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Original Article

Identification and isolation of immunodominant proteins of *Naja naja (Oxiana)* snake venom

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ABSTRACT

Snake venom is a complex mixture of proteins, peptides, enzymes, carbohydrates, and minerals. They contain a variety of chemicals with pharmacological and toxicological properties. The innate immune system is the first line of defense against toxins and microbes. Antibacterial and anticancer proteins produced by snake venom have recently attracted significant attention due to their relevance to bacterial diseases and the potential of being converted into new therapeutic agents. However, the production of anti-snake venom from large mammals is proven to be low-yielding and arduous. The aim of the present study was to investigate and isolate immunodominant proteins of *Naja oxiana* snake venom. Identification was performed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Subsequently, four sharp protein bonds of 14, 22, 32, 65 kDa were appeared in nitrocellulose paper. In the next step, the identified proteins were isolated directly by electro-elution from preparative gel electrophoresis. Results showed that immunodominant proteins of (14, 22, 32, and 65 kDa) with high immunogenicity had high immunoreactivity with antiserum. To the best our knowledge, these proteins can be candidates for preparing a specific anti-venom against *Naja oxiana* and antimicrobial immunodominant proteins, as well as designing antimicrobial peptides
Keywords: *Naja naja,* Immunodominant protein, Antivenom, Electro-elution

Identification et isolation des protéines immunodominantes de Naja naja (Oxiana) venin de serpent

Résumé: Le venin de serpent est un mélange complexe de protéines, peptides, enzymes, hydrates de carbone et les minéraux. Ils contiennent une variété de produits chimiques ayant des propriétés pharmacologiques et toxicologiques. Le système immunitaire inné est la première ligne de défense contre les toxines et les microbes. Les protéines antibactériennes et anticancéreuses produites par le venin de serpent ont récemment attiré une attention particulière en raison de leur rapport avec les maladies bactériennes et du potentiel de conversion en de nouveaux agents thérapeutiques. Cependant la production de venin anti-serpent des grands mammifères s'est révélée de faible rendement et ardu. L'objectif de la présente étude était d'étudier et d'isoler les protéines immunodominantes du venin de serpent Naja oxiana. L'identification a été effectuée par 15% électrophorèse sur gel de polyacrylamide de dodécyl sulfate de sodium (SDS-PAGE) et analyse de Western Blot. Par la suite, quatre liaisons protéiques nettes de 14, 22, 32 et 65 kDa sont apparues dans du papier de nitrocellulose. Dans l'étape suivante, les protéines identifiées ont été isolées directement par électro-élution à partir d'une électrophorèse en gel préparatoire. Les résultats ont montré que les protéines immunodominantes de14, 22, 32 et 65 kDa ayant une immunogénicité élevée avaient une immunoréactivité élevée avec un antisérum. Au mieux de notre connaissance, ces protéines peuvent être candidates à la préparation d'un anti venin spécifique contre Naja oxiana et des protéines immunodominantes antimicrobiennes, ainsi qu'à la conception de peptides antimicrobiens.
Mots-clés: Naja naja, Protéine immunodominante, Antivenom, Electro-élution

INTRODUCTION

Snake bite is a serious health problem in many tropical and subtropical regions (Kini and Evans, 1990). For instance, a recent report by World Health Organization (WHO) estimated that ~2.5 million snakebite incidents occur around the world, resulting in as many as 125,000 deaths annually. The majority of these victims inhabit in tropical regions (Chippaux, 1998). In addition, a large number of survivors may suffer from physical disability. Therefore, since 2009, snakebite has been classified as a neglected tropical disease (WHO, 2010). Intravenous administration of anti-venom directed against the most toxic venom components is recommended to rescue victims. Anti-venom, composed of immunoglobulins, is usually produced by immunized animals such as horses, rabbits, or camels with crude venom. Ideally, snakebite management should not only reduce mortality, but also minimize complications due to tissue damage. Venom toxins from snakes inhabiting in different geographical regions may vary significantly in composition (Gutiérrez et al., 2009) as a result of prey adaptations, or diet-related functions (Barlow et al., 2009). Such variations in toxin composition not only alter the activities of venom, but also challenge anti-venom efficacy. It is, therefore, important to determine geographical venomic profiles and optimize the venom immunogen used for anti-venom production to generate cross-protection, which is more efficient (Laustsen et al., 2015; Tan et al., 2015). Accordingly, previous studies demonstrated that some components of snake venom have beneficial attributes in the treatment of various pathophysiological conditions. Enzymes from cobra venom are assumed to be beneficial in the treatment and prevention of Parkinson's and Alzheimer's diseases (Barker et al., 2000). Thus, elucidation of specific proteomic profiles of snake venoms can have vast implications for medicine. Snake venoms are mainly composed of proteins and peptides, which possess a variety of biological activities. Snake venoms are broadly divided into three categories based on toxicity from envenomation. These categories are (i) haemotoxins, promoting hemorrhaging primary to extensive local swelling and necrosis, (ii) neurotoxins, which disable muscle contraction, paralyze the heart, and hinder respiration, and (iii) cardiotoxins, which elicit specific toxicity to cardiac and muscle cells, causing irreversible depolarization of cell membranes (Hati et al., 1999). Cobra is one of the world's most poisonous snakes and is indigenous of the countries of Asia, Africa, and Oceania. Cobra venom is mainly categorized as a cytotoxin (Yang, 1996). During the last several years, the field of proteomics has evolved considerably (Binz et al., 2003). With regard to the availability of genomic sequences, advances in medical sciences, development of computational methods, power of two-dimensional electrophoresis (2DE), and various chromatographic techniques to separate complex mixtures of proteins, it is now possible to globally identify proteins expressed in a cell under a given set of conditions. Several reports have described the analysis of snake venom using proteomic strategies (Rioux et al., 1998; Nawarak et al., 2003). At the first Swiss Proteomics Society congress (SPS'01), a total of eight laboratory groups participated in an exercise examining protein identification using different mass spectrometric approaches (Binz et al., 2003). One of the samples for the exercise was snake venom from the Brazilian snake, *Bothrops jararaca*, provided by Dr. D. C. Pimenta (Institute Butantan, Sao Paulo, Brazil). In the present study, we chose cobra *N oxiana*, one of the major species of snake found in the northeast of Iran. At first, identification of immunodominant proteins of the venom of *N. oxiana* snake was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis; in the next step, the identified proteins were isolated directly from preparative gel electrophoresis by electro-elution. Our results can help with evaluation of immunogenicity of immunodominant proteins of to prepare a specific antivenom for *Naja oxiana* in further studies.

MATERIALS AND METHODS

Lyophilized crude venom of *Naja naja (oxiana)* was a kind gift from Department of Poisonous Animal, Razi Vaccine and Serum Research Institute, Karaj, Iran. The serum of hyper-immunized horses was provided by Razi Vaccine and Serum Research Institute, Karaj, Iran. Rabbit anti-horse–IgG (whole molecule)–peroxidase conjugate was prepared from Sigma Co.

Chemical reagents. Ammonium persulfate (APS), Tween 20, acrylamide, SDS, and tetramethylethylenediamine (TEMED) were obtained from Bio-Rad (USA); phosphate buffered saline (PBS) was acquired from Merck Co. Bovine serum albumin (BSA) and chloro-1-naphthol were prepared from Fluka Co. Hydrogen peroxidas was obtained from Sigma Co (USA).

Determination of protein concentration. Protein concentration was determined by Lowry's method, using BSA as standard (Lowry et al., 1951).

SDS-PAGE. Electrophoresis was carried out on 15% polyacrylamide gel by the method of Laemmli (Walker, 2002). Crude venom (20 µg) was mixed with an equal volume of sample buffer, and molecular weight marker was separately loaded into the wells. The wells were filled with running gel buffer. The output voltage was 110 V for 75 min. After the run, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma Co), and then it was distained. Molecular weight of proteins of crude venom was estimated according to the band patterns of the obtained marker proteins.

Gel diffusion assay. Gel diffusion assay using serum of hyper-immunized horse against *N. oxiana* snake venom was performed. The existence of polyclonal antibody in horse serum was tested by Ouchterlony's immunodiffusion assay using crude venom as the antigen. Furthermore, 2% agarose (Sigma Co.) gel in sterile PBS was produced. The agarose and PBS mixture was gently pipetted into plate and was allowed to cool on a smooth surface. Sets of five wells pentagonally with the sixth one in the center were arranged. The wells on the outside plate were filled with solution of 1:2, 1:4, 1:8, and 1:16 horse serum. The well in the center was filled with crude venom solution as antigen. The gel was placed in an incubator at 37 °C for 30 min and then kept at room temperature for 24 h. Precipitation lines among central wells with 1:2, 1:4, and 1:8 dilution of antibody was clearly observed (Deshpande, 2012).

Western blotting. The proteins of venom were denatured and separated on 15% SDS-PAGE gel, then transferred onto nitrocellulose membrane (Sigma Co.) by semi-dry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked with 3% bovine serum albumin (BSA) and incubated at room temperature for 90 min. The membrane was washed in several changes of PBST (phosphate buffered salt tween) buffer and the membrane was incubated with diluted horse anti-venom solution (1:20 diluted with PBST buffer) at 4°C overnight. Thereafter, the membrane was washed in several changes of PBST buffer and incubated with appropriate horseradish peroxidase (HRP)-conjugated rabbit anti-horse secondary antibody (1:10,000 dilution with PBST buffer) for 90 min at room temperature. After washing off unbound secondary antibody with PBST buffer, the bonds were visualized with substrate buffer (18 mg 4-chloro-1-naphthol (Fluka), 24 ml PBS, and 3 µL hydrogen peroxidase (Schindelin et al., 2012).

Isolation of immunodominant proteins identified by Western blot. Electro-elution.

In the present study, Bio-Rad model 422 electro-eluter was used to recover four sharp bond immunodominant proteins that were identified by Western blot and separated by SDS-PAGE electrophoresis. Briefly, after running a standard 15% preparative gel electrophoresis, the gel was stained with Coomassie blue to visualize the protein bands. The expected (14, 22, 32, and 65 kDa) protein bonds were cut out and diced into smaller slices and placed into six tubes. The tubes were filled with elution buffer (Tris/Glycin buffer was also used for gel electrophoresis). Then, silicone adaptors were filled with elution buffer and checked for leakage and air bubbles. The lead was attached and the elution conditions were set (8 to 10 mA/glass tube) for 5 h on a magnetic stirrer. The Per chamber was removed and the eluted proteins were pipetted from the membrane.

Next, they were dialyzed in elution buffer without SDS (overnight at 4 °C) in order to remove SDS from the isolated proteins; finally, it was concentrated with polyethylene glycol (PEG).

RESULTS AND DISCUSSION

Determination of protein concentration. Protein concentration of crude *N. oxiana* venom was determined 16 mg/ml by BSA standard protein using Lowry's method.

SDS-PAGE. Figure 1 shows the results of SDS-PAGE (15%), which determined the molecular weight of proteins of *N. naja* venom using the protein ladder (14, 20, 21.2, 22, 25, 32, 34, 37, 50, 65, 75, and 150 kDa). Proteins were defined by database searching to reveal the desired protein profile.They were homogeneous as judged by SDS-PAGE, and respectively, with molecular mass of acidic phospholipase A2 4 (PLA2), cytochrome b, cytochrome c oxidase subunit 1 (COI), ATP synthase subunit a, snake venom serine protease (NaSP), NADH-ubiquinone oxidoreductase chains (1, 2), L-amino-acid oxidase, zinc metalloproteinase-disintegrin-like (K-like and atragin), hepatocyte growth factor–like protein, Crumbs-like 1, in UniProt protein database (NCBI research), which is in agreement with previous reports (Oliveira et al., 2002; Huang et al., 2015) .

Gel diffusion assay. Figure 2 shows hyper-immunized serum of horse with dilutions of crude, 1:2, 1:4, and 1:8 precipitated with crude venom. The extreme and the least cross-reactivity of the horse serum with crude venom were respectively related to crude and 1:2 dilutions. 1:8 dilution of horse serum, cross-reactivity of which with the venom is shown more clearly in Figure 2, is suggested in this study and the results were investigated with this dilution.

Western blot analysis. Western blot analysis on antigen at the dilution 1:20 with serum hyper-immunized-horse (Figure 3) shows four sharp bands as 14, 22, 32, and 65 kDa molecular weights. Therefore, in this study we identified four novel antigens with high immunogenicity and immunoreactivity with small molecular weight (≤65 kDa) to develop anti-venom against *N. oxiana* in Iran. These four sharp bands indicated immunodominant proteins.

Identification of Isolation of (14, 22, 32, 65 kDa) immunodominant proteins by electro-elution. The electro-elution method was used for isolation of 14, 22, 32, and 65 kDa immunodominant proteins. Electro-elution of proteins from gel is the method used to recover proteins resolved by electrophoresis in polyacrylamide gel by transferring the protein molecules out of gel by means of an electric field (Dunn et al., 2004). Only four protein bonds with 14, 22, 32, and 65 kDa molecular weights were manifested in the Coomassie staining gel of the isolated proteins achieved from electro-elution. Then, the concentrations of the isolated proteins (14, 22, 32, and 65 kDa) were determined respectively as 1.3 .7, 0.7, and 1 mg/ml by Lowry's assay.

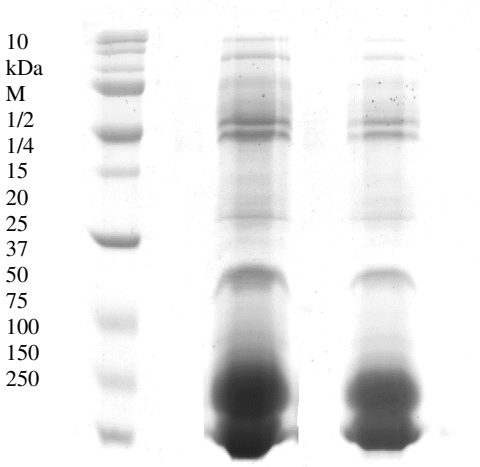

Figure 1. Separation of *N. oxiana* venom proteins by SDS-PAGE (15%): 1) molecular weight markers, 2) crude venom (dilution 1:2), 3) crude venom (dilution 1:4)

Snake venoms consist of numerous toxic components that cause haemorrhagic, coagulopathic, and neurotoxic pathologies in envenomed patients. Because of the inter-specific and intra-specific variations in toxin composition of snake venoms, treatment of envenoming is best achieved by administration of polyspecific anti-venom. Monospecific anti-venoms are used preferentially if the envenomed patient identifies the snake responsible for the symptoms (monospecific

Echis viper anti-venom in West Africa). (Petras et al., 2011).

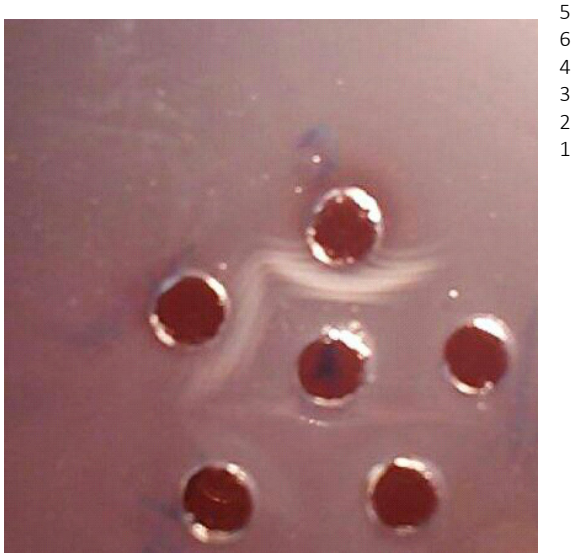

Figure 2. Gel diffusion assay using serum of hyper-immunized-horse against *N.oxiana* snake venom: 1) crude serum of hyper-immunized-horse, 2) serum of hyper-immunized-horse dilution (1:2), 3) serum of hyper-immunized-horse dilution (1:4), 4) serum of hyper-immunized-horse dilution (1:8), 5) serum of hyper-immunized-horse dilution (1:16), and 6) crude venom

Polyspecific anti-venoms are prepared from the sera of horses or sheep immunized with increasing doses of whole venoms from the most medically important snake species within a defined region. Anti-venoms, therefore, contain antibodies to the majority of venom components, irrespective of their toxicity. While this comprehensive responsiveness satisfies the polyspecific requirements of antivenoms, it also means that anti-venoms contain numerous redundant antibodies specific to non-toxic venom antigens that dilute the efficacy of the toxin-specific antibodies (Theakston and Reid, 1983). Among the venomous snakes, cobra (*Naja*) is one of the most common biters in both Asia and Africa. Its bite delivers potent three-finger neurotoxins (NTXs) that cause rapid onset of neuromuscular paralysis, leading to respiratory failure, and death. Cobra venom also contains other three-finger toxins such as cardiotoxins (CTXs, a.k.a. cytotoxins) and phospholipases A2 (PLA2s) (Guan et al., 2010). Though these toxins have a lower degree of lethality, they are also responsible for severe tissue necrosis causing permanent physical disabilities. Many studies were performed to shed light on the structure and function relationships of cobra venom toxins (Tong et al., 2012). As of now, three-dimensional structures of most toxic components including NTXs (X. Lou *et al.,* 2004) and PLA2s (White, 1990) from *Naja atra*, are available. Shuting *et al.* (2004) performed proteomic characterization of the snake venom of *N. naja,* and in another study, Chellapandi *et al.* (2008) extracted proteins of Indian cobra, and determined the molecular weight of purified venom proteins through SDS-PAGE, the results of which were similar to our findings (Figure 1).

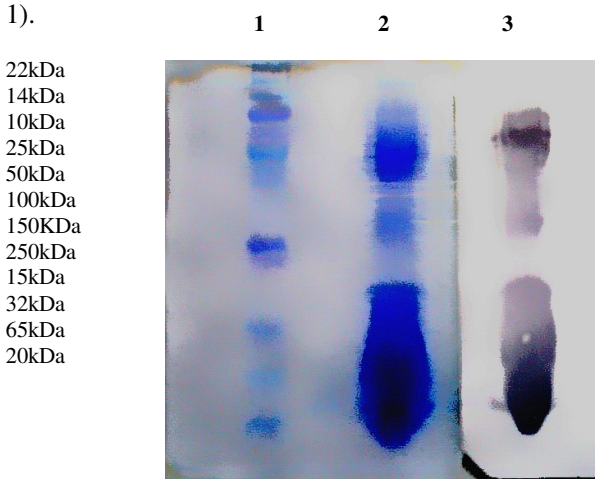

Figure 3. Western blot analysis on *N. oxiana* venom: 1) molecular weight markers, 2) crude venom from *N. oxiana* snake after being transferred onto nitrocellulose membrane by SDS-PAGE (15%) Coomassie brilliant blue R-250 stain, and 3) immunological reactivity of antibody and crude venom from *N. oxiana* snake

Harrison *et al. (*2003) studied snake venom toxins to generate toxin-specific antibodies with polyspecific cover. In that study, *N. naja* snake venom was included to assess the specificity of the antibody. They found that JD9-specific antibody reacted strongly with 50-60 kDa proteins from *B. jararaca* venom, but *N .naja* was not reactive to this antibody. Since metalloproteases from *N. naja* species shared antigenic domains with analogous molecules in venoms, they were reactive to JD9 antisera. In addition, Hsuan-Wei Hung *et al.* (2015) reported CTXs (8980-9323 Da), acidic

phospholipase A2 (PLA2 16,013 Da), and zinc metalloproteinase-disintegrin-like (K-like and atragin respectively SVMP 66,292 Da and 69,180 Da) extracted from *Naja atra* with high immunogenicity and protective potential to immunize mice against snakebite of individual *Naja atra*. Accordingly, it is important to identify immunogenicity of proteins of *Naja oxiana* venom. In the present study, we investigated and isolated four novel proteins of *Naja oxiana* snake venom with molecular weights of 14 kDa, 22 kDa, 32 kDa, and 65 kDa that reacted strongly to horse antibody. Immunodominant proteins were homogeneous as judged by SDS-PAGE, and respectively with molecular mass of acidic phospholipase A2 4 (14,015 Da with AC.NO:Q6T179 and 16,013 kDa, AC.NO: P00598), cytochrome C oxidase subunit 1 (COI) (22,915 Da, AC.NO: A0A0A7PA27), snake venom serine protease (Nasp) (31,137 Da, AC.NO A8QL53), zinc metalloproteinase-disintegrin-like (*K-like and atragin*) (66,292 Da with AC.NO D3TTC1 and 69,180 Da, AC.NO D3TTC2) in UniProt protein database (NCBI research), which was in line with previous reports (Oliveira et al., 2002; Huang et al., 2015). The proteins isolated from *Naja oxiana* with high immunogenicity are important for the defensive mechanism of innate immunity system. For example, there is a family of secreted phospholipase A2 with low molecular weights (13-15 kDa) (Oliveira et al., 2002; Wei et al., 2006; Zhong et al., 2006) that are able of killing bacteria. We reported four immunodominant proteins with molecular weights of 14, 22, 32, and 65 kDa from elapid snake that can be included to assess a potential specific monovalent anti-venom or antiserum candidate against *Naja oxiana* venom in Iran. Therefore, four immunodominant proteins could be used to provide a better anti-venomic approach for snakebite management in Iran and other countries (Africa, Asia, and Oceania), which have serious problem of snakebites. In addition, we suggested a simple and economical method to prepare suitable immunogens for saving snakebite victims from venomic components.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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