Research Paper:

Two Proteins From Snake Venom Have Potent Antibacterial Effects Against Bacillus Anthracis and Streptococcus Pneumoniae

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ABSTRACT

Background: Antibacterial proteins are widely expressed in snake venoms. Previously, we have isolated two immunodominant proteins with molecular weights of 14 and 65 kD from the snake venom of Naja naja oxiana (N. oxiana). It was demonstrated that they had potent inhibitory effects against gram-positive bacteria, S. aureus and B. subtilis but were less effective against gram-negative bacteria, such as E. coli and P. aeruginosa. This study aimed at investigating the potential antibacterial effects of the two proteins against Bacillus anthracis and Streptococcus pneumoniae.

Methods: The proteins were identified by SDS-PAGE and western blot analysis, and isolated by Gel Electrophoresis (Electro-elution). The antibacterial effects were tested against the strains of Bacillus anthracis and Streptococcus pneumoniae, using broth microdilution and disc-diffusion assays. For comparison, the antibacterial effects of standard antibiotics, such as Gentamicin, Ampicillin, Penicillin, Amoxicillin and Ciprofloxacin were also tested on the same B. anthracis and S. pneumoniae batches under identical laboratory conditions.

Results: The two proteins showed high immunogenicity and strongly inhibited the growth of gram-positive bacteria, B. anthracis, and to a lesser extent S. pneumoniae.

Conclusion: The isolated proteins demonstrated strong antibacterial effects against Gram-positive bacteria, B. anthracis and S. pneumoniae, in addition to their previously known effects against S. aureus and B. subtilis.

Keywords: Naja naja oxiana; Immunodominant proteins; Antimicrobial effects; Pathogenic & Gram-positive bacteria

Introduction

Potent antimicrobial agents inhibit the growth of microorganisms at low concentrations. Antibiotics include not only chemically-synthesized drugs but also naturally occurring compounds from various toxins derived from animals and microorganisms [1]. Snake venoms contain proteins and peptides with a variety of biological properties [2, 3]. These venoms are divided into three categories based on their toxicity:

A. Hemotoxicity, promoting hemorrhage, extensive local edema and tissue necrosis,

B. Neurotoxicity, inhibiting skeletal muscle contractions and respiratory funtion, and

C.) Cardiotoxicity, causing irreversible depolarization of cardiac cell membranes, leading to the paralysis of heart muscle [4, 5].

Cobra is one of the world's most poisonous snakes with cytotoxic venom, indigenous to countries in Asia, Africa, and Oceania [4, 5]. Previous studies have demonstrated that L-amino acid oxidase [6, 7], lectin [1] and phospholipase A2 [8] have antibacterial properties. Antibiotics, derived from venoms and microorganisms, can be alternatives for treating bacterial infections besides chemically synthesized drugs [2]. It is known that naturally occurring antibacterial proteins are widely expressed in snake venoms [3, 9]. A variety of proteins and peptides from the venoms have been identified and investigated that are highly potent in killing a wide range of bacteria [3, 10].

In a preliminary study, we investigated the antibacterial properties of the venom from cobra N. oxiana, one of the major snake species found in northeastern Iran [10]. That study demonstrated the antibacterial effect of several proteins isolated from the venom of N. oxiana snake, with varying molecular weights of 14, 22, 32 and 65 kilodalton (kD). Specifically, we tested these proteins against several strains of bacteria, including Gram-positive S. aureus, B. subtilis and P. aeruginosa, and Gram-negative E. coli, using broth microdilution and disc-diffusion assays [10]. This paper reports the antibacterial effects of the two isolated proteins (14 & 65 kD) from the same venom against Gram-positive bacteria, B. anthracis and S. pneumoniae. The findings confirm the broad antibacterial efficacy and potency of these venom proteins.

Materials and Methods

The lyophilized crude venom of N. oxiana was a kind gift from the "Poisonous Animal Department" at Razi Vaccine and Serum Research Institute (Karaj, Iran). Also, the sera of hyper-immunized horses was provided by Razi Institute, while the whole molecule, rabbit anti-horse IgG peroxidase conjugate was purchased from Sigma Aldrich (Taufkirchen, Germany).

Micro-organisms: The Gram-positive bacteria including B. anthracis (ATCC 2397) and S. pneumoniae (ATCC 2072) were obtained from Razi Type Culture Collection (Karaj, Iran).

Standard antibiotics: The standard preparations of antibiotics were used at the following concentrations per milliliter of distilled water: Ampicillin (10 µg), Gentamicin (10 µg), Penicillin (10 µg), Ciprofloxacin (5 µg), and Amoxiclav (25 µg).

Chemical reagents: Ammonium Persulfate (APS), TWEEN 20 viscous liquid, acrylamide, SDS and TEMED were obtained from Bio-Rad (Hercules, CA, USA); Phosphate Buffered Salin (PBS) was obtained from Merck (Kenilworth, NJ, USA), Bovine Serum Albumin (BSA), chloro-1-naphthol from Fluka Chemicals (Buchs, Germany), and hydrogen peroxidase purchased from Sigma Aldrich (Taufkirchen, Germany).

Determination of protein concentrations: Protein concentrations were determined by the Lowry method, using BSA as the standard, by the method previously described [10-12].

SDS-PAGE: Electrophoresis was carried out on a 15% polyacrylamide gel by the method of Laemmli as previously described [10, 13].

Western blotting: The venom proteins were denatured, separated by 15% SDS-PAGE gel electrophoresis, and transferred onto nitrocellulose membrane (Sigma Aldrich; Taufkirchen, Germany) by semi-dry transfer cell (Bio-Rad; Hercules, CA, USA) as previously described [10].

Evaluation of immunodominant proteins: A Bio-Rad model 422 electro-eluter was used to recover four sharp bands of immunodominant proteins that were identified by western blot and separated by SDS-PAGE electrophoresis, as previously described [10].

Disc diffusion: Disc-diffusion assay was performed by the method of Bauer et al., [14] as previously described [10]. The experiments were performed in four replicates.

Broth micro-dilution assay: Broth micro-dilution assay was carried out according to the protocol established by the National Committee for Clinical Laboratory Standards (NCCLS, 2000) as previously described [10].

Results

Determination of protein concentration: The protein concentration of the crude Naja Naja venom was determined to be 16 mg/mL with Bovine Serum Albumin (BSA) as the standard.

SDS-PAGE: Based on the results from a 15% SDS-PAGE, the molecular weights of the proteins in Naja Naja venom was determined to be 14, 20, 21.2, 22, 25, 32, 34, 37, 50, 65, 75 and 150 kD, using the protein ladder.

Table 1. Antibacterial activity of the immunodominant proteins

Immunodominant Protein or Antibiotic	Bacillus Anthracis	Streptococcus Pneumoniae
Immunodominant protein 1*	16.5 ± 0.50	7.1 ± 0.36
Immunodominant protein 2 Ω	15.76 ± 0.25	7.43 ± 0.40
Ampicillin	19.26 ± 0.64	24.13 ± 0.41
Gentamicin	35.88 ± 0.34	10.13 ± 0.32
Penicillin	10.66 ± 0.28	16.13 ± 0.20 0.0±0.0
Ciprofloxacin	30.88 ± 0.34	20 ± 0.80
Amoxiclav	19.04 ± 0.16	24.2 ± 0.13

 Each number represents the Mean±SD of inhibition zone in mm (n=4)

*14 kD; Ω 65 kD

Table 2. Antibacterial activities of the immunodominant proteins from N. oxiana

Bacteria	Immunodominant Proteins and Standard Antibiotics	MIC (μg/ml)
Streptococcus pneumoniae	Immunodominant protein 1 *	>120
	Immunodominant protein 2 Ω	>170
	Ampicillin	25
	Gentamicin	120
	Penicillin	50
	Ciprofloxacin	10
	Amoxiclav	25
Bacillus anthracis	Immunodominant protein 1*	31
	Immunodominant protein 2 Ω	35
	Ampicillin	25
	Gentamicin	10
	Penicillin	120
	Ciprofloxacin	<5
	Amoxiclav	25

* 14 kD; Ω 65 kD

Western blot analysis: Based on a western blot analysis of the antigen at a 1:20 dilution with serum hyper-immunized- horse, 2 sharp bands were visualized the molecular weights of 14 and 65 kD. These were highly immunogenic and immunoreactive, and were chosen for our study.

Evaluation of the immunodominant proteins: The immunodominant proteins, isolated by electro-elution and Coomassie blue staining, had molecular weights of 14 and 65 kD. The concentrations of these isolated proteins were determined to be 1.3 and 1 mg/mL, respectively, by the Lowry assay [11].

Disc diffusion: Table 1 represents the antibacterial activity of the two crude proteins isolated from N. Oxiana (14 & 65 kD). It also presents the various antibiotic

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activities against Gram-positive bacteria, B. anthracis and S. pneumonia on Moeller Hinton agar, tryptic soy broth and blood agar plates which revealed the inhibitory zones for B. anthracis and S. pneumonia, respectively. These proteins support the innate defense and immune mechanisms against Gram-positive bacteria and snake-bite venoms.

Broth microdilution assay of the immunodominant proteins: Table 2 represents the Minimum Inhibitory Concentration (MIC) values of the immunodominant proteins and of the standard antibiotics against the two bacteria, B. anthracis and S. pneumonia.

Discussion

Previous studies [3, 9, 10] have demonstrated that a variety of snake venoms possess a wide range of anti-bacterial effects. Generally, antibacterial proteins are cationic in nature [15] and it is believed that they exert their bactericidal effects through increasing the permeability of the bacterial membranes, by destabilizing the lipid bilayer membranes of the bacteria [16-18]. Similarly, the bactericidal mechanism of anionic antimicrobial proteins against Gram-positive bacteria, such as S. aureus, involves interactions with the membrane lipids' head groups [17]. In addition to increasing the membrane's permeability, antibacterial proteins and peptides destroy bacteria by inhibiting macromolecular biosynthesis [19] and interacting with specific molecular components within the bacteria [20].

Studies have suggested that the interaction of autolytic enzymes with peptides and proteins may lead to bacterial death [10, 21, 22]. The mechanisms of bactericidal and membrane damaging effects have already been confirmed at ultrastructural levels [22]. Lee, et al. [22] isolated L-amino Acid Oxidase (LAAO), a 65kD protein from the venom of King Cobra snake (Ophiophagus hannah) and demonstrated that the LAAO had greater bactericidal potency against Gram-positive bacteria than Gram-negative ones. Hung, et al. [23] determined the molecular weights of the cytotoxins to range between 8980 and 9323 Daltons. Specifically, various components had the following molecular weights: acidic phospholipase A2,16,013 Da; the zinc metalloproteinase, disintegrin-like and Atragin 66,292 and 69,180 Da, respectively.

These immunogenic and immunoreactive proteins have been extracted from Naja atra snake venom.

In a previous study [10], we identified several immuno-dominant proteins (14, 22, 32 & 65 kD) in the venom of N. oxiana cobra snake. As an extension to that study, we investigates the antibacterial effects of the two highly immunogenic proteins (14 & 65 kD) against Gram-positive

bacteria S. aureus and B. subtilis. The MIC values of these proteins against these bacteria were comparable with those of the tested antibiotics. The only exception was Ciprofloxacin, which was far more potent antibacterial agent than the two isolated proteins (Table 2).

Results from our study on the antibacterial activity of the 65kD protein strongly confirmed the findings of previous studies [10, 21, 22]. However, these proteins were only slightly effective against the two Gram-negative bacteria, P. aeruginosa and E. coli. Also, the highly immunodominant 22 and 32 kD proteins showed slight antibacterial activity against the Gram-positive and Gram-negative bacteria compared to those reported for the two potent proteins in the current study. The results suggest that the 14 and 65kD proteins were more potent agents against the Gram-positive bacteria, S. aureus and B. subtilis than the Gram-negative E. coli and P. aeruginosa [10]. Hence, the reason for undertaking the current study to further explore the antibacterial potencies of the two proteins.

It is hoped that the findings of this study provide future directions for the development of better anti-venomic and antibacterial agents toward the management of patients with snakebites in Iran and other nations in Africa, Asia and Oceania. These nations have had serious problems with the management of such patients and numerous drug-resistant poisonous organisms. In addition, the results of this study add new knowledge for the development of simple, economical and naturally occurring therapeutic agents, leading ultimately to saving of lives for many patients suffering from snakebite envenomation in Iran and elsewhere.

Conclusions

The two isolated, immune-dominant proteins from N. Oxiana snake venom with the molecular weights of 14 and 65kD possess potent bactericidal effects against Gram-positive bacteria, B. anthracis and to a lesser extent S. pneumonia. These proteins show MIC values comparable to those of standard antibiotics, such as Ampicillin, Gentamicin, Penicillin, Ciprofloxacin and Amoxiclav. Elucidation of the proteins' mechanisms of action on various bacteria and other microorganisms at cellular and molecular levels awaits future research.

Ethical Considerations

Compliance with ethical guidelines

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