

Proteomic Analysis of *Latrodectus tredecimguttatus* Venom for Uncovering Potential Latrodectism-Related Proteins

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ABSTRACT: Black widow spider is one of the most poisonous spiders in the world. Up to now, there have been few systematic analyses of the spider venom components, and the mechanism of action of the venom has not been completely understood. In this work, we employed combinative proteomic strategy to analyze the venom collected from living adult spider *Latrodectus tredecimguttatus* by electrical stimulation. The experiments demonstrated that the venom is primarily composed of high molecular weight proteins and has high abundance proteins around 100 kDa. The content of peptides and proteins with low molecular weight is low. A total of 75 nonredundant venom proteins with distinct function were unambiguously identified. Besides the known black widow spider venom proteins including latrotoxins, a variety of hydrolases and other proteins with special activity were found in the venom, such as proteinase, phospholipase, phosphatase, nuclease, fucoselectin, venom allergen antigen 5-like protein and trypsin inhibitor, and so on. Their possible biological actions and relationship with latrodectism were discussed. The results help to understand the complexity and action mechanism of *L. tredecimguttatus* venom. © 2008 Wiley Periodicals, Inc. J Biochem Mol Toxicol 22:328–336, 2008; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20244

KEYWORDS: *Latrodectus tredecimguttatus*; venom; protein; electrostimulation; latrodectism

INTRODUCTION

The notorious black spider (genus *Latrodectus*) belongs to the family of comb-footed spiders (Theridiidae: Arthropoda, Arachnida, Araneae) [1,2]. It is responsible for a number of spider bites and envenomation around the world [3]. Its venom is a complex mixture of components with diverse biological actions. Many of these components are biologically active proteins and peptides, which play a number of adaptive roles: paralyzing, immobilizing, killing, liquefying prey, and restricting competitors. The elucidation of specific proteins and peptides in its venom has vast implications in the treatment of latrodectism and in neurobiology and pharmaceutical research.

Over the past 30 years, some pioneers found that the venom components of the spider of genus *Latrodectus* cause a massive transmitter release from diverse nerve terminals [4–7]. Some researchers attempted to employ gel-filtration followed by ion exchange chromatography to separate the venom from *L. mactans tredecimguttatus*. But they did not obtain completely pure component. However, their research results clearly indicated that some of fractions contain several proteins selectively toxic to vertebrates, insects, or crustaceans [8,9]. Up to now, at least seven different latrotoxins have been isolated from the venom of *L. mactans tredecimguttatus* spider by means of such as ion exchange chromatography and hydrophobic chromatography [10,11]. All the studied latrotoxins are large acidic proteins (pI ~ 5.0–6.0) with molecular weights ranging from 110 to 140 kDa. Most of them are targeted against insects and are called latroinsectotoxins (LITs; α -, β -, γ -, δ -, ϵ -LIT) [10]. The most studied latrotoxin is α -latrotoxin (α -LTX), which exerts a lethal effect on vertebrates by inducing a massive neurotransmitter release. α -LTX acts both in the presence and in the absence of Ca^{2+} , with the two activities exhibiting different features [12,13]. To date, the primary structures of four genes encoding, respectively, four latrotoxins,

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α -LTX, α -LIT, δ -LIT, and α -LCT, were determined in the spider *L. mactans tredecimguttatus* by cDNA or intronless genomic cDNA and sequenced [14–17]. In addition, Volkova et al. isolated two low molecular mass proteins LMWP and LMWP2 from black widow spider venom, and the two proteins were inactive to mammals and insects [18]. Akhunov et al. studied kininase and two bradykinin-potentiating peptides of the *L. tredecimguttatus* venom. They characterized the kininase as a thiol endopeptidase, which cleaves internal peptide bonds at the proline carboxyl end. The two bradykinin-potentiating peptides prolong depressor effects of bradykinin, stimulate histamine releasing from cells, and decrease blood pressure in rats [19,20].

Although several specific components in the venom of *L. mactans tredecimguttatus* have been isolated and characterized, there were few systematic analyses of protein components in the venom. Global analysis of spider venom presents a challenge for analytical and identifying techniques, which demand approaches that effectively separate and identify a complex mixture of venom proteins and peptides with varying charge, size, and abundance in spider venom. With the availability of 1DE (one-dimensional gel electrophoresis), 2DE (two-dimensional gel electrophoresis), and multiple-dimensional chromatography to separate complex mixture of proteins, advances in MS (mass spectrometry) and the development of computational methods, it is now possible to have virtually all the venom components visualized and identified with given sufficient effects and resources.

The clinical symptoms of black widow spiders are complex, mainly including muscular pain and tremors, profuse perspiration, itching erythema, paraesthesiae, increased lacrimal, nasal and salivary secretions, and hypertension [21,22]. Obviously, the intoxication after a bite by *L. tredecimguttatus* must be related to other venom components except for latrotoxins. To gain comprehensive insight into the complexity of black widow spider venom and investigate whether there are any other protein components in the venom that may be involved in the latrotoxicity except for the known venom components such as latrotoxins, the present study utilized combined strategy to analyze the venom of *L. tredecimguttatus* indigenous to some areas of the Xinjiang Uighur Autonomous Region of China. The strategy included the following two different approaches: (i) one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis plus capillary column LC coupled with tandem mass spectrometry (SDS/PAGE-CapLC-MS/MS) and (ii) shotgun digestion plus fractionation and capillary column LC coupled with tandem mass spectrometry (SDF-CapLC-MS/MS). Protein database search was made for venom protein identification. After dereplication, a total of 122 matched pro-

teins were obtained, of which 75 proteins have distinct function, and the rest are mostly matched to predicted or hypothetical proteins with uncertain or unknown function. The experimental results help to understand the complexity and action mechanism of *L. tredecimguttatus* venom.

MATERIALS AND METHODS

Materials

Trypsin (proteomics sequencing grade), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), acrylamide, *N,N'*-methylene bisacrylamide, glycine, trihydroxymethyl aminomethan, sodium dodecyl sulfate (SDS), and SDS-PAGE protein standards were obtained from Bio-Rad Laboratories (Hercules, CA). Bromophenol blue, *N,N,N',N'*-tetramethylethylenediamine, was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Phenylmethylsulfonyl fluoride was obtained from Ameresco (Solon, OH). HPLC-grade acetonitrile (ACN) was purchased from Hunan Fine Chemistry Institute (Changsha, Hunan, People's Republic of China).

Venom Collection

Venom was collected from female spider *L. tredecimguttatus* using an electropulse stimulator. Briefly, one person held a spider on a small venom collection table and placed a plastic strip between its chelicerae and mouth. There was a piece of aluminum vertically fixed on the table. The end of spider abdomen was made to contact the sheet metal to expand the contact area of output electrode. Another person made the two output electrodes of stimulator contacted the root parts of cheliceral claws and the sheet metal, respectively. Physiological saline was used to enhance the electrical contact. When appropriate electrical stimulation of 25–50 V and 15–40 Hz, adjusted as desired, was applied, venom was expressed on the plastic strip, and then the venom was collected from the strip and freeze-dried.

SDS-PAGE

SDS-PAGE of the venom was performed basically according to the method of Laemmli [23] under denatured conditions in an 11.5% polyacrylamide slab gel. Venom sample (50 μ g of protein) was denatured and reduced in SDS loading buffer and boiled for 3 min. The venom solution was centrifuged for 10 min at room temperature (25°C) at 10,000 $\times$ g, and the supernatant was loaded into gel well. SDS-PAGE was run at 25 mA

on polyacrylamide stacking gel and at 50 mA on separating gel. After completion of electrophoresis, the separated proteins in gel were visualized by Coomassie brilliant blue G-250. A low molecular weight calibration kit was used as a standard molecular weight marker for the proteins.

In-Gel Digestion

For in-gel digestion, gel slices from SDS/PAGE were carefully excised and cut into pieces, successively destained with 50% ACN. After the dye (Coomassie brilliant blue) was completely removed, the gel pieces were washed with 25 mM NH_4HCO_3 (pH 8.0) and dehydrated with pure ACN. After being dried in a SpeedVac concentrator, the gel-bound proteins were reduced with 10 mM DTT at 57°C for 1 h and alkylated by 55 mM IAA in the dark at room temperature for 45 min. Then the gel pieces were sequentially washed with 25 mM ammonium bicarbonate in water, 25 mM ammonium bicarbonate in water/ACN (1:1 v/v), and pure ACN. After freeze-dried in a SpeedVac concentrator, the dry gel pieces were reswollen with 30 μL of 25 mM NH_4HCO_3 containing 0.2 μg of trypsin. Digestion was carried out at 37°C for 19 h. For enriching peptides, peptides released during the digestion were sequentially extracted in 50 μL of 30% ACN containing 0.1% TFA, 50 μL of 50% ACN containing 25 mM NH_4HCO_3 , 50 μL of 70% ACN containing 25 mM NH_4HCO_3 by sonication for 20 min. The combined extracts were evaporated to 2 μL in a SpeedVac concentrator and stored at -80°C until use.

Shotgun Digestion and Fractionation by Liquid Chromatography

For direct digestion, 200 μg of venom powder was dissolved in 20 μL of 0.5% SDS buffer and boiled for 3 min. The proteins in the sample were reduced with 10 mM DTT at 56°C for 1 h and alkylated by 55 mM IAA in the dark at room temperature for 45 min. The venom sample was diluted to 200 μL with a solution containing 30 mM NH_4HCO_3 . Finally, tryptic digestion was carried out by 4 μg of trypsin and incubation at 37°C for 20 h. The enzymatic digestion was stopped by acidification using diluted acetic acid.

For decreasing the complexity of sample, the digested peptides were fractionated on a reversed-phase C8 column (2.0 mm $\times$ 150 mm, Agilent, Santa Clara, CA) using a Waters HPLC system (model Alliance) with a 996-photodiode array detector. Mobile phase A was 0.1% aqueous TFA, and mobile phase B was ACN containing 0.1% TFA. After the sample was loaded and desalted, peptides were eluted for 15 min with 10%,

15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 100% mobile B, respectively. The flow rate was maintained at 0.2 mL/min, and effluent absorbance was recorded at 215 nm. Eluates were separately collected and lyophilized, stored at -20°C until CapLC-MS analysis.

CapLC-MS/MS Analysis

Tryptic peptides obtained from the fractionation of direct digestion and the in-gel digestion of SDS/PAGE gel slices were separately injected into a capillary LC system (Waters) and first desalted and preconcentrated on a C18 PepMapTM precolumn (0.3 mm $\times$ 5 mm; LC Packings). The peptides were then eluted onto a C18 column (75 μm $\times$ 15 cm; LC Packings, Sunnyvale, CA) coupled to a quadrupole time-of-flight (Q-TOF) microhybrid mass spectrometer (Q-TOF microTM, Waters/Micromass, Manchester, UK) equipped with a micro-mass nano-ESI source. The gradient profile consisted of a linear gradient from 5% to 50% B (0.1% formic acid/4.9% H_2O /90% ACN, v/v/v) over 65 min into A (0.1% formic acid/4.9% ACN/95% H_2O , v/v/v), followed by a 10-min gradient to 85% B and then a 10-min gradient to 95% B. The flow rate was 2 μL /min. Eluted peptides were detected in positive ion MS mode and data-dependent MS/MS mode. The data-dependent mode was used for survey scans (m/z 400–1400) to choose up to three most intense precursor ions (with charge states ≥ 2). For collision-induced dissociation mass spectrometric (MS/MS) analysis, collision energy was chosen automatically as a function of m/z and charge. Collision gas was argon, the temperature of heated sample source was 85°C, and electrospray voltage was 3,000 V.

Data Analysis and Bioinformatics

The tandem mass spectrometry (MS/MS) data were acquired by software MassLynx 4.0 software (Waters), deisotoped and converted (by Waters ProteinLynx Global server 1.0 software) to "peak list" (pkl) files, which include the mass values, intensity (at least 5 counts per second) and charge of precursor ions. Background subtraction was set at 10%. Smooth was three times with "smooth window" (channels) 2.0 in the Savitzky Golay mode. The pkl files were analyzed using a licensed copy of the Mascot 2.0 program (MatrixScience Ltd., London, UK) on a 2.6-GHz Pentium-4 personal computer with 1 GB of RAM, which compared the files against NCBI nr with animal species restriction. Search parameters allowed for carbamidomethylation of cysteine, phosphorylation of serine, threonine, and tyrosine (variable modification), one missed trypsin cleavage, and a 1.0-Da mass accuracy. To ensure the accuracy of

protein identification, we selected the candidate proteins with individual score greater than threshold according to peptide summary report of Mascot search results at a 95% confidence level ($P < 0.05$). Furthermore, de novo sequencing was employed to validate the identification of venom proteins. The theoretical molecular weight, isoelectric point (pI), and other related information of matched proteins were retrieved from Mascot output files and their superlinked databases.

RESULTS

Sample Treatments Prior to CapLC-MS/MS Analysis

In this work, two different sample treatment methods were separately employed prior to CapLC-MS/MS analysis: SDS/PAGE plus in-gel digestion and in-solution digestion plus fractionation.

SDS/PAGE image of the venom in the first sample treatment method is shown in Figure 1. It can be seen that major components of the venom are distributed

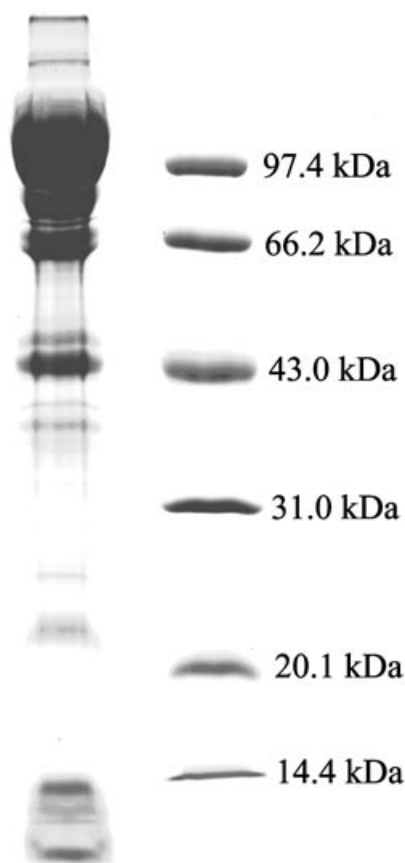


FIGURE 1. SDS-PAGE gel image of the venom collected from living adult *L. tredecimguttatus* by electrical stimulation.

in the molecular weight range of higher than 60.0 kDa as well as around 43.0 kDa. The proteins of highest abundance were of around 100 kDa. There are few proteins distributed in other ranges. To identify the separated proteins effectively, protein bands were excised from the SDS/PAGE gel, and subsequently digested with trypsin. The resulting tryptic peptides were extracted from the gel for subsequent mass spectrometric analysis.

In the second sample treatment method, the venom was directly in-solution digested with trypsin. Peptide mixture resulting from the digestion was fractionated on a reversed-phase C8 column with various isocratic gradients using a Waters HPLC system. The representative elution profiles are shown in Figure 2. Chromatograms show that peptides were eluted at most

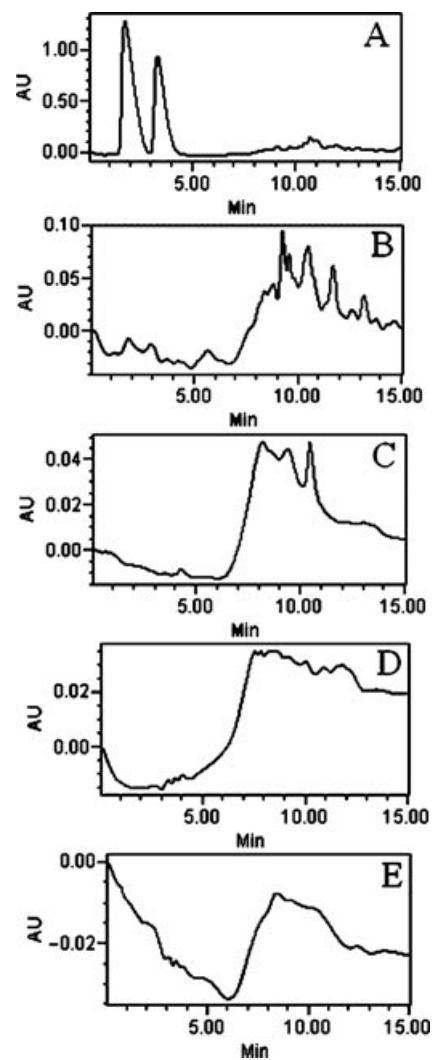


FIGURE 2. Partial elution profiles of peptide mixture from C8 column with isocratic ACN gradients. A: 10%; B: 20%; C: 30%; D: 40%; E: 50%.

isocratic gradients, so that the complexity of the sample was greatly reduced. In addition, it can be seen that when the concentration of ACN reached up to 50%, no significant amount of peptides was eluted. Each fraction was separately collected and lyophilized before analysis by CapLC-MS/MS.

Mass Spectrometry Analysis and Venom Protein Identification

All of the peptide samples obtained in the two sample treatment methods mentioned above were separately subject to the CapLC-MS/MS analysis, during which the peptide samples were further separated by reversed-phase C18 CapLC (capillary column liquid chromatography) and online detected and sequenced with the coupled Waters Q-TOF micro mass spectrometer. As a result, SDS/PAGE-CapLC-MS/MS and SDF-CapLC-MS/MS made 89 and 58 different matching proteins at a 95% confidence level ($P < 0.05$), respectively. After strict dereundance, there are 122 matching proteins, of which 75 venom proteins have distinct function, and 47 proteins are mostly predicted or hypothetical proteins with uncertain or unknown function (see the supplementary table). Although some venom proteins were matched based on one single peptide, more than 80% of the proteins were matched based on two or more peptides. Among the 75 proteins unambiguously identified, 31 (41.3%) were identified only by

SDS/PAGE-CapLC-MS/MS strategy, 22 (29.3%) were identified only by SDF-CapLC-MS/MS strategy, and 22 (29.3%) were shared by the two strategies. It can be concluded that these two strategies were complementary to one another and the combinative use of them favors the global analysis of venom proteins with different physiochemical properties.

In addition, to further validate the matched venom proteins, de novo sequencing plus Sequence Query against the protein database was performed. For example, fucosectin was one of the venom proteins matched with a Mowse score of 46 by using raw MS/MS data. In its further validation, the MS/MS spectrum of a peptide (at m/z 861.71²⁺) was de novo sequenced (Figure 3). The sequence was identified as KALSPVIPENLSSK, and the corresponding protein was identified with the Sequence Query method as fucosectin with a Mowse score of 177. In view of the significance threshold for identification by the Sequence Query was 77, fucosectin was further validated at a high confidence level. Another validation example is shown in Figure 4, which also further validated the identification of Fa2h protein.

For delineating the significantly matched proteins, these proteins were broadly classified into six groups according to their biological functions, including predicted and hypothetical functions (see Table 1 and the supplementary table): (i) known proteins of black widow spider venom, (ii) hydrolases, (iii) other enzymes, (iv) proteins of unknown function, (v) proteins with binding function, and (vi) other proteins.

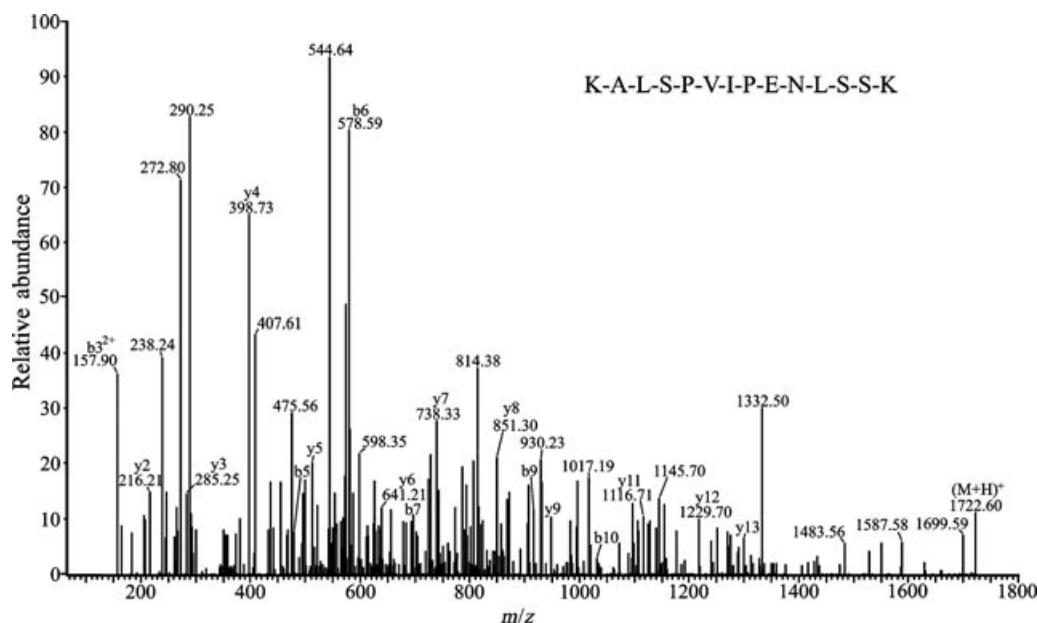


FIGURE 3. MS/MS spectrum for precursor ion 861.71²⁺. The sequence was identified as KALSPVIPENLSSK and the corresponding protein was fucosectin with a Mowse score of 177. The significance threshold was 77. S4, S12, and S13 were phosphorylated, and, however, all of them had neutral losses 97.9769 when the MS/MS was performed.

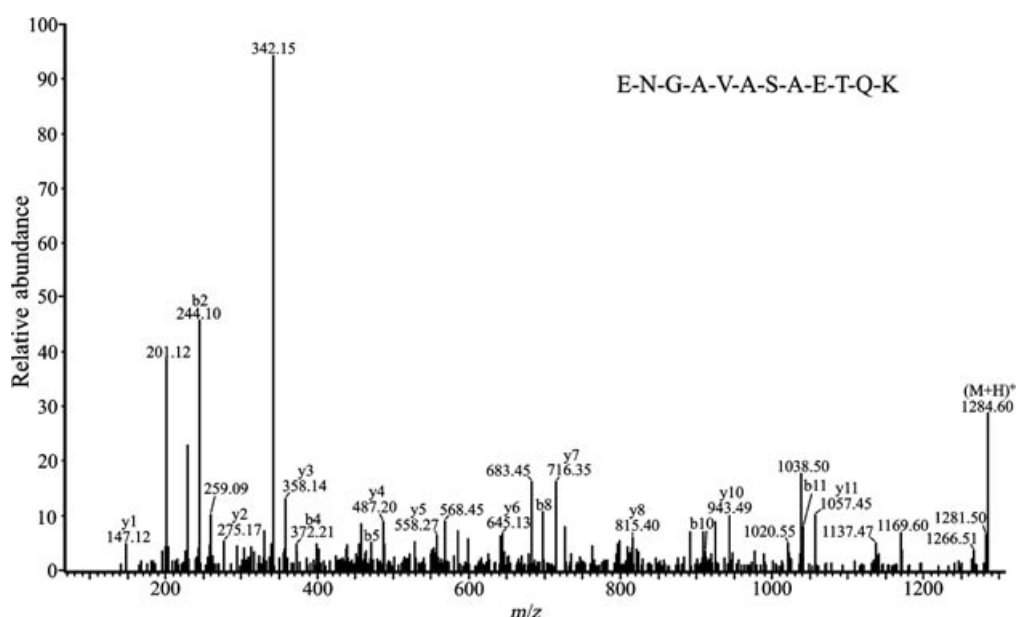


FIGURE 4. MS/MS spectrum for precursor ion 642.73^{2+} . The sequence was identified as ENGAVASAETQK, and the corresponding protein was Fa2 h protein with a Mowse score of 153. The significance threshold was 77. T10 was phosphorylated, and, however, it had a neutral loss 97.9769 when MS/MS was performed.

Obviously, this classification is not strict due to the multiple or predicted function of a protein.

Of the 122 matched proteins, 10 proteins (accounting for 8.20%) had been reported in the literature and belong to the known proteins of black widow spider venom, including main black widow spider toxins alpha-latrotoxin, delta-latroinsectotoxin, alpha-latrocrustotoxin precursor, and so on. These proteins were unambiguously identified by both of the two identification strategies (Table 1 and the supplementary table). Most of them were identified with high score and matched the peptide number. Taking alpha-latrotoxin for example, the Mascot search results obtained with

the raw MS/MS data suggested that individual ions scores greater than 44 indicate identity or extensive homology ($P < 0.05$). The score of alpha-latrotoxin was 1816, and the number of peptides matched was 43.

Thirteen matched venom proteins were hydrolases, including five proteinases (stejnefibrase 2, 20 S proteasome alpha 5 subunit, AT14086p, metalloproteinase I, and serine protease HTRA2), four phosphatases (mKIAA0606, Hro-Cdc25, PTPRB protein, and protein phosphatase 1H), three nucleases, and one phospholipase. Besides, there were 20 other enzymes found in the venom, such as kinases, transferases, and phosphorylases, and so on. This indicated that the venom of *L. tredecimguttatus* was rich in metabolic enzymes.

Twenty-three proteins (18.9%) with a binding function were found in the venom. These proteins could bind nucleic acid, protein, ATP, ribonucleotide, and so on, suggesting the diversity of the action of venom components to some extent. In the group of "other proteins," there were mainly cellular constituents, ion channel proteins, and the like. It is worth noting that there are several venom proteins with special activities classified into this group, such as leech-derived trypsin inhibitor trypsin complex, venom allergen antigen 5-like protein and fucoselectin, and so on. In addition, a total of 25 (20.5%) identified proteins were of unknown function, whose functions could not be retrieved from the databases available and need to be further investigated.

TABLE 1. Classification of Venom Proteins Matched Using Two Different Strategies

Classification	Method 1 (%) ^a	Method 2 (%) ^b	Unique Proteins
Known proteins of black widow spider venom	10 (11.2)	10 (17.2)	10 (8.2)
Hydrolases	8 (9.0)	6 (10.3)	13 (10.7)
Other enzymes	12 (13.5)	11 (19.0)	20 (16.4)
Proteins of unknown function	18 (20.2)	7 (12.1)	25 (20.5)
Protein with binding function	11 (12.4)	15 (25.9)	23 (18.9)
Other proteins	30 (33.7)	9 (15.5)	31 (25.4)
Total	89 (100)	58 (100)	122 (100)

^aSDS/PAGE-CapLC-MS/MS.

^bSDF-CapLC-MS/MS.

DISCUSSION

To date, the lack of sufficient quantities of venom has limited the detail elucidation of black widow spider venom. The main method of venom collection, particularly in the early stage of the research, is to homogenate the dissected venom glands followed by extraction [9,24,25]. Obviously, the venom obtained using this method is unavoidably contaminated by various amounts of nonvenomous cellular components. In this work, venom was collected from living adult *L. tredecimguttatus* by electrical stimulation. The collected venom is not contaminated with histiocytic proteins and other components. Therefore, it is more representative of the pure venom and is more suitable for the identification of venom proteins.

The complexity of spider venoms has long been appreciated by researchers in the fields of toxicology and medicine. However, it is the recent advances in protein separation and mass spectrometry techniques that allow virtually all venom components to be effectively separated and identified. Although proteomic techniques have been developed extensively in recent years, there is no unique method that can universally be utilized for all proteomic analyses [26]. Because of the variety of proteins, a method having strong advantages for some proteomes may have disadvantages for others. Owing to that proteins in spider venom span a wide range of molecular weights and biochemical properties, analysis by a single method could result in loss of proteins in separation as well as identification. Combined strategy for proteomic analysis can enhance the rate and reliability of protein identification. Therefore, we employed two combinative methods to make a multifaceted analysis of the total venom proteome of *L. tredecimguttatus*.

In the present experiment, a total of 122 nonredundant proteins were significantly matched in database searching by using complementary strategy including de novo sequencing. Almost all of the proteins of black widow spider venom that have been reported in the literature were unambiguously identified, indicating that our identification strategy was reliable. Besides those known proteins, a range of enzymes were detected in the venom, including proteinase, phosphatase, phospholipase, nucleases, phosphorylases, and so on, which suggested that *L. tredecimguttatus* venom was an enzyme-rich venom. This is in accordance with our previous experimental results, which indicated that the venom showed activities of multiple hydrolases including protease, DNAase, and phosphatase, and so on [27].

Up to now, the mechanism of action of black widow spider venom has not been completely understood. It

was speculated that the intoxication after a bite by *L. tredecimguttatus* must be related to, except for latrotoxins, other venom components. Literature survey indicated that protease, phospholipase, phosphatase, nuclease, and some other enzymes have been described in several snakes and spiders such as *Elapidae*, *Viperidae*, and *Loxosceles* venoms. Some hydrolases, particularly the proteases such as metalloproteases and serine proteases, were demonstrated to have some participation in the noxious effects of the venoms [28–30]. The pathogenic properties of *Loxosceles* venoms are even attributable to the presence of proteolytic enzymes that degrade basement membrane molecules including laminin, entactin, type IV collagen, and heparan sulfate proteoglycan [31–36]. Several extracellular matrix molecules such as fibronectin, fibrinogen, entactin, and heparan sulfate proteoglycan have been described as targets for degradation evoked by proteases present in *L. intermedia* venom [34]. Therefore, the hydrolases such as 20 S proteasome alpha 5 subunit and stejnefibrase 2 found in the *L. tredecimguttatus* venom might also play important roles in the latrodectism. At the same time, other venom proteins identified in the venom such as fucosectin, venom allergen antigen 5-like protein and trypsin inhibitor were also speculated to be related to the actions of *L. tredecimguttatus* venom. Taking fucosectin for example, a large number of c-type lectin-like proteins were found in *Cerastes cerastes* venom and were considered to contribute to the action of the venom [37]. Among the matched proteins, many proteins have the binding function. Obviously, the presence of these proteins in the venom extends its scope and model of action. In addition, there are 47 proteins being matched to predicted protein, hypothetical protein, and protein of unknown function, respectively. They are also potential active components of the venom. However, their biological effects in the venom to be determined in another study.

Interestingly, some proteins that appeared not likely to be present in the venom were identified, such as membrane proteins (e.g., olfactory receptor MOR13–5 and voltage-gated potassium channel subunit Kv10.1), transcription factors, elongation factors, and hemocyanins, and so on. Because of to the careful operation in the venom collection by electrical stimulation, it was speculated that these proteins were not from the contamination by other body fluids, but from the normal secretory epithelial cells of venom glands, because the secretory epithelial cells of the venom glands periodically burst and expel their contents in the lumen of the glands [38]. As regard to hemocyanins, it has generally been accepted that hemocyanins were the particular components of blood and lymphatic liquid from many arthropod species. However,

hemocyanins that were identified in the *L. tredecimguttatus* venom were also found in the venoms of several distinct species of spiders such as *Ornithoctonus huwena* Wang and *Chilobrachys jingzhao* [39,40]. Therefore, these proteins should be the normal constituent components of spider venom.

SUPPLEMENTARY MATERIAL

Additional information on the “venom proteins matched using two different strategies” as the supplementary table is available from the corresponding authors on request (wang_xianchun@263.net and liangsp50@yahoo.com.cn).

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