Journal of Protein Chemistry, Vol. 19, No. 3, 2000

Indentification of Venom Proteins of Spider *S. huwena* on Two-Dimensional Electrophoresis Gel by N-Terminal Microsequencing and Mass Spectrometric Peptide Mapping

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Received May 15, 2000

Venom proteins of the spider *Selenocosmia huwena* were separated by two-dimensional gel electrophoresis, with the separation in the first dimension on a wide range of immobilized pH (3–10) gradients. Over 300 protein spots were presented on a silver-stained 2D gel. The protein spots with molecular weight >10 kDa were analyzed, after electrotransferring to polyvinyldene difluoride (PVDF) membrane, by N-terminal microseqencing. Some of the silver-stained protein spots with molecular weight over 10 kDa were analyzed and identified by employing an improved procedure of mass spectrometric peptide mapping, including (1) in-gel reduction, alkylation, and enzymatic digestion; (2) extraction and desalting by using the pipette tip containing a small C18 microcolumn (Ziptip™); and (3) direct MAIDI-TOF mass analysis and protein database searching. Several known toxins such as HWTX-I, HWTX-II, HWTX-IV, and SHL-I were identified and some new components were found among these protein spots.

KEY WORDS: MALDI-TOF mass; proteome; spider venom; 2D-PAGE.

1. INTRODUCTION

The major components of spider venom are proteins and polypeptides with different biological activities including neurotoxic, enzymatic, cytotoxic, necrotic, and hemagglutinic activities (Liang *et al.,* 1993*a, b;* Grishin, 1999) Some of the components found in spider venom are of interest as tools for studying neurophysiology and as potential lead structures for insecticides and pharmaceuticals. Due to limits in the sensitivity and resolution of commonly used methods of separation, the complexity and diversity of components in spider venom are usually underestimated. Many compounds with low abundance are often neglected. Two-dimensional polyacrylamide gel electrophoresis is the most efficient method for separation of several hundred protein molecules on the basis of difference in their pIs and molecular masses (O'Farrell, 1975). The introduction of immobilized pH gradient first-dimensional strips (Bjellqvist *et al.,* 1982) and advances in image analysis, protein sample handling, N-terminal microsequencing, and mass spectrometry, together with algorithms for searching sequence databases, present a technology well suited for global and detailed studies of proteins from a certain cell type, tissue, or body fluid, such as venom, of an organism (Bini *et al.,* 1997; Cordwell *et al.,* 1995).

The Chinese bird spider, *Selenocosmia huwena,* distributed in the hilly areas of Yunan and Guangxi in the south of China, was recently identified as a new species (Wang *et al.,* 1993) and is one of the most venomous

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[3] Abbreviations: **HWTX,** huwentoxin; **SHL-I,** *Selenocosmia huwena* lectin-I; **MALDI-TOF,** matrix-assisted laser desorption ionization–time of flight; **PVDF,** polyvinyldene difluride; **2D-PAGE,** two-dimensional polyacrylamide gel electrophoresis; **SDS,** sodium dodecyl sulfate; **IPG,** immobilized pH gradient; **DTT,** dithiothreitol.

0277-8033/00/0400-0225$18.00/0

spiders in China. In our previous work, we have demonstrated that *S. huwena* venom contains a mixture of compounds with different types of biological activities, and several protein and polypeptide components were isolated and characterized, such as HWTX-I, -II, -III, -IV and SHL-I (Zhang and Liang, 1993; Liang and Pan, 1995; Shu and Liang, 1999).[3] In order to investigate the venom of this spider systematically and globally, we here report our initial attempts to apply MAIDI-TOF-MS and microsequencing for the characterization of the 2D resolved proteins of the venom of the spider *S. huwena.*

2. MATERIAL AND METHODS

2.1. *S. huwena* Venom Preparation

The venom from adult female *S. huwena* was collected as described (Liang *et. al.*, 1993*a, b*) and immediately freeze-dried.

2.2. High-Resolution 2D PAGE

Two-dimensional electrophoresis was performed using the IPG-SDS-PAGE system according to Bjellqvist *et al.* (1993). One mg of the lyophilized *S. huwena,* dissolved in 8 M urea, 4% W/V CHAPS, 40 mM Tris-Base, 65 mM dithiothretol (DTT), and trace amounts of bromophenol blue, was loaded on a 13-cm linear Immobiline strip, pH range 3–10 (Pharmacia). Voltage was set at 300 V for the first 4.5 h, then stabilized at 3000 V for 9.5 h (total about 30 kV h). The second-dimensional run was carried out on 9–15% polyacrylamide linear gels (13 cm $\times$ 20 cm $\times$ 1.0 mm) at 40 mA/gel constant current, for approximately 5 h until the dye front reached 1 cm from the bottom of the gel. Both runs were performed at 10°C. The gels were then stained with Coomassie blue to visualize protein spots or stained with silver using the following procedure.

2.3. Silver Staining of 2D Gel for In-gel Digestion

After the gel was run, it was soaked for 30 min in fix solution (45% MeOH, 5% acetic acid) and then rinsed in water for 30 min. Then the gel was sensitized with 0.02% sodium thiosulfate for 1–2 min. After rinsing with two changes of water (1 min each), the gel was incubated in chilled 0.1% $AgNO_3$, solution for 30 min at 4°C. The solution was discarded and the gel was rinsed with two changes of water (1 min each). The gel was then developed with a solution of 0.04% formaldehyde, 2% Na_2CO_3 solution on a shaking table. Finally, the development was quenched when sufficient staining was obtained (usually after 1–5 mim) by discarding develop solution and addition of 1% acetic acid.

2.4. N-Terminal Sequencing

For N-terminal sequence determination the Coomassie blue-stained gels were electroeluted onto PVDF membranes (Pharmacia) according to Matrudaira (1987). Protein spots were cut from a single membrane and were analyzed using a 475 gas-phase protein sequencer from Applied Biosystem.

2.5. In-Gel Protein Alkylation and Digestion

In-gel protein alkylation and digestion was performed according to Shevchenko *et al.* (1996) and Jensen *et al.* (1999). The silver-stained gel was washed with water (two changes, 10 min each) and the spots of interest were cut as close to the protein spots as possible to a piece of roughly the same size as from a nonprotein-containing region of the gel for use as a control. The excised piece was then cut into roughly 1-mm^3 tubes and was transfered to a clean 0.5-ml microfuge tube. After the gel particles were washed with water/acetonitrile 1:1 (V/V), all liquid was removed and enough acetonitrile was added to cover the gel particles. After the gel pieces had shrunk, the acetonitrile was removed and the gel pieces were rehydrated in 0.1 M NH_4HCO_3 for 5 min. Then an equal volume of acetonitrile was added and the tube was incubated for 15 min before being dried in a vaccum centrifuge. The gel particles were then allowed to swell in 10 mM dithothreitol/0.1 M NH_4HCO_3, and incubated for 45 min at 57°C to reduce the protein. The liquid was replaced with the same volume of freshly prepared 55 mM iodoacetamide, 0.1 M NH_4HCO_3 solution and was then incubated for 30 min at room temprature in the dark. The iodoacetamide solution was removed and the gel was washed with 0.1 M NH_4HCO_3 and acetonitrile. The gel particles were completely dried in a Speed Vac and were then rehydrated with digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl^2$, and 12.5 ng/μl of typsin) and were incubated at 37°C overnight. After tryptic digestion, the peptides were extracted with 2 $\times$ 50 ml 5% TFA/50% acetonitrile and then were freeze-dried.

2.6. Desalting of Peptides with C18 ZipTip™

The ZipTip™ pipette tips, which contain C18 spherical silica (15 mm, 200 Å pore size) in a 0.6-ml bed vol-

umn from Millipore, were used for the desalting of the peptides. The tips were first wetted with 50% acetonitrile in water, then were equilibrated with 0.1% TFA. Addition of 20 ml 0.1% TFA/water dissolved the peptides. The peptides were bound to ZipTip™ by fully depressing the pipette plunger to a dead stop. Samples were aspirated and dispensed for 10 cycles. Then the tips were washed with 0.1% TFA twice. The peptides on the tips were eluted out using 5 ml of elution buffer (saturated a-cyano-4-hydroxy-cinnamic acid in 50% ACN:0.1% TFA, 1:1).

2.7. MAIDI-TOF Mass Spectrometry of the Peptides

One to 2 ml of desalted sample was pipetted directly onto the target plate of the mass spectrometer. MAIDI-TOF mass spectra of peptide mixtures were obtained on a Bruker ProFlex-III time-of-flight mass spectrometer with a delayed extraction ion source that was equipped with a nitrogen laser of 337 nm. The acceleration voltage was set to 20 kV. The pressure in the TOF analyzer was $\sim 4 \times 10^{-7}$ mbar. The spectral data were calibrated with a fragment of adrenocorticotropic hormone (2466.74 Da) and HWTX-I (3750.35 Da).

2.8. Database Searching

Identification of proteins via peptide mass fingerprints in protein sequence databases was performed using the Peptide Search software tool developed by Jensen *et al.* (1997, 1999) and downloaded from their homepage (http://www.mann.embl-heidelberg.de). The database used for all searches was the nonredundant protein sequence database (nrdb) maintained at the European Bioinformatics Institute (EBI). The search program accepts the following inputs: (1) series of measured peptide molecular masses, (2) the achieved mass accuracy, i.e., expected mass deviation in percentage or daltons, (3) the number of peptide matches required for a protein to be considered as a candidate, and (4) the mass range of proteins to be considered in the search.

3. RESULTS AND DISCUSSION

A representative silver-stained 2D polyacrylamide gel of the crude venom of *S. huwena* is shown in Fig. 1. The first dimension was an isoelectric focusing Immobiline gel ranging from pH 3 to 10 as indicated along the horizontal axis, and the second dimension is molecular weight as determined by relative mobility in an SDS–polyacrylamide gel, shown on the vertical axis. The total protein amount of the sample loaded on the first dimension was 1 mg. Approximately 350 spots were counted after background subtraction within the window of pI 3.5–9 and molecular mass 1–100 kDa, using the PDQuest software (Bio-Rad). Experimental error estimated by the number of mismatching spots was found to involve on the average 10 spots per gel when comparing six different electrophoresis runs of the same venom preparation and 25 spots per gel when comparing three different preparations. Upon transfer onto PVDF membranes and Coomassie blue staining, about 150 spots became visible, with good preservation of the overall electrophoretic pattern. As shown in Fig. 1, over 50 spots with relatively high abundance were located in the region below the molecular weight 10 kDa. There are more protein spots in the basic region (pI>7) than that in the acidic region. At the present stage of our work, 18 spots, after transfer to PVDF membrane, were analyzed by micosequencing on the 475 gas-phase protein sequencer. Among them, 12 spots have readable sequences. A sequence could not be obtained because of blocked N-terminus for the remaining 6 spots. The N-terminal sequences were used as queries to search the database. Spots 1, 3, 6, and 11 were assigned to known *S. huwena* toxins: HWTX-I, HWTX-II, HWTX-IV, and SHL-I, respectively (Table I). The N-terminal sequence of spot 13 is 100% identical to that of *S. huwena* lectin-I (SHL-I), but the molecular weight, about 25 kDa is much higher than that of SHL-I, indicating that SHL-I is most probably a fragment of the protein in spot 13. Consequently we named the protein in spot 13 as the precursor of SHL-I. The N-terminal sequences of spots 4, 5, 7, 8, 10, and 14, which have molecular weight below 5 kDa, did not have any identical sequences in the database. They should be considered new components of the *S. huwena*

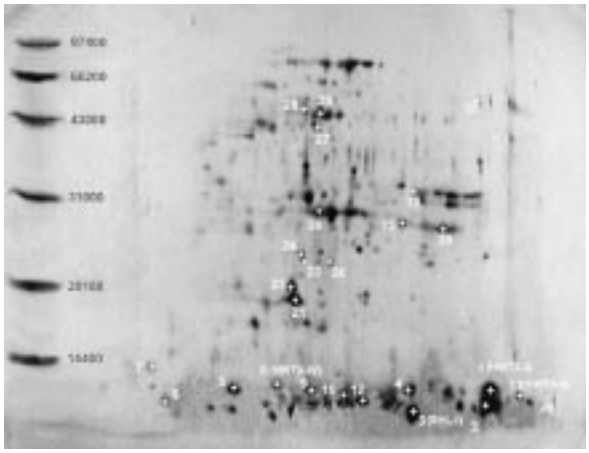

Fig. 1. Protein map of the venom from the spider *Selenocosmia huwena* obtained by 2D electrophoresis.

Table I. Proteins from the Venom of the Spider *S. huwena* Identified by N-Terminal Microsequencing and MALDI-TOF-MS Peptide Mapping

Spot no.	Database accession no.	Protein name	MS (kD)	pI	Peptide masses matched	N-terminal sequence determined
1	Swissprot: p56676	Huwentoxin-I	3.7	8.9	No data	ACKGVFDACTPGLNE—
2	Swissprot: p56676	Huwentoxin-I	3.7	8.9	No data	ACKGVFDACTPGLNCCPNR—
3	Genbank: 5822308	*S. huwena* lectin-I	3.5	8.1	No data	GCLGDKCDYNNGCCSG—
4	No accession number	New component	3.6	7.9	No data	GIELQVPCDE—
5	No accession number	New component	3.6	4.4	No data	ACRKQIGDHCK
6	No accession number	Huwentoxin-IV	3.8	4.6	No data	ECLEIFKACNEANDC
7	No accession number	New component	9.5	3.8	No data	EWCRQACC—
8	No accession number	New component	3.5	4.1	No data	ECSSYLGCGE—
11	No accession number	Huwentoxin-II	3.8	9.2	No data	LFECSFSCEQEKEG—
13	No accession number	ShL-I precursor	28.0	8.1	No data	GCLGDKCDYNNGCCSG—
21	Genbank: M18374	Trypsin inhibitor	22	5.2	1475.289 1493.100 1584.657 1708.290 1853.883	24.9% sequence coverage
22	Swissprot: P46279	DNA-directed RNA polymerase II, 19-kD polypeptide	20	6.5	1982.915 2011.002 2504.006 2594.796	32.4% sequence coverage
23	Swissprot: Q24251 D chain	ATP synthase	20	8.7	1493.911 1708.151 1839.521 1903.681	30.3% sequence coverage
24	Swissprot: P42074	DNA-directed RNA polymerase gamma chain	23	6.9	1351.446 1367.764 1595.895 1687.946	25.1% sequence coverage
29	Swissprot: p02546	Lamin C	57	6.8	1314.60 1485.13 1631.67 1648.71 1914.92 2507.36 1176.81	20.8% sequence coverage
33	Sptrembl: O40666	Hemagglutinin	36	9.3	1309.305 1995.522 2706.301 3370.877 1639.482	28.9% sequence coverage
51	Sptrembl: O16316	C05C8.8 protein	46	9.4	1900.96 1992.375 2609.509 2701.756 2870.178 2929.230 2547.525	31.0% sequence coverage

venom. Several of these small proteins probably belong to the same three-dimensional structure family of HWTX-I and SHL-I because they have about the same Cys residue location in the sequence and similar molecular weight as HWTX-I and SHL-I (Qu *et al.*, 1997; Lu *et al.*, 1999).

Some protein spots in silver-stained 2D gel were analyzed and identified by employing an improved procedure of mass spectrometric peptide mapping. Although recently literature has demonstrated that identification of silver-stained proteins from electrophoretic gels is feasible by enzymatic digestion in conjunction with mass spectrometry, problems remain with detection limits of commercial mass spectrometers and, perhaps more significantly, sample preparation. In our procedure, the en-

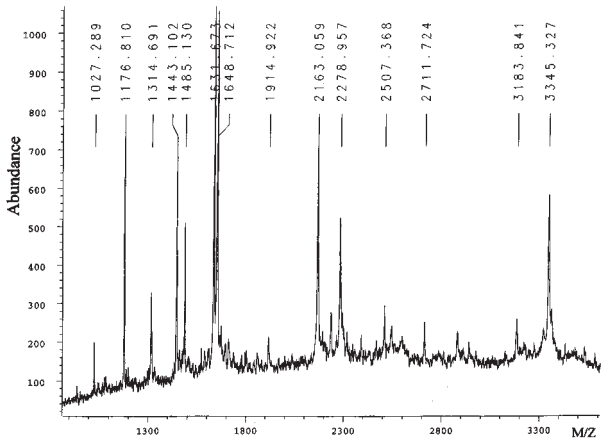

Fig. 2. MALDI-TOF spectrum of tryptic peptides of protein spot 29 obtained from one piece of silver-stained 2D PAGE gel of *S. huwena* venom.

zymatic digested peptides of protein spots were extracted and desalted by using a pipette tip containing a small C18 microcolumn (ZipTip™ from Millipore). The quality of the mass spectrum and detectability of peptides is enhanced by employing ZipTip™ as shown in Fig. 2.

Total peptide mixtures of 12 protein spots were analyzed by MAIDI-TOF-MS and the resulting peptide masses were used for identity searches with the Peptide Search software. The results are summarized in Table I. Eight protein spots were found to be likely candidate, in the database based on their peptide masses; in at least four peptide masses match with mass accuracy 0.08% along with close agreement with the molecular weight and the pI. Since the genome of *S. huwena* has not been determined and only small amounts of protein and DNA sequences of spider are available in the database, the identification of the protein spots of *S. huwena* is difficult. The results in Table I list the likeliest candidates rather than precisely identified proteins. Due to the low sequence coverage (less than 40%) of the matched peptides, the results in Table I need to be corroborated by additional evidence such as data on partial sequences or amino acid composition.

In summary, silver-stained 2D gel electrophoresis demonstrated that the crude venom of the spider *S. huwena* contains at least 350 different proteins and polypeptides. A number of proteins, including the known *S. huwena* toxins HWTX-I, -II, -IV and SHL-1 and a precursor of SHL-1, were identified by N-terminal microsequencing. The silver-stained spots on 2D gel can be identified by an improved procedure of mass spectrometry peptide mapping. Using the C18 ZipTip™ pipette tip for the desalting of the peptides increases the sensitivity of the MAIDI-TOF analysis. The peptide mass fingerprints of several spots on the 2D gel of *S. huwena* venom were used for identification and homologue searching. Although the most likeliest candidates in the database to match the spots were found, precise and confident identification needs more evidence.

ACKNOWLEDGMENTS

This work is supported by National Natural Science Foundation of China under contract No. 39990600 and a grant from the United Research Centre of Life Science of Hunan Province.

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