JOURNAL OF INTEGRATED OMICS

A METHODOLOGICAL JOURNAL

HTTP://WWW.JIOMICS.COM

Electrophysiological and Proteomic Studies of *Protobothrops mangshensis* Venom Revealed Its High Bioactivities and Toxicities

Yu Liu[1,2,#], Xiaojuan Wang[2,#], Zhe Wu[1], Ping Chen[2], Yang Xiang[1], Dongsong Nie[1], Jianmin Yi[1], Jizu Yi[1,*], and Zhonghua Liu[2,*]

[1]College of Chemistry and Chemical Engineering, Hunan Institute of Science and Technology, Yueyang, 414006, China; [2]College of Life Sciences, Hunan Normal University, Changsha, 410081, China; [#]Those authors contributed equally to this study.

ABSTRACT

Snake venoms contain rich components having medical and biotechnological values. The proteomic characterization of snake venom proteome has thus potential benefits for basic research, clinical diagnosis, and development of new drugs for a variety of diseases. *Protobothrops mangshensis* is a monotypic genus of pit viper known only from Mountain Mang in Hunan Province of China, and represents the largest and the most spectacular snake among Asian venomous snakes. The venom of *Protobothrops mangshensis* exhibits a high coagulant activity on bovine and human fibrinogen and human plasma, a high phosphodiesterase activity and an arginine ester hydrolytic activity. In this study, the *Protobothrops mangshensis* venom was analyzed by 2D-gel electrophoresis separation, subsequently by in-gel digestion, MS/MS identification, and enzymatic activity analysis. Our results demonstrated that *Protobothrops mangshensis* venom comprised highly functional proteins and/or enzymes, and each of these proteins displayed multiple isoforms separated in 2-DE. Approximately 59.4% of the identified total 143 proteins had enzymatic activities and 24.5% were involved ion channels, representing highly complex and extensive bioactivities of the snake venom. The identified toxins included six protein families: serine proteinases, L-amino acid oxidase, phospholipases A2, C-type lectin-like proteins, cysteine-rich secretory proteins and metalloproteinase-disintegrin, and were correlated well with the clinical manifestations by *Protobothrops mangshensis* bite such as coagulopathy, oedema, hypotensive and tissue damaging effects. Electrophysiological studies showed that the snake venom inhibited tetrodotoxin-resistant (TTX-R) Na+ currents. All our results in this study provided the first functional proteomics of *Protobothrops mangshensis* venom.

Keywords: Snake venom; Venomics; Protein family; 2-D electrophoresis; Proteomics.

1. Introduction

A snake venom fluid contains varieties of proteins and peptides with different biological activities including enzymatic, cytotoxic, neurotoxic, and hemagglutinic activities [1]. Some of the components found in a snake venom sample are of interests as tools for studying neurophysiology and/or as potential lead structures for new drug development. Recently there were several reports that snake venoms were used in drug development for novel human disease therapeutics. These included the antihypertensive drug captopril, the molecule modeled from the venom of the Brazilian arrowhead viper (*Bothrops jaracusa*) [2]; the anticoagulant Integrilin (eptifibatide), one heptapeptide derived from a protein found in the venom of the American southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*) [3]; Ancrod, one compound isolated from the venom of the Malaysian pit viper (*Agkistrodon rhodostoma*) for treating heparin-induced thrombocytopenia and stroke [4]; and alfimeprase, one novel fibrinolytic metalloproteinase for thrombolysis derived from southern copperhead snake (*Agkistrodon contortrix*) venom [5]. Due to the limits of the sensitivity and resolution of commonly used methods for protein separation, the complexity and

*Corresponding author: Dr. Jizu Yi, College of Chemistry and Chemical Engineering, Hunan Institute of Science and Technology, Yueyang, 414006, China, phone number:+86-730-8640122, fax: +86-730-8640122, e-mail adress: Jizu_Yi@Yahoo.com; Dr. Zhonghua Liu, College of Life Sciences, Hunan Normal University, Changsha, 410081, China, phone number: +86-731-88872556, fax: +86-731-88861304, e-mail adress: liuzh@hunnu.edu.cn

diversity of components in snake venom were usually underestimated. Many compounds with low abundance were often neglected. Two-dimensional polyacrylamide gel electrophoresis (2-DE) was one of efficient methods for separating several hundred proteins based on the differences in their pIs and molecular masses. The immobilized pH gradient (in the first-dimensional) gel strip-based 2-DE separation, followed by image analysis, in-gel digestion, digested protein sample handling, and mass spectrometry (MS) analysis together with bioinformatic algorithms for searching sequences in databases, present currently a typical proteomic method well suitable for global and detailed studies of the entire proteome from a certain cell type, or a tissue of an organism, or a body fluid such as a snake venom. The progress in MS technology has dramatically accelerated the application of proteomics during recent years. Venoms from various venomous animals have been analyzed using MS technologies such as MALDI-TOF and LC/ESI-Q-TOF [6–10]. These studies have demonstrated the advantages of using proteomic approaches in venom mapping [8–10], therapeutic target screening, and species' classification [11]. *Protobothrops mangshensis* was firstly discovered in the forests on Mount Mang at elevations from 700 to 1300 m, located at Pingkeng District, Yizhang County, Hunan Province of China [12, 13]. To date, the known place where these snakes live was restricted to this mountain area ranging only a few tens of square kilometers [12, 13]. An adult *Protobothrops mangshensis* grows to body length of about 2.0 m, weight of about 2-4 kg, and is known to prey mainly on rodents [12]. It was also named as *Trimeresurus mangshanensis*, *Zhaoermia mangshanensis* or *Ermia mangshanensis* [14, 15], but recently reclassified as the genus *Protobothrops* based on its mitochondria gene analysis [16].

The crude venom from a *Protobothrops mangshensis* contained a wide variety of proteins and peptides, and was highly lethal to mice with an intraperitoneal LD_{50} of 4.2 mg/kg body weight [17]. The envenoming elicited coagulopathy, local hemorrhage, inflammation, edema, blood blisters, myonecrosis, and severe pain on human victims [17]. The treatment of an envenomation by a snake bite was critically dependent on the availability of effective antivenoms. Detailed knowledge of the identities and relative amounts of the different toxins in the given venom was thus required for generating immunization protocols to elicit toxin-specific antibodies showing greater specificity and effectiveness than conventional methods relying on the immunization of large mammals with whole venom. Here, we reported an electrophysiological and proteomic study of the venom collected from *Protobothrops mangshensis*. Our results demonstrated that the venom contained a variety of enzymes and proteins including serine proteinases, L-amino acid oxidase, phospholipases A2, C-type lectin-like proteins, cysteine-rich secretory proteins, metalloproteinase-disintegrin, and more others. The venom toxin composition was correlated with the clinical manifestation of the *Protobothrops mangshensis* bite, explaining its pathological effects such as coagulopathy, oedema, as well as hypotensive and tissue damaging effects. Electrophysiological results showed that the venom fractions inhibited tetrodotoxin-resistant (TTX-R) Na^+ currents, but not on tetrodotoxin-sensitive (TTX-S) Na^+ currents. Together, these results provided a detailed characterization of the venom proteome of the snake for a deeper understanding the biological effects of the venom, and served as a starting point for further structure-function studies of individual toxins.

1. Materials and methods

1.1 Chemicals

Sephadex G-75, IPG (pH 3–10), DryStrips (3–10 linear), cover fluid, agarose, and colloidal Coomassie Brilliant Blue (CBB) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Dithiothreitol (DTT), iodoacetamide, trypsin, and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, USA). Acrylamide, bis-acrylamide, urea, thiourea, glycine, tris, α-Cyano-4-hydroxycinnamic acid (CHCA), and sodium dodecyl sulfate (SDS) were from of Amresco (Solon, OH, USA). Acetonitrile (ACN, chromatogram grade) and other chemicals (analytical grade) are from Sinopharm Group (Shanghai, China). Deionized water prepared with an Aquapro high-end water treatment solution provider system (Ever Young, China) was used for all buffers in this study.

1.2 Snake venom

Protobothrops mangshensis venom was collected from two adult female snakes maintained in Mangshan Institute of Snakes. The snakes were manually restrained and venom fluids were collected with an opened glass container. Approximately 2.0 ml of yellowish collected venom liquid was lyophilized immediately at cold temperature to minimize possible preanalytical variability during the sample handling [18, 19]. Total ~ 1200 mg dry powder of the venom sample was obtained and stored at 4℃ until usage.

1.3 Electrophysiological studies

Whole cell sodium currents were recorded from rat dorsal root ganglion (DRG) cells at room temperature. Recording pipettes were made from borosilicate glass capillary tubing, and their resistances were 1-2 megaohms when filled with internal solution containing: 135.0 mM CsF, 10.0 mM NaCl in 5.0 mM HEPES at pH 7.0. External bath composition was: 30 mM NaCl, 5.0 mM CsF, 25 mM D-glucose, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20.0 mM triethanolamine-chlorine, and 70 mM tetramethylammonium in 5.0 mM HEPES (pH 7.4). Ionic currents were filtered at 10 kHz and sampled at 3 kHz on EPC-9 patch clamp amplifier (HEKA Electronics). Linear capacitive and leakage currents were subtracted by using a P/4 protocol. Experimental data were acquired and

analyzed by the software program pulse+ pulsefit 8.0 (HEKA Electronics, Germany). The toxin dissolved in external solution until desired concentrations was applied onto the surface of experimental cells by low pressure injection with a microinjector (IM-5, Narishige, Japan).

1.4 *Separation of venom proteins by two-dimensional gel electrophoresis (2-DE)*

2-DE was performed according to the method described previously [20]. IEF was carried out on an IPGphor system (Amersham Pharmacia Biotech, USA). Venom protein samples (300 mg) pooled from protein fractions collected after gel-filtration chromatography were first mixed with a rehydration solution containing 8 M urea, 2 M thiourea, 4.0 % CHAPS, 20 mM Tris-base, 18.0 mM DTT, and traces of bromophenol blue in 0.5 % (v/v) IPG buffer to give a total volume of 350 mL, and applied to IPG dry strips (pH 3-10, 180×30×0.5 mm) for the rehydration up to 14 hrs. IEF was then carried out at step-n-hold conditions: 500 V for 1 h; 1000 V for 1 h; and 8000 V for 6 hrs. at 50 Ma/strip. After focusing, the strips were soaked for 20 min in reduction solution (6 M urea, 30 % glycerol, 2 % SDS, and 125 mM DTT) followed by 20 min in alkylation solution (6 M urea, 30 % glycerol, 2.0 % SDS, and 125 mM iodoacetamide). The second dimensional separation with SDS-PAGE based on the molecular size differences was performed in 10 % polyacrylamide gels using a Protean II system (Bio-Rad, Hercules, CA, USA). The gels were then stained with CBB G250 and scanned using ProXPRESS 2D Proteomic Imaging System (Perkin-Elmer, Waltham, MA, USA). Protein spots were detected and analyzed using PDQuest software Version 6.1 (Bio-Rad).

1.5 *In-gel protein digestion and MS analyses using ESI-Q-TOF and MALDI-TOF-TOF MS*

The CBB-stained protein spots were excised and digested in-gel with trypsin as described previously [21]. Peptide mixtures from in-gel digestion were extracted and analyzed by an ESI-Q-TOF MS/MS (Waters, Milford, MA, USA) with the nanoelectrospray for ionization as described previously [22]. We selected the candidate peptides for identifications based on the probability-based Mowse scores (total score) which exceeded their thresholds indicating a significant (or extensive) homology ($p < 0.05$), and referred them as "hits" as defined by Matrix Science, Ltd. (London, UK). Proteins identified with two or more peptides matched with a Mowse score greater than 40 of each were validated without further investigations. Those with at least two peptides matched with a score less than 40 and greater than 20 of each peptide were systematically checked manually to confirm or cancel the MASCOT suggestion. For a protein identified by only one matched peptide, the Mowse score of the MS/MS search must exceed 40, and the peptide sequence was checked manually to confirm the protein ID.

Tandem mass spectra acquired on an ESI-Q-TOF mass spectrometer were interpreted de novo using masslynx 4.0 software (Waters). Contiguous stretches of seven or more amino acid residues with a 100% confidence call using the software's default parameters were collected and matched to the NCBI non-redundant protein database using the protein BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

The tryptic digestion peptide mixture from a 2-DE gel spot without a matched result by ESI-Q-TOF MS/MS were loaded onto an AnchorChip target plate (Bruker-Daltonics, Germany) according to previous report [23]. Peptide identification was performed using a MALDI-TOF-TOF mass spectrometer (UltraFlex I, Bruker-Daltonics, Germany) equipped with a nitrogen laser (337 nm) and operated in reflector/delay extraction mode for both peptide mass fingerprint (PMF) and MALDI-TOF-TOF MS methods. The MS spectra were obtained by a fully automated mode through the flexControl software (Bruker-Daltonics). An accelerating voltage of 25 Kv was used for PMF. The peaks with S/N $\geqslant$ 5 and resolution $\geqslant 2500$ were selected for further TOF-TOF MS analysis from the same target. The acquired PMF MS and peptide TOF-TOF MS spectra were combined and searched using Mascot (Matrix Science) with peptide mass tolerance of 50 ppm, and MS/MS tolerance of 1.0 Da. The protein identifications were accepted when the total Mowse score of the "hit" exceeded the threshold significance score more than 70 ($p < 0.05$).

2. Results and Discussion

2.1 *Effects of Protobothrops mangshensis venom on ion channel currents*

It was widely accepted that voltage-gated sodium channels (VGSCs), voltage-gated potassium channels (VGPCs), and voltage-gated calcium channels (VGCCs) existed in rat dorsal root ganglia (DRG) neurons. By whole-cell patch-clamp recording，we first investigated the effects of the venom from *Protobothrops mangshensis* on voltage-gated Na+，K+and Ca2+channels in adult rat DRG neurons. We observed that *Protobothrops mangshensis* venom had no evident effect on tetrodotoxin-sensitive (TTX-S) Na+ currents (Fig. 1A). However, it was able to inhibit tetrodotoxin-resistant (TTX-R) Na+ currents and the inhibition was dose-dependent (Fig. 1B). The venom had also no evident effect on delayed rectifier potassium current (Fig. 1C) and low voltage-activated calcium current (Fig. 1D), but a little effect on high voltage-activated calcium current (Fig. 1E). The results indicated that the venom components blocked effectively on TTX-R sodium channels and less effectively on high voltage-activated calcium channels. To further confirm this effect, we separated the venom by a gel-filtration chromatography and collected the fractions (I, II, III and IV) for their individual tests (Fig. 2). We observed that fractions I and II had no significant effect on TTX-R sodium currents (Figs. 2B & 2C) and the
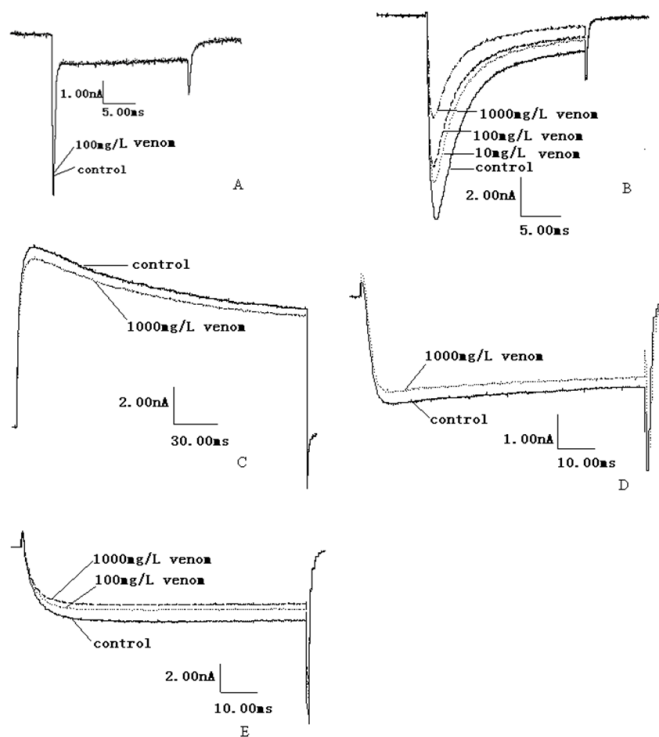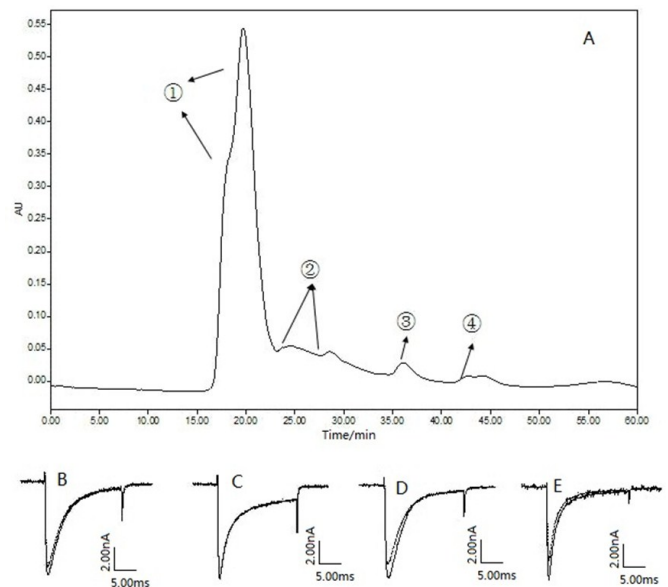
Fig. 1. Effects of *Protobothrops mangshanensis* crude venom on voltage-gated ion channels on rat DRG cells. (A) The TTX-S Na⁺ currents were elicited by 50 ms voltage steps to -10 mV from a V_h of - 80mV. The currents were almost unaffected by 100 mg/L venom (n=4); (B) TTX-R sodium currents were elicited by depolarizing the cell from a holding potential of – 80 mV to -10 mV. The duration of the test pulses was 50 ms. 10 mg/L venom caused a significant decrease in peak current amplitude. The inhibition was dose-dependent. (C) The delayed rectifier K⁺ currents were elicited by a 30 ms depolarization to +30 mV from a V_h of - 80mV. The currents were almost unaffected by 1,000 mg/L venom. (D) Low voltage-activated currents were induced by a 150 ms depolarizing potential of -30 mV from a holding potential -90 mV, and current traces were not changed before and after the application of 1,000 mg/L venom. (E) High voltage-activated currents were elicited by 150 ms depolarizing voltage of 0 mV from a holding potential of 40 mV, and current traces were slightly changed before and after the application of 1,000 mg/L venom.

inhibitory effects of fractions III and IV increased on TTX-R sodium currents (Figs. 2D & 2E). The results demonstrated that the TTX-R sodium channel blockers presented within the composition of the fractions III and IV.

2.2 *Proteomic profile of venom proteins of Protobothrops mangshensis*

Due to the limits in the detection sensitivity and resolution of commonly used methods for protein separation, the complexity and diversity of snake venom proteins were traditionally underestimated. Many protein components with low abundance were often omitted in previous investigations. Here, we used a proteomic strategy to obtain more comprehensive identifications of the snake venomome (or venom proteome). The venom was first separated by 2-D gel electrophoresis (Fig. 3), followed by in-gel trypsin digestion, and MS as well as MS/MS analyses.
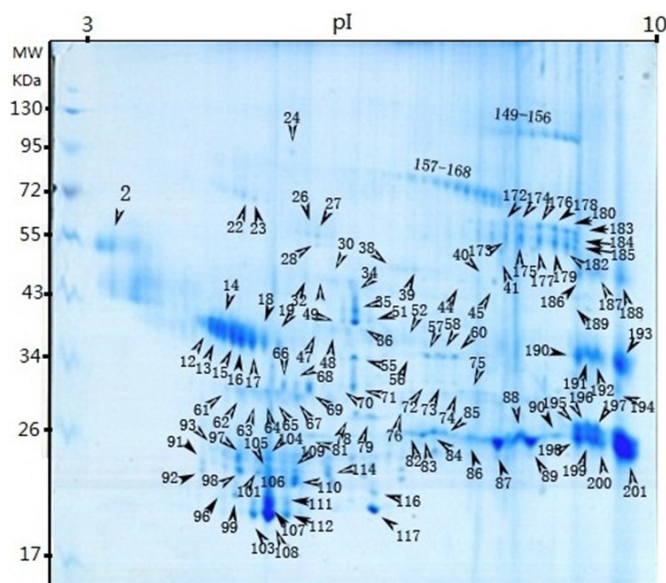

Fig. 2. Effects of *Protobothrops mangshanensis* venom fractions after Gel filtration chromatography on TTX-R sodium currents. The current traces were not changed before and after the application of fractions ① (B) and ② (C), but decrease moderately after adding fraction ③(D) and fraction④ (E). The results showed that the TTX-R sodium channel blockers present in these two fractions.

Due to the absence of a snake venom database, the LC MS/MS and MALDI TOF-TOF MS spectra were searched against the theoretical peptide MS of Metazoa proteins in NCBI and Swiss-Prot databases, with total 43 proteins being identified from Swiss-Prot. Some spots from 2-DE displayed high-quality ESI-Q-TOF MS/MS spectra but resulted in no matched sequences. For those spots the spectra were subjected to BLAST from NCBI, and 100 more proteins were identified. We categorized the identified proteins according to their functions on the basis of universal GO annotation terms, and found that 59.4 % of the total proteins were enzymes including types of proteinases, oxidases, phospholipase, and son on; 24.5 % were ion channel proteins; 9.8 % behaved as cellular binding and structural proteins; 3.5 % were signal transport proteins, and only 2.8 % other proteins were unknown in their functions. The fact that the greatest percentage (~ 60 %) of the identified proteins was enzymes and the second (~ 25 %) were associated with ion channels indicated that the biological activities of a venom fluid were extremely intensive and extensive.

2.3. *Variability, bioactivity and toxicity*

The identified toxins were found mainly belonged to the following six protein families: phospholipases A2, L-amino acid oxidases (LAAOs), C-type lectins, and cysteine-rich secretory proteins (CRISP), metalloproteinase-disintegrin, and serine proteases (see Supplementary material, Table 1). Specifically, among the identified venom serine proteinases (VSP) included VSP 1 (spots # 61, 62, 63, 64, 70, 97, 98, 101),

Fig. 3. 2-DE map of proteins from *Photobothrops mangshanensis* venom. Protein spots were visualized by CBB G 250 staining. The spots analyzed by MS were designated by numbers; their identifications were listed in Table 1.

VSP 2 (spot 194), VSP 2A (spot 188), and VSP 2C (spots 67, 68, 78, 79) (Fig. 3 and Supplementary material, Table 1) in addition to several other thrombin-like serine proteinases (see bellow), implying its high proteolytic activities of the snake venom fluid.

Coagulopathy was one of the major toxic effects exerted by the *photobothrops mangshanensis*. The venom proteins revealed by our proteomic analysis were in accordance with this clinically observed blood clotting disorders caused by a snake bite. Indeed, several thrombin-like proteases associated with blood clotting cascade were identified, among which were pallabin (spot 56), flavoxobin (spot 65), mucrofibrase-5 (spots 67, 75, 81), halystase-like proteinase (spots 191, 193) and conortrixobin (Fig. 3 and Supplementary material, Table 1). Furthermore, multiple isoforms of the thrombin-like serine proteases were separated by 2-DE and many of these toxins were found in the middle part of the gel (Fig. 3). As one representative of such thrombin-like serine proteinases, contortrixobin was isolated and identified from multiple spots (# 63, 65, 66, 67, 68, and 70 on Fig. 3). The multiple isoforms of a single protein were observed commonly in this study, demonstrating the high variability of the *Protobothrops mangshensis* venom proteins.

In terms of its structure and functions, contortrixobin had strong homology with snake venom serine proteases acting on either fibrinogen or other blood coagulation components. The interaction of contortrixobin with chromogenic substrates indicated a higher specificity for arginine than lysine in the primary subsite and a faster attack to ester than amides [24]. Interestingly, contortrixobin preferentially released fibrinopeptide B from human fibrinogen and activated blood coagulation Factors V and XIII with a rate 250-500-fold lower than human alpha-thrombin. However

contortrixobin did not induce the aggregation of thrombocytes, the increase of intracytoplasmatic calcium ions in platelets, and the activation of Factor VIII [24].

However, halystase-like proteinases (spots 191, 193 on Fig. 3) were expected to have the activity similar to that of other thrombin-like snake venom serine proteases: to hydrolyze the fibrinogen chains at the sites different from thrombin and kininogen producing bradykinin, which leaded to hypotension. No coagulation effect on the human plasma by the identified halystase-like proteases was expected [25].

The coagulation factor IX/factor X-binding proteins were also isolated and identified (Fig. 3, spots 103, 104, 106, 107, 108, 109, and 111). The coagulation factor IX/factor X-binding protein (IX/X-bp) from the venom of *Trimeresurus flavoviridis* was reported to be a heterogeneous two-chain protein, and the structure of each chain was similar to that of the carbohydrate-recognition domain of C-type lectins. The analysis of the binding properties of IX/X-bp revealed that it bound to the gamma-carboxyglutamic acid (Gla)-containing domains of factors IX and X [26]. Here, we also identified galactose-binding lectins (Fig. 3, spots 82, 83, 84, 85, and 86), which recognized the specific carbohydrate structures and agglutinated a variety of animal cells by binding to cell-surface glycoproteins and glycolipids. A galactose-binding lectin was a calcium-dependent protein which showed a high hemagglutinating activity [27].

We also identified zinc metalloproteinase-disintegrin jerdonitin from the photobothrops mangshanensis venom (Fig. 3, spots 44, 49, 52, 58, 73, 74) (also see Supplementary material, Table 1). This protease inhibited ADP-induced human platelet aggregations and belonged to the class II of snake venom metalloproteinases (SVMPs) (P-II class). Different from other P-II class SVMPs, the metalloproteinase and disintegrin domains of this natural protein were not separated. It has two additional cysteine residues located separately in the spacer domain and disintegrin domain. The cysteine residues probably formed a disulfide bond which bound the metalloproteinase and disintegrin domains together during posttranslational processes [28].

L-amino acid oxidase (LAAO) was another major component of the *Photobothrops mangshanensis* venom. Eleven isoforms of this enzyme were detected after 2-DE and MS/MS analysis of tryptic peptides (Fig. 3 and Supplementary material, Table 1). The proteins isolated from the spots 172, 174, 175, 176, 177, 178, 180, 181, 183, 184 and 185 had closely same molecular masses of approximately 58 kDa, a typical mass of LAAO, but different isoelectric points in the alkaline region. Although the knowledge about the biological roles of LAAOs was limited, it was reported that these enzymes induced apoptosis, hemorrhagic effect and cytotoxicity [29]. These activities could be attributed to the high number of LAAO isoforms in the venom. With the similar molecular weight, these multiple isoforms of the LAAO, as well as other protein isoforms, were generated by either post-translation modifications or *in vivo* mutual
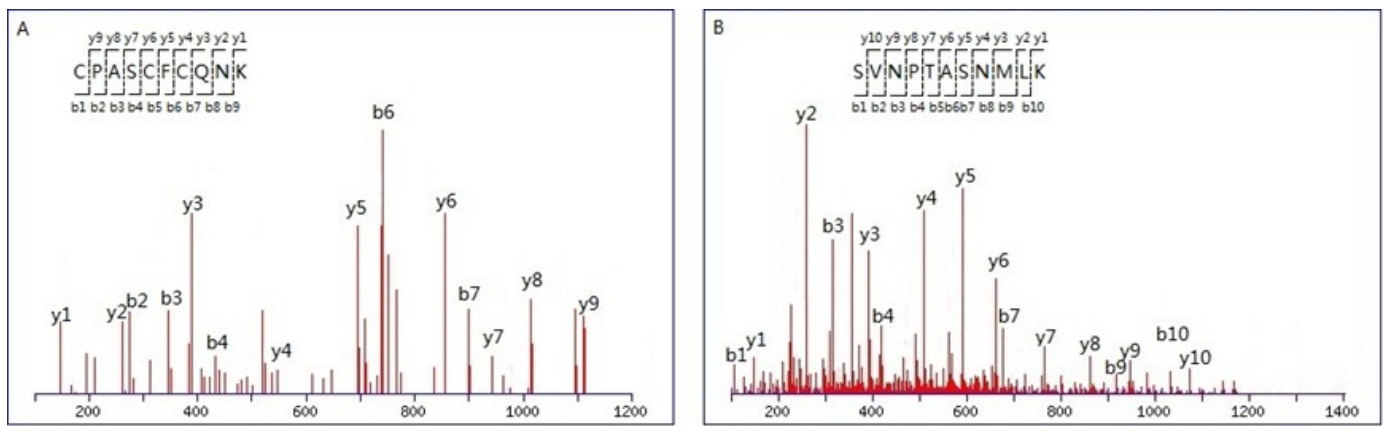
Fig. 4. Typical tandem ESI-MS spectra of two trypsin-digested peptides of Triflin from *Protobothrops mangshanensis* venom. The identification of the Triflin (spot # 57 in Fig. 3) was confirmed with acquired fragment spectra of two peptides: (A) CPASCFCQNK (FM:1270.39) from 636.20 (M/z) and (B) SVNPTASNMLK (FM: 1160.57) from 581.29 (M/z), with both peptides doubly charged. Only corresponding y-ion and b-ion were indicated.

modifications by the intrinsic venom enzymes. This high protein/enzyme isoforms of the snake venom observed also with many other protein spots in 2-DE (Fig. 3) demonstrated not only the high biological variability but also the complex and multiple functionalities of the venom proteins.

Phospholipase A_2s (PLA$_2$s), as another example, were also multifunctional enzymes that exerted multiple pharmacological activities: presynaptic/postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, antiplatelet, convulsant, hypotensive and oedema-inducing effects [30]. Seven PLA$_2$s were found in the pooled *Protobothrops mangshanensis*, including an acidic E6-PLA$_2$, another acidic R6E7-PLA$_2$, two basic N6-PLA$_2$s, two basic R6-PLA$_2$s, and one R49-PLA$_2$ homolog in the previous report [31]. However only R49-PLA$_2$ without enzymatic activities was isolated and characterized from an individual male *Protobothrops mangshanensis* specimen [32]. In this study, several PLA$_2$ components were also identified (Spots 195, 197, 198, 199, 200 in Fig. 3 and see Supplementary material, Table 1). We also did not detect any PLA$_2$ enzymatic activity in this *Photobothrops mangshanensis* venom sample. The amino acid sequences from *Photobothrops mangshanensis* toxin and other R49-PLA$_2$ or K49-PLA$_2$ homologs were aligned with each of 121 or 122 a.a. residues including seven conserved cysteine residues which could cross-linked the proteins by inter or intra molecular disulfide bonds. With the sequence identity of >80%, they were highly expected structurally similar to each other. Since Asp48 was responsible for direct binding to the active site calcium ion, the PLA$_2$s with basic substitutions at this position could not bind calcium ion which was necessary for its hydrolytic activities. Notably, the R49-PLA$_2$s were found only in Protobothrops venoms, and thus could serve as venom markers of this genus. Furthermore, the R49-PLA$_2$s were functionally identical to the K49-PLA$_2$ subfamily. R49-PLA$_2$ from *Photobothrops mangshanensis* could bind membrane and induced edema and myonecrosis in mice [32], thus contributed significantly to the symptom elicited by

Photobothrops mangshanensis envenoming. Given the fact that R49-PLA$_2$s are especially rich in *Photobothrops mangshanensis* venom, certain anti-inflammatory drugs can be potential therapeutic agents in treating the snake bite.

Among many other identified proteins, Triflin, as one of cysteine-rich secretory proteins (CRISPs), was also detected and identified from multiple spots: 57, 86, 87, 88, 89 and 90 (Figs. 3 & 4). CRISPs were found in the venoms of a wide variety of snake species [33], such as ablomin from Japanese Mamushi snake (*Gloydius blomhoffi*, formerly *Agkistrodon blomhoffi*) [34], latisemin from Erabu sea snake (*Laticauda semifasciata*) [33], ophanin from King Cobra (*Ophiophagus hannah*) [35], piscivorin from Eastern Cottonmouth (*Agkistrodon piscivorus*) [35] and triflin from Habu snake (*Trimeresurus flavoviridis*) [36], with each of these proteins named after the snake specie in which it was discovered. It was reported that triflin reduced high potassium-induced smooth muscle contraction and blocked the L-type calcium channels [34], another reason that venoms were toxic.

3. Conclusions

In this study, we provided both electrophysiological and proteomic investigations to the toxicities and protein contents of *Protobothrops mangshensis* venom. Among the total of 143 proteins identified by MS/MS analysis of the tryptic peptides of the protein spots from 2-DE separation, ~ 59.4% proteins were various enzymes and ~24.5% were ion channel proteins. Many enzymes were found and identified to be with multiple isoforms observed in 2-DE. These results demonstrated a great variability of the snake venom proteins and enzymes, and correlated with high biological activities of the snake venom fluid, contributing to its extremely complex and highly toxic properties. Although many of the bioactivities of the identified proteins were based on similarity comparison of protein sequences and phylogenetic analysis, these results together with the electrophysiological study provided the first functional proteomics of

Protobothrops mangshensis venom.

4. Supplementary material

Acknowledgements

References

1. Fox, J.W., Serrano, S.M.T., Snake venoms and coagulopathy. Toxicon 2005, 45, 951-967.

2. Cushman, D.W., Ondetti M.A. Design of angiotensin converting enzyme inhibitors. Nature Medicine 1999, 5, 1110–1113.

3. O'Shea, J.C., and Tcheng, J.E., Eptifibatide: a potent inhibitor of the platelet receptor integrin glycoprotein IIb/IIIa. Expert Opinion on Pharmacotherapy 2002, 3, 1199–1210.

4. Burger, K.M., and Tuhrim S., Antithrombotic trials in acute ischaemic stroke: a selective review. Expert Opinion on Emerging Drugs 2004, 9, 303–312.

5. Toombs, C. F., Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis. Haemostasis 2001, 31, 141–147.

6. Escoubas, P., Mass spectrometry in toxinology: a 21st-century technology for the study of biopolymers from venoms. Toxicon 2006, 47, 609–613.

7. Escoubas, P., Corzo, G., Whiteley, B.J., Célérier, M.L., Nakajima, T., Matrix-assisted laser desorption/ionization time -of-flight mass spectrometry and high-performance liquid chromatography study of quantitative and qualitative variation in tarantula spider venoms. Rapid Communications in Mass Spectrometry 2002, 16, 403–413.

8. Favreau, P., Menin, L., Michalet, S., Perret, F., Cheneval, O., Stöcklin, M., Bulet, P., Stöcklin, R., Mass spectrometry strategies for venom mapping and peptide sequencing from crude venoms: case applications with single arthropod specimen. Toxicon 2006, 47, 676–687.

9. Legros, C., Celerier, M.L., Henry, M., Guette, C., Nanospray analysis of the venom of the tarantula Theraphosa leblondi: a powerful method for direct venom mass fingerprinting and toxin sequencing. Rapid Communications Mass Spectrometry.

10. Guette, C., Legros, C., Tournois, G., Goyffon, M., Célérier, M.L., Peptide profiling by matrix-assisted laser desorption/onization time-of-flight mass spectrometry of the Lasiodora parahybana tarantula venom gland. Toxicon 2006, 47, 640–649.

11. Richardson, M., Pimenta, A.M., Bemquerer, M.P., Santoro, M.M., Beirao, P.S., Lima, M.E., Figueiredo, S.G., Bloch, C.Jr., Vasconcelos, E.A., Campos, F.A., Gomes, P.C., Cordeiro, M.N., Comparison of the partial proteomes of the venoms of Brazilian spiders of the genus Phoneutria. Comparative. Biochemistry Physiology Toxicology & Pharmacology 2006, 142, 173–187.

12. Zhao, E.M., Chen, Y.H., Description of a new species of the genus Trimeresurus. Sichuan Journal of Zoology 1990, 9, 11–12 (in Chinese).

13. David, P., Tong, H., Translations of recent descriptions of Chinese pitvipers of the Trimeresurus complex (Serpentes Viperidae), with a key to the complex in China and adjacent areas. Smithsonian Herpetological Information Service 1997, 112, 1–29.

14. Wuster, W., Peppin, L., Pook, C.E., Walker, D.E., A nesting of vipers: Phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes). Molecular Phylogenetics Evolution 2008, 49, 445-459.

15. Gumprecht, A., Tillack, F., Proposal for a replacement name of the snake genus Ermia. Russian Journal of Herpetology 2004, 11, 73-76.

16. Castoe, T.A., Parkinson, C.I., Bayesian mixed model and the phylogeny of pitvipers (Squamata: Serpentes). Molecular Phylogenetics Evolution 2006, 39, 91-110.

17. Mebs, D., Kuch, U., Coronas, F.I., Batista, C.V., Gumprecht, A., Possani, L.D., Biochemical and biological activities of the venom of the Chinese pitviper Zhaoermia mangshanensis, with the complete amino acid sequence and phylogenetic analysis of a novel Arg49 phospholipase A2 myotoxin. Toxicon 2006, 47, 797–811.

18. Yi, J., Craft, D., Gelfand, C.A., Minimizing preanalytical and analytical variability of plasma peptidomics using MALDI-TOF MS. Methods of Mol. Biol. 2011, 728, 137-49.

19. Yi, J., Changki K., Gelfand, C.A., Inhibition of Intrinsic Proteolytic Activities Moderates Preanalytic Variability and Stability of Human Plasma, J. of Proteome Res., 2007, 6, 1768-81.

20. Huang, L.Y., Li, B.X., Luo, C., Xie, J.Y, Chen, P., Liang, S.P., Proteome comparative analysis of gynogenetic haploid and diploid embryos of goldfish (Carassius auratus). Proteomics 2004, 4, 235–243.

21. Hellmann, U., Wemstedt, C., Gonez, J., Heldin, C.H., Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Analytical Biochemistry 1995, 224, 451-455.

22. Cao, R., Li, X., Liu, Z., Liang, S.P. Integration of a two-phase partition method into proteomics research on rat liver plasma membrane proteins. Journal of Proteome Research 2006, 5, 634-642.

23. Gobom, J., Schuerenberg, M., Mueller, M., Theiss, D., Lehrach, H., Nordhoff, E., Alpha-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. Analytical Chemistry. 2001, 73, 434-438.

24. Amiconi, G., Amoresano, A., Boumis, G., Brancaccio, A., De Cristofaro, R., De Pascalis, A., Di Girolamo, S., Maras, B., Scaloni A., A novel venombin B from Agkistrodon contortrix contortrix: evidence for recognition properties in the surface around the primary specificity pocket different from

thrombin. Biochemistry 2000, 39, 10294-10308.

25. Matsui, T., Sakurai, Y., Fujimura, Y., Hayashi, I., Oh-Ishi, S., Suzuki, M., Hamako, J., Yamamoto, Y., Yamazaki, J., Kinoshita, M., Titani K., Purification and amino acid sequence of halystase from snake venom of Agkistrodon halys blomhoffii, a serine protease that cleaves specifically fibrinogen and kininogen. European Journal of Biochemistry 1998, 252, 569-575.

26. Atoda, H., Yoshida, N., Ishikawa, M., Morita, T., Binding properties of the coagulation factor IX/factor X-binding protein isolated from the venom of Trimeresurus flavoviridis. European Journal of Biochemistry 1994, 224, 703-708.

27. Hirabayashi, J., Kusunoki, T., Kasai, K., Complete primary structure of a galactose-specific lectin from the venom of the rattlesnake Crotalus atrox. Homologies with Ca^{2+}-dependent-type lectins. Jounal of Biology Chemistry 1991, 266, 2320-2326.

28. Chen, R.Q., Jin, Y., Wu, J.B., Zhou, X.D., Lu, Q.M., Wang, W.Y., Xiong, Y.L., A new protein structure of P-II class snake venom metalloproteinases: it comprises metalloproteinase and disintegrin domains. Biochemical Biophysical Research Communications 2003, 310, 182-7.

29. Du, X.Y., Clemetson, K.J., Snake venom L-amino acid oxidase isoenzyes. Toxicon 2002, 40, 659-65.

30. Huang, M.Z., Gopalakrishnakone, P., Chung, M.C.M., Kini, R.M., Complete amino acid sequence of an acidic, cardiotoxic phospholipase A2 from the venom of Ophiophagus Hannah (king cobra): a novel cobra venom enzyme with "pancreatic loop". Archives Biochemistry Biophysics 1997, 338, 150–6.

31. Tsai, I.H., Chen, Y.H., Wang, Y.M., Comparative proteomics and subtyping of venom phospholipases A_2 and disintegrins of Protobothrops pitvipers. Biochimica Biophysica Acta 2004, 1702, 111-119.

32. Mebs, D., Kuch, U., Coronas, F.I., Batista, C.V., Gumprecht, A., Possani, L.D., Biochemical and biological activities of the venom of the chinese pitviper Zhaoermia mangshanensis, with the complete amino acid sequence and phylogenetic analysis of a novel Arg49 phospholipase A2 mytotoxin. Toxicon 2006, 47, 797-811.

33. Yamazaki, Y., Morita., Structure and function of snake venom cysteine-rich secretory proteins. Toxicon 2004, 44, 227–231.

34. Yamazaki, Y., Koike, H., Sugiyama, Y., Motoyoshi, K., Wada, T., Hishinuma, S., Mita, M., Morita, T., Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. European Journal Biochemistry 2002, 269, 2708–2715.