Identification and molecular cloning of insecticidal toxins from the venom of the brown spider *Loxosceles intermedia*

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**Abstract**

The venom of *Loxosceles intermedia* was investigated for the presence of insecticidal toxins active against *Spodoptera frugiperda* (Lepidoptera: Noctuidae), an insect that has caused great reductions in corn production in Brazil. A combination of gel filtration (Sephadex G-100) and ion-exchange chromatography (Carboxymethyl Cellulose, CM 52) resulted in four major fractions that were submitted to biological assay. Fraction 4 was further purified by a reverse phase HPLC (C18 Column) resulting in peptides active against *Spodoptera frugiperda*. Three new potential insecticidal toxins named LiTx\(^1\), LiTx\(^2\) and LiTx\(^3\) were identified. The partial amino terminal sequences of these peptides were obtained and used to clone the corresponding cDNAs with the help of degenerate oligonucleotides. The amino acid sequence deduced from the cDNA of LiTx\(^1\), LiTx\(^2\) and LiTx\(^3\) revealed mature proteins of approximately 7.4, 7.9 and 5.6 kDa.

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1. **Introduction**

Spider venoms are complex mixtures of neurotoxins, proteins and low molecular weight organic molecules used to capture different prey (Escoubas et al., 2000) especially insects. An important group of chemical components found in spider venoms are the acylpolyamine-like toxins. This group appears to have high insecticidal activity, being responsible for the immediate insect paralysis observed during predation, a phenomenon mediated by the blockade of neuromuscular transmission (Parks et al., 1991). Other components used as toxic tools are polypeptides of 3000–8000 Da, which can be vertebrate or insect specific. The major targets of these low molecular mass toxins are the ionic channels present on cell membranes (Gomez et al., 2002). Spider venoms also possess high molecular weight neurotoxins which can promote massive neurotransmitter release from presynaptic endings of insects, vertebrates or crustaceans (Grishin, 1998). The enormous diversity of toxins in spider venoms shows they are a rich source...
of biologically active components which, more than good tools for pharmacological research, may be very useful in the future as biopesticides. During the last decades, a large number of insecticidal spider peptides have been identified, purified, characterized and cloned (Figueiredo et al., 1995; Corzo et al., 2000; Penaforte et al., 2000).

The small brown spider L. intermedia, commonly found in Southern Brazil, is known by its increasing medical importance in the country (Ministry of Health, 1998). Its venom contains numerous active components which contribute to the development of a characteristic dermonecrotic lesion observed after the spider’s bite. Tambourgi et al. (1998) reported proteins with haemolytic, dermonecrotic and spingomyelinase activity. The discovery of two proteins from L. intermedia venom, both characterized as being metalloproteinas with proteolytic action, was described by Feitosa et al. (1998). In 2000, Veiga et al. identified two high molecular weight serine-proteases with gelatinolytic activity probably involved in the harmful effects of the venom. Recently, Kalapothakis et al. (2002); Pedrosa et al. (2002) reported the identification, molecular characterization and expression of dermonecrotic proteins found in spiders of the genus Loxosceles. However, by the best of our knowledge, no insecticidal peptides have been reported to date for L. intermedia venom. In the present paper we report the molecular cloning of three insecticidal toxins isolated from the venom gland of L. intermedia and called LiTx × 1, LiTx × 2 and LiTx × 3.

2. Material and methods

2.1. Venom

10 mg of L. intermedia venom in 0.15 M saline solution (1.0 mg/ml) were provided by “Laboratório Interdisciplinar de Pesquisa em Animais Peçonhentos” of the Federal University of Paranaí.

2.2. Insecticidal activity

Groups of 30 Agrotis ipsilon, Diatraea saccharalis, Spodoptera cosmoides and Spodoptera frugiperda larvae were injected with 10 μl distillate water containing different concentrations (0.5; 0.75; 1.12; 1.69; 2.53; 3.80 μg/g body weight) of L. intermedia venom. The LD50 observed 24 hs after injection was determined by Probit analysis (Finney, 1971). Fractions obtained by ion-exchange chromatography and reversed-phase HPLC were assayed for insecticidal activity against S. frugiperda by injecting a 10 μl aqueous sample (1 μg) of each fraction into larvae weighing 100 mg. The lethality of column fractions was determined using six sample (1 ml) of each fraction into larvae weighing 100 mg. Positive control larvae received L. intermedia crude venom under the same conditions.

2.3. Fractionation of L. intermedia venom

L. intermedia venom was fractionated by gel filtration chromatography (Sephadex G-100) according to the purification procedure described by Barbaro et al. (1992). The fraction that did not show dermonecrotic activity in biological assays with rabbits (Barbaro et al., 1992) was submitted to ion-exchange chromatography (Carboxymethyl Cellulose, CM 52) as previously described (Kalapothakis et al., 2002). After the insecticidal biological assay, the most lethal fraction to S. frugiperda was further purified by reversed-phase HPLC (Shimadzu LC-10AD equipped with a Shimadzu SPD-M10A diode array detector) using a C18 Shimadzu Shim-pack column (4.6 × 150 mm, 5 μm particle diameter, 100 Å pore diameter). The fractions were eluted with a linear gradient of 0–42% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min. The eluate was monitored by absorbance at 190 nm.

2.4. Edman sequencing

Insecticidal fractions obtained by reversed-phase HPLC were sent to New Castle Protein, University of New Castle, Callaghan, Australia for Edman degradation sequencing.

2.5. General molecular biology

Standard recombinant DNA techniques (plasmid purification, electrophoresis, DNA quantification, etc.) were carried out as described by Sambrook et al. (1989).

2.6. cDNA library screening

The cDNA library was constructed with mRNA extracted from the venom glands of L. intermedia as described by Kalapothakis et al. (2002). A total of 800 plasmids containing cDNA inserts were used for dot blot screening as described by Kalapothakis et al. (1998). In the present study the molecular screening of LiTx × 1, LiTx × 2 and LiTx × 3 was based on the peptide sequences of these toxins (XXHGDGSKGGATKXPDD-LiTx × 1; AGKGEVGKKGYEADD-DLiTx × 2 and XXI-KYGRDWGSPHGLPS-LiTx × 3) using degenerate probes: A) 5'-CAYGGNGAGGNNWNAAR-3'; B) 5'-TAYTAYGARGCNGAYGAYA-3' and C) 5'-AARTAYGGNAYMGNTGG-3' corresponding to the nucleotide sequence encoding amino acids 3–8 (HGDGSK) of LiTx × 1; 11–17 (YYEADD) of LiTx × 2 and 4–9 (KYGRDW) of LiTx × 3. The oligonucleotides synthesized by Operon Technologies (1000 Atlantic Avenue, Alameda, CA 94501) were 32P-end labelled using T4 polynucleotide kinase as described by Sambrook et al. (1989).
2.7. DNA sequencing and computer analysis

DNA sequencing reactions of selected plasmids were performed on both strands using the ABI Prism 310 Genetic analyser (Applied Biosystems). Deduced amino acid sequences were analyzed with software tools (ProtScale, Compute pl/Mw, Dialign, PredictProtein, Prosite) provided by the ExPASy World Wide Web server (Gasteiger et al., 2003) (http://www.expasy.org). A search for similar sequences in the database was performed on the basis of the amino acid sequence of each toxin using PSI-BLAST software (Altschul et al., 1997).

2.8. Similarity analysis

To assign putative specific functions to LiTx 1, LiTx 2 and LiTx 3, we conducted an exploratory analysis of amino acid sequence similarity to other known insecticidal spider peptides (Eisen, 1998). We chose preferentially insecticidal toxins whose mechanisms of action have been characterized. Once the toxin sequences were chosen, a multiple alignment was performed using Clustal X (Thompson et al., 1997) and a pairwise distance among sequences was computed with a Poisson correction for multiple amino acid substitutions (Nei and Kumar, 2000). A similarity tree was constructed based on a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. Gaps were pairwise deleted. The reliability of the resulting tree was assessed by a bootstrap resampling method with 500 random replicates. All analyses carried out on the basis of the alignment were performed using the MEGA 2.1 software (Kumar et al., 2001).

3. Results and discussion

We have investigated the lethality of L. intermedia crude venom for highly destructive pests that attack roots, stems and leaves of a wide range of vegetables, fruit and non-food crops: S. frugiperda (corn), S. cosmioides (soybean, apple, bean, etc), A. ipsilon (corn, tobacco, tomato, etc), and D. saccharalis (sugarcane). The LD$_{50}$ found was 0.90, 1.80, 0.71, 1.39 ± 0.34 µg g$^{-1}$ for S. frugiperda, S. cosmioides, A. ipsilon and D. saccharalis, respectively.

The fractionation of L. intermedia venom by gel filtration resulted in three major fractions named P1, P2 and P3 (Fig. 1A). While P1 and P2 were found to be able to induce dermonecrotic lesions in rabbits (data not shown), P3 revealed no such activity, being further purified by ion-exchange chromatography. Of the four major fractions obtained in this step (Fig. 1B), F4 proved to be the most lethal to S. frugiperda, inducing flaccid paralysis. The components of F4 were resolved by reverse phase HPLC and fractions in which insecticidal activity remained were named LiTx 1, LiTx 2 and LiTx 3 (Fig. 1C).

The partial amino acid sequence of these peptides was obtained by Edman degradation. The results showed approximately 17 residues for each neurotoxic peptide as being XXHGDGSKGGATKXPDD, AGKGEVKKG GYYEADD and XXIKYGDRWGSHPHGLPS for LiTx 1, LiTx 2 and LiTx 3, respectively. Their partial amino acid sequence were used to design degenerate oligo probes and clone the corresponding cDNAs using the strategy of plasmid dot blot screening (Cardoso et al., 2003). The plasmids from 800 PCR selected clones isolated from the cDNA library of a L. intermedia venom gland (Kalapothakis et al., 2002) were transferred to a nylon membrane and hybridized with specific oligonucleotides (See Section 2). Positive clones (46) were detected and, after a confirmatory screening, 10 clones were further analyzed by DNA sequencing. cDNAs coding for LiTx 1, LiTx 2 and LiTx 3 were isolated (Figs. 2A–C). Comparing the mature peptide amino acid sequence obtained by Edman degradation and the one obtained on the basis of the cDNA, LiTx 1 showed one discrepancy at position 10 (G for C), LiTx 2 two discrepancies: the first at position 10 (G for C) and the second at position 17 (D for W) and, in contrast, LiTx 3 showed the same sequence obtained by peptide and DNA sequencing. We are confident that all the discrepancies found are artifacts from the Edman degradation method and the cDNA sequencing corresponds to the exact sequence for the three peptides analysed.

The three cDNAs coding for LiTx 1, LiTx 2 and LiTx 3 have the same overall organisation, encoding a precursor peptide with 3 or 4 segments in the following sequence: a signal peptide, a propeptide, the mature toxin and a C-terminal peptide. The presence or absence of the last segment is uncertain and only the complete sequencing of the isolated peptide could reveal the precise C-terminal of the mature toxins. However, it is known that toxins from spiders and scorpions are frequently modified at the C-terminal position by post-translational processing of the immature toxins (Martin-Eauclaire et al., 1994; Kalapothakis et al., 1998).

The sequence characterization of the cDNA coding for LiTx 1, LiTx 2 and LiTx 3 revealed the presence of open reading frames encoding mature polypeptides of 66, 69 and 53 amino acids, pl of 8.76, 8.11 and 8.76, and molecular weight of 7431.63, 7920.11 and 5648.49 Da, respectively (not considering the removal of residues in the C-terminal peptide and the presence of disulphide bonds).

The amino acid sequence analysis of mature toxins LiTx 1, LiTx 2 and LiTx 3 by PROSITE revealed that all of them may present N-myristoylation sites (LiTx 1: GSKGCA; LiTx 2: GVGGCY; LiTx 3: GSPHGL, GCTMGV and GCDWSK) and protein kinase C phosphorylation sites (LiTx 1: SRR and THR; LiTx 2: SCR and THR; LiTx 3: SKK). In addition, LiTx 1 and LiTx 2 may present amidation sites...
Fig. 1. Purification of LiTx × 1, LiTx × 2 and LiTx × 3. (A) *L. intermedia* venom was applied to a Sephadex G-100 column and the fractions obtained were assayed for dermonecrotic activity by intradermal injection in rabbits. (B) Negative fraction for dermonecrotic activity (P3) was applied to a CM 52 column and fractions obtained were assayed for insecticidal activity on *Spodoptera frugiperda* larvae. (C) Positive fraction for insecticidal activity (F4) was applied to a C18 HPLC column and LiTx × 1, LiTx × 2 and LiTx × 3 insecticidal peptides purified.
LiTx\(1^1\): KGKR; LiTx\(1^2\): RGKR), and only LiTx\(1^1\) may present casein kinase II phosphorylation sites (LiTx\(1^1\): TKPD and STSE). In contrast to other animal toxins, little is known about post-translational modifications in spider polypeptide toxins (Escoubas et al., 2000). Amidated C-terminal residues were reported for \(\delta\)-palutoxins (Corzo et al., 2000), curtatoxins II and III (Stapleton et al., 1990) and \(\mu\)-agatoxin III (Skinner et al., 1989), among other toxins. The addition of an \(\Omega\)-palmitoyl group to a C-terminal threonine in the PLTX-II toxin from Plectreurys tristis was described by Branton et al. (1993). However, no report of phosphorylation or \(N\)-myristoylation is observed in literature for spider toxins.

LiTx\(3^1\) has presented significant similarity with metallothioneins in protein/protein PSI-BLAST search (best BEXPECT of \(2 \times 10^{-15}\)). Yoshioka et al. (1994) suggest that metal chelates play an important role in the insecticidal activity of clavamine, the main insecticide component in Nephila clavata spider venom. This fact, if extended to other spider toxins, would help us to explain the existence of metallothionein-like proteins in spider venoms.

We may hypothesize that these proteins regulate the venom's toxicity. The toxicity dependence of divalent metal ions (Mg\(^{2+}\), Ca\(^{2+}\)) for several Loxosceles proteins related to the dermonecrotic action has already been described by many authors (Tambourgi et al., 1998; Feitosa et al., 1998).

Furthermore, PSI-BLAST also showed some similarity among toxin LiTx\(1^1\), curtatoxin II, curtatoxin III and \(\mu\)-agatoxin III, peptides known to cause irreversible paralysis in lepidopterous insects (Skinner et al., 1989; Stapleton et al., 1990).

Here we rely on sequence similarity as an indicator of putative means of action of the described peptides. The resulting tree (Fig. 3) had good overall bootstrap support (\(\geq 50\%\)). LiTx\(3^1\) clustered with the most internal tree groups, which are formed by toxins that are known or appear to act on Na\(^+\) channels (Corzo et al., 2000; Grishin, 1999; Adams et al., 1989) and was closely related to \(\delta\)-palutox3 (Corzo et al., 2000). Since similar sequences may reflect functional similarity, then it is possible that LiTx\(3^1\) interacts on Na\(^+\) channels. LiTx\(1^1\) and LiTx\(1^2\) were in an intermediate position between the branch of toxins that...
Fig. 2 (continued)
act on Na\(^+\) channels and all \(\omega\) toxins, which act upon Ca\(^{2+}\) channels (Wang et al., 1999; Adams et al., 1990). Although there is a tenous indication of proximity between LiTx\(^{2+}\), LiTx\(^{2-}\) and toxins active on Na\(^+\) channels, due to the lack of statistical support (bootstrap values lower than 50%), no conclusion can be reached for LiTx\(^{2+}\) and LiTx\(^{2-}\).

Finally, the present investigation illustrates the potential of the *L. intermedia* venom as a source of insecticidal toxins for biotechnology applications. Our group is already working with the production of the recombinant toxins LiTx\(^{2+}\), LiTx\(^{2-}\) and LiTx \(\times 3\) using the baculovirus expression system, which would contribute to expand the understanding of this powerful venom and its mechanism of action.

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**References**


