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Identification of an antiviral component from the venom of the scorpion *Liocheles australasiae* using transcriptomic and mass spectrometric analyses

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Abstract

Scorpion venom contains a variety of biologically active peptides. Among them, neurotoxins are major components in the venom, but it also contains peptides that show antimicrobial activity. Previously, we identified three insecticidal peptides from the venom of the *Liocheles australasiae* scorpion, but activities and structures of other venom components remained unknown. In this study, we performed a transcriptome analysis of the venom gland of the scorpion *L. australasiae* to gain a comprehensive understanding of its venom components. The result shows that potassium channel toxin-like peptides were the most diverse, whereas only a limited number of sodium channel toxin-like peptides were observed. In addition to these neurotoxin-like peptides, many non-

disulfide-bridged peptides were identified, suggesting that these components have some critical roles in the *L. australasiae* venom. In this study, we also isolated a component with antiviral activity against hepatitis C virus using a bioassay-guided fractionation approach. By integrating mass spectrometric and transcriptomic data, we successfully identified LaPLA₂-1 as an anti-HCV component. LaPLA₂-1 is a phospholipase A₂ having a heterodimeric structure that is N-glycosylated at the N-terminal region. Since the antiviral activity of LaPLA₂-1 was inhibited by a PLA₂ inhibitor, the enzymatic activity of LaPLA₂-1 is likely to be involved in its antiviral activity.

Keywords

bioactive peptide; glycosylation; hepatitis C; phospholipase; venom gland

1. Introduction

Scorpions use their venom to capture their prey and defend themselves against predators. For this purpose, scorpion venom is composed of a variety of biologically active peptides (Ahmadi et al., 2020). Among them, neurotoxins are the most effective components in capturing their prey, such as insects. These toxins can immediately stop the movement of prey by acting on their ion channels (Schwartz et al., 2012, Smith, J. J. et al., 2013). In addition, these neurotoxins often show selective toxicity between insects and mammals, which is conferred at the ion channel level (Gordon et al., 2007, Housley et al., 2017).

Scorpion toxins acting on ion channels are peptides cross-linked with multiple disulfide bonds (Quintero-Hernandez et al., 2013). These peptides can be classified into two groups based on their molecular size. The long-chain group consists of peptides with 60-80 amino acid residues, which include toxins that act on Na^+ channels. The short-chain group consists of peptides with 20-50 amino acid residues, which include toxins that act on K^+ , Ca^{2+} , and Cl^- channels. The scorpion venom also contains many peptides without disulfide bonds. Most of these peptides show antimicrobial activity (Almaaytah and Albalas, 2014), some of which also exhibit insecticidal activity and/or synergistically enhance the activity of other toxins (Miyashita et al., 2010, Wullschleger et al., 2005). In addition to peptides, enzyme proteins are present in the scorpion venom, although their contribution to the biological activity of the venom remains unclear (Smith, J.J. and Alewood, 2015). Because of these diverse components, scorpion venom has been studied as a rich source of bioactive molecules, which provide useful information for the development of novel pesticides and drugs (Ghosh et al., 2019, Smith, J. J., et al., 2013). Scorpion toxins can also serve as an important probe for the study of ion channels (Herzig et al., 2020, Zhao, Y. et al., 2019).

Traditionally, the search for bioactive peptides/proteins has been conducted mainly relying on the bioassay-guided isolation procedure, in which high performance liquid chromatography (HPLC) fractionation and bioassay is repeated until a single active

component is obtained (Vetter et al., 2011). This approach has led to the discovery of a number of novel bioactive molecules with unique structures. However, it is often difficult to identify minor components in the venom using this approach. In such cases, cDNAs obtained by reverse transcription of the mRNAs in the venom gland have been amplified by polymerase chain reaction (PCR) using a primer constructed based on a partial sequence to determine the entire structure (Quintero-Hernandez et al., 2011). Recently, the advent of next-generation high-throughput sequencing technologies allows us to obtain sequences of all mRNAs expressed in the venom gland (transcriptome), which provides a comprehensive understanding of structures of venom components (Oldrati et al., 2016). Furthermore, the combination of transcriptome analysis with proteome analysis has accelerated the determination of mature structures of each component in the venom (Fu et al., 2018, Walker et al., 2020). Although bioactive components can be estimated based on their structural similarity with reported molecules using this approach, biological functions of many components remain unclear due to the absence of similar molecules reported. In this regard, the classical bioassay-guided approach is still effective for the discovery of bioactive components with unique structural characteristics, and its combination with transcriptome analysis should accelerate the structural determination.

It is known that infection with hepatitis C virus (HCV) often causes liver disease, including cirrhosis and hepatocellular carcinoma. Although highly effective direct-acting antivirals can cure the vast majority of HCV infections, in the absence of a vaccine, there is a continued demand for antiviral drugs against HCV. Of the various enemies and pathogens scorpions cope with, virus represents one of major threats, and actually the scorpion venom contains antiviral components to prevent infection via venom glands (da Mata et al., 2017, El-Bitar et al., 2015, Yacoub et al., 2020). In this context, the scorpion venom has been studied as one of the promising sources of antiviral molecules. Previously, we identified three insecticidal peptides from the venom of the *Liocheles australasiae* scorpion based on toxicity against insects (Juichi et al., 2019, Matsushita et al., 2009,

Matsushita et al., 2007). However, the venom has not been evaluated for other biological activities, including antiviral effects. Thus, in this study, we first performed a transcriptome analysis of the venom gland of *L. australasiae* to gain a comprehensive understanding of its venom components. Using this information coupled with the bioassay-guided approach, we identified a new component with antiviral activity against HCV from the venom of *L. australasiae*.

2. Materials and methods

2.1. Biological materials

The scorpions *L. australasiae* were collected in Ishigaki Island, located at the southern end of the Ryukyu Islands in Japan. They were reared in the laboratory under humid conditions at 25°C and fed crickets. The venom was obtained by mechanical stimulation as previously reported (Miyashita et al., 2007). The venom secreted on Parafilm was dissolved in aqueous 2% acetic acid and filtered, which was lyophilized and stored at –80°C.

2.2. RNA extraction and sequencing

The telsons were dissected from six specimens anesthetized on ice and placed in a glass tube of a micro tissue grinder. The total RNA was extracted using RNAiso Plus (Takara Bio, Kusatsu, Japan) and further purified using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The integrity of the RNA was verified using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The mRNA was isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). A cDNA library was prepared from the purified mRNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs). The 100 bp paired-end sequencing was performed on an Illumina HiSeq2500 platform (San Diego, CA, USA). The short-read data were deposited to the Read Archive of DDBJ (accession number DRA010798).

2.3. *De novo assembly and functional annotations*

After adapter- and quality-trimming using the software TagDust (Lassmann et al., 2009) and Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit), the cleaned reads were assembled into contigs with Trinity (2.06) (Haas et al., 2013) and Bridger (r2014-12-01) (Chang et al., 2015) software using the standard protocol. Coding regions were predicted by TransDecoder (2.0). The predicted sequences obtained using two different assembly software were separately submitted to similarity searches using the BLASTP program against a database containing only sequences identified from scorpion venom (the UniProt Animal Toxin Annotation Project) to annotate the functions of identified peptides and proteins through the local BLAST tool (Altschul et al., 1990). Coding regions of each component were further inspected and corrected manually by comparing them with the reported sequences. Multiple sequence alignments were performed using MAFFT online (Katoh et al., 2019) and Clustal Omega (Madeira et al., 2019).

2.4. *HPLC purification*

The crude venom dissolved in distilled water was applied to a C4 semi-preparative column (10 × 250 mm, Grace Vydac, Deerfield, IL, USA). The column was eluted with 0.1% trifluoro acetic acid (TFA) in water (solvent A) and 0.08% TFA in acetonitrile (solvent B) at a flow rate of 2 mL/min, using a linear gradient of 5–60% of solvent B over 55 min. Elution was monitored by the UV absorbance at 215 nm. Fractions were collected every 5 min during gradient elution. Each fraction was submitted to the antiviral activity test after lyophilization, and the fraction showing activity was then applied to a C18 microbore column (1.0 × 250 mm, Grace Vydac). The column was eluted with solvent A and B at a flow rate of 0.05 mL/min, using a linear gradient of 20–50% of solvent B over 60 min. Each HPLC peak was individually collected and lyophilized. The most active fraction was further purified on the same C18 microbore column using a different solvent system. The column was eluted with 0.1% formic acid in water (solvent C) and 0.1%

formic acid in acetonitrile (solvent D) at a flow rate of 0.05 mL/min using a linear gradient of 5–55% solvent D over 50 min. The purity was checked by liquid chromatography/mass spectrometry (LC/MS) analysis as described below.

2.5. Mass spectrometric analysis

LC/MS and LC/MS/MS measurements were carried out in a positive ion mode on an LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ion source. Precursor ions were manually selected, and a collision-induced dissociation (CID) spectrum was obtained by using argon as a collision gas. Reversed-phase (RP)-HPLC separation was performed on a C18 microbore column (TSKgel ODS-100V 3 μ m, 1.0 $\times$ 150 mm, Tosoh, Tokyo, Japan). The column was eluted with solvent C and solvent D at a flow rate of 0.05 mL/min, using a linear gradient of 5–70% of solvent D over 65 min. The mass scale was calibrated externally using sodium trifluoroacetate cluster ions.

2.6. Enzymatic digestion

The protein was dissolved in a buffer containing 0.2 M Tris-HCl (pH 8.5), 6 M guanidine hydrochloride and 10 mM dithiothreitol (DTT), and the mixture was incubated at 50°C for 1 h. The reaction mixture was then mixed with iodoacetic acid (20 mM final concentration) and incubated at 28°C for 1 h. The solution was diluted three-fold with water, and the Cys-alkylated protein was digested with endoproteinase Lys-C (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or Glu-C (FUJIFILM Wako Pure Chemical Corporation) at 37 °C for 18 h at a peptide/enzyme ratio of 20:1 (w/w).

2.7. Deglycosylation

The protein was dissolved in a solution containing 0.5% SDS and 40 mM DTT, and the mixture was incubated at 100°C for 10 min. The solution was diluted two-fold with a buffer containing 50 mM sodium acetate and 1% NP-20, then mixed with 5 units of

PNGase A (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h.

2.8. Cell culture and viruses

Huh7it-1 cells were cultivated in Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with fetal bovine serum (Biowest, Nuaille, France), non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Thermo Fisher Scientific). Cells were grown at 37°C in a 5% CO₂ incubator. The J6/JFH1-P47 strain of HCV (El-Bitar, et al., 2015), the Trinidad 1751 strain of dengue virus (DENV) (El-Bitar, et al., 2015), the Nakayama strain of Japanese encephalitis virus (JEV) (Song et al., 1999), and the CHR3 strain of herpes simplex virus type 1 (HSV-1) (Aoki-Utsubo et al., 2018) prepared in Huh7it-1 cells were used in this study. Infectivity of the stock virus was 1.5×10^6 , 9.0×10^5 , 5.0×10^5 , and 2.0×10^4 cell-infecting units (CIU)/ml for HCV, DENV, JEV and HSV-1, respectively.

2.9. Antiviral activity test

Antiviral activity was tested according to two different experimental procedures as described below.

(i) Pretreatment of virus with venom components before and during virus inoculation (pretreatment). This procedure is used to determine virucidal (neutralizing) activity of a test sample (Aoki-Utsubo, et al., 2018, El-Bitar, et al., 2015). In brief, Huh7it-1 cells were seeded in 24-well plates (1.6×10^5 cells/well). A fixed amount of the virus (either 10-fold diluted or undiluted stock virus) was mixed with serial dilutions of the whole venom or separated HPLC fractions of the venom and inoculated to the cells for 2 h. The cells were then washed with medium to remove the residual virus and venom components, and further cultured in medium. Culture supernatants were obtained at 24–48 h post-infection and titrated for virus infectivity as described previously (El-Bitar, et al., 2015). After 24 h, the virus-infected cells were washed with phosphate-buffered saline (PBS), fixed with

4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After being washed three times with PBS, the cells were incubated with UV-inactivated HCV-infected patient's serum for 1 h, followed by incubation with FITC-conjugated goat anti-human IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The cells were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 5 min, and HCV-infected cells were counted under a BX53LED-43FLD fluorescence microscope (Olympus Corporation, Tokyo, Japan). Virus and cells treated with medium served as controls. Percent inhibition of virus infectivity by the samples was calculated by comparing with the controls, and 50% inhibitory concentrations (IC₅₀) were determined. Experiments were performed in duplicate and repeated three times.

(ii) Treatment of virus-infected cells after virus has been entered the cells (post-entry treatment). This procedure is used to determine viral replication-inhibiting activity of a test sample in the infected cells (Aoki-Utsubo, et al., 2018, El-Bitar, et al., 2015) In brief, cells were inoculated with virus in the absence of the venom components for 2 h. The virus-infected cells were cultured in medium containing serial dilutions of the venom components. The virus-infected cells cultured in medium without the venom components served as a control. After 48 h, RNA was extracted from the cells and subjected to reverse transcription-quantitative PCR (RT-qPCR) as described below. Experiments were performed in duplicate and repeated three times.

2.10. Reverse transcription-quantitative PCR (RT-qPCR)

Total cellular RNA was extracted from the cells using NucleoSpin RNA extraction kit (TaKaRa Bio, Inc.) according to the manufacturer's instructions. RNA (1 µg) was reverse transcribed using a GoScript Reverse Transcription system (Promega) with random primers. The cDNA products were subjected to quantitative real-time PCR analysis using Power SYBR Green PCR Master kit (Thermo Fisher Scientific Corp.) and a StepOne qPCR system (Thermo Fisher). The primers used to amplify an NS5A region of the HCV

genome were 5'-AGACGTATTGAGGTCCATGC-3' (sense) and 5'-CCGCAGCGACGGTGCTGATAG-3' (antisense) (Deng, L. et al., 2011). As an internal control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression levels were measured using primers 5'-GCCATCAATGACCCCTTCATT-3' (sense) and 5' TCTCGCTCCTGGAAGATGG-3'. Relative quantity of HCV NS5A cDNA was calculated and expressed as an arbitrary unit for each sample.

2.11. Cytotoxicity test

Cytotoxicity of LaPLA₂-1 was evaluated using the WST-1 reagent (Roche, Mannheim, Germany) as reported previously (El-Bitar, et al., 2015). Briefly, Huh7it-1 cells plated in each well of a 96-well plate (0.1 ml/well) were treated with serial dilutions (0.1 to 1,000 ng/ml) of LaPLA₂-1 at 37 °C for 24 h. Untreated cells served as a control. After this treatment, 10 µl of the WST-1 reagent was added to each well, and the cells were cultured for 4 h. The WST-1 reagent is converted to formazan by living cells. The amount of formazan, which correlates with the number of living cells, was determined by measuring the absorbance at 450 and 630 nm using a microplate reader. The percent cell viability compared to the untreated control was calculated, and the 50% cytotoxic concentration (CC₅₀) was determined.

2.12. Phospholipase A₂ activity test

L-α-Phosphatidylcholine (from egg yolk, Nacalai Tesque, Kyoto, Japan) was suspended in a buffer containing 0.1 M Tris-HCl (pH 8.0), 10 mM CaCl₂, and 0.2% TritonX-100 at a concentration of 2 mg/ml. The reaction was started with the addition of the PLA₂ solution to the buffer (total 100 µl) at 37°C. After incubation for 10 min at 37°C, the mixture was analyzed by LC/MS to quantitate the amount of 1-palmitoyl-*sn*-glycero-3-phosphocholine (P-lysoPC, *m/z* 540.3, [M+HCOOH-H]⁻) generated by the reaction. Conditions used for LC/MS analysis (LCMS-8030, Shimadzu) were as follows: ionization, ESI-negative; column, COSMOCORE 2.6C₁₈ (2.1×75 mm, Nacalai Tesque);

flow rate, 0.3 mL/min; mobile phase, solvent C and solvent D; gradient, 35–95% of solvent D over 30 min. The amount of P-lysoPC generated in the absence of phospholipase A₂ (PLA₂) was subtracted from those generated in each experiment. Experiments were repeated three times. PLA₂ activity was expressed relative to that of PLA₂ from honeybee (*Apis mellifera*) venom (Sigma-Aldrich, St. Louis, MO, USA).

3. Results

3.1. Transcriptome analysis

A total of 253,691,496 raw reads were obtained by sequencing. After adapter- and quality-trimming, the clean reads were assembled in a de novo fashion using Trinity, which resulted in 133,715 contigs with N50 of 849 bp (224–14,739 bp), and Bridger software, which resulted in 98,577 contigs with N50 of 1447 bp (201–21,016 bp). BLASTP search was performed for predicted coding regions against a database consisting of reported sequences identified from scorpion venom. This resulted in identification of 77 transcripts encoding peptides and proteins similar to those of scorpion venom as shown in Fig. 1-5 and Table S1 and S2.

3.2. Components identified by venom gland transcriptome analysis

3.2.1. Non-disulfide bridged peptides

Scorpion venoms are rich in non-disulfide bridged peptides (NDBPs) (Almaaytah and Albalas, 2014). NDBPs are known to display diverse biological functions to defend from pathogen infection via the venom gland by showing antimicrobial activity and/or to capture prey by showing insecticidal activities (Almaaytah and Albalas, 2014, Dias et al., 2018, Miyashita, et al., 2010). NDBPs are classified into five subfamilies based on the sequence similarity and the molecular size. In this study, we identified nine transcripts coding for NDBPs (Fig. 2a); two transcripts (LaNDBP2-1 and LaNDBP2-2) sharing sequence similarity to those of group 2 consisting of long-chain multifunctional peptides, two transcripts (LaNDBP3-1 and LaNDBP3-2) to those of group 3 consisting of medium-

length antimicrobial peptides, and five transcripts (LaNDBP4-1, LaNDBP4-2, LaNDBP4-3, LaNDBP4-4, and LaNDBP4-5) to those of group 4 consisting of short antimicrobial peptides.

3.2.2. Invertebrate defensins

Defensins are cationic peptides stabilized with three disulfide bonds, which are observed in a wide variety of organisms, including plants, insects, and mammals (Holly et al., 2017, Yi et al., 2014). These peptides play an important role in innate immunity by showing antimicrobial activity. Peptides similar to defensins are also found in scorpion venom (Cheng et al., 2020, Harrison et al., 2014, Meng et al., 2016). Although a limited number of scorpion venom peptides were reported as a defensin, a similar structural motif exists in potassium channel toxins as described below (Zhu, S. Y. et al., 2014). In this study, we found three transcripts (LaDefensin1, LaDefensin2, and LaDefensin3) coding for invertebrate defensins, which share sequence similarity to AbDef-1 that was identified from the venom of *Androctonus bicolor* (Fig. 2b) (Zhang et al., 2015).

3.2.3. Potassium channel toxin-like peptides

Potassium channel toxins (KTx) are cysteine-rich peptides that act on potassium channels (Jimenez-Vargas et al., 2017). To date, a large number of KTx peptides have been identified from scorpion venom. KTx peptides are currently classified into seven groups (α -, β -, γ -, δ -, ϵ -, κ -, and λ -KTx) based on the sequence similarity and disulfide-bonding patterns (Chen, Z. Y. et al., 2012, Jimenez-Vargas, et al., 2017). In this study, we identified 18 transcripts coding for KTx peptides, and the majority of them were α -KTx peptides (La-alphaKTx1, La-alphaKTx2, La-alphaKTx3, La-alphaKTx4, La-alphaKTx5, La-alphaKTx6, La-alphaKTx7, La-alphaKTx8, and La-alphaKTx9, Fig. 3a). α -KTx adopts a typical fold consisting of an α -helix and three β -strands stabilized by three or four disulfide bonds (CS- $\alpha\beta$ fold). We also found three transcripts (La-betaKTx1, La-betaKTx2, and La-betaKTx3) coding for β -KTx peptides (Fig. 3b). These peptides adopt

a CS- $\alpha\beta$ fold as observed in α -KTx peptides, but they have an additional α -helical structure without disulfide bonds at the N-terminal region. La-betaKTx1 and La-betaKTx2 correspond to LaIT2 and LaIT3, which were previously identified from the *L. australasiae* venom at a peptide level, respectively (Juichi, et al., 2019, Matsushita, et al., 2009). Five transcripts (La-kappaKTx1, La-kappaKTx2, La-kappaKTx3, La-kappaKTx4, and La-kappaKTx5) coding for κ -KTx peptides were also found (Fig. 3c). Unlike α - and β -KTx peptides, κ -KTx peptides do not adopt a CS- $\alpha\beta$ fold, but instead they have two parallel α -helices stabilized with two disulfide bonds. The inhibitory activity of κ -KTx on potassium channels is known to be relatively weak compared with that of α -KTx peptides. Furthermore, one transcript (La-deltaKTx1) coding for δ -KTx was identified, which has a similar structure with Kunitz-type protease inhibitors consisting of two antiparallel β -strands and an α -helix stabilized by three or four disulfide bonds (Fig. 3d). Some δ -KTx peptides, such as BmKTT-2 from the *Mesobuthus martensii* venom, are known to show inhibitory activity on both potassium channels and proteases (Chen, Z. Y., et al., 2012).

3.2.4. Disulfide-directed hairpin peptides

Peptides with disulfide-directed hairpin (DDH) motif were identified from the venom of the limited number of scorpion species (Horita et al., 2011, Smith, J. J. et al., 2011). Although DDH peptides contain only two disulfide bonds, their structures are similar to the inhibitory cystine knot (ICK) motif, which is stabilized by three disulfide bonds. LaIT1, an insecticidal peptide previously identified from the venom of *L. australasiae*, is the first example of DDH peptides (Matsushita, et al., 2007). In this study, two transcripts (LaDDH1 and LaDDH2) coding for DDH peptides were identified, in which LaDDH1 corresponds to LaIT1 (Fig. 4a).

3.2.5. Sodium channel toxin-like peptides

Sodium channel toxins (NaTxS) adopt a CS- $\alpha\beta$ fold as observed in α -KTx peptides,

but their sequences are relatively long (58-76 residues) (Housley, et al., 2017, Quintero-Hernandez, et al., 2013). NaTx peptides were identified mainly from the venom of Buthidae scorpions, and their sodium channel modulating activity is thought to be responsible for the relatively high toxicity of Buthidae scorpion venom (Cid-Urbe et al., 2019, de Oliveira et al., 2015, Luna-Ramirez, K. et al., 2015, Ward et al., 2018, Zhao, R. M. et al., 2010, Zhong et al., 2017). In addition to ion channel modulating activity, several members of this family are known to induce adipocyte lipolysis by forming a homodimer (Soudani et al., 2005, Zhu, S. and Gao, 2006). In the present study, two transcripts (LaLAP1 and LaLAP2) coding for the peptides similar to lipolysis-activating peptides were identified (Fig. 4b). However, these peptides could have other biological functions because the total number or the position of Cys residues are different from those of the known lipolysis-activating peptides (Zhu, S. and Gao, 2006).

3.2.6. Serine protease inhibitor-like peptides

Animal venom generally contains protease inhibitors probably for protecting the venom components from enzymatic degradation. Two types of inhibitors having different structural motifs (Kunitz- and Ascaris-type) are known to exist in scorpion venom (Chen, Z. Y. et al., 2013, Ranasinghe and McManus, 2013). In this study, we found one transcript (La-deltaKTx1) coding for Kunitz-type inhibitor-like peptides, which was classified as δ -KTx peptides as described above (Fig. 3d). In addition, we found eight transcripts (LaAPI1, LaAPI2, LaAPI3, LaAPI4, LaAPI5, LaAPI6, LaAPI7, and LaAPI8) coding for Ascaris-type inhibitor-like peptides (Fig. 4c). Interestingly, LaAPI1 contains only eight Cys residues that can form four disulfide bonds, whereas a typical Ascaris-type motif is stabilized by five disulfide bonds.

3.2.7. La1-like peptides

La1 was previously identified as the most abundant peptide in the *L. australasiae* venom (Miyashita, et al., 2007). To date, peptides similar to La1 have been identified

from the venom of diverse scorpion species, although their biological significance remains unknown. La1-like peptides adopt a single domain von Willebrand factor type C (SVWC) motif (Sheldon et al., 2007). Some peptides adopting this motif based on the position of Cys residues are classified as SVWC peptides. In the present study, we identified eight transcripts (La1, La1-1, La1-2, La1-3, La1-4, La1-5, La1-6, and La1-7) coding for La1-like or SVWC peptides (Fig. 5).

3.2.8. Enzymes

In scorpion venom, enzymes have not been recognized as major bioactive components. However, recent progress in venom gland transcriptome analysis revealed that there are various enzymes, such as phospholipase, metalloprotease, serine protease, and hyaluronidase, in the venom (Bordon et al., 2015, Carmo et al., 2014, Gao, R. et al., 2008, Krayem and Gargouri, 2020). In this study, we identified three transcripts (LaPLA₂-1, LaPLA₂-2, and LaPLA₂-3) coding for phospholipase A₂, and eight transcripts (LaSP1, LaSP2, LaSP3, LaSP4, LaSP5, LaSP6, LaSP7, and LaSP8) coding for serine protease (Table S2). In addition to these enzymes, we identified one transcript (La-alpha-amylase) coding for α -amylase. Since only a limited number of α -amylase sequences have been identified from scorpion venom at a transcript level, there is little information on their biological functions.

3.2.9. Other proteins

We identified 10 transcripts (LaCRVP1, LaCRVP2, LaCRVP3, LaCRVP4, LaCRVP5, LaCRVP6, LaCRVP7, LaCRVP8, LaCRVP9, and LaCRVP10) coding for cysteine-rich secretory proteins, and three transcripts (LaVP1, LaVP2, and LaVP3) coding for proteins with an insulin-like growth factor binding motif (Table S2). Although these proteins are widely observed in scorpion venom, their biological functions remain unknown (Amorim et al., 2019, de Oliveira, et al., 2015, Yang et al., 2014).

3.3. Isolation of an anti-HCV component

We first evaluated anti-HCV activity of the venom of *L. australasiae*. The result showed that the venom has a significant inhibitory activity against HCV infection at extremely low concentrations ($IC_{50} = 0.01 \mu\text{g/ml}$). To isolate a component responsible for the anti-HCV activity, the venom was separated using RP-HPLC on a C4 column (Fig. 6a). Each fraction was tested for anti-HCV activity, and the fraction eluting at 37-42 min was found to be active. This fraction was further separated using a C18 column (Fig. 6b). The HPLC peak eluting at 25.5-26.5 min showed the most significant activity, and the main component in this peak was found to be responsible for anti-HCV activity (Fig. 6c). Mass spectrometric analysis revealed the molecular mass of this component as 13,079.8 Da.

3.4. Structure determination of the anti-HCV component

The anti-HCV component was subjected to reduction and alkylation reactions to examine the number of disulfide bonds in this molecule. LC/MS analysis showed that the molecule was separated into two parts after the reactions, indicating that the anti-HCV component consists of two subunits cross-linked with disulfide bonds (Fig. 7a). Molecular masses of each subunit after alkylation of Cys residues were determined as 1145.2 Da (small subunit) and 12,524.7 Da (large subunit). A total increase of the molecular mass after Cys-alkylation was approximately 590 Da $[(1145.2 + 12,524.7) - 13,079.8 = 590.1]$. Considering a mass shift due to carboxymethylation of Cys residues (118 Da per disulfide bond), we determined that the anti-HCV component contains five disulfide bonds $(118 \times 5 = 590)$.

To obtain partial sequence information from the anti-HCV component, MS/MS de novo sequencing analysis was performed for the small subunit. As shown in Fig. 7b, the sequence of the small subunit was determined as KCVAHWKES. This sequence was searched from all transcripts obtained in this study, and the anti-HCV component was identified as LaPLA₂-1, one of the phospholipase A₂ proteins. As observed in other

scorpion PLA₂s, LaPLA₂-1 forms a heterodimeric structure through processing. However, a sequence region that corresponds to the large subunit could not be determined based on its observed molecular mass (12,524.7 Da). It was assumed that the large subunit undergoes some types of post-translational modifications (PTMs). To determine a mature structure of the large subunit of LaPLA₂-1 including PTMs, it was digested with Lys-C or Glu-C, which was analyzed by LC/MS (Table 1). The N- and C-terminal sites of the large subunit were identified based on the sequences of two Lys-C digests (LIFPGTK and CFVLDCD), because these peptides cannot be generated by Lys-C digestion (–IHQR/LIFPG– and –VLDCD/KRRF–). The calculated molecular mass of the deduced sequence of the large subunit is 11,826.2 Da, which is still 698.5 Da lower than the measured value (12,524.7 Da). When the LC/MS data of Lys-C and Glu-C digests were carefully examined, the molecular masses of two digested peptides (AANYSDLGSAETDK and LIFPGTKWCGAGDKAANYSDLGSAE) were found to be 698 Da higher than those calculated without modifications. These peptides share the same sequence region containing an N-linked glycosylation consensus motif (NXS) (Marshall, 1972). This suggests that the Asn residue in this region is glycosylated. To confirm the existence of an N-linked glycan at this position, the protein was treated with PNGase A, which can cleave the N-glycan moiety from the Asn residue (Plummer and Tarentino, 1981). LC/MS analysis showed that the measured molecular mass of the large subunit after removal of N-glycan was identical to the calculated one (11,304.9 Da, Fig. S1). The large subunit without N-glycan was then treated with Lys-C after reduction and alkylation of Cys residues. The existence of the peptide containing an Asp residue instead of the N-glycosylated Asn residue was confirmed by LC/MS analysis (Table 1). To obtain structural information of the N-glycan moiety, MS/MS analysis was performed for the Lys-C digested peptide containing the N-glycan moiety. As shown in Fig. 8, several fragment ions formed through regular and successive losses of 203 and 145 Da, which correspond to an N-acetyl hexose and a deoxyhexose, respectively, were observed in the product ion spectrum. Based on the fragmentation pattern and the molecular masses, the

N-glycan moiety is supposed to consist of two N-acetyl hexoses and two deoxyhexoses. Considering the N-glycan structures and the biosynthetic pathways reported for arthropods including scorpions and insects (Hassani et al., 1999, Staudacher et al., 1992, Walski et al., 2017), the N-glycan structure in LaPLA₂-1 was estimated as shown in Fig. 8. Homology searches revealed that LaPLA₂-1 shares high sequence similarity to hemilipin from *Hemiscorpius lepturus*, HgPLA₂ from *Hadrurus gertschi*, and phaiodactylipin from *Anuroctonus phaiodactylus* (Fig. 9) (Jridi et al., 2015, Schwartz et al., 2007, Valdez-Cruz et al., 2004).

3.5. Antiviral activity of LaPLA₂-1

LaPLA₂-1 inhibited infectivity of HCV in a concentration-dependent manner from 0.1 to 100 ng/ml (Fig. 10). Antiviral activity of LaPLA₂-1 against several viruses was further examined. Accordingly, IC₅₀ values of LaPLA₂-1 against HCV, DENV and JEV, which belong to the family *Flaviviridae*, were 2.0, 3.4 and 5.7 ng/ml, respectively (Table 2). On the other hand, LaPLA₂-1 did not exhibit virucidal activity against HSV-1, which belongs to the family *Herpesviridae* even at a much higher concentration (1,000 ng/ml) (Fig. 10 and Table 2). To determine whether the post-entry step of the viral life cycle is affected by LaPLA₂-1, possible effect of post-entry treatment with LaPLA₂-1 on virus replication in infected cells was examined. The result demonstrated that post-entry treatment with LaPLA₂-1 barely exhibited anti-HCV activity even at a high concentration (100 ng/ml), whereas pretreatment exhibited highly potent anti-HCV activity (Table S3).

To examine whether the virucidal activity of LaPLA₂-1 against HCV is associated with its enzymatic activity, the effect of PLA₂ inhibitor on the anti-HCV activity was evaluated. The result obtained revealed a dramatic decrease of the virucidal activity in the presence of manoalide, the PLA₂ inhibitor (Lombardo and Dennis, 1985). In addition, LaPLA₂-1 exhibited the significant phospholipase activity at a comparable level that was observed for bee venom PLA₂ (Fig. 11). These results suggest that the virucidal activity of LaPLA₂-1 against HCV is closely associated with its enzymatic activity. In the present study, the

concentration ranges of LaPLA₂-1 that showed virucidal and PLA₂ activities appear to be different. However, this may be due to differences in states of the substrate (membrane versus solution) and incubation time used for measurements (2 h versus 10 min) between virucidal and PLA₂ activity tests.

We also evaluated the toxicity of LaPLA₂-1 to the host cells. LaPLA₂-1 showed no cytotoxic effect at concentrations of up to 100 ng/ml and only a marginal cytotoxic effect at 1,000 ng/ml (Table S4), suggesting that observed LaPLA₂-1 action is specific to the viruses.

4. Discussion

4.1. Peptides identified by venom gland transcriptome analysis

Previously, we isolated and characterized four peptides (LaIT1, LaIT2, LaIT3, and La1) from the venom of the scorpion *L. australasiae*, mainly based on toxicity against insects (Juichi, et al., 2019, Matsushita, et al., 2009, Matsushita, et al., 2007, Miyashita, et al., 2007). In the present study, we performed the transcriptome analysis of the venom gland of *L. australasiae* to comprehensively understand the components in the venom. This resulted in the identification of 77 transcripts coding for peptides and proteins similar to those from the venom of other scorpion species. Among them, KTx-like peptides, including insecticidal toxins LaIT2 and LaIT3, were more diverse than other families of venom components (Fig. 1). On the other hand, only three NaTx-like peptides were identified in this study. This is consistent with the previous observations obtained by mass spectrometric analysis (Miyashita, et al., 2007). The number of the components with molecular masses of 6,000-9,000 Da, which may correspond to NaTx-like peptides, detected at the peptide level was relatively small in the *L. australasiae* venom when compared with that observed in the Buthidae scorpion venom. On the other hand, the number of the components with molecular masses of 3,000-5,000 Da, which may correspond to KTx-like peptides, was relatively large at the peptide level. A similar trend was noted in the transcriptome analysis of the venom glands of *Hadogenes troglodytes* of

the family Hormuridae (Zhong, et al., 2017). The study showed the existence of many KTx-like peptides in the venom, whereas no NaTx-like peptides were found. It is known that NaTx peptides have been found mainly from the venom of Buthidae scorpions (Cid-Uribe, et al., 2019, de Oliveira, et al., 2015, Luna-Ramirez, K., et al., 2015, Quintero-Hernandez, et al., 2013, Ward, et al., 2018, Zhao, R. M., et al., 2010, Zhong, et al., 2017). This suggests that sodium channels are not a main target of the venom components in non-Buthidae scorpions.

LaIT2 and LaIT3 belong to the β -KTx peptide group, which is structurally characterized by the α -helical region attached to the N-terminal of a CS- $\alpha\beta$ fold. In addition to these toxins, we found another peptide having a structure similar to β -KTx peptides (Fig. 3b). This peptide (La-betaKTx3) has the sequence similar to scorpine-like peptides, such as SC11 from *Urodacus yaschenkoi*, which are classified as one of the subfamilies of β -KTx peptides (Luna-Ramirez, Karen et al., 2016, Luna-Ramirez, K. et al., 2013). Since scorpine-like peptides are known to show antibacterial activity, La-betaKTx3 is likely to have a similar biological function.

LaIT1 is the first insecticidal toxin identified from the *L. australasiae* venom (Matsushita, et al., 2007). In the present study, another peptide (LaDDH2) that has the sequence similar to LaIT1 was found (Fig. 4a). Two basic residues (R13 and R15) that are important for the expression of the insecticidal activity of LaIT1 are conserved in LaDDH2, suggesting that this peptide may also have insecticidal activity (Horita, et al., 2011). To date, peptides similar to LaIT1 have been identified from the family Hormuridae (*L. waigiensis* and *Opisthacanthus cayaporum*) (Silva et al., 2009, Smith, J. J., et al., 2011) and the family Hemiscorpiidae (*Hemiscorpius lepturus*) (Kazemi-Lomedasht et al., 2017). This suggests that LaIT1 and its related peptides have been evolved independently in limited scorpion species.

La1 was previously identified as the most abundant component in the *L. australasiae* venom, although its biological function remains unknown (Miyashita, et al., 2007). Unlike LaIT1, peptides similar to La1 have been found in a wide variety of scorpion

species from seven families; Buthidae (Zeng et al., 2013, Zhao, R. M., et al., 2010), Hemiscorpiidae (Kazemi-Lomedasht, et al., 2017), Hormuridae (Silva, et al., 2009, Zhong, et al., 2017), Scorpionidae (Abdel-Rahman et al., 2013, Deng, Y. C. et al., 2018, Diego-Garcia et al., 2012, Luna-Ramirez, K., et al., 2015), Superstitioniidae (Santibanez-Lopez et al., 2016), and Vaejovidae (Quintero-Hernandez et al., 2015, Romero-Gutierrez et al., 2018). Particularly, many La1-like peptides were observed in the venom gland transcriptome of *H. troglodytes* (Zhong, et al., 2017). Therefore, it is possible that La1-like peptides are abundant in the venom of Hormuridae scorpions, as observed in this study (Fig. 5). La1 adopts an SVWC motif, which has been observed in peptides from a wide variety of arthropods, including insects (Sheldon, et al., 2007). Recently, it has been reported that SVWC peptides may play a role in protection against entomopathogenic fungi in the epidermis of the silkworm and in basal AMPs expression of bumblebee (Han et al., 2017, Wang et al., 2017). Although the mechanisms of action of these peptides are still unknown, La1 might play a role in preventing pathogenic infections in the venom glands as well.

In addition to the peptides having disulfide bonds, many NDBPs were identified in this study (Fig. 2a). These peptides generally show membrane-disrupting activity by forming an amphipathic α -helical structure (Harrison, et al., 2014). Among five subfamilies of scorpion NDBPs, peptides of four subfamilies were found in this study. The reasons for the presence of the various types of NDBPs in the venom are unknown, but they are likely to have different biological functions. For example, four NDBPs isolated from *Isometrus maculatus* show different antimicrobial spectra, and some of them also exhibit insecticidal and hemolytic activities (Miyashita et al., 2017). Furthermore, some NDBPs may have a synergistic effect on other neurotoxins in venom to enhance their activity (Gao, B. et al., 2018). Defensins are another type of antimicrobial peptides observed in a wide variety of organisms (Holly, et al., 2017, Yi, et al., 2014). In this study, three defensin-like peptides were found (Fig. 2b). Since invertebrate defensins have the same disulfide-bonding pattern as peptides that act on potassium channels, it is

also possible that defensins identified in this study could show some ion channel-modulating activity (Meng, et al., 2016).

A number of peptides similar to serine protease inhibitors were also found in the present study (Fig. 4c). Kunitz-type protease inhibitors have been found in many organisms (Mishra, 2020). The peptides having a Kunitz-type motif have been reported to be present in the venom of various scorpion species (Ranasinghe and McManus, 2013). In addition to Kunitz-type peptides, Ascaris-type protease inhibitors have also been identified from the scorpion venom (Chen, Z. Y., et al., 2013). These peptides are likely responsible for preventing degradation of venom components by proteases of its own or other organisms. In addition, some of the protease inhibitor peptides from scorpion venom are known to act on potassium channels. This is thought to be a result of divergent evolution. For example, BmKTT-2, a Kunitz-type peptide identified from *Mesobuthus martensii*, shows inhibitory activity on Kv1.3 channels as well as inhibition of trypsin activity (Chen, Z. Y., et al., 2012). Since La-deltaKTx1 identified in this study (Fig. 3d) shares sequence similarity to BmKTT-2, it could show potassium channel inhibition activity.

4.2. Anti-HCV component in the *L. australasiae* venom

In the present study, we found that the *L. australasiae* venom has a potent virucidal activity against HCV and successfully identified LaPLA₂-1 as an anti-HCV component in the venom using a bioassay-guided approach. PLA₂ enzymes can be found in a wide variety of organisms, including snake and bee venom (Dennis et al., 2011). They catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids. PLA₂ molecules can be classified into six types, and scorpion venom PLA₂ (scvPLA₂) belongs to group III of secreted PLA₂ (sPLA₂) (Krayem and Gargouri, 2020). This group also includes PLA₂ from bee venom (bvPLA₂). Some of sPLA₂ molecules are known to exhibit antiviral activity, and their enzymatic activity is suggested to be associated with antiviral activity (Chen, M. et al., 2017). For example, PLA₂

molecules from snake venom (svPLA₂) specifically act on *Flaviviridae* viruses. This is likely because they can degrade the viral envelope membranes, particularly those derived from endoplasmic reticulum (ER). In fact, LaPLA₂-1 efficiently inhibited the infection of *Flaviviridae* viruses such as HCV, DENV and JEV (IC₅₀ = 2.0, 3.4 and 5.7 ng/ml, respectively). A comparable degree of virucidal activity was reported for PLA₂s obtained from snake venom (Teixeira et al., 2020). However, no significant antiviral activity was observed for a *Herpesviridae* virus such as HSV-1, which has the plasma membrane (PM)-derived envelope. This suggests that LaPLA₂-1 has the same mechanism of action as svPLA₂ (Chen, M., et al., 2017). It has been shown that phaiodactylipin can hydrolyze phosphatidylcholine more preferably than phosphatidylethanolamine and phosphatidylserine (Valdez-Cruz, et al., 2004). Since the ER-budded viruses contain a relatively high proportion of phosphatidylcholine than PM-budded viruses (Callens et al., 2016), LaPLA₂ may have a similar substrate preference as observed for phaiodactylipin. The possible action specificity of LaPLA₂-1 on the ER membrane implies that it may have no adverse effect on the host cells, which is further supported by its low cytotoxicity with CC₅₀ being >1,000 ng/ml. Although PLA₂s, in general, are known to be cytotoxic and inflammatory or neurotoxic, LaPLA₂-1 or its derivative(s) might be a good candidate as a lead compound for the development of an antiviral drug against HCV, DENV and other viruses budding from the ER membranes.

It is known that the Ca²⁺-binding motif (XCGXG) and the catalytic center with a His-Asp dyad are important for the expression of enzyme activity of PLA₂ (Dennis, et al., 2011). In the structures of scvPLA₂s, these motifs are conserved except for phaiodactylipin, in which one of the Gly residues in the Ca²⁺-binding motif is missing (Fig. 9). The structure of scvPLA₂s is further characterized by the formation of a heterodimer consisting of a large and a small subunit cross-linked with a disulfide bond, although its relevance to biological function remains unknown. It is also known that many bvPLA₂s contain N-glycans, most of which are core-fucosylated (Kubelka et al., 1993). Glycosylation of scvPLA₂ molecules has been investigated particularly for

phaiodactylipin (Valdez-Cruz, et al., 2004). Three N-glycosylation sites were recognized in the structure of phaiodactylipin, one of which is located at the N-terminal region of the large subunit, as observed in LaPLA₂-1 (Fig. 9). Hemilipin, HgPLA₂, and bvPLA₂ also contain one or two possible N-glycosylation sites in their sequences, although the presence of glycans is not experimentally examined in hemilipin and HgPLA₂. Among the N-glycosylation sites in these PLA₂ molecules, that at the N-terminal region of the large subunit is commonly observed. Since this site is close to the catalytic center, it may play an important role in enzymatic and/or antiviral activity.

5. Conclusion

Scorpion venom is known to be a rich source of bioactive peptides and proteins. Previously, we identified three insecticidal peptides from the venom of the *L. australasiae* scorpion, but the activities and structures of the other venom components remained unknown. In the present study, we performed a transcriptomic analysis of the venom gland of *L. australasiae* to elucidate a comprehensive picture of the venom components. As a result, 77 transcripts coding for venom peptides and proteins, including four previously reported peptides, were successfully identified. Among them, KTx-like peptides were the most diverse, suggesting that these peptides play a crucial role in this venom. On the other hand, a relatively small number of NaTx-like peptides were found. This is consistent with previous findings that NaTx-like peptides are predominantly found in the venom of Buthidae scorpions. In addition, a relatively large number of peptides without disulfide bonds were identified, suggesting that these peptides have some critical functions in the *L. australasiae* venom.

These transcriptome data facilitated the structural determination of the anti-HCV component in the venom, which was obtained by a bioassay-guided approach. Mass spectrometric analysis revealed that this component is one of the PLA₂s (LaPLA₂-1). This is the first report of a PLA₂ with antiviral activity from scorpion venom, although PLA₂s from snake and bee venom are known to show antiviral activity. Since the anti-HCV

activity of LaPLA₂-1 was inhibited by a PLA₂ inhibitor, the enzymatic activity of LaPLA₂-1 is likely to be involved in its expression of the anti-HCV activity. It is known that scvPLA₂s, including LaPLA₂-1, adopt a heterodimeric structure and are likely to be post-translationally modified by N-glycosylation at the N-terminal region. Elucidation of the relationship between the characteristic structures and enzymatic/antiviral activity of svcPLA₂s will be a subject of future research.

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Table 1 List of the peptide fragments generated by enzymatic digestions

Peptide sequence	Molecular mass (Da)		Mass difference
	Obs.	Calc.	
<i>Lys-C digestion</i>			
LIFPGTK	774.46	774.47	−0.01
WCGAGDK	793.30	793.31	−0.01
AANYSDLGSAAETDK	2209.90	1511.68	698.22
CCRAHDHCDNIAAGETK	2016.74	2016.78	−0.04
YGLENSYWFTK	1406.63	1406.66	−0.03
CEESFRNCLAEAK	1614.64	1614.67	−0.03
FSYFNVYGPK	1220.57	1220.59	-0.02
CFVLDCD	929.30	929.32	-0.02
<i>Lys-C digestion after PNGase A treatment</i>			
AADYSDLGSAAETDK	1512.69	1512.67	0.02
<i>Glu-C digestion</i>			
LIFPGTKWCGAGDKAANYSDLGSAAE	3397.39	2699.25	698.14
TDKCCRAHDHCDNIAAGETKYGLE	2823.04	2823.16	-0.12
NSYWFTKLNCKCEE	1879.70	1879.78	-0.08
SFRNCLAE	996.40	996.44	-0.04
TTAKFVKFSYFNVYGPKCFVLDCD	2907.22	2907.36	-0.14

Table 2 Virucidal activity of LaPLA₂-1

Virus	IC ₅₀ (ng/ml) ^a
HCV	2.0 ± 0.3
HCV (+ manoalide)	>500
DENV	3.4 ± 0.6
JEV	5.7 ± 0.8
HSV-1	>1,000

^a The data represent means ± SEM from three independent experiments.

Table S1 Sequences of peptides identified by the transcriptome analysis of the venom gland.

Name	Sequence	Similar peptide (Accession number, species)
Non-disulfide-bridged peptide		
LaNDBP2-1	MNIKVVLVVCLITLLVTEQVEGGWFSRIKSLAKKAWKSNLAKDLRKMAGKAARNYAAKVLNVSPEEQVQLDNLLRYLD	Vejovine (F1AWB0, <i>Vaejovis mexicanus</i>)
LaNDBP2-2	MNGKVLVVCLIVAMLVMEPAEAGIWGWIKKTAKKVWNSDIANQLKNKALNAAKNYVAEKVGATPAEAGQMPFDEFMDIYYS	Con22 (L0GBQ6, <i>Urodacus yaschenkoï</i>)
LaNDBP3-1	MQYKTFLVIFLAYLLVTEEAQAFWWALAKGAAKLLPSVVGAFTRKREIEKIFDPYQSSLDLEMERFLRQLQ	Heterin 2 (AGK88595, <i>Heterometrus spinifer</i>)
LaNDBP3-2	MKLRTLMIFFLAYLIVTDEAEAFWGFALAKAAKLLPSLFSSKKEKEKREIEHFFDPYEKELDSELDQLLYELQ	Heterin 2 (AGK88595, <i>Heterometrus spinifer</i>)
LaNDBP4-1	MKIQLAILVITVVLQMQLVQTEAGFWGKLWEGVKNVIGKRGLRNKDQLDDLFDSDLSADAKLLREMFK	OcyC2 (C5J887, <i>Opisthacanthus cayaporum</i>)
LaNDBP4-2	MKIQLAILVITVVLQMQLVQTEASFWGKLWEGVKSAGKRGLRDVDQMDDLFDSDLSADAKLLREIFK	OcyC2 (C5J887, <i>Opisthacanthus cayaporum</i>)
LaNDBP4-3	MKNQFVILLIAIVFLQLFSQSEAFLSALWGVAKSLFGKRGLKNLDQLDDLFDGEVSEADLDFLKELMR	UyCT3 (L0GCI6, <i>Urodacus yaschenkoï</i>)
LaNDBP4-4	MKAQIVLLVITVVLQMFAQSEAGFWGKLWEGVKSAGKRGLRNLDQFDDLEFDSDLSADAKLLKEIFK	UyCT1 (L0GCV8, <i>Urodacus yaschenkoï</i>)
LaNDBP4-5	MRLVSLTPLFLILLIAVDYQCQSFPLLISLIPSAISALKKLGKRSTDFQRQLDFQRRYLNSDLDFDLDELEEFLDQLPDY	VpAmp1.0 (ALG64974, <i>Vaejovis punctatus</i>)
Invertebrate defensin		
LaDefensin1	MKAIATLLLLIVAFSVLEFGIVDAGFGCPFNRYQCHSHCQSIKRRGGFCAGTFRTTCTCYKSK	AbDef-1 (AIX87626, <i>Androctonus bicolor</i>)
LaDefensin2	MKAIATLLLLLIVAFSILEDGIVDAGFGCPFNRYQCHSHCRSIGRRGGYCSGPFRFTCTCYK	AbDef-1 (AIX87626, <i>Androctonus bicolor</i>)
LaDefensin3	MKAIATILLLLAIFSILEFGIVGAGFGCPHNRYQCHSHCQSIGRNGGYCGGTFRTTCTCYNSGGPTSSP	AbDef-1 (AIX87626, <i>Androctonus bicolor</i>)
Potassium channel toxin-like peptide (alpha)		
La-alphaKTx1	MNAKFIYILLLTAVMFALYEASVPPNIPCQVTNQCPKPCREATGRPNNSKCINGRCKCYG	Urotoxin (P0DL37, <i>Urodacus yaschenkoï</i>)
La-alphaKTx2	MNTKFVFLLLVISTLMPTFDASAEDISCSSSKECYDPCEEETGCSSAKCVGGWCKCYGCRG	Urotoxin (P0DL37, <i>Urodacus yaschenkoï</i>)
La-alphaKTx3	MNRNFVFLLLLIVTLMPLDAATEDINCDNWRDCLKPCKDETGCPNSKCEEGNCLCYGCNRLTV	Urotoxin (P0DL37, <i>Urodacus yaschenkoï</i>)
La-alphaKTx4	MNKKFIFLLLVTTLMPMFDAATEAISCSNPNDCREPCKKQTGCSGGKCMNRKCKCHRCNG	OckTx2 (Q6XLL8 <i>Opisthophthalmus carinatus</i>)
La-alphaKTx5	MNAKLVCIVLLTAVMFAPDEASLPPIRIPCYVSKDCRKPCLYLTGTPRSKCINRRCKCYG	Hemitoxin (P85528, <i>Hemiscorpius lepturus</i>)
La-alphaKTx6	MNKPFCAIFLVVLMFAVSVLPAESTGGCPVDSLCKSYCKSNKFGTEGKCDGTSCKCAIG	LmKTx8 (A9QLM3, <i>Lychas mucronatus</i>)
La-alphaKTx7	MNKLACYILICVMVSCLFKVPVAEGISAGCPLTAKLCTIYCKKHRFGREGKCIGPTRFRCKCYV	LmKTx8 (A9QLM3, <i>Lychas mucronatus</i>)

La-alphaKTx8	MRLVILLMLTTLVLAVGAPLGGAKCSSSTQCTRPCRYAGGTHGKCMNGRCRCYG	St20 (P0DP36, <i>Scorpiops tibetanus</i>)
La-alphaKTx9	MELKYLLVLLAVTCLVSCQDNSLLPSGSCSRTGICMESCAPFLYQPKYHRRCPAGYVCCTLIY	Kbot55 (P0DL62, <i>Buthus occitanus tunetanus</i>)
Potassium channel toxin-like peptide (beta)		
La-betaKTx1 (LaIT2)	MAKHLIVMFLVIMVISSLVDCAKKPFVQRVKNAASKAYNKLKGLAMQSQYGCPIISNMCEDHCRRKKMEGQCDDLDCVCS	Previously identified
La-betaKTx2 (LaIT3)	MQAQFTVLLLLVLVTLCSGGILREKYFHKAADALTSNIPIPVVKDVLKSAANQMIRKIGKVQACAFNKDLAGWCEKSCQEAE GKKGYCHGTCKCKGKPIDYRK	Previously identified
La-betaKTx3	MDTKLSILVFLCVVVIASCSWISEKRIQKALDEKLPKGFIQGAAKAIVHKFAKNQYGCLADMVKGSCDRHCQETESTNGVCHG TKCKCGIGRVY	SCI1 (L0G8Z0, <i>Urodacus yaschenkoi</i>)
Potassium channel toxin-like peptide (delta)		
La-deltaKTx1	MASQFLLFCIVLIAVNPLVYSKGGCRLPPETGLCYAYFERYYYDPSSNNCKMFVYGCGGNSNNFVSKACLARCAN	BmKTT-2 (P0DJ50, <i>Mesobuthus martensii</i>)
Potassium channel toxin-like peptide (kappa)		
La-kappaKTx1	MKPSTSAYALLLVLTFGIITSGVFAVPMDEENTFEVEKRGNSCMEVCLQHEGNVAECEKACNKG	HeTx203 (P0DJ34, <i>Heterometrus petersii</i>)
La-kappaKTx2	MKPSTSAFILLLVLTFGIITSGVSAIPMDEENTFEEQKRDSACVEVCLHHEGNVAECEEEACKKS	HeTx203 (P0DJ34, <i>Heterometrus petersii</i>)
La-kappaKTx3	MKLLPLLVLIIICALMANEAFCDQGARERSENLEDTRDLVQKPCRIVCSENMRKCIRRCTLGR	HelaTx1 (P0DJ41, <i>Heterometrus laoticus</i>)
La-kappaKTx4	MKPSSFAIALILVFLGFTNAVSGEYAESISGDRMERAERAGCRIRCLQFTDDFEKCRKLCG	PcavC10 (AEX09227, <i>Pandinus cavimanus</i>)
La-kappaKTx5	MKLLPLLLVILVCALLPNEAFCDQSAVERSESLEEVSREIVKRSCKRVCSGTRRTKKCMQCKSQPGR	HelaTx1 (P0DJ41, <i>Heterometrus laoticus</i>)
DDH peptide		
LaDDH1 (LaIT1)	MNFATKVFFLLLAVAVIAIVAGEEDDSWFEQNEESDTERDFPLSKEYETCVRPRKCQPPLKCNKAQICVDPKKGW	Previously identified
LaDDH2	MNCAIKVSFLLLAITVIFSVAGGEGDNSFEQREENDTERDLPLSKKHESCVRPRKCRPPLRCNKAHICVESKKGWRIPVISWVS KTKIMFSE	LaIT1 (P0C5F2, <i>Liocheles australasiae</i>)
Sodium channel toxin-like peptide		
LaLAP1	MNTGEHFASLIIFLLLLGENPCLGDGGWPMRIDGNYYLCYEEKPAELYCKRACKLHKASQSSCSYHWKYMSWYCYCEDLKK GYTRDRGLKKGGGHQFSES	LVP1-alpha (P0CI45, <i>Lychas mucronatus</i>)
LaLAP2	MLMVIYIATLIPLILQGECRAKDDHPRNFEGNCYRCKYPDKSGYCEAICKMHKAETGYCSRSNLFCYCKGIEDKYVSARDFLEP	BmLVP1-alpha (Q6WJF5, <i>Mesobuthus martensii</i>)
La1-like peptide		
La1	MGGTLKHLLLVCLIVVCSSSLCLGFGESCIAGRFIVPLGQQVTDQRDCALYKCVNYNKKFALETKRCATVNLKSGCKTVPGGA GAAFPSCCPMVTCKG	Previously identified
La1-1	MKHLHAAVLLVCLSICALPSLTLGAGESCKVGSLVIPVGQKKFDKPSCAEYECSTEFNRVLLKAITCATLNTKGKGCKSVPGKS PNSFPDCCPTILCRGEQWNH	La1-like protein 15 (L0GB04, <i>Urodacus yaschenkoi</i>)

La1-2	MKIVCTLVPLVFACIANAHVLTERTCRTHTGVLKNGEEWADPNHCSIYRCTIYSGEAELQGLTCATYRVPPNCEFVRGRGKFY PSCCPTVMCKP	OcyC11 (C5J895, <i>Opisthacanthus cayaporum</i>)
La1-3	MALRFVLAFLLPCLVLGSNEPAKFISYKNDMLGPLVEGKCKVGNDKMIEQGGTWYRDDYCEKIYCLRTGTLGHMEVWGCAPV APLPNCTVHHSGLYPNCCSGEIVCEENPDTKSDVEMAEIIRALLQSERK	MeVP-7 (ABR21061, <i>Mesobuthus eupeus</i>)
La1-4	MFNIVTITLLWSCTCIALCHSYGETCQAGELTIPLDNEKQDPEACTLYKCTMFAGRVVLNLTLCAPQEPRRGCRNVDSPVELPF PDCCPLVVCNVQPLGSK	HsVx1 (K7WMX6, <i>Heterometrus spinifer</i>)
La1-5	MRTLVPVAVFLLALIVAAMASHKDPYHRNCPIGNKDLSDGEEWADQQRVCVKYKQIRGPDAALLLTRCPVSGIYPPDKCREIAG KGNFPDCCPKLQCD	MmKTx1-like (XP_02321219, <i>Centruroides sculpturatus</i>)
La1-6	MSRADKAFVGTLIAIFLVCSLTNAYSSLKRQKYGSGAPCVDHLGNSNRKFDDVWYDNESCEEHTCIKYRGIPHVQIYGCIAAVAS PGCELVKGSGSYPDCEEEIC	TxLP9 (ABY26691, <i>Lychas mucronatus</i>)
La1-7	MKTWEARFYIFLVALVTITFVESYVYLVLPQDPGAVVCIGKDRVSHKPGDVWYDDSKCERLTCGHSSGGLVIDGAGCGSISVQD GCKLVPGVGSYPSCCPSPVC	Toxin-like protein 14 (L0GCW8, <i>Urodacus yaschenko</i>)

Serine protease inhibitor-like peptide

LaAPI1	MKSWTYRLCFLILFLVCVNCTRPVLSPEECTRPGEFFTTAGSYCPLTCDNYKNPPIICSLIGIVGCECSPELVRDERSGNCVDTS DCTED	SjAPI (P0DM55, <i>Scorpiops jendeki</i>)
LaAPI2	MKGFAFLVLTFFVVFGDKEQECEDPNAEFRRCNTACPITCANMDNPPNICTLQCVIGCACKEGYYKNDDGLCVHPEGC	SjAPI (P0DM55, <i>Scorpiops jendeki</i>)
LaAPI3	MKALLLLLSFVVIHSAKSQEDLGGDEHAQSCLLPNEVWDKCGPSCPPSCIGVIEPGTLCSTECTPGCFCREGLVRTKRGSCIPP KACRNERETKL	SjAPI-2 (P0DM56, <i>Scorpiops jendeki</i>)
LaAPI4	MKGSKPLCFLYLVLWTSVKCTRWASSSEECTRPGEAFTSCGTDCPITCANYENPPEFCNYMCVIGCECTSGLVRDEGSGN CVNPSQCGP	HtPi1 (AOF40217, <i>Hadogenes troglodytes</i>)
LaAPI5	MKRNLVLLAFVLLLFGSIFEKCSAQRRGGADRRCRRPGEVFMNCGPSCPLTCDNYQNPPTVCTLQCVIGCFRRGLVRDTRRG GCVRPSQCRR	HtPi1 (AOF40217, <i>Hadogenes troglodytes</i>)
LaAPI6	MKRNLALSALFVLLFCSALDNCEAQRGGYDRTCQLEGEVFTRCGTACPLTCDNYKNPPEVCTLQCIIGCVCGRGWVKDTRRG RCVRPSQCRR	HtPi2 (AOF40218, <i>Hadogenes troglodytes</i>)
LaAPI7	MKSNLVLFAFVLLLFCVLEICTAQRVVDRSCRGAGEVFTRCGTACPLTCDNYRNPPRFCTRQCIIGCACRRGWVKATRGG GCVRPSQCRR	HtPi2 (AOF40218, <i>Hadogenes troglodytes</i>)
LaAPI8	MAKTAFGVVLCMFVLAQSAPQYPGTFGCDEDKQFVRCLPPCPVTCKSILNTPCTLLLPRCTPGCGCRGGKILDNAGKCVFP ADCRRG	CtAPI (P0DM57, <i>Chaerilus tricostatus</i>)

Table S2 Sequences of proteins identified by the transcriptome analysis of the venom gland

[illegible]

LaSP5	MAISLLVKYAILWISLAVLSSAQSSALPDRRPNSEVFPETVRDSPRCRTADGSPGSCLKASECRDVFQRGTLPLLCYWEDNQPI VCCADRSAAISSENLEPQTGCGKSERKPTTRSPTIAGGWISQPSAWPVMVAILTSNLGEKFLCGGTLVSQKYVLTAAHCFRR NGVDQRRIPVARFLVRVGSTENNQGTAYRIRIMIHEDYRVNQHYNDIAVIEVNDPISLSSSVRPICLPSELQGRSVVGREVVV VGWGDQSFGGIRDNLREVNISVIDRDTCEAYNELSSRSIPNGITSQFMCAGDPEGGDACQADSGGPLMMFSPSQWSIVG VVSFGYGCAHKGFPGVYTQVSSYLNWIKDKTDL	HLClotting-factor1 (API81376, <i>Hemiscorpius lepturus</i>)
LaSP6	MAMKYFVLFISTNALLAASFLPAKEENRIFKGREANEGEFPWMVFIRLTDELNCSGFLVSHNYVVTAAHCMIRSVTDMKGVVGS VDREQDNMLEFEKFKVIHPEYNSTFHGDVALLKLKRPLEFTSLIKPICIGKKKSFINPGNEVLQMGWGRDRNDSAVVSKILKVTN VGSVMSEEHCHTFLGMINFTSIGRICVKNGEVEGVCEGDSGGPLVYKDSEHGNVAVGLSSFGFYLNCSVTNENPEIFTSTAYF SNWIAENVEDSVCVIG	Chymotrypsin-like protease-1 (ABR21038, <i>Mesobuthus eupeus</i>)
LaSP7	MFKSFELIYLAIACIGSGSVIFAKNCDDCILITSCPGAVALAVHAKNAKTEDLIKHSLCSLEKVNGLPKVVCCSEFPAPQLDNHPN LELLPKDCGEIEGSRIVGGEVAKLYEFPWMVLISYDTRIGREFLCGGSLISPLYVLTAAHCVHGRKIAGVRIGDYDWRSKIDCEK DTNLCESYYQDIGVSERLPHPDYQGGPVVRNDIALLRLRPVNLTVKNAGVICLPVTKELRERRLDTEQVTVAGWGITENNTAS SVLLKVNLPVHSGEMCRAYYGRNSKEDTTKNILCAGVLGKDSCKGDSGGPLMLEGNYDNVFKFIQYGIVSYGPSQCGSNFPG VYTDVSSFMKWILDNIKP	HLClotting-factor2 (API81377, <i>Hemiscorpius lepturus</i>)
LaSP8	MKCF AFLIFSSQLFPAVPFQVEEKTRIFGGREANDGEFPWMVFIRLSAEWNCGGFLISPSYVLTAAHCVKGSSVTDMRGVVGS VDREQQDMLEFKKYQIHPEYGPKRWRNADLALLELRTPLGFTDLIKPICIGKKTSFTRPGNAVLQMGWGRDRSDAVVTKKLK VTEVGNLMSRCDCHRFESINIVDIQLNGRLCVKNREVEGVCEGDAGSPLVYQYAESSHVAIGIVSAGFYVNCSTNENPEIYT DLAYYSDWIIRTVDIPII	chymotrypsin-like protease-1 (ABR21038, <i>Mesobuthus eupeus</i>)
Alpha-amylase		
La-alpha- amylase	MIVDCLLLWFVWSVHCSYHEPNTQAGRSVLVHLFEWRWKDIAEECETFLGPYGGGVQVSPANENGIWEPHWNSVIRRP WFERYQPVSYKIATRSNGESEFRDMVRRCCNAGVRIYVDAVINHMTGDIGRGKGTGGSDFDGALQYYGVYPGPSDFNGRD QCPSGSGDIENYQDKYQVRNCRSLGLADLNLKEYVRDKIVEYLNFLIDIGVAGFRVDASKHMMWPGDLKIIYEKLKNLNTIYFP VHRRPFIYQEVIDLGGGEAVKAEETDLGRVTEFRFGKHLGDVIRKNNYQRLKYLKNFGQDWGMVSGSNALTFIDNHDNRG HGAGGFGSILTFESRMYKMAVAFMLAWPYGLPRVMSSYNWPRYVENGGKDNWIGPPHDDEYNTKPVVRNPDLTCGNGW VCEHRWHQIYSMVKFHNVA GFEPVDFWWDNDYQQIAFGRKGKGLAINNENHNLDQTLPTGLPPGTCDVISGKLEGDKCT GRSVKVEQDGKAKIFIDNSWEDPMLAIHVEAKLNDVLHNGNTGRNRNG	Pancreatic alpha-amylase-like (XP_023225708, <i>Centruroides sculpturatus</i>)
Cysteine-rich venom protein		
LaCRVP1	MMHFILTCILFPQVIHLNGVVAKEIKYIFYSES DYCVLGERDCNTSIYAEDFLWPHNSNRAYMASGDVPELSASNMLKLEWDQN LANIAQRAAEQCIDDYPTTFHGKVECRQPDSSAVINFSWKQSQNLNENAKRIEERIFEWTEYIKYRYNDSSLHFYRGTGIFEDL WAKVVQATTWKVGCGLADSDAVNHDMEHYTEVISCLYENTKLQPGDEIYKLGSPCSACPLGTRCIPGSNLCEVEPGNCPIP GNAKQDCAKNTERTNRET LWKCDLK KYGEECEIVPSCSLLWSVDQQGNFKSISVTRDCVSANVFIKRIRIGKPSCFTFQYIKE GNRNEPSDTVVMGMVFNLSEGDIIQVAKGEDATTWTDVMIDIPWTGVDIQVGVARSFNDNFRKQILMKDFHVS DSACL RD TEHFKPNLH	HLAllergen3 (API81354, <i>Hemiscorpius lepturus</i>)
LaCRVP2	MFIPLALLTAIRLSLRDRCCYAEFLANPSVVWETETVCPTTPTYAGSVKSKRNANPGTIPLSDQDKMEIVNEHNKYRGQVSP PAANMRYMIYNEDVAYAAQLWANGCQFRHGRPQGSKFQKLVGQNIYKGPSGSYSFYMLRLWHNEVRDFDMKSQQCNATMC GHYLMVWAESFLIGCGQSHCRGKRRSYNLFVCHYYKAFESFHPYKVGPRCSMCDAEAGGFCYNNTCVSKEDCEREGWPC	HLAllergen1 (API81352, <i>Hemiscorpius lepturus</i>)

	ECNLKCHNCGILDESTCTCKCPPGWDYQDCSQNCSDSSDYCGKEDGFEGWVGCEMLEDGYYKTHYCRKMCETCEVITEGN KEKTCGGELCDKGYVLDNSNCNCRRLCPGPECYFTYEDNGVAYRVTSLLTVIIAGIYALWQGQEPKPG	
LaCRVP3	MEKNVLWLIVLHLSLIWVKSQRDVGIKFTGNLSSAWVHKLRVDYRLKLRRVARLASMMNDSRLEVIRLHNLRYSMVAPPAA DMEYMAWDDRLASLSQQWAECKWRHGNPRHDFPTGLGQNLRYGSVRSVALAIRLWYEEHVDYRYHNLSCNPKKQCGHY TQMVWSKTHLVGCGVKNGCWKGFKHYIVCNYWPSHYKGEKPFIEGRPCSLCNSTKSGLCWNKSCVSRSQCEEYKLDSCSD LICHNCGKLDREKCTCICKDGWKGVDCEPCVDTMEDCSLYHCYIHEYSRNHPCLKSCNVCKPVNADDLRNSCCDGVSCPH GYVLDLADIPCECKPLCPGPKCGSLLHGPYFTLMLFVLLLLKCSAL	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
LaCRVP4	MEWTFVCSIMLSFAPLWGTQFETERINVSYSWVHYGGMSRRARATSMMKDPDKLEITRLHNSYRSMVSPSAVDMEYMEW DDRIASVSQQWADRCTWKHSKDTAIADFPDGLGENLYRGWSSSPAYAMNLWYSEYTHYDIQNTSCKPGKVCGHYTQMVWS KSRLVGGCVKEGCWRDYRYIYCNYSPPGNYKGEKPYQIGRSCSHCHSGSGLCSNKSCVDRSQCDKYNLDCSCDLVCLNC GELNRHSCTCKCKEGWKGVDCSEPCQDAQENCLVSACSYYERWPRGSHPCCKCNVCKSVTPDSVQNTCCDGVTCPPGEI LDLSEPCCKKLCSGPKCQSSLHEPHSLFLLVAMSIFLRCYAR	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
LaCRVP5	MAAVAVALVVLWITVTSGSANNDTCAERYTNITPEHTMCKSTNENCTFVRSSGEVFEEQLLRTHNLIRNSIRKYVGKKYHLATN MKLMVWDDLEYEMARLHSLQCVEEPCDCLCHQIGDFPVEQNFVAVKTFKSSEVDGNGPIRRLQAVIEEWAAELRSYDCDVVKV FRNTEGLPTNWTNIFRATTMLVGCASMTFLTDEPGTFKEVYVCNYGPANLTEGEEIYRTGRKPCSECEDDEGCDTEFKHLCFP ADMEEEENIPEETELHNTFARNMVTKKFPRQRRRYLWNTDVARSYLDRRTENWRTTGGGVTGSYPDYRTGNWNGNTGEGVTR SSHTYQCKRKSETSQLSESSILVMEQQGF	HLAllergen7 (API81358, <i>Hemiscorpius lepturus</i>)
LaCRVP6	MEIFLAIPCLLTAFLPSISNERCSDSFGNYDQALFREGTRDESQISWWTTTPISKETGDKTRTSEEKECEPELYQRYSRNHTFC QVSNCDIIRKGVTEEDKNIILEFHNSLRSLKASGMEKRYCSLPSAANMMQIEWDDELA AVAEAHAE LCVYGHDRGKRAVESFS VGQNLMLYGGDIKRWGDADGWYKEEVCFYSPEKNSPFRSGIYGHFTQVTWATTWKIGCGFASYRKNKGKVEALYTCNYGPS GNVREGRHYIVGEPSCQCPPNTECSTEDPGLCKSKTCDGPQMRPPSEDFILFCDFSHEDEECNKVKVNGSREFSTRHIYT GNYKTVVNLNAGESITIDLGAQNDDGGICTFVYSRFGPNNAKDAPGSVMEIKYESPNILPVPPRTIGPYGPTFFMAGTHMSYHGE LQNTLTLEGALEGAEPQYFDIKMWGIRKGDCLMSLESD	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
LaCRVP7	MEIFLAIPCLLTAFLPSISNERCSDSFGNYDQALFREGTRDESQISWWTTTPISKETGDKTRTSEEKECEPELYQRYSRNHTFC QVSNCDIIRKGVTEEDKNIILEFHNSLRSLKASGMEKRYCSLPSAANMMQIEWDDELA AVAEAHAE LCVYGHDRGKRAVESFS VGQNLMLYGGDIKRWGDADGWYKEEVCFYSPEKNSPFRSGIYGHFTQVTWATTWKIGCGFASYRKNKGKVEALYTCNYGPS GNVREGRHYIVGEPSCQCPPNTECSTEYPGLCKSKTCDGPQMRPPSEDFILFCDFSHEDEECNKVKVNGSREFSTRHIYT GNYKTVVNLNAGESITIDLGAQNDDGGICTFVYSRFGPNNAKDAPGSVMEIKYESPNILPVPPRTIGPYGPTFFMAGTHMSYHGE LQNTLTLEGALEGAEPQYFDIKMWGIRKGDCLMSLESD	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
LaCRVP8	MDLLFAISCLLAFLPCICNKRCNSNIYGKYDQGTLSKGARDETFLSSWRNVIPINIERGEKTQFSEKCEPELYQRY SADHTFCK KSTCSIIQKGVTEDEKNTILKFHNSLRSLKASGKEARYSKTPLPSAANMMQLEWDNELAAVAE AHAE LCLYDHDANEQRAVEN FPVGQNLMMQYNGDIRKWGDADMWYKDEVCFYSPQYNSPFNSGDFGHFSQVTWATTWKIGCGFTSYRKDGSEKALYTCNYG PAGNVPGGRHYIVGKPCSQCPPNTECSTEYPGLCKSKTSDGPQMKRPSEDFILFCDFSQADPEECKKV/KVSGSREFSTRHI YTGDYKTVVNLNAGESISIDVGKAQNTGGICPFVYARFGPNNAKDAPVGSVVEIQFSAPNIVPMPPSTVTPLGSSFFVAGTHMMYD GELQSVLTLKAEQGAQPQYFDVKAWGVVRKGSCTSLGPK	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
LaCRVP9	MEVTSRPVTEANPVIQPLLTWKWTHLYICARKRIRAIINLHFLT VETANMNLTLVVSILCLALFTCQVVYSQRCPEIYQRFSEDH TYCKHSTCQVIKSGVTDQDKKIILDMHNSYRNKVALGQEDTPQRQPPAANMLQMEWDDDELARIAQAHANLCKFEHDSPDQRQ	HLAllergen1 (API81352, <i>Hemiscorpius lepturus</i>)

	<p>VENFNVGQNLFISSMTQVIDWNKTAMWYTWEIKHYYPQYREPFASGPYLHLSQMIWADTWKVGCGVAVYYDNNERRDKVLY TCNYGPGGNQIGQKVYTAGKPCSQCCKNTQCSSEYKGLCKSRTPDGPQQDTTRNPDDFLLYCDFSNNEEDRACKNVQISGLR QFETRKIYGGYKIAILKGGESITFKLGKAKDSRGICPFIYGSFGPNRAGDAKQSAVSIGFAASGLIFGDPIKIDYGSSEDFWPIGM HMQYDQEMESTIKLEAYPGAPPQYFKIKAFGIGKGKCPKF</p>	
LaCRVP10	<p>MDFLLAISCLLAFLPCNSNHLCELYGEYDEDSDEDPSSSEWEEIPTDEERGEKSQISERKECELYQRYSTNHTFCKESTC DIIRRGITEEDKNTILEFHNSLRSLASGKEARYSQTPLPAAANMMQMEWDDLAABAQAHAELCINFHEPDDQREVENFPVG QNLMQFDYDIKSWESAIEWYKEEVCFYSEEYNPFNSGIFGHFSQLTWATSWKVGCGFASYRVNGSEKGLHTCNYGPPGNV AGGRHYIVGEPCSRCPNTECSTEYPGLCKSKTSDGPQMKRPSSDFILFCDFSQEDPEECDGVKVNKSREFSTRHLYIGDY KTVVLNAGESISIDLGAQNTGGICPFVYARFGSNNAKDPAGSVLEIQFDAPNMWSMPPIRVNPNYGDSFQVAGVQMMYDDEL QSILTFKAEEGAQPKQYFDVKAWGIRRGSCETSLDPEDEN</p>	HLAllergen2 (API81353, <i>Hemiscorpius lepturus</i>)
Insulin-like growth factor binding protein		
LaVP1	<p>MGKFLIALFLFGVTVSALGLSCRPCGTYQCPPLPRCPVGVKDACFCCQVCAKGLNERCGGPWNISGRGRLKCFKQAA DAIGVCRKV</p>	Venom insulin-like growth factor binding protein-1 (ABR21044, <i>Mesobuthus eupeus</i>)
LaVP2	<p>MALRFCFITLLLLGVILGAMTLRCRQCGTYECPAPENCPVGKVKDICNCCDECGKNLGEECGGAWDYMGKCGKGLRCFKEP VEGDPFNAKGTCR</p>	Venom insulin-like growth factor binding protein-1 (ABR21044, <i>Mesobuthus eupeus</i>)
LaVP3	<p>MWFRLVLFVLVTSIYSLSCPCHELDLEEF CGPPP KDCPLGLTLDACACCQVCFLTEGEVCGGPWDVNGKCGAGLTCVKPPG QSDFVFDQSDGVCKKQ</p>	Venom insulin-like growth factor binding protein-1 (ABR21044, <i>Mesobuthus eupeus</i>)

Table S3 Comparison of inhibition of HCV infection between pretreatment and post-entry treatment with LaPLA₂-1

LaPLA ₂ -1 (ng/ml)	Inhibition (%)	
	Pretreatment	Post-entry treatment
100	>99.9	8 ± 6
10	92 ± 1	<0.1
1	16 ± 23	<0.1
0.1	<0.1	n.t. ^a

^a n.t., not tested.

Table S4 Cytotoxicity of LaPLA₂-1.

LaPLA ₂ -1 (ng/ml)	Cell viability (%)
1000	79
100	100
10	100
1	100

Figure 1. Proportion of the components identified by venom gland transcriptome analysis

(a) NDBP

LaNDBP2-1	MNIKVVVLVVCLITLLVTEQVEGGWFSRIKSLAKKAWKSNLAKDLRKMAGKAARNYAACKVLNVSPREEQVQL--DNLLRYL--D	100 (%Id)
LaNDBP2-2	MNGKVLVVCLIVAMLVMEPAEAGIWGWIKKTAKKVWNSDIANQLKNKALNAKNYVAEKVGATPAEAGQMPFDEFMDIYY-S	37
Vejovine	MNAKTLFVVFLIGMLVTEQVEAGI <u>WSSIKNLASKAWNSDIGQSLRNKAAGANKFVADKIGVTPSQASMTLDEIVDAMYD</u>	42
LaNDBP3-1	MQYKTFVLVIFLAYLLVTEEAQAFWWALAKGAACKLLPSVVGAFTR-KREIEKIFDPYQSSLDLEMERFLRQLQ	100
LaNDBP3-2	MKLRTLMVIFLAYLIVTDEAEAFWGFLAKAAKLLPSLFSSKKEKEKREIEHFFDPYEKELDSELDQLLYELQ	56
Heterin2	MQYKTFVLVIFLAYLLVTEEAALAFWGALAKGALKLIPSLVSSFTKKDKRALKNIFDPYQKNLDLELERLLSQLQ	75
LaNDBP4-1	MKIQLAILVITVVLQMQLVQTEAGFWGKLWEGVKNVIGKRGRLRNKDQLDD-LFSDLSADADAKLLREMFK	100
LaNDBP4-2	MKIQLAILVITVVLQMQLVQTEASFWGKLWEGVKSAGKRGRLRDVDQMDD-LFSDLSADADAKLLREIFK	90
LaNDBP4-3	MKNQFVILLILAIVFLQLFSQSEA-FLSALWGVAKSLFGKRGRLKNDQLDD-LFDGEVSEADLDFLKELMR	48
LaNDBP4-4	MKAQIVLLVITVVLQMFAQSEAGFWGKLWEGVKSAGKRGRLNLDQFDDELFDSDLSADADAKLLKEIFK	80
OcyC2	MKTQFAILMIAVVLQMQLVQTEGILGKIWEGVKSILIGKRGRLKLDQLDD-TFSDLSADADVKKLLREMFK	78
LaNDBP4-5	MRLVSLTPLFLILLIAVDYCSFP-FLLSLIPSAISALKKLGRSTDFQRQLDFQRRYLNSDLDFDLDELEEFLDQLPDY	100
VpAmp1.0	MKLINLVPVFFVLIIVVDYCHSLPFFLLSLIPSAISAIKKIGKRSVESQRYVDLNR-----DLEQDLQELQDFLDQISEH	58

(b) Invertebrate defensin

LaDefensin1	MKAIATLLLLILVAFSVLEFGIVDAGFGCPFNRYQCHSHCQSIKRRGGFCAGTFRTTCTCYKSK-----	100
LaDefensin2	MKAIATLLLLLVAFSILEDGIVDAGFGCPFNRYQCHSHCRSIGRRGGYCSGPFRFTCTCYK-----	85
LaDefensin3	MKAIATILLLLAASFIFLFGIVGAGFGCPHNRYQCHSHCQSIGRNGGYCGGTFRTTCTCYNSGGPTSSP	74
AbDef-1	MKTIVLLFVLALVFCTLEMGVVEAGFGCPFNQGRCHRHCRSIGRRGGYCRGIFKQTCACYRK-----	55

Figure 2. Multiple sequence alignment of NDBPs (a) and invertebrate defensin peptides (b) identified in this study and comparison with their similar peptides. Mature regions that were previously reported are underlined. Cys residues in the reported and putative mature regions are shaded in yellow. %Id represents the percentage of sequence identity.

(a) Alpha-KTx

La-alphaKTx1	MNAKFIIYILLLTAVMFALYEASVPPNIP	<u>CQVTNQCPKPCREATGRPNSK</u>	<u>CINGRC</u>	<u>KCYG</u>	-----	100 (%Id)								
La-alphaKTx2	MNTKFVFLLLVISTLMPTFDAS-AEDIS	<u>CSSSKECYDPC</u>	<u>EEETGCSSAK</u>	<u>CVGGWCKCYG</u>	<u>CRG</u>	---								
La-alphaKTx3	MNRNFVFLLLLIVTLMPLDAA-TEDIN	<u>CNDNRDCLKPC</u>	<u>KDETGCPNSK</u>	<u>CEEGNCLCYG</u>	<u>CNRLTV</u>	42								
Urotoxin	MNAKLIYLLLVVTTMMLTFDFTTQAGDIK	<u>CSGTRQ</u>	<u>WGPCKKQTT</u>	<u>CNSKCMNGK</u>	<u>KCYGCVG</u>	48								
La-alphaKTx4	MNKKFIFLLLVVTTLMPMFDA-ATEAIS	<u>C</u>	<u>SNPND</u>	<u>C</u>	<u>REPCKKQTG</u>	<u>CSGGKCMNRK</u>	<u>CKCHRCNG</u>	100						
OcKTx2	MNAKFIFLLLVVTTMMLLPDTQGA	<u>EVIKCRTPKDCADP</u>	<u>CRKQTG</u>	<u>CPHGKCMNRT</u>	<u>CRCNRC</u>	-G	61							
La-alphaKTx5	MNAKLVCIVLL-TAVMFAPDEASLPPIRIP	<u>CYVSKD</u>	<u>CRKP</u>	<u>CLYLTGT</u>	<u>PRSKCINRRC</u>	<u>KCYG</u>	---	100						
Hemitoxin	MNTKFIFLFLVVTTMMLFDTTAVEA--	<u>IKCTLSKDCYSP</u>	<u>CKKETG</u>	<u>CPRAK</u>	<u>CINRNC</u>	<u>KCYG</u>	<u>CSG</u>	48						
La-alphaKTx6	MNKPFC	AI	FLVVLIMFAVSVLPAES--TGG	<u>CPV</u>	<u>DSL</u>	<u>CKSY</u>	<u>CKSNKFGTEGK</u>	<u>CDGTS</u>	---	<u>CKCAIG</u>	100			
La-alphaKTx7	MNKLACYILICVMVSLFKVPVAEGI-SAGC	<u>PLTAKLCTIYCKK</u>	<u>HRFGREGK</u>	<u>CIGPTRFRCKCYV</u>							44			
LmKTx8	MNKVCFVVVLVLFVALAAYVSP	<u>IEGVPTGGCPLSDSL</u>	<u>CAKYCKSHK</u>	<u>FGKTGRCTGPNKMK</u>	<u>CKLV</u>						49			
La-alphaKTx8	--MRLV	<u>II</u>	<u>LL</u>	<u>MTT</u>	<u>LV</u>	<u>LA</u>	<u>VGAPLGGA</u>	<u>CKSSSTQ</u>	<u>CTRP</u>	<u>CRYAGGTHGK</u>	<u>CMNGRC</u>	<u>CRY</u>	--G	100
St20	MKMSI	<u>VI</u>	<u>II</u>	<u>LL</u>	<u>FT</u>	<u>CL</u>	<u>IATNGA--SGTK</u>	<u>SGSPE</u>	<u>CVKFC</u>	<u>RTKG</u>	<u>CRNGK</u>	<u>CMNRS</u>	<u>CKYLCS</u>	49
La-alphaKTx9	MELKYLLVLLAVTCLVSCQDNSLLPSGSC	<u>SRTGI</u>	<u>CMES</u>	<u>CAPFLYQPKYHRR</u>	<u>CPAGYVC</u>	<u>CTLIY</u>								100
Kbot55	-----	<u>AGSMDS</u>	<u>CSETGV</u>	<u>CMKA</u>	<u>SERIRQ</u>	<u>VENDNK</u>	<u>CPAGE</u>	<u>CICTT</u>	--					39

(b) Beta-KTx

La-betaKTx1 (LaIT2)	MAKHLIVMFLVIMVISSLVDCAKKPFVQRVKNAASKAYNKLKGLAMQSQYG	CPIISNM	CDHC	RRKKMEGQ	C	DLLD	CVCS					
La-betaKTx2 (LaIT3)	MQAQFTVLLLLVLVTLCSCGGILREKYFHKAADALTSNIPIPVVKDVLKSAANQMIRKIGKVQQA	CAF	NKDLAGW	CEKS	C	QEAE	GKKGY	CHG	TK	CK	CGKPIDYRK	
La-betaKTx3	MDTKLSILVFLCVVVIASCSWISEKRIQKALDEKLPKGF	IQGA	AKAIVHKFAKNQYG	CLADMDVKGS	CDRH	C	QETESTNGV	CHG	TK	CK	CGIGRVY	100
SC11	MNTKFTVLIFLGI-VVSYGWITEKKIQKVLDEKLPNGFIKGA	AKAVVHKLAKSEYG	CMMDISWNKD	CQRH	C	QSTEQKDGI	CHG	MK	CK	CGKPRSY		60

(c) Kappa-KTx

La-kappaKTx1	MKPSTSAYALLLVLTFGIIT--SGVFAVPMDEENTFEVEKRGNS	<u>MEV</u>	<u>CLQ</u>	<u>HEGNVAE</u>	<u>CEKAC</u>	<u>NKG</u>		100
La-kappaKTx2	MKPSTSAFILLLVLTFGIIT--SGVSAIPMDEENTFEEQKRDSAC	<u>VEV</u>	<u>CLH</u>	<u>HEGNVAE</u>	<u>CEEAC</u>	<u>KKS</u>		78
HeTx203	MKTSGTVYVFLLLAFGIFTDISSACSEQMDDSDSYEVEKRGNA	<u>C</u>	<u>IEV</u>	<u>CLQHTGNPAE</u>	<u>CDKAC</u>	<u>DK</u>	-	59
La-kappaKTx3	MKLLPLL-VILIICALMANEAFCDQGARER-SENLED-TRDLVQK	<u>PC</u>	<u>RIV</u>	<u>CSENMR--KC</u>	<u>IRROT</u>	<u>L</u>	--G--R	100
La-kappaKTx5	MKLLPLLVLIVCALLPNEAFCDQSAVER-SESLEEVSREIVKRS	<u>CKRV</u>	<u>CSGTRRTKKCMQK</u>	<u>CKSQPG--R</u>				58
HeLaTX1	MKLLPLLVLIVCAILPDEASCDQSELERKEENFKDESREIVKRS	<u>CKKE</u>	<u>CSGSRTTKKCMQK</u>	<u>CNREHGHGR</u>				47
La-kappaKTx4	MKPSSFAIALILVLFLGFTNAVSGEYAESI---SGDRME---RAERAG	<u>CRIR</u>	<u>CLQFTDDFEK</u>	<u>CRKL</u>	<u>CG--</u>			100
PcavC10	MK-SSLVVASLLILFVVSINDYTSVYAQSTHEESGSKMERRARIK	<u>RAG</u>	<u>CVIK</u>	<u>CLQFTPELEK</u>	<u>CRKL</u>	<u>CGLK</u>		50

(d) Delta-KTx

La-deltaKTx1	MASQFLLFCIVLIAVNPLVYSKG-G	RLPPETGL	CYAYFERYYYDPSSNN	CKMFVYGG	CGGNSNNFVSKKAC	LARCAN---	100				
BmKTT-2	MMNVITVVGIIILSVVCTISDAEGVD	CTLP	SDTGR	CAYFIRYFYNQKAGEC	CQKFVYGG	CEGNSNNFLT	TKSD	CK	KQ	CSPGKC	42

Figure 3. Multiple sequence alignment of KTx peptides identified in this study and comparison with their similar peptides. Mature regions that were previously reported are underlined. Cys residues in the reported and putative mature regions are shaded in yellow. %Id represents the percentage of sequence identity.

(a) DDH

LaDDH1 (LaIT1)	MNFATKVFLLLLAVAVIAIVAGEEDDSWFEQNEESDTERDFPLSKEYETCVRPRKCQPPLKCNAQICVDPKKGW-----	100 (%Id)
LaDDH2	MNCAIKVSFLLLAITVIFSVAGGEGDNSFEQREENDTERDLPLSKKHESCVRPRLCRPPLRCNKAHICVESKKGWIPVISWVSKTKIMFSE	71
Phi-LITX-Lw1a	MNFATKVSLLLLAIIVIVIVEGEGDSWFEHEESDTERDFPLSKEYESCVRPRCKPPLKCNAQICVDPNKGW-----	84
OcyC10	MNFATKIVILLLLVAALILAVTSEKGDSSSDNEAKETEGELPLSDFYGCVRPKCKPHLKNAAQICVFPKTGR-----	56

(b) NaTx

LaLAP1	MNTGEHFASLIIFLLLLGENPCLGDGGWPMRIDGNYYCYEYEEKPAELYCKRACKLHKASQSSCSYHWKYSWYCYCEDLK-----KGYTRDRGLKKGGGHQFSSES	100
LVP-1-alpha	MNI-KLFCFLSILISLTGLSLSGDDGNYPIDANGNRYSC--GKLGENEFCCLKVCKLHGVKRGYCYF-----FKCYCELLKDKDIQFFDAYKTYCKN-----SRI	26
LaLAP2	-MLMVIYIATLIPLILQGECAKDD---HPRNFEGNCYRCKYPDKSGYCEAICKMHKAETGYCSRSNLCYCKGIEDKYVS-----ARDFLEP---	100
BmLVP1-alpha	MMKFVLFGMIVILFSLMGSIRGDDDPGNYPTNAYGNKYCTILGENEYCRKICKLHGVTYGYCYNRS--CWCEKLEDKDVTIWNAVKNHCTNTILYPNGK	33

(c) Ascaris-type protease inhibitor

LaAPI1	MKS--WTYRLCFLILFLVCV--NCT-RPVLSPEEC	TRPGEFFTTAGSYCPLTCDNYKN--P-PIICSLIGIVGCEC	SPELVDRERSGN-CVDTSDCTED----	100	
LaAPI2	MKG-----FAFLVLTFFVVF-----GDKEQEC	EDPNAEFRRONTAC	PITCANMDN--P-PNICTLQCVIGCACKEGYKND-DGL-CVHPEGC-----	33	
LaAPI3	MKA--LLLLLSFVVIH--SAKSQEDLGGDEHAQS	CLLPNEVWDKCGPSCPPSC	IGVIE--P-GTLCSTECTPGCFCREGLVRTK-RGS-CIPPKACRNERETKL	25	
LaAPI4	MKGSKPLCFLYLVLVLLWTSV--KCT-RWASSSEEC	TRPGEAFTSCGTDCPITCANYEN--P-PEFCNYMCVIGCECT	SGLVRDEGSGN-CVNPSQCGP-----	51	
LaAPI5	MKR--NLVLLAFVLLLFSGIFEKCSAQRGGADRR	ORRPGEVFMNCGPSCPLTCDNYQN--P-PTVCTLQCVIGCF	RRGLVRDTRRG-CVRPSQCR-----	42	
LaAPI6	MKR--NLALSALFVLLFCSALDNCEAQRGGYDRT	CLEGEVFTRC	GTACPLTCDNYKN--P-PEVCTLQCIIGCV	GRGWKDTRRGR-CVRPSQCR-----	40
LaAPI7	MKS--NLVLFVAFVLLLFCSVLEICTAQRVVDRS	RGAGEVFTRC	GTACPLTCDNYRN--P-PRFCTRQCIIGC	ACRRGWKATRRGG-CVRPSQCR-----	40
LaAPI8	MAKT-----LAFGVVLCMFVLAQSAPQYPGTFG-C	-DEDKQFVRLPPCPVTCKSILNTTP-CTLLLP	RC	TPGCGRGGKILDN-AGK-CVFPADCRRG-----	22
SjAPI	MKW--GALLCIFGFLAFCSVLDRLGWIPDIWQK	CSKNEEFQCGSSCPETCANHKN--PEPKSCAAVCFVGV	CKPGFIRDDLKGSICVKPEDCSK-----	34	

Figure 4. Multiple sequence alignment of DDH (a), NaTx (b), and Ascaris-type protease inhibitor peptides (c) identified in this study and comparison with their similar peptides. Mature regions that were previously reported are underlined. Cys residues in the reported and putative mature regions are shaded in yellow. %Id represents the percentage of sequence identity.

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La1      MG-----GTLKHL LLVCLIVVCSSSLCLGFG-----ESC IAGRF-IVPLGQQVTDQRD CALYK VNYNKKFALETKR CATVNL-K-SG CKTVPGGAGAAFPS CCP-MVT CKG----- 100 (%Id)
La1-1    MK-----HLHAAVLLVCLICALPSLTLGAG-----ESC KVGSL-VIPVGQKKFDKPS CAEYE STEFNRVLLKAIT CATLNT-KGKG CKSVPGKSPNSFPD CCP-TIL CRGEQWNH----- 42
La1-2    MK-----IVCTIVPLVF-----ACIANAHVLT-----RT CRHTTG VILKNGEEWADPNH CSIYR CTISGEAELQGLT CATYRV-P-PN CEFVRGRGKF-YPS CCP-TVM CKP----- 25
La1-3    MA-----LRFVLAFLLPCLVLGSNEPAKFISYKNDMLGPLVEGK CKVGNDKMIEQGGTWYRDDY CEKIY CLRTGTLGHMEVWG CAPVAPLN-PN CTVVHHSGL--YPN CCSGEIV CEENPDTKKSDVEMAEIIRALLQSERK 17
La1-4    MF-----NIVTITLLWSCTCIALCHSYG-----ET CQAGEL-TIPLDNEKQDPEA CTLYK TMFAGRVVLNLTLT CAPQEP-R-RG CRNVDSPELPPFD CCP-LVV CNVQPLGSK----- 32
La1-5    MR-----TLVPAVFLLALIVAAMASHKDPYH-----RNC PIGNK-DLS DGE EWADQQR CVKYK QIRGPDAALLLTR CPSVGIYPPDK CREIAGKGN--FPD CCP-KLQ CD----- 28
La1-6    MSRADKAFVGTLLIAIFLVCSLTNAYSSLKRQKYGSG-----APC VDHLGSNRKFDVWYDNES CEEHT CIKYRGIPHVQIYG CIAAVA-S-PG CELVKGS GS--YPD CCE-EEI C----- 19
La1-7    MKTWEARFYIFLVALVTITFVESYVYLVPQDPGA-----VVC IGKDRVSHKPGDVWYDDSK CERLT CGHSSGGLVIDGAG CGSISV-Q-DG CKLVPGVGS--YPS CCP-SPV C----- 19
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Figure 5. Multiple sequence alignment of La1-like peptides identified in this study. Mature regions that were previously reported are underlined. Cys residues in the reported and putative mature regions are shaded in yellow. %Id represents the percentage of sequence identity.

Figure 6. HPLC purification of an anti-HCV component from the venom. (a) First separation using a C4 column. (b) Second purification using a C18 column. (c) Final purification using a C18 column. Asterisks indicate fractions showing anti-HCV activity.

Figure 7. LC/MS analysis of the anti-HCV component after reduction/alkylation reactions. (a) TIC chromatogram of the reaction mixture. (b) Product ion spectrum of the small subunit.

Figure 8. Product ion spectrum of the Lys-C digest containing N-glycosylation and its estimated glycan structure (dashed square).

LaPLA ₂ -1	MVFIFLAVLSGLVTLTSHSTAVQREMHHVHFEPLPGQRDSWPVARAALVNLATKSETGRE--	
Hemilipin	MTFLILTILATVTPSLYSHVVQRELRVNFEPLAGQRDSWPVARAAMVTFDARSEKARE--	
HgPLA ₂	MSLIIVLVISVL---SADAVLSMDNELYLNLPSQRSSWPVARAVRMQFSKRSEGGRESR	
Phaiodactylipin	-----	
BvPLA ₂	-----	
LaPLA ₂ -1	-FSDCRMNSIDEIAREGAVLSRYEIKRVSKEEMRSLEKRCSSSGIHQRLIFPGTKWCG	VVV
Hemilipin	-FSECRMINSMHLSRELMDSPHETVKRASKEEMDDLVRCSGSAEGRSWFIWPDTKWCG	
HgPLA ₂	KMQGCQILESINDIAREALRTPRHTTKRISKDEMEFFEGRCLSVGESER--TVLGTKWCG	
Phaiodactylipin	-----MVKRVSKEEMDALERSCSQPFEERFLIVSGTKWCG	
BvPLA ₂	-----MQVVLGSLFLLLL-----S-TSHGWQIRDRIGDNELEERLIYPGTWCG	
LaPLA ₂ -1	AGDKAANYS DLG SAAETDKCRAHDHCDN-IAAGETKYGLENSYWFTKLNCCEESFRNC	vv
Hemilipin	PGTDAKNES DLG P-LEADKCCRTHDHCY-IGAGETKYGLTNKSF FT KLNCCEA AF DQC	▲▲
HgPLA ₂	AGNEAANYS DLG YFNNVDRCCREHDHCDN-IPAGETKYGLKNEGTYTMMNCKCEKAFDKC	
Phaiodactylipin	NNNTAAANYS DLG F-LEADKCCRDHDHCDH-IAAGETKYGLENKGLFTILNCDCEA AF DHC	
BvPLA ₂	HCNKS SG PNELGRFKHTDACCRT HD MC P DVMSAGESK HGL TNTASHTRLSCDD DK FYDC	
LaPLA ₂ -1	LAEAKKKET-----TAKFVKFSYFNVYGPKCFVLDCKRRFEM--SRK-----	
Hemilipin	LKESIDRAEGS--AKSSMEGLHSFYFNTYSPECYEVKCSRKRDAE--CTN-----	
HgPLA ₂	LSDISGYFTRK-----AVSAVKFTYFTLYGNGCYNVKCENGRSPSNECPN-----	
Phaiodactylipin	LKEISNNVT TD IRQKGAENVWREYFQWYNANCYRLYCKDEKSAR--DEA-----	
BvPLA ₂	LKNSADTIS-----SYFVGKMYFNLDITCKYKLEHPVTGCGERTEGRCLHYTVDKSK	
LaPLA ₂ -1	- <u>CA</u> VAHWKESRRG-----	100 (%Id)
Hemilipin	-GIAIWKDSYKS-----	49
HgPLA ₂	-GVAEYTG ETG =LGAKVINFGK	41
Phaiodactylipin	- <u>CT</u> NQYAVVKK NFT VQ-----	46
BvPLA ₂	<u>PRVYQWF</u> DLRKY-----	26

Figure 9. Multiple sequence alignment of LaPLA₂-1 and its related PLA₂ molecules. Mature regions that were experimentally determined are underlined. Cys residues in the mature regions are shaded in yellow. Open triangles indicate the Ca²⁺-binding motif. Closed triangles indicate the catalytic dyad. N-glycosylation motif is shown in bold. The N-glycosylation sites that were experimentally confirmed are shown in red. %Id represents the percentage of sequence identity.

Figure 10 Representative concentration-inhibition curves for LaPLA₂-1 on HCV (●), DENV (×), JEV (▲), and HSV-1 (○) infection. Each point represents means $\pm$ SEM.

Figure 11. Comparison of PLA₂ activity between bvPLA₂ and LaPLA₂-1

Figure S1 Mass spectrum of LaPLA₂-1 after deglycosylation