separate species from *L. tredecimguttatus* [36]. When sequencing was attempted using toxin resolved on SDS-PAGE and then transblotted to

PVDF membrane, LMWP was not detected. This suggests that SDS-PAGE followed by transblotting results in separation of the high (α -LTX) and low molecular mass (LMWP) proteins. We were unable to determine any similar sequences for LMWP from any other *Latrodectus* venoms either when on blot or direct sequencing of toxin were attempted.

Attempts at N-terminal sequencing of α -LTX from the remaining vertebrate-active fractions of *L. hasselti*, *L. mactans*, or *L. hesperus* venom also failed when attempted in-solution or when transblotted onto PVDF membrane, suggesting that these toxins may be N-terminally blocked [39]. Given that the N-terminal residue of α -LTX-Lt1a is glutamate, N-terminal block may result from the conversion to pyroglutamate [49], which is resistant to N-terminal sequencing by Edman degradation [50]. As a result, the vertebrate-active peaks of *L. hasselti*, *L. mactans*, *L. lugubris* and *L. hesperus* venoms were subjected to SDS-PAGE and on-blot pyroglutamate aminopeptidase digestion in an attempt to cleave any N-terminal pyroglutamate. This method was unsuccessful suggesting that either the toxin may not have been present in sufficient quantities to be detected by this sequencing method, or that alternative post-translational modifications that interfere with Edman sequencing may be present [50].

3.6 MS-MS protein sequencing of latrotoxins

To derive a partial amino acid sequence of α -LTX homologs from *Latrodectus* venoms, MS-MS protein sequencing of tryptic-digest fragments of the vertebrate-active fractions was carried out. Initially, MS-MS sequencing was used to analyze tryptic-digest peptides from *L. hasselti* and *L. hesperus* vertebrate-active anion-exchange fractions *f7* and *f6*, respectively. Interestingly, the peptides sequenced were highly homologous with α -LIT and δ -LIT [9, 10] and *not* α -LTX (Supplementary Fig. S2). Results are summarized in Table 1. This most likely arose as a result of significant cross-contamination with the immediately preceding latroinsectotoxin anion-exchange FPLC fractions (*f6* and *f5*, respectively). However, it would not be possible to further separate these contaminants by size-exclusion FPLC or gel electrophoresis due to the similarity in the molecular masses of the toxins. Hence, further purification of α -LTX from the vertebrate-active anion-exchange FPLC fractions from *L. hasselti* (*f7*), *L. hesperus* (*f6*), and *L. mactans* (*f6*) venoms was achieved using C18 RP-HPLC (Fig. 5D-F). Reverse-phase HPLC was not used earlier in the purification process as we had previously found that the mobile phase inactivated crude venom and venom fractions making it impossible to use the CBCNM bioassay to determine which fraction was vertebrate-active.

C18 RP-HPLC fractionation of the vertebrate-active anion-exchange fraction from *L. mactans* and *L. hesperus* revealed nine and seven sub-fractions, respectively (Fig. 5D-E). For *L. hesperus*, three fractions contained proteins with a molecular mass of 120 kDa when separated on SDS-PAGE. Two of these were excised and subjected to a 16 h tryptic digestion. MS-MS analysis of two of

these peptide fragments revealed sequences homologous to α -LTX in *f6.6*, and δ -latroinsectotoxin in *f6.4* (not analyzed further in view of previous amino acid sequence analysis). Although only two peptide sequences (22 amino acids in total) were determined, they exhibited 100% identity with α -LTX-Lt1a (Fig. 6, Table 1).

For *L. mactans*, C18 RP-HPLC fractionation and subsequent SDS-PAGE analysis revealed two fractions (*f6.8* and *f6.9*) containing a molecular mass of 120–130 kDa. Following tryptic digestion, both fractions contained peptide fragments with high homology with α -LTX-Lt1a. Fraction 6.8 revealed 13 peptides containing 150 amino acids with 94.6% identity and 98% similarity with α -LTX-Lt1a. Fraction 6.9 returned 10 peptides containing a total of 119 amino acids with 91.6% identity and 95.7% similarity with α -LTX-Lt1a (Fig. 6, Table 1). A number of peptides sequenced from these two fractions were identical. It is interesting that two α -LTX homologs were purified from *L. mactans* anion exchange vertebrate-active fraction by RP-HPLC. This suggests that there might be two distinct α -LTX isoforms in *L. mactans* venom with similar but not identical primary structure.

Finally, *L. hasselti f7* yielded nine RP-HPLC fractions (Fig. 5F). Two fractions (*f7.7* and *f7.9*) yielded 120–130 kDa bands when separated on SDS-PAGE. These were excised, subjected to tryptic digestion, and analyzed using MS-MS. No peptides could be identified in *f7.7*. However, *f7.9* yielded four peptide sequences (total of 54 amino acids) that yielded a 100% sequence match with α -LTX-Lt1a [8] (Fig. 6, Table 1).

3.7 Determination of the DNA sequence of *Latrodectus hasselti* α -LTX gene

It was difficult to reconcile the 100% identity of the *L. hasselti f7.9* peptide fragments and α -LTX-Lt1a with the observed lack of neutralization using the anti- α -LTX-Lt1a monoclonal antibody 4C4.1. We therefore decided to construct a *L. hasselti* genomic DNA library in order to be able to determine the complete sequence of the gene encoding the α -LTX-like protein. The choice of *Latrodectus* species was based on the ready availability of *L. hasselti* and the fact that it is a medically significant spider, associated with significant morbidity; this spider is estimated to be responsible for at least 5,000 envenomations per year in Australia [2].

Determination of the DNA sequence of α -LTX-Lh1a from the genomic DNA of this spider was performed in a stepwise fashion along the α -latrotoxin gene. This process resulted in the definitive determination of 3845 bases, covering the complete mature toxin sequence, including a three base insertion (bases +10–12, Fig. 7). Comparison with α -LTX-Lt1a revealed that the DNA sequence also covered 35% of the signal peptide and 81% of the C-terminal propeptide region of α -LTX-Lt1a [11]. There was 95.1% sequence identity between the DNA sequence encoding α -LTX-Lh1a with that encoding the mature α -LTX-Lt1a toxin (Fig. 7).

Translation of the genomic DNA sequence for α -LTX-Lt1a yielded a mature toxin comprising 1180 amino acid residues with a predicted molecular mass of 132,514 Da and a *pI* of 5.72. 1097 (93%) of these residues are identical to those in the mature α -LTX-Lt1a toxin, which increases to 93.2% if three residues identical to a variant of α -LTX-Lt1a (AAC78471 in EMBL-Bank CDS) are included (V41, V143 and D1097). Of the 83 amino acid substitutions, 34 are conservative and 49 non-conservative, resulting in an overall 93.8% similarity with α -LTX-Lt1a (Fig. 8). Importantly the deduced polypeptide contained all four partial amino acid sequences previously sequenced by tandem MS of *f7.9*. Furthermore, the mature α -LTX-Lh1a also contains two hydrophobic segments in its N-terminal region, HS1 (residues 35–60) and HS2 (219–238) homologous to similar segments found in α -LTX-Lt1a (Fig. 8) and δ -LIT [10].

Analysis of the deduced amino acid sequence revealed the presence of 22 imperfect repeats (R1–R22) at positions 439–1180 (Fig. 8). These comprise the ankyrin-like repeat motif previously described in α -LTX-Lt1a [8] and other latrotoxins [9, 10, 16] and divide the mature toxin into two domains: the N-terminal domain II (50,026 Da, 38%) and the ankyrin-rich domain III (82,506 Da, 62%). Ankyrin repeats are a sequence motif found in proteins with various functions including transcription initiation, cell cycle regulation, cytoskeletal integrity, ion transport, and cell-cell signaling [51]. These repeats have a consensus sequence as shown in Supplementary Fig. S2A [52, 53]. The 33-residue ankyrin repeats in α -LTX-Lh1a have a highly conserved length, except for R1, R16 and R17 (32 residues); similarity to the consensus sequence ranges from 9% (R16) to 61% (R3 and R13). Analogous to α -LTX-Lt1a, repeats R1, R16 and R17 are atypical, imperfect repeats (Supplementary Fig. S2B). Finally, the putative binding site for 4C4.1 was derived from the DNA sequence for α -LTX-Lh1a (nucleotide bases 3156–3177). Importantly, α -LTX-Lh1a contains non-conservative F1009D and S1011N substitutions in the binding epitope when compared to α -LTX-Lt1a.

4. DISCUSSION

Comparative Western blot analysis of *Latrodectus* spp. and *S. grossa* venoms using a polyvalent anti- α -LTX-Lt1a antibody revealed that all venoms contained proteins that are antigenically similar to those found in *L. tredecimguttatus*, in particular a 130 kDa band consistent with α -LTX. The pattern of expression is similar to that seen in Western blots employing polyvalent red-back spider antivenom, an equine-derived antibody composed of purified IgG-F(ab')₂ fragments produced from the venom of *L. hasselti* [32, 46]. These findings suggested the presence of an α -LTX-Lt1a-like protein in all *Latrodectus* spp. and *S. grossa* venoms. The subsequent identification of α -LTX and LIT sequences from *Latrodectus* venoms, other than *L. tredecimguttatus*, provides the first confirmation that α -LTXs are widely expressed in theridiid spiders.

Using non-degenerate primers based on the DNA sequence of α -LTX-Lt1a and MS-MS peptide sequences from *L. hasselti* RP-HPLC fractions, we were also able to determine the complete sequence of the novel α -LTX-Lh1a from the venom of the red-back spider *L. hasselti*. The primary structure of α -LTX-Lh1a has the same domain structure as α -LTX-Lt1a. α -LTX-Lh1a contains two furin-like endopeptidase cleavage sites coded for by a cluster of 4 basic residues: 'RMRR' at the N-terminus (-4 to -1) and 'KFRR' at the C-terminus (1177-1180) followed by an acidic amino acid (glutamate). These conform to the $(K/R)\Phi_{1-3}(K/R)_{0-1}R$, consensus sequence for proteolytic cleavage of α -LTX-Lt1a and LITs where K and R are lysine and arginine, respectively, and Φ is any hydrophobic/aromatic residue [54]. These furin sites are believed to be important for removal of the signal peptide and C-terminal propeptide in α -LTX-Lt1a [11]. Therefore α -LTX-Lh1a appears to be produced as a preprotoxin in a similar fashion to α -LTX-Lt1a. Unfortunately most of the nucleotide sequence upstream of the N-terminus of the mature protein could not be determined and therefore we cannot draw any conclusions about the size of the signal peptide and its homology to that of α -LTX-Lt1a. We sequenced 160 bases in the domain IV propeptide region but unfortunately we were unable to sequence to the stop codon 201 residues downstream of the furin cleavage site in α -LTX-Lt1a. However, within domain II and III, the cysteine residues important for structural stability and activity of latrotoxins [20, 55] as well as the two hydrophobic segments in domain II, were all conserved. In particular, the second hydrophobic segment (HS2) is highly conserved between α -LTX-Lh1a and all latro(insect/crusta)toxins (see [10]) and may, therefore, be involved in an important aspect of function in all latrotoxins.

Despite the fact that all the venoms tested appeared to be antigenically similar when probed with polyclonal anti- α -LTX antibodies, the anti- α -LTX-Lt1a monoclonal antibody 4C4.1 failed to recognize venom from *L. hasselti*, *L. hesperus*, *L. mactans*, and *S. grossa*. This was noted in both Western blots and in the inability of 4C4.1 to prevent the rapid muscle contracture and loss of twitch tension produced by these latter venoms in the CBCNM preparation. Thus, even though all *Latrodectus* and *S. grossa* venoms appear to contain α -LTX-like proteins, the epitope required for binding of the 4C4.1 antibody appears to be present only in the toxin from *L. tredecimguttatus*, or is at least significantly modified in the α -LTX-like toxins from other *Latrodectus/Steatoda* venoms. The putative binding site for 4C4.1 was determined directly by tandem MS from *L. mactans* f7.9 (α -LTX-Lm1a) and derived from the DNA sequence for α -LTX-Lh1a. Both α -LTX-Lm1a and α -LTX-Lh1a contain a non-conservative F1028D substitution when compared to α -LTX-Lt1a. In addition, α -LTX-Lh1a also contains a non-conservative S1030N substitution. These substitutions explain the lack of binding of 4C4.1 to *L. hasselti* and *L. mactans* protein bands in Western blots, and also the lack of neutralizing activity of the antibody in the chick nerve-muscle preparation. *L. hesperus* α -LTX-Lhe1a is also likely to contain similar substitutions that underlie the ineffectiveness of 4C4.1 in Western blots and neutralization assays.

The actions of α -LTX-Lh1a to cause rapid spontaneous neurotransmitter release, leading to an inhibition of neurotransmission at the chick neuromuscular junction, are indistinguishable from that of α -LTX-Lt1a (Fig. 4G-I). This results from the known action of LTXs to cause massive vesicle exocytosis [4-6]. This action is consistent with the clinical reports of latrodectism and animal studies using *L. hasselti* venom, including reports of muscle fasciculation, tremor, muscle paralysis, tachycardia, priapism, hypertension, salivation and diaphoresis resulting from excessive neurotransmitter release from motor and autonomic nerve endings [2, 32]. Since α -LTX-Lt1a shares a very high degree of sequence homology with α -LTX-Lh1a, and probably α -LTX-Lm1a and α -LTX-Lhe1a, this indicates that the subtle differences in structure between these α -LTX homologs do not significantly alter the intense neurotransmitter release seen *in vitro* with all *Latrodectus* venoms ([32] and Fig. 4G-I). These findings therefore provide strong support for the widely held assumption that the clinical symptoms of latrodectism are due to the presence of α -LTX homologs in all theridiid venoms.

The *in vitro*, *in vivo* and clinical observations of the effectiveness of red-back spider antivenom in the treatment of latrodectism from a wide range of *Latrodectus* species [32-35] suggests that it may therefore be possible to use a single antivenom worldwide for the treatment of widow-spider envenomation. This is likely to impact less on regions that already have their own antivenom to treat envenomation by local *Latrodectus* species [56], but could potentially benefit many regions of the world where specific antivenoms to local widow spiders are not available. Given the success of red-back spider antivenom in neutralizing the activity of a variety of *Latrodectus* venoms [32], together with the efficacy of the monoclonal antibody 4C4.1 [31] and Fab antibody fragment FM1 [57] (also see [58]) in neutralizing the activity of whole *L. tredecimguttatus* venom or α -LTX-Lt1a, there has been some interest in the development of monoclonal antibody therapy for the treatment of widow spider antivenom. The advantages of this approach include the virus- and sterile-free methods of production and the high purity of recombinant proteins lacking contaminant animal proteins derived from animal sera. Also, when compared to the large molecular mass IgG-based polyvalent antivenoms, Fab fragment antibodies are likely to diffuse more rapidly due to their smaller molecular mass, and distribute more extensively due to a larger apparent volume of body distribution. These may result in more rapid inactivation of circulating toxin and an earlier recovery from persistent symptoms of latrodectism.

However, as evidenced by neutralization studies in the CBCNM preparation, the monoclonal antibody 4C4.1 is unlikely to have any utility as an antidote in the treatment of latrodectism from any species other than *L. tredecimguttatus*. Monoclonal antibodies may not bind or neutralize α -LTXs from other species, reflective of the highly homologous but not identical primary structure of α -LTXs across the family Theridiidae. Furthermore, in view of the mechanism of action of the 4C4.1 antibody, it is unlikely that it would be an effective treatment for established envenomation.

Pescatori et al. have shown that while 4C4.1 exerts its effect through prevention of tetramerization of α -LTX-Lt1a prior to its insertion into cell membranes, once α -LTX-Lt1a tetramer ion channels have been formed the antibody is incapable of reversing α -LTX-Lt1a pore formation [30]. Hence, polyclonal anti- α -LTX antibodies targeting multiple epitopes on the toxin are likely to be more effective at preventing and reversing the toxicity of widow-spider venoms. Furthermore, in view of the greater expense in production of specific antitoxins, production of polyclonal antivenoms reared in animals exposed to crude venoms remains the least expensive option for the production of clinically useful antivenoms.

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Table 1: Sequence similarities for latrotoxins from *L. hasselti*, *L. hesperus*, and *L. mactans* determined by MS-MS

Known sequence (<i>L. tredecimguttatus</i>)		MS-MS Sequence comparison	
		% Identity (identities/total)	% Similarity (similarities/total)
α -latrotoxin ¹	<i>L. hasselti</i>	100 (54/54)	100 (54/54)
	<i>L. hesperus</i>	100 (22/22)	100 (22/22)
	<i>L. mactans</i>	94.6 (148/150)	98 (147/150)
α -latroinsectotoxin ²	<i>L. hasselti</i>	100 (151/151)	100 (151/151)
δ -latroinsectotoxin ³	<i>L. hasselti</i>	88.7 (63/71)	91.5 (65/71)
	<i>L. hesperus</i>	91.2 (133/148)	93.2 (135/148)

Similarities and identities are based on comparisons with the known sequences of *L. tredecimguttatus* latrotoxins. ¹ Kiyatkin et al., 1990, ² Kiyatkin et al., 1993, ³ Dulubova et al., 1996.

Figure legends

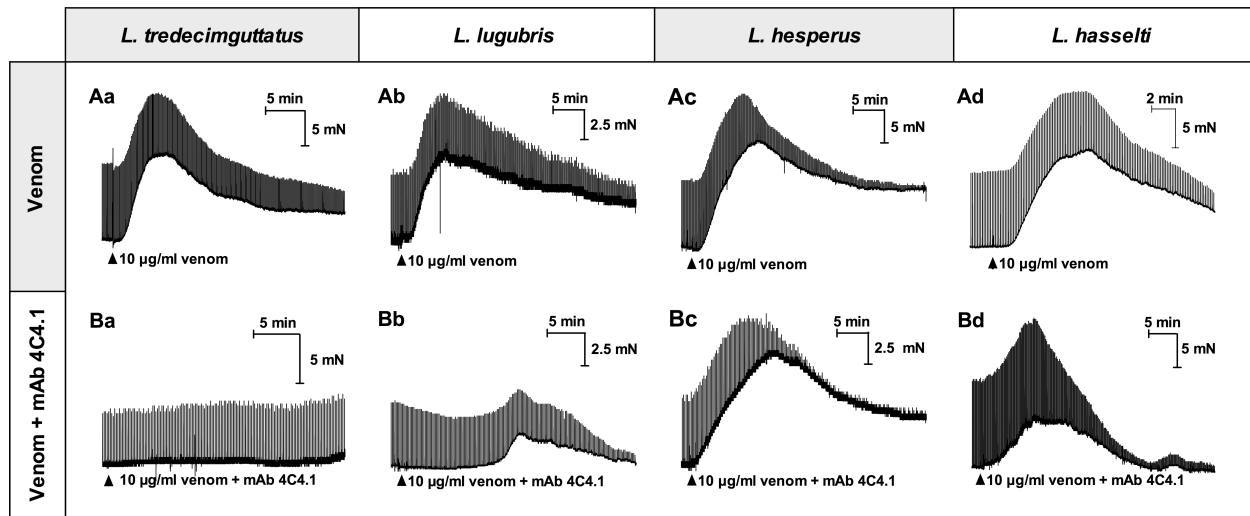


Fig. 1. Lack of cross-reactivity of the monoclonal antibody 4C4.1 as judged by its ability to prevent the neurotoxicity of *Latrodectus* spp. venoms in the isolated CBCNM preparation. (A) Typical effects of 11 µg/ml *L. tredecimguttatus* venom (a), 10 µg/ml *L. mactans* (b), 11 µg/ml *L. hesperus* (c), and 11 µg/ml *L. hasselti* (d) venom on indirectly-stimulated twitch contractions in the CBCNM preparation. (B) Typical effects of the same venoms recorded after a 5 min pre-incubation of venom with 1 ml (5 µg) of the monoclonal antibody 4C4.1.

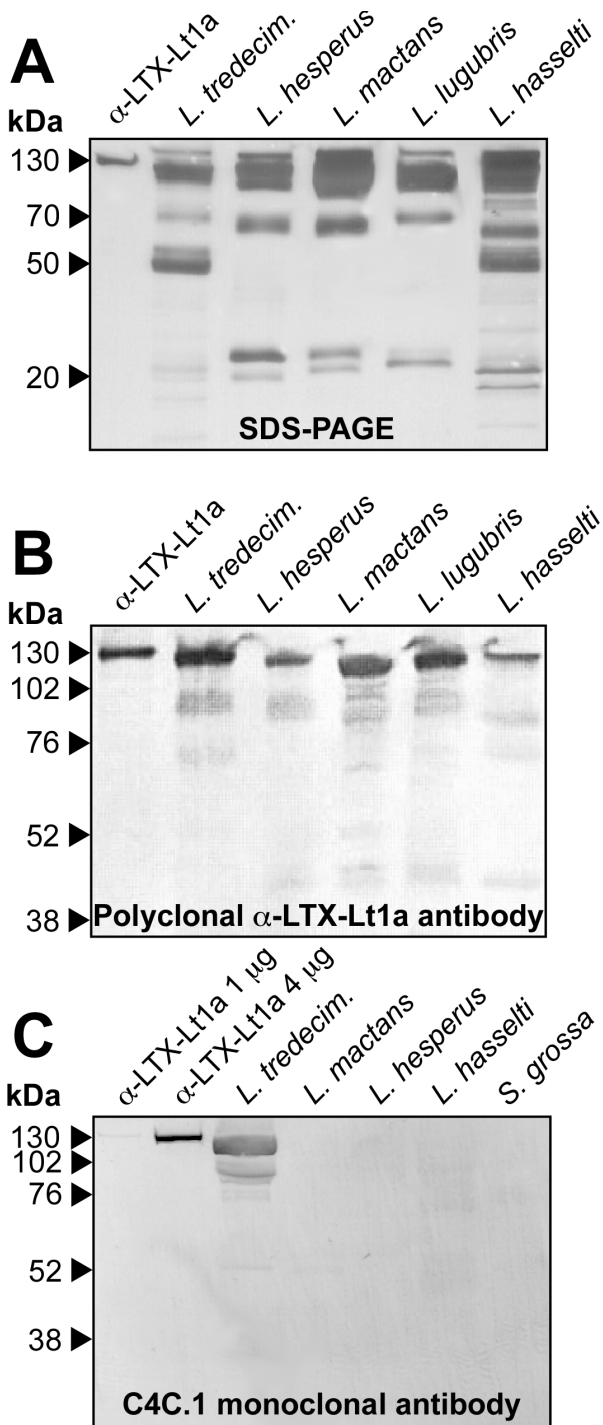


Fig. 2. SDS-PAGE gel and Western blots of α -LTX-Lt1a and theridiid spider venoms. (A) SDS-PAGE gel run under reducing conditions showing α -LTX-Lt1a and protein components of *Latrodectus* spp. venoms. Note a consistent protein band in the venom lanes at \sim 130 kDa, consistent with α -LTX-Lt1a. (B-C) Western blot analyses of α -LTX-Lt1a and theridiid venoms employing polyvalent anti- α -LTX antibody (B) and the monoclonal antibody 4C4.1 (C) as the primary antibodies. Note that purified α -LTX-Lt1a as well as the 130 kDa protein band in *L. tredecimguttatus* venom are recognized by both antibodies.

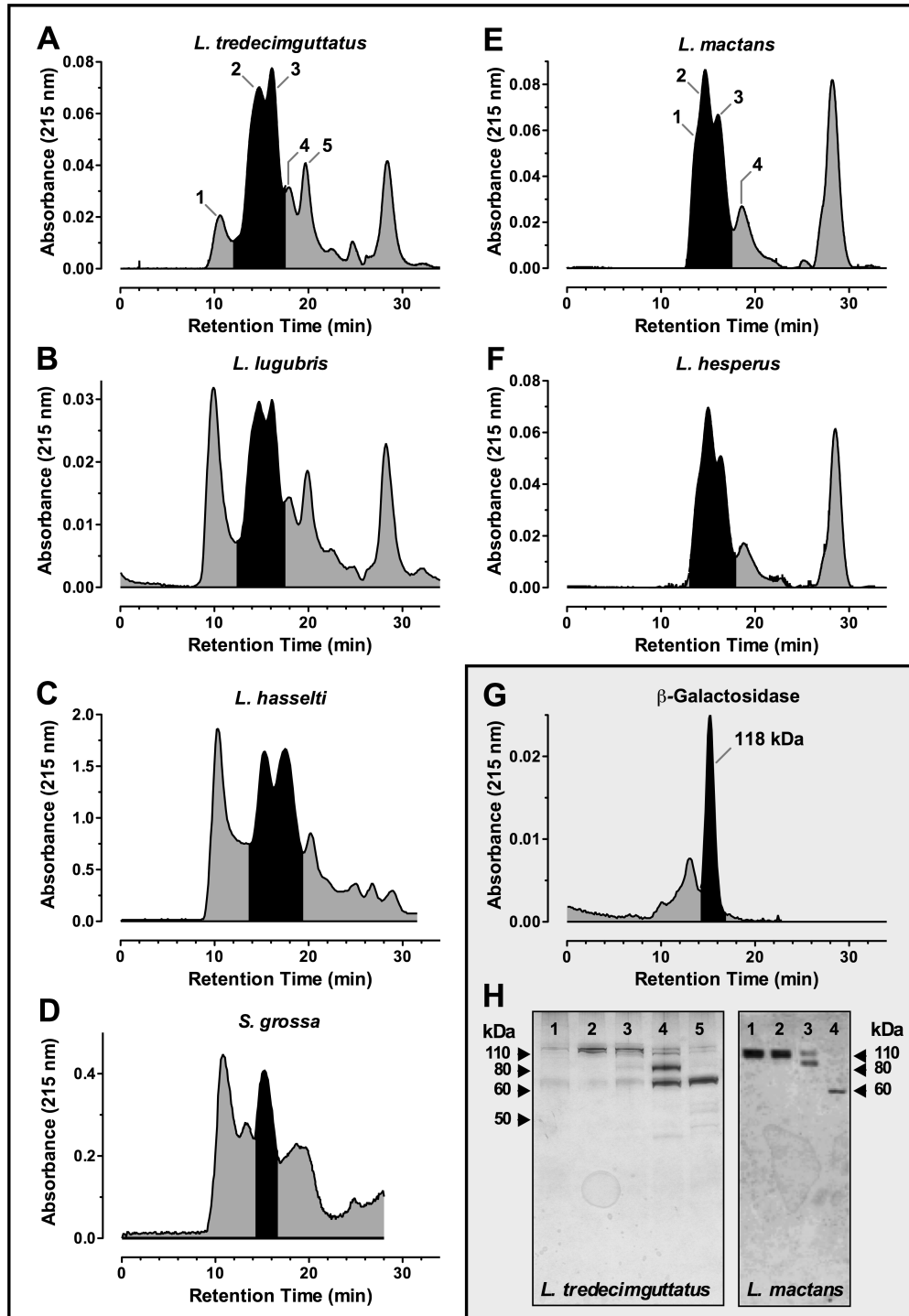


Fig. 3. Chromatographic fractionation of theridiid spider venoms. (A-F) Size-exclusion FPLC chromatograms of *Latrodectus* and *S. grossa* venoms. The black shaded portion of the chromatogram indicates fractions with vertebrate activity in the CBCNM preparation. (G) Chromatogram obtained for the molecular mass marker β -galactosidase (118 kDa) under the same elution conditions (H) SDS-PAGE gels run under reducing conditions of *L. tredecimguttatus* and *L. mactans* size exclusion fractions. Lanes correspond to the peaks marked in panels A and E, respectively.

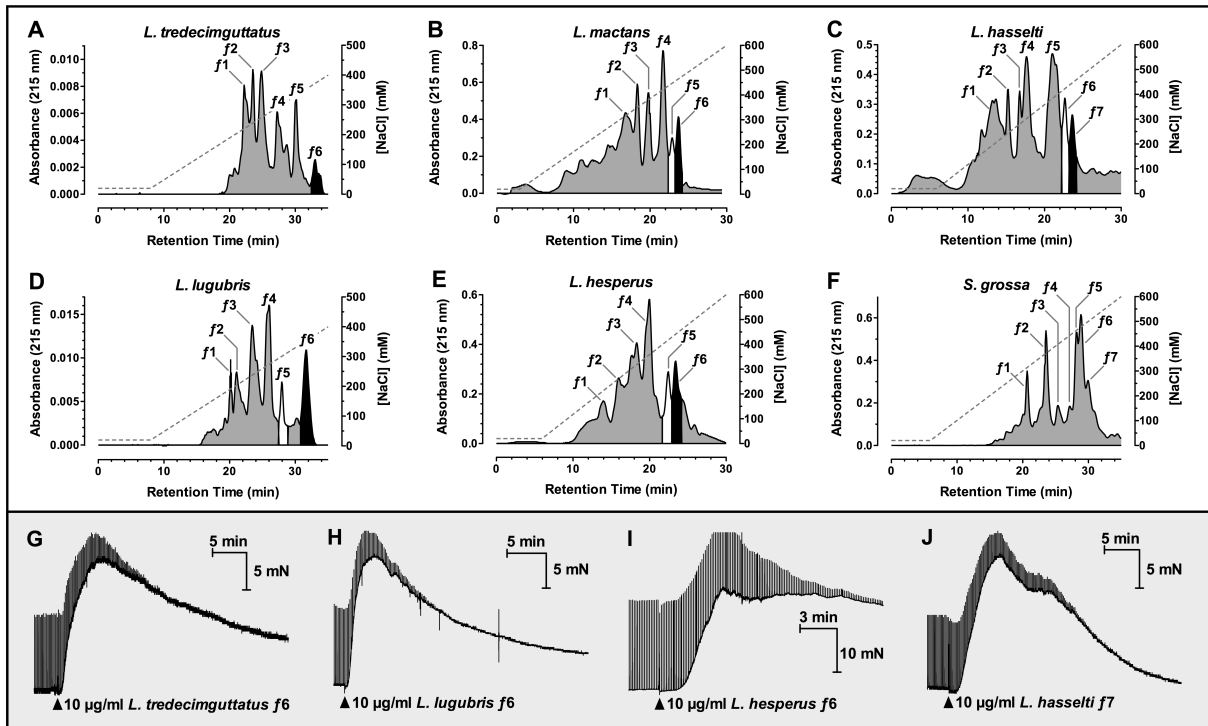


Fig. 4. Anion-exchange FPLC separation of vertebrate-toxic intermediate-mass protein fractions from theridiid spider venoms. (A-F) Anion-exchange FPLC chromatograms recorded at 215 nm (left-hand ordinate axis) using a linear NaCl gradient (right-hand ordinate axis). The final eluting peaks shaded in black (*f6* or *f7*) indicate fractions with vertebrate toxicity. The preceding peaks shaded in white (*f5* or *f6*) indicate fractions with insect toxicity. Note the similarity in chromatographic profiles and the overlap of adjacent peaks. The anion-exchange profile obtained for *S. grossa* venom is markedly different to that obtained for the *Latrodectus* venoms. Additionally, *S. grossa* fractions did not exhibit any activity in the chick bioassay. (G-J) Effect of 10 µg/ml *f6* or *f7* fractions from anion-exchange FPLC on indirectly-stimulated twitch contractions in the CBCNM preparation.

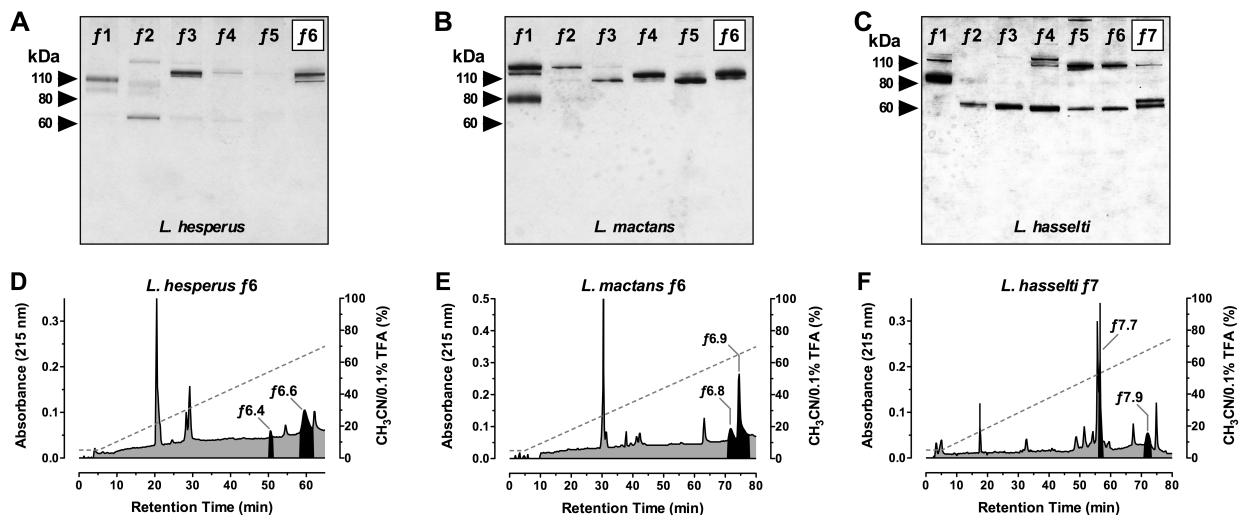


Fig. 5. Electrophoretic and reverse-phase separation of vertebrate-toxic anion-exchange FPLC fractions. (A-C) Typical SDS-PAGE gels run under reducing conditions of anion-exchange FPLC fractions. Lanes correspond to the peaks marked in Fig. 4. (D-F) Analytical C18 RP-HPLC chromatograms obtained for vertebrate-toxic anion-exchange FPLC fractions (*f6* or *f7*) recorded at 215 nm (left-hand *y*-axes) using a linear 0.1% TFA/acetonitrile gradient (right-hand ordinate axes). Peaks highlighted in black were submitted for MS-MS analysis.

α-LTX	α-LTX-Lt1a	44	63	165	183	195	209				
	α-LTX-Lha1a	...I VGT I A A A A M A V T H V A S G RL S D Y I L K L E E P N G I L L H F KA P L F S I I E E G Y A V P K ...							
	α-LTX-Lm1a	...I VGT I A A A A M T V T H V A S G RL S D Y I L K L E E P N G I L L H F KA P L F S I I Q E R Y A V P K ...							
	α-LTX-Lt1a	...S S L V G T G T S N N E G L L D RN G V D E M L N E KY A V Q Y A S E T L F S KD L Y N A A S N P D ...						
	α-LTX-Lha1a	...S S L V G T G T S N N E G L L D RN G V D E M L N E KY A V Q Y A S E T L F S KD L Y N T A N N P D ...						
	α-LTX-Lm1a	...S S L V G T G T S N N E G L L D RN G V D E M L N E KY A V Q Y A S E T L F S KD L Y N A A S N P D ...						
	α-LTX-Lhe1a	...S S L V G T G T S N N E G L L D RN G V D E M L N E KY A V Q Y A S E T L F S KD L Y N A A S N P D ...						
	α-LTX-Lt1a	448	453	560	571	619	629	699	707	843	851
	α-LTX-Lha1a	S A V G F KL M E S P E I N I N E RD D W P V A S T L L GS S N V N V N L KN S D T P L N L A ...					
	α-LTX-Lm1a	S A V G F KL M E S P E I N I N E RD D W P V A S T L L GS S N V N V N L KN S D T P L N L A ...					
	α-LTX-Lhe1a	S A V G F KL M E S P E I N I N E RD D W P V A S T L L GS S N V N V N L KN S D T P L N L A ...					
	α-LTX-Lt1a	862	873	920	927	1141	1148	1154	1163		
α-LTX-Lha1a	...Y F I D Q G A D I N T RN G H L N I V KY G I D V T I RT A L D I A I D A K ...							
α-LTX-Lm1a	...Y F I D Q G A D I N T RN G H L N I V KY G I D V T I RT A L D I A I D A K ...							
α-LIT	α-LIT-Lt1a	154	165	367	374	582	601	620	629		
	α-LIT-Lha1a	...L V I E T I E N I A T KL S I S I S F KT D V T Q T L I D I T E I D L N A Q D KI L I R I N T N A V I ...						
	α-LIT-Lt1a	630	652	659	687						
	α-LIT-Lha1a	...N I K S K V G L T P L H L A T L Q N N L S V S KG A Y L N D G D A N G M T P L H Y A A M T G N L E M V D F L ...								
	α-LIT-Lt1a	704	714	751	760	939	959	1118	1122		
	α-LIT-Lha1a	...K W T P L H L A I L F KQ L V I E L L A KT F F D L A I E N G R L N I V A F A V E KY I A A R ...						
δ-LIT	δ-LIT-Lt1a	29	40	410	421	482	491	558	567		
	δ-LIT-Lha1a	...D E E D G E M T L E E RF S E W S E P F T V Q GA V D E A E T T L I E KI N H G A D V N A K ...						
	δ-LIT-Lt1a	806	816	842	846	882	890				
	δ-LIT-Lha1a	...D N L T P L H S A A KT A L H LI N D D L D M V R ...							
	δ-LIT-Lt1a	216	240	295	304	325					
	δ-LIT-Lha1a	...E P S G N S L S S A L N E L L D K N N Y A I P KV A I I F D D F K S S L T G G D D G L I D N V I E V L N T V K ...								
	δ-LIT-Lt1a	697	718	785	803						
	δ-LIT-Lha1a	...V S I N S V T E N N N W T P L H F A I Y F KK E P E I A V V L I E N G A D I E A R ...								
δ-LIT-Lt1a	909	918	943	958							
δ-LIT-Lha1a	...T S I M L I V Q K L L L E L Y N Y F I N N Y A E T L D E E A L F N R L D E Q G K L E L A L I I M H N K ...										

Fig. 6. Comparison of the MS-MS peptide sequences from RP-HPLC separation of the vertebrate-toxic anion-exchange fraction from *Latrodectus* spp. venoms. The amino acid sequences were determined by MS-MS sequencing following overnight (16 h) digestion with trypsin. Sequences are aligned against the known sequences of α -LTX-Lt1a, α -LIT-Lt1a and δ -LIT-Lt1a from *L. tredecimguttatus* (light gray) [8-10]. Identities are boxed in dark gray, conservative substitutions are in gray italic text. Isoleucine and leucine residues were assigned from the known sequences.

α-LTX-Lha1a
α-LTX-L1fa

141 120 101

CGACTGCTGTTTTCGTTTTAACAGTTGCTCGAGATTTTG

SLTX1F SLTX1F

100 80 60 40 20

GGTTTATATATACCTATATTTGCACTTAAGAGTATCAAAATGATTTCTGTAGGAGAAATATGGAACGCGCTAATCATTCGTTAGTGGCAATGAGACGA

1 20 40 60 80 100

GAAGGAGAAG AAGACTTAACTTTAGAGGAAAAGCAGAACTATGCTCAGAAATAGAATCCAAACAAAATATGTAGATATAGGTTTCCAATATTATTGGCG

GAAGGAGAAGATTTTAACTTTAGAGGAAAAGCAGAACTATGCTCAGAAATAGAATCCAAACAAAATATGTAGATATAGCTTCCAATATTATTGGCG

101 120 140 160 180 200

ACCTTTCTCTCTTCCAATAGTTGGTAAAATCGTAGGACCATAGCTGCAGCCGCTATGGCAGTCACCCATGTTGCTTCTGGGAGGTTAGATATCGAGCA

ATCTTTCTCTCTTCCAATAGTTGGTAAAATCGTAGGACCATAGCTGCAGCCGCTATGGCAGTCACCCATGTTGCTTCTGGGAGGTTAGATATCGAGCA

201 220 240 260 280 300

GACGTTAGGGGTTGCGAGTACGTAACCTTTTCGATCAGATTAAGGAAATCTAGAAAGAGGTTCAACGAAATAGACAGGAAGTTGGAAGTCAACAGTCCG

GACGTTAGGGGTTGCGAGTACGTAACCTTTTCGATCAGATTAAGGAAATCTAGAAAGAGGTTCAACGAAATAGACAGGAAGTTGGAAGTCAACAGTCCG

301 320 340 360 380 400

GCCTTTGAGGAAATTAACCTGGTAGAGAAAAGTATATCCCGGTTAGAAAAACACGGAAGCAGATGAATAAGAGGTTTGGATGAAGTCATGAGGTTCA

GCCTTTGAGGAAATTAACCTGGTAGAGAAAAGTATATCCCGGTTAGAAAAACACGGAAGCAGATGAATAAGAGGTTTGGATGAAGTCATGAGGTTCA

401 420 440 460 480 500

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TTCAGGACGCAAAAGTTTCCCACTTTGTTTGC AAAATCAATATTTTGC AAGATATTTTGATACTGAAAAGGAAAGAAATCCGTGGTTTAAAGTTAAAGTGA

501 520 540 560 580 600

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TTATATCTTAAAGCTTGAAGAACCAATGGCATACTACTTTCATTTCAAGAGTGCAGAACTCCAAAGGATGATAGTTTACAAAGCTCCTTTATTCAGTATT

601 620 640 660 680 700

ATTCAAGAAAGATATGCAAGTACCCAAATCTATTGATGACGAATAGCTTTTAAAGTTCCTATGCAATGCTGTATGTTACCCAGACATATGCTCGGTTA

ATTCAAGAAAGATATGCAAGTACCCAAATCTATTGATGACGAATAGCTTTTAAAGTTCCTATGCAATGCTGTATGTTACCCAGACATATGCTCGGTTA

701 720 740 760 780 800

TGTTTTTCTCTAGAACAACTCTTTTCCGTTAACCATTATTACGAGAAAGGAGACTTAGAAAAGTATGATGAATAC TTTAATAGCTTAAATAATGT

TGTTTTTCTCTAGAACAACTCTTTTCCGTTAACCATTATTACGAGAAAGGAGACTTAGAAAAGTATGATGAATAC TTTAATAGCTTAAATAATGT

801 820 840 860 880 900

ATTTTTAGATTTTAAATCGTCACTTTGGGTACCGGCACATCTAATAAGTGAAGTCTCTTGATAGAGTGCCTCAAGTTTTAGTACTGTAAAGAATAGC

ATTTTTAGATTTTAAATCGTCACTTTGGGTACCGGCACATCTAATAAGTGAAGTCTCTTGATAGAGTGCCTCAAGTTTTAGTACTGTAAAGAATAGC

901 920 940 960 980 1000

GAGTTCTTAGGACTTGAAA AAAATGGTGTAAATGAAATGCTGAACGAGAAAATAAATTTTAAATAAAAATAAGGTAAGAAATCGAAGGCCAAACAAAGAA

GAGTTCTTAGGACTTGAAA AAAATGGTGTAAATGAAATGCTGAACGAGAAAATAAATTTTAAATAAAAATAAGGTAAGAAATCGAAGGCCAAACAAAGAA

1001 1020 1040 1060 1080 1100

TGACATTTGAGTGAGACACCCAGAAAATTTTGCACAAATTTCAATTTGACAAAGATATAACAACTCCCATCGGAGATTTGAGAGAGATGGTCTGTAAGTACGTTA

TGACATTTGAGTGAGACACCCAGAAAATTTTGCACAAATTTCAATTTGACAAAGATATAACAACTCCCATCGGAGATTTGAGAGAGATGGTCTGTAAGTACGTTA

1101 1120 1140 1160 1180 1200

CGCTGTACAATACGCAAGTGAAACTTTATTTTCAAAATCAGTCAATGGTCTGATCCCGTGGTGTGAGAGAGAAAAGCATGCCCTACTCTCCGAATGCCA

CGCTGTACAATACGCAAGTGAAACTTTATTTTCAAAATCAGTCAATGGTCTGATCCCGTGGTGTGAGAGAGAAAAGCATGCCCTACTCTCCGAATGCCA

1201 1220 1240 1260 1280 1300

GTGGACCAAAACCGAAGAAAATACCTTTGTTTTCAGAAAATTTGACAGCAGCAAACTCAACTGGTTGGCGAAAATCACACCATACC AATCAAATTTTCATCG

GTGGACCAAAACCGAAGAAAATACCTTTGTTTTCAGAAAATTTGACAGCAGCAAACTCAACTGGTTGGCGAAAATCACACCATACC AATCAAATTTTCATCG

1301 1320 1340 1360 1380 1400

ACATTTGATAGAGATCTTTATAATACCGCTAATAATCTGATTCAGCTGTAGGTTTAAAGGAATTCACGAAATTAATTTATGATGGCGCTAATATTAGAGC

ACATTTGATAGAGATCTTTATAATACCGCTAATAATCTGATTCAGCTGTAGGTTTAAAGGAATTCACGAAATTAATTTATGATGGCGCTAATATTAGAGC

1401 1420 1440 1460 1480 1500

AACCTTTCGAAACAGGAAGAACAGTTTTTCATGCCGACGCTAAAAGTGGCAATTCATAGGATTTATGATCGGATTAACGTTTATAGTAAAATCTAACGAACATA

AACCTTTCGAAACAGGAAGAACAGTTTTTCATGCCGACGCTAAAAGTGGCAATTCATAGGATTTATGATCGGATTAACGTTTATAGTAAAATCTAACGAACATA

1501 1520 1540 1560 1580 1600

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AACCAACAGATAAAAAAGGTTACACTCCTATTTCATGAGCTGACAGATAGTGGAAACGCTGGTATTGTTAATCTCTTAAATACAAAAGAGGTGTATCAATAA

1601 1620 1640 1660 1680 1700

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1701 1720 1740 1760 1780 1800

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TATAAATGAAAGAGATAAAGATGGATTACGCCCTACATTTAGCTGTTGGGGTGGTGAAAGAATTTTGGAGGCATTCAAAAACAAATCCGATTATGAT

1801 1820 1840 1860 1880 1900

TTAAATGCCAAGTCTAATAAGGGTTTAACTCCTTTCCATCTGGCAATCATTAAGGACGATTGGCCAGTGCCAGTACTTTATAGGAAGCAAAAAGGTTG

TTAAATGCCAAGTCTAATAAGGGTTTAACTCCTTTCCATCTGGCAATCATTAAGGACGATTGGCCAGTGCCAGTACTTTATAGGAAGCAAAAAGGTTG

1901 1920 1940 1960 1980 2000

ATGTCATATGCTGTCGATGAAAACAACATGACAGCATTGACATATGACAGCATTGTTGGGATACTTAGAAAACACAAAACAAATGATTAAC TTAAGAATAA

ATGTCATATGCTGTCGATGAAAACAACATGACAGCATTGACATATGACAGCATTGTTGGGATACTTAGAAAACACAAAACAAATGATTAAC TTAAGAATAA



Fig. 7. Alignment of the nucleotide sequence of α -LTX-Lh1a with the known sequence of α -LTX-Lt1a from *L. tredecimguttatus*. The α -LTX-Lh1a sequence was determined from a gDNA library created from female *L. hasselti* venom glands. Identities are boxed in light gray. The two upward arrowheads indicate the N-terminus (AAG) and C-terminus (GAG) of the mature toxin based on sequence homology with α -LTX-Lt1a. Labels above and below the sequences indicate the forward ('F', black) and reverse ('R', gray) primers used for PCR. The W at 3585 and 3624 indicates a nucleotide base of either A or T.

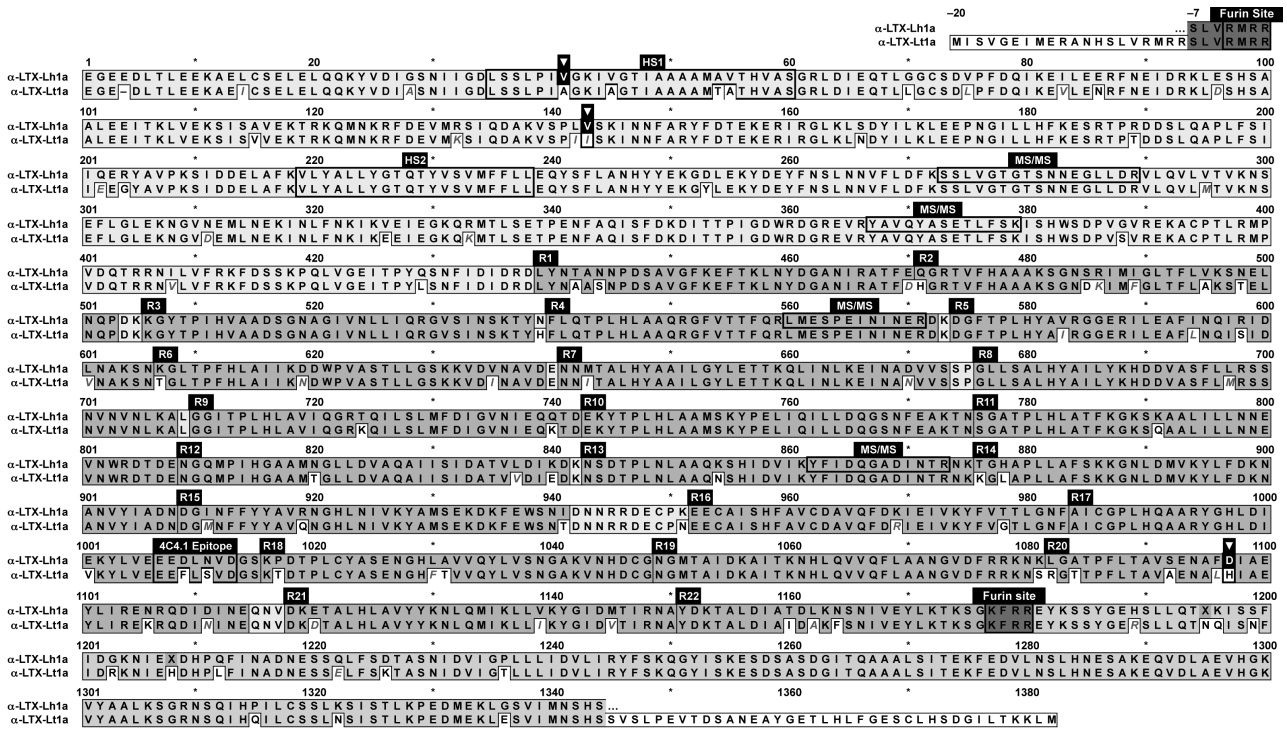


Fig. 8. Comparison of the deduced amino acid sequences of α -LTX-Lh1a with α -LTX-Lt1a. Identities are boxed in gray, conservative substitutions are in gray italic text. Proteins are numbered from the N-terminus of the mature α -LTX-Lh1a based on homology with α -LTX-Lt1a. Note insertion of a glutamate (E) residue at position 4 in α -LTX-Lh1a. The location of the conserved furin-like endopeptidase cleavage sites for the signal sequence (–20 to 0) and C-terminal propeptide (1281–1382) [11] are highlighted and delineate the mature toxin. The three residues marked with arrowheads denote residues in α -LTX-Lh1a that are identical to a variant of α -LTX-Lt1a (AAC78471 in EMBL-Bank CDS). Sequences marked ‘MS-MS’ denote residues also sequenced by MS-MS analysis. Positions of the 22 ankyrin-like imperfect repeats (R1-R22), hydrophobic segments (HS1 and HS2, [10]) and monoclonal antibody 4C4.1 binding epitope (residues 1007–1113; [30]) are marked above the sequences. Residue marked ‘X’ in the C-terminal propeptide region indicate unknown residues due to uncertainties (A or T) in the one of the bases in the corresponding nucleotide triplet: position 1208 is N (corresponding residue in α -LTX-Lt1a) or K while position 1195 is H (corresponding residue in α -LTX-Lt1a) or Q.

SUPPLEMENTARY FIGURE LEGENDS

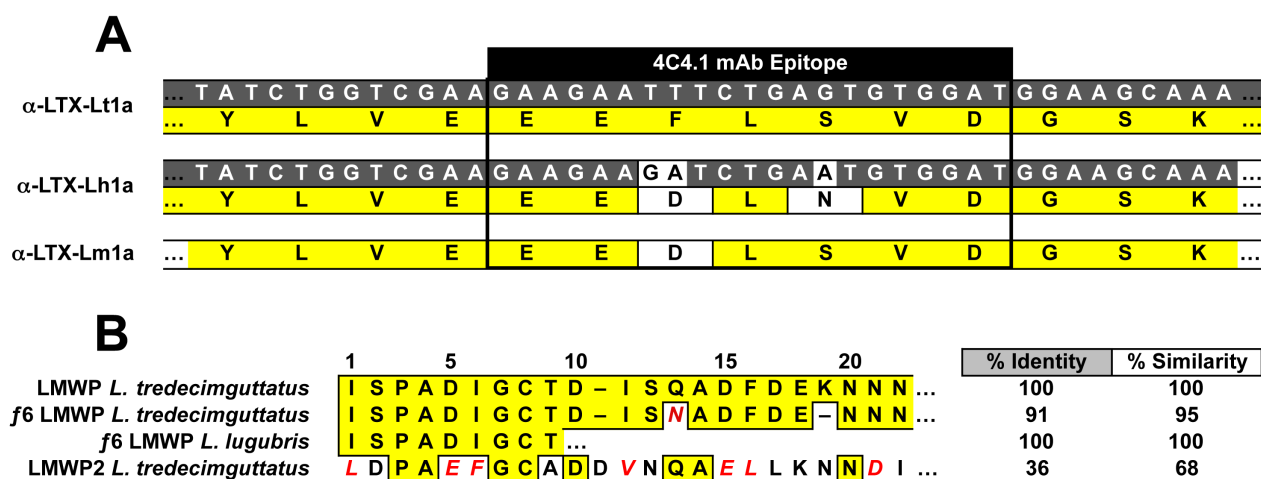


Fig. 1S. Sequence comparisons of (A) α -LTX-associated low molecular weight proteins, and (B) the binding epitope on α -LTX for monoclonal antibody 4C4.1. Identities are boxed in gray, conservative substitutions are in gray italic text. (A) Alignment of the N-terminal partial sequences of two novel LMWPs from the anion-exchange FPLC *f6* of *L. lugubris* and *L. tredecimguttatus* venom with known LMWPs. The two novel sequences were determined by Edman degradation. Note that LMWP from *L. lugubris* is 100% identical to the known sequence of LMWP from *L. tredecimguttatus*. (B) Alignment of the binding epitope for monoclonal antibody 4C4.1 on α -LTX from *L. tredecimguttatus* with the corresponding sites in *L. hasselti* and *L. mactans*. The binding epitope for *L. mactans* was determined from MS-MS sequencing.

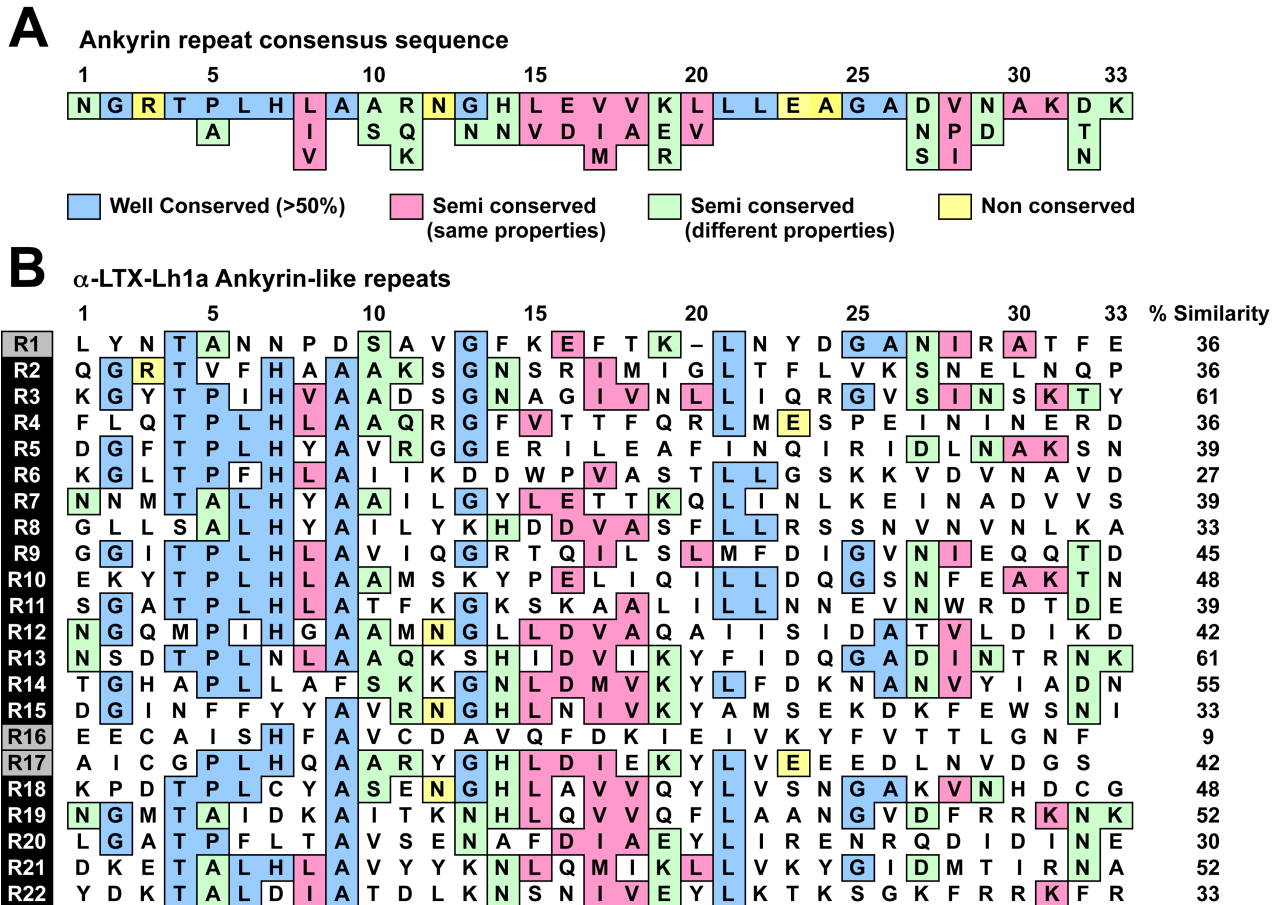


Fig. 2S. Identification of ankyrin-like repeats in α -LTX-Lh1a. (A) Consensus sequence for the 33-residue ankyrin repeat determined from a large number of proteins with ankyrin repeats. Each position is color-coded according to the level of conservation described previously [52, 53]. Blue indicates a residue that is normally present >50% of the time in ankyrin repeats in a wide variety of proteins. Positions are coloured red if residues occur with a higher frequency than any other residues and residues had the same physicochemical property (e.g., hydrophobic). Positions were colour-coded green if residues occurred with a higher frequency than any other residues but the group had different physicochemical properties. Positions that are poorly conserved in the consensus sequence were colour-coded yellow and assigned to the amino acid detailed in [53]. (B) Identification of the 22 ankyrin-like repeats (R1–R22) in α -LTX-Lh1a. Note the imperfect repeats R1, R16 and R17. The percentage similarity with the consensus sequence is indicated at the right of the sequences.