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# Isolation and identification of a sodium channel-inhibiting protein from eggs of black widow spiders

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ABSTRACT

The eggs of black widow spider (*L. tredecimguttatus*) have been demonstrated to be rich in biologically active components that exhibit great research value and application foreground. In the present study, a protein toxin, named Latroeggtoxin-II, was isolated from the eggs using the combination of gel filtration, ion exchange chromatography and reversed-phase high performance liquid chromatography. Electrospray mass spectrometric analysis indicated that the molecular weight of the protein was 28.69 kDa, and Edman degradation revealed that its N-terminal sequence was ESIQT STYVP NTPNQ KFDYE VGKDY—. After being abdominally injected into mice and *P. americana*, the protein could make the animals especially *P. americana* display a series of poisoning symptoms. Electrophysiological experiments demonstrated that the protein could selectively inhibit tetrodotoxin-resistant $Na^+$ channel currents in rat dorsal root ganglion neurons, without significant effect on the tetrodotoxin-sensitive $Na^+$ channel currents. Using multiple proteomic strategies, the purified protein was shown to have only a few similarities to the existing proteins in the databases, suggesting that it was a novel protein isolated from the eggs of black widow spiders.



## 1. Introduction

The venoms of many kinds of spiders such as black widow spider (*Latrodectus tredecimguttatus*) are known to contain a multitude of components with different biological activities. Among them, many active components are of interests as tools for the studying neurophysiology and as potential lead structures for insecticides and pharmaceuticals [1–3]. However, it may not be widely known that the black widow spider, different from many other poisonous animals such as snake, scorpion and some other spider species that have toxins only in the venoms secreted by their poisonous glands, has toxic components not only in its poisonous glands, but also throughout its body, including the legs and abdomen, and even in the eggs and the newborn spiders [4–6]. Study of the toxins in the materials other than the poisonous glands and the investigation of the possible relationship between these toxins and those in the venom of the spider have important theoretical and practical significances. Therefore, from very early on, the toxicity of black widow spider eggs and its possible relationship with that of the venom have aroused great interest in many researchers [6–9]. However, no systematic researches on this special biological material have been reported so far, which may be in large part due to the limited amounts of the material and the complexity of egg protein composition that complicate the research process [4,8]. In an attempt to gain insight into the toxicity of the eggs, our previous work using a proteomic strategy analyzed the protein composition of the black widow spider eggs and probed into its relationship with egg toxicity. It was found that the protein composition of the eggs was more complex than that of the venom secreted by poisonous glands and there were only a few similarities between the protein compositions of the two materials, suggesting that the eggs have a toxic mechanism different from that of the venom [10]. Moreover, a neurotoxic protein (named Latroeggtoxin-I) with a molecular weight of 23.752 kDa was purified from the eggs by gel filtration combined with ion exchange chromatography. Utilizing the determined N-terminal sequence of the protein, protein database searching with the sequence query method of Mascot engine indicated that there was no matched sequence from any known proteins, and BLAST analysis showed that no significant similarity was found between the purified protein and the existing proteins, demonstrating that the protein was a novel protein

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purified from the eggs of black widow spiders [11]. In the present work, another active protein was isolated from the black widow spider eggs using a combinative purification strategy. The egg extract was first fractionated with gel filtration chromatography and then the desired fraction was further separated using an ion exchange chromatography. Finally, the protein was further purified by the reversed-phase high performance liquid chromatography (RP-HPLC) with a C4 column. The main physicochemical and biological properties including molecular weight, N-terminal sequence and its effect on the $Na^+$ channel currents in rat dorsal root ganglion (DRG) neurons were determined. Database search indicated that the purified protein, named Latroeggtoxin-II, was a novel toxic protein from the eggs of black widow spiders.

## 2. Materials and methods

### 2.1. Chemical materials

Molecular sieve gel Sephacryl™ S-200 was purchased from GE Healthcare. An ion exchanger TOYOPEARL DEAE-650M was from TOSOH Corporation. Acetonitrile (ACN), trifluoroacetic acid (TFA) and TPCK trypsin were from Sigma. Acrylamide, Bis, Tris, glycine, and SDS-PAGE protein standards were from Fermentas. Ammonium persulfate, urea, agarose, glycerol, bromophenol blue and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were from Amersham Pharmacia Biotech. All other chemicals were of HPLC or analytical grade.

### 2.2. Preparation of egg extract

The soluble components in the black widow spider eggs about 1–2 weeks before hatching of newborns were extracted according to the methods previously described [4,11]. Briefly, the eggs were homogenized in a neutral PBS buffer of weak ionic strength or $ddH_2O$ with a mortar and pestle. The ratio of eggs to buffer was about 1:3 (w/v). The homogenate was cleared by centrifugation at 10,000 × *g* for 10 min at 4 °C and the supernatant was collected. The pellet was repeatedly homogenized and extracted twice. The supernatants were pooled, lyophilized or appropriately concentrated in vacuum and stored at −20 °C till further use.

### 2.3. Extract fractionation

The fractionation of egg extract was performed with a gel filtration chromatographic column (2.6 cm i.d. × 60 cm long) packed with Sephacryl™ S-200 on a Waters™ 650 Advanced Protein Purification System equipped with a model 485 detector. After the extract sample was loaded to the column initially equilibrated with a dilute PBS buffer solution, the components in the sample were eluted with the buffer solution at a flow rate of 2.5 ml/min. The optical density of eluate was monitored at 280 nm and the fractions were separately collected. After being appropriately concentrated, the molecular weight distributions of proteinous components in the respective fractions were analyzed with SDS-PAGE and preliminary toxicity detection was performed.

### 2.4. Anion exchange chromatography

The fraction of interest from gel filtration chromatography was further separated with a TOYOPEARL DEAE-650M anion exchange column (5 mm i.d. × 10 cm long) on the Waters™ 650E Advanced Protein Purification System. After the column was sequentially washed with buffer A (50 mM Tris–HCl, 1.0 M NaCl, pH 8.5) and buffer B (50 mM Tris–HCl, pH 8.5), the sample was loaded and then eluted by gradually increasing the concentration of NaCl in the buffer B. The optical density of eluate was monitored at 280 nm using a Waters™ 486 tunable absorbance detector. The protein composition of the resulting fractions was analyzed with SDS-PAGE.

### 2.5. Reversed-phase HPLC

The desired fraction from anion exchange chromatography was desalted and further purified using an XBridge™ BEH300 C4 reversed-phase column (4.6 mm × 150 mm, Waters, USA) on a Waters HPLC system (Model Alliance) with a 996-photodiode array detector. Mobile phase A was 0.05% TFA, and mobile phase B was ACN containing 0.05% TFA. After the sample was loaded, the column was eluted to remove the salts and further separate the absorbed proteins using a gradient as follows: 0–10 min, 100% A, 10–60 min, 0–50% B; 60–65 min, 50–100% B, followed by 100% B for 5 min. The flow rate was 1.0 ml/min. Effluent absorbance was recorded at 280 nm. The resulting fractions were separately collected and lyophilized. SDS-PAGE was employed to detect the purity of the protein sample of interest.

### 2.6. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [12] under denaturing conditions on a 10% polyacrylamide slab gel to detect the protein molecular weight distribution as well as the purity of samples. The samples were dissolved in a loading sample buffer (100 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 200 mM DTT, a trace of bromophenol blue) and boiled for 3 min. After centrifugation at 10,000 × *g* for 10 min, the proteins in the supernatants were loaded and separated through the gel electrophoresis. The resolved proteins in the gel were fixed with 10% acetic acid/40% methanol and visualized by staining with Coomassie brilliant blue G-250.

### 2.7. Biological and electrophysiological assays

In order to determine whether the purified protein had toxicity, abdomen-injection of the protein into mice and American cockroaches and the electrophysiological experiments were performed. Whole-cell patch-clamp technique was employed to detect the possible effect of the purified protein on the ion channels in rat dorsal root ganglion (DRG) neurons. DRG neurons were acutely dissected from 30 day old Sprague-Dawley rats of either gender and maintained in short-term primary culture according to the methods described previously [13,14]. Suction pipettes (2.0–3.0 MΩ) were made of borosilicate glass capillary tubes. Ion channel currents in experimental DRG neurons were recorded at room temperature (20–25 °C). When the effect of the purified protein on the sodium currents in DRG neurons was investigated, the pipettes solution (pH 7.2) contained (in mM):CsCl 145, $MgCl_2$•6 $H_2O_4$, HEPES 10, EGTA 10, glucose 10, ATP 2, whereas the external solution (pH 7.4) contained (in mM):NaCl 145, KCl 2.5, $CaCl_2$ 1.5, $MgCl_2$•$6H_2O$ 1.2, TEA-Cl 90, HEPES 10, EGTA 10, glucose 10. In order to investigate the effect of the purified protein on the sodium currents of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistent (TTX-R) sodium channels, the DRG neurons with diameter of >40 or <20 μm were used, respectively, because the larger cells tended to express TTX-S sodium channels whereas the smaller ones tended to express TTX-R sodium channels [15]. 0.2 μM TTX was used to inhibit TTX-S sodium currents to obtain TTX-R sodium currents [16].

All studies with laboratory animals were conducted in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals" and applicable institutional national law.