Toxicon 125 (2017) 13–18

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Identification of snake venom allergens by two-dimensional electrophoresis followed by immunoblotting

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ARTICLE INFO

Article history:
Received 10 October 2016
Received in revised form
10 November 2016
Accepted 16 November 2016
Available online 17 November 2016

Keywords:
Snake venom
Allergen
Allergenomics
Serine proteinase
Metalloproteinase

ABSTRACT

This allergic reaction to snake venom was described to occur in patients after recurrent exposure through bites in amateur and professional snake handlers, which might be underestimated and contribute to fatal snakebites in victim, independently from the toxicity of the venom itself. Few allergens were identified from snake venoms by normal SDS-PAGE, which cannot separate the snake venom completely. In the present study, we identified nine potential allergens by two-dimensional (2D) electrophoresis followed by immunoblotting (named as allergenomics) in *Protobothrops mucrosquamatus* venom. By multidimensional liquid chromatography-ion trap mass spectrometry (MDLC-ESI-LTQ-MS/MS) analysis, six allergens showed sequence similarity to snake venom serine proteinases. Other allergens showed sequence similarity to snake venom metalloproteinase. These allergic reactions to snake venom allergens might contribute to fatal snakebites in victim, independently.

1. Introduction

Envenomation by snakebite is a common and generally harmful, environmentally and occupationally neglected tropical disease and highly relevant public health problem, estimating to cause around 50,000 deaths annually around the world (Cruz et al., 2009). Snake venoms are mixtures of proteins, toxins and enzymes that may cause coagulopathy, neurotoxicity, myotoxicity, hypotension and tissue necrosis (Alape-Girón et al., 2009). In addition to their direct toxic effects, snake venoms also activate the victims' normal tissues and cells to release some harmful components to cause secondary injury. For example, many snake venom components could activate mast cells directly and cause inflammatory cascade (León et al., 2011; Stone et al., 2013; Wei et al., 2009a, 2009b, 2006a, 2006b).

Besides the direct and secondary injury caused by snake venom, there are some reports about allergic sensitization to snake venom (Wadee and Rabson, 1987; Reimers et al., 2000; Prescott and Potter, 2005; de Medeiros et al., 2008; Madero et al., 2009; de Pontes et al., 2016). The allergic reaction to snake venom was described to occur in patients after recurrent exposure through bites in amateur and professional snake handlers (Wadee and Rabson, 1987; de Medeiros et al., 2008; Madero et al., 2009). The snake venom allergy might be underestimated as it contributes to fatal snakebites in victim, independently from the toxicity of venom itself (Wadee and Rabson, 1987; Reimers et al., 2000; Prescott and Potter, 2005; de Medeiros et al., 2008; Madero et al., 2009; de Pontes et al., 2016). Madero et al. identified 4 IgE-binding bands of about 60, 28, 14 and 7 kDa in the Bothrops extract by SDS-PAGE followed by immuno-blotting methods (Madero et al., 2009). Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) analysis demonstrates that 14-kDa protein has similarity with snake venom phospholipase A_2 and the 60- and 28-kDa proteins shows significant similarity with snake venom metalloproteinases (Madero et al., 2009). Recently, de Pontes et al. identified crotoxin as an

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http://dx.doi.org/10.1016/j.toxicon.2016.11.251
0041-0101/

allergen to cause occupational anaphylaxis in one patient who worked with *Crotalus durissus terrificus* venom for 4 years (de Pontes et al., 2016). All these evidences suggested that snake venom contained allergens which could cause serious allergic reactions in sensitive patients who had the experience to meet the snake venom.

Snake venoms comprise of complex mixtures of toxins, which largely belong to a few major protein families (Fry et al., 2009, 2008). These snake venom families have similar molecular weight, which cannot be separated from each other by normal SDS-PAGE. Indeed, two-dimensional (2D) electrophoretic analysis of snake venom provides a more realistic view of venom complexity (Calvete, 2014; Calvete et al., 2007). On the other hand, allergen-targeted proteomics based on 2D electrophoresis followed by immunoblotting (named as allergenomics), have been used for comprehensive identification and/or quantification of potential allergens that bind to IgE (Di Girolamo et al., 2015; Nakamura and Teshima, 2013). The aim of the present study is to identify the potential allergen in snake venom (*Protobothrops mucrosquamatus*) in occupational snake venom handler by allergenomics method.

2. Materials and methods

2.1. Chemicals

Chemicals for electrophoresis, including acrylamide, bis-acrylamide, SDS and Tris–HCl (pH 8.8) were purchased from GE Healthcare Life Sciences China (Shanghai, China). Dithiothreitol (DTT) and iodacetamide (IAM) were purchased from Promega (Beijing) Biotech Co (Beijing, China). Water from a Millipore Milli-RO4 reverse osmosis system was used for making all solutions.

2.2. Patients' sera and preparation of Protobothrops mucrosquamatus venom

Sera from *Protobothrops mucrosquamatus* snake venom handlers who had worked or studied in the department of animal toxicology, Kunming Institute of Zoology, Chinese Academy of Sciences were collected and stored in aliquots at −70 °C. The allergic response to snake venom was noticed by their clinical histories. Sera from non-allergic individuals were used as controls. The study protocol was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent for the use of blood samples were obtained from all participants before study entry according to the declaration of Helsinki.

2.3. 2D gel electrophoresis

For total protein separation, the immobilized pH gradient (IPG) strips (pH 3–10, linear, 13 cm, Bio-Rad) were rehydrated passively for 13 h. The voltage settings for isoelectric focusing (IEF) in the Protean system (GE EttanIPGphor 3) were 2 h at 250 V, 1 h at 1000 V, 3 h at 8000 V, and then keep at 8000 V until a total of 50000 Vh was reached. After IEF and equilibration, the second dimensional SDS-PAGE gels of 12.5% were run at 2 W/gel for 2 h and 12 W/gel for 1.5 h using Multiphor system (GE Ettan DALT six). The gels were visualized by colloidal Coomassie brilliant blue staining.

2.4. Western blotting

For each sample, duplicate 2D gels were run under the same conditions. One gel was subjected to colloidal Coomassie staining to visualize the protein spots and analyze the spots using mass spectrometry. The other gel was transferred onto PVDF membrane (Bio-Rad, Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell) at 4 °C at 1 mA/cm^2 for 1.5 h. After electro transfer step, all transferred protein spots on PVDF membranes were stained temporarily with Ponceau S solution and then scanned. These Ponceau S-stained images served as reference gel images to match spots to Coomassie-stained protein spots.

The transferred PVDF membranes were blocked for overnight at 4 °C with blocking solution [3% BSA in TBST (Tris-buffered Saline with Tween 20)], and incubated with sera from *Protobothrops mucrosquamatus* allergic persons respectively for 5 h at 37 °C at a dilution of 1:5 in TBST. After washing four times in TBST, the membranes were incubated with monoclonal anti-human IgE Ab (Promega, 1:2500 dilution) for 2 h at 37 °C. The protein spots were then detected with an enhanced chemiluminescence kit (Super-SignalTM West Pico substrate; Pierce Biotechnology) for 5 min before scanning. Image analysis software PDQuest (Bio-Rad) was used to match Coomassie-stained spots in gels to Ponceau S-stained spots in PVDF membrane or Ponceau S-stained protein spots to immunoblotting spots in the same PVDF membranes. Since no mass spectra could be obtained from proteins blotted onto PVDF membranes, protein spot identities were assigned by matching the chemiluminescence images with Coommassie-stained gels run in parallel.

2.5. Peptide analysis by multidimensional liquid chromatography-ion trap mass spectrometry (MDLC-ESI-LTQ-MS/MS)

The stained protein spots matching the immunoblotting signals were cut out of the gels. In-gel digestion of protein spots followed the procedures described previously (Yang et al., 2010). Mass spectra analysis was performed by Shanghai applied protein technology company (Shanghai, China). We used SEQUEST to search the theoretical sequence databases and identify the best matches to the spectra. Then we compared these peptides with the results that SEQUEST has identified from the UniProt database (Eng et al., 1994).

3. Results

3.1. 2D gel electrophoresis of Protobothrops mucrosquamatus

Protobothrops mucrosquamatus venom was subjected to 2D gel electrophoresis. Fig. 1 shows the 2D gel profile of the *Protobothrops mucrosquamatus* snake venom, in which 83 distinct protein spots

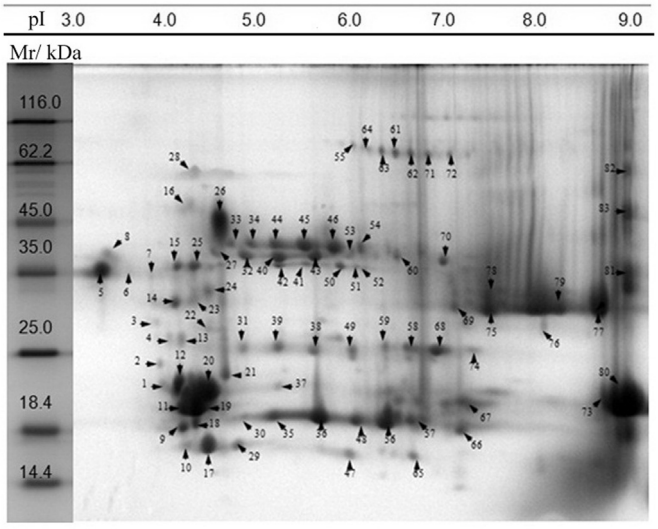

Fig. 1. 2D gel profile of the *Protobothrops mucrosquamatus* snake venom. The arabic number represents serial number of the spots.