ORIGINAL ARTICLE

Spider venom toxins, its purification, solubilization, and antimicrobial activity

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Abstract

Aim: Purification of spider venom *Crossopriza lyoni* toxins on a Sepharose CL-6B 200 column and evaluation of antimicrobial susceptibility in infectious bacterial pathogenic strains. **Materials and Methods:** Solubilization of spider venom toxins in Triton X-100 (0.1%), phosphate-buffered saline, and trichloroacetic acid.For obtaining purified toxins/ peptides, and determination of their molecular weight of toxins, poison gland homogenate was loaded on a gel filtration column and 135 regular fractions were eluted at constant rate of 5mL/min. Antimicrobial potential was determined in serial microdilution assays and agar disc diffusion method in presence of spider toxin peptides. **Results and Discussions:** The elution pattern of purified and homogenized mouth poison glands displayed two major peaks at 280 nm. The first one was eluted in fraction No. 43–51 while the second one after fraction no. 61–90. From gel filtration chromatography, total yield of protein obtained was 67.3%. Low-molecular-weight peptides (6–64 kDa) were obtained in spider venom. These were subjected to employ for antimicrobial study that revealed possible interaction between different concentration ranges of spider venom toxins. In serial microdilution assays, low minimum inhibitory concentration values 3.75 µg/ml were obtained in the presence of spider toxins. By agar disc diffusion method, the diameter of inhibition zones in mm in the presence of spider toxin at a concentration range of 98.56–1.92 µg/ml in *Escherichia coli, Salmonella typhi,* and *Vibrio cholera* obtained was 17.86 ± 0.21 mm, 16.16 ± 0.21 mm, and 18.66 ± 0.21 mm, respectively. **Conclusion:** In the present investigation, spider toxins have generated lytic effect in bacterial cells that results in heavy cell death. No doubt spider toxins can be used as alternate of broad-spectrum antibiotics.

Key words: Antibacterial potential, *Crossopriza lyoni,* inhibition zone diameter, venom protein

INTRODUCTION

Spiders are venomous animals belong to family Araneae (Arachnida). These bear toxin-secreting venom glands in their mouth. Spiders use envenomation for hunting the prey mainly insects and for making territorial defense. Spider bite is a major problem in many Asian, African, and other tropical countries. Dwelling spiders spin web of threads in corners of houses to catch the insects. They make instant stinging to paralyze insects simultaneously. Spider venom is a rich source of various short peptides which show neurotoxic, cytotoxic, hemotoxic, and insecticidal activity. This structural and functional diversity in spider toxins was evolved during long evolutionary timescale that is used employed to paralyze and kill the prey or to deter a predator from the territory.[1] Spider toxins are short basic peptides of 32–76 amino acids in length and possess 2–4 disulfide bridges. Spider venom contains a mixture of biologically active compounds with diverse biological activities. Its venom toxins are enzymes, proteins, acids, salts, carbohydrates, amino acids, and biogenic amines.[2] Spider toxins impose multiple biological effects such as muscle and respiratory paralysis, integumentary, neuroinflammatory, and neurotoxicity in small animals. In animals, visible symptoms of spider envenomation are lacrimation, hypertension, salivation, sweating, scratching, and agitation followed by spastic paralysis of the posterior and anterior extremities. Spider toxins (JZ TX-V) inhibit ion channels present on nerve cell membrane[3] and show platelet aggregation in blood circulatory system.[3] Spider

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toxins impose irreversible paralysis in lepidopteran insects by massive transmitter release, generate insecticidal effect, and kill insect larvae at a very low dose.[4] Spider venom toxins generate necrotizing skin lesions, do cell necrosis, and display systemic reactions and impose death in animals.[5]

Spider toxins are also good antibacterial agents as they were found active against positive bacteria such as *Staphylococcus aureus* /methicillin-resistant *S. aureus* [6] and show bacteriostatic effect against number of bacterial strains such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *S. aureus* and fungistatic effect against *Candida tropicalis* and *Candida krusei*.[2] These animal origin natural products show more potency against human pathogens and are less toxic than synthetic antibiotics. Lycosin-II isolated from the venom of the spider *Lycosa singoriensis* displays potent bacteriostatic effect against drug-resistant bacterial strains.[7] Spider venom toxin peptides interact with ligand-gated channels and modulate the activity of neuronal ion channels and receptors located on cell membrane. Spider toxins show inhibition of voltage-gated ion channels,[8] and especially target vertebrate or invertebrate voltage-gated potassium (Kv), calcium (Cav), or sodium (Nav) channels. They also target specific Na (V) channel subtypes and show analgesic effects. Spider venom peptides bind on purinergic receptors, channels such as acid-sensing ion channels, mechanosensitive channels and transient receptors, and potassium channels. Venom toxins generate various channelopathies including epilepsy, arrhythmia, and paralytic myotonic effect with severe pain.[9]

Toxin peptides can also become a good source of strong antimicrobials and can replace broad-spectrum antibiotics which are highly toxic and show multiple biological effects and responsible for drug resistance in microbes.[10] Spider venom is concerned to be a potential source of modulators for all of drug targets. These are proved much valuable tool for the investigation of structure and function of channels and for drug development.[11] These are good therapeutic agents for the treatment of cardiovascular disorders, chronic pain, and inflammation.[12] Though, in the past various anti-microbial toxins were reported from different spider toxin species.[13,14] which show lytic activity against Gram-positive and Gram-negative bacteria.[15] In the present investigation, venom from long tail spider *Crossopriza lyoni* was isolated, purified, and evaluated for its antibacterial susceptibility against three important microbial diseases pathogens, i.e., *Salmonella typhi, Vibrio cholera,* and *Escherichia coli.*

MATERIALS AND METHODS

Microbial Culture

Laboratory culture of bacterial strains *E. coli* (ATCC 25922), *S. typhi* (MTCC 98), and *V. cholera* (MTCC 3906) was maintained using Luria broth (2% w/v) culture medium for 4 days at 37°C before use, and 100 µl of the overnight culture was mixed in the test as well in control. Bacterial cultures were stored at 4°C and subcultured after 7 days in solid agar medium [Figure 1a and 1b] .

Solubilization of Venom Gand Homogenate

The living spider *C. lyoni* were collected from different region of Gorakhpur city. The collected spider was immobilized by quick freezing at −20°C. The venom chelicera glands were taken out by cutting at mouth part of spider and venom gland was homogenized in phosphate buffer saline (50 mM, pH 7.2) with the help of power homogenizer. This homogenate was centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatant was used as crude venom toxin of spider.

The field-collected spider *C. lyoni* in 400 numbers were anesthetized with chloroform and dissected in cold phosphate-buffered saline (PBS). Two venom glands with white and curved sacs located in the cephalothorax and open at the base of chelicerae, end in sharp, and chitinous fangs were taken out from its mouth part. These were homogenized in glass-glass homogenizer in 5 ml of different solubilizing buffer agents such as Triton X-100, trichloroacetic acid (TCA) 10%, Tris+EDTA, PBS, and absolute alcohol separately [Figure 2]. Homogenate was centrifuged at 10,000 rpm for 10 min at 4°C

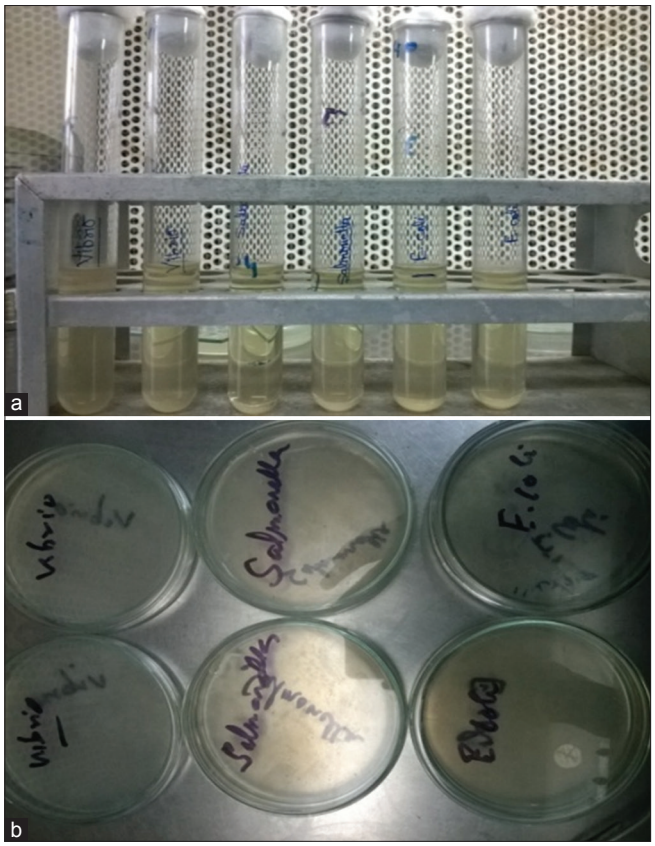

Figure 1: (a) Microbial culture in Luria broth medium. (b) Subculture in solid agar medium

and supernatant was taken out and venom protein present in supernatant was estimated by the Lowry's method.[15]

Purification of Venom Protein of Spider Toxins

Proteins were eluted on a Sepharose CL-6B-200 a double cavity gel filtration column[16] with sintered disc filtered in the bottom having a height of 1 m in 25 mm diameter. A known volume, i.e., 5 ml of toxin protein solubilized in PBS was loaded in the column, and the flow rate between 1 ml/min was maintained by a continuous buffer supply in a cold room. Eluted fraction collected at a fixed time interval using a Pharmacia fraction collector, and the values of protein concentration in different eluted fraction were plotted on graph; 260 nm, 280nm and 540 nm [Figure 3a-3d]. Column was tightly held by clips and held erect withstand. The eluted fractions containing venom protein were pooled and lyophilized to a desired concentration of the venom proteins. Dialysis bag made of cellulose membrane was boiled for 10 min in a large volume of 2% sodium bicarbonate and 1 mM EDTA and then rinsed the membrane thoroughly in distilled water inside and outside before use. The lyophilized venom protein was filled in the dialyzing bag and dialyzed again three changes of phosphate buffer (50 mM, pH 7.2) to remove the excess salt from the lyophilized protein venom solution of *C. lyoni*.

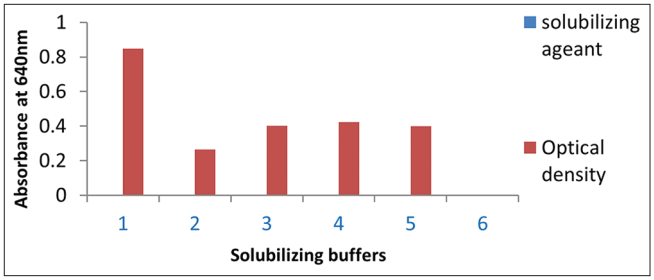

Figure 2: Solubilization of whole gland from *Crossopriza lyoni* in different buffers. The absorbance of solubilizing protein was taken at 640 nm. Solubilizing buffer on X-axis is 1 - Triton X-100, 2 - phosphate buffer, 3 - 10% TCA, 4 - Tris+EDTA, and 5 - absolute alcohol.

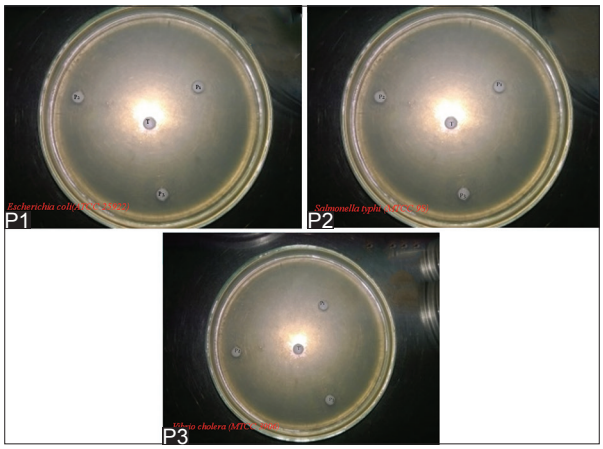

Figure 3: Inhibitory effects of *Crossopriza lyoni* (T=98.56–1.92 µg/ml) on the growth of different bacterial strains compared with P1 - tetracycline, P2 - ampicillin, and P3 - cyprofloxacine

Antimicrobial Assays

Bioassays were performed for the evaluation of antimicrobial activity of purified spider venom toxins test by agar disc diffusion method. In this test, sterile filter paper discs (Whatman No. 1) of 6 mm in size were coated with different concentrations of spider venom toxins solubilized in phosphate buffer saline (pH 6.9) [Figure 4]. Inoculums size was adjusted to 10^6 colony-forming units (CFU/ml). It was spread evenly on agar plate surface by a sterile rubber pad. Each toxin was assayed in triplicate. Sterile distilled water was used as negative control. Tetracycline, ampicillin, and ciprofloxacin were used for comparison. Plates were incubated for 24 h at 7°C and diameter of inhibition zones was measured (NCCLS).[17]

For determination of antimicrobial susceptibility in purified spider venom toxins tests in the liquid medium were conducted according to the method of Amsterdam's method.[18] Serial microdilution method was employed for making dilution of spider toxins up to 10^{-10} using Luria broth, final concentrations ranged from 58.57 to 0.229 mg/ml, and assays were done in triplicate. Minimum inhibitory concentration (MIC) values were the lowest concentration of the spider toxins where no turbidity was observed in the culture flask after 24 h incubation at 37°C for three infectious bacterial species, and it was standardized in terms of absorbance at 600 nm in spectrophotometer. For the determination of minimal bactericidal concentration (MBC), inoculums size was adjusted to 10^6 CFU/ml in sterile agar plates and determined again after incubation at 37°C for 24 h in all test and control discs. The lowest concentration at which no visible growth was obtained in agar plates was considered as MBC values. For evaluation of inhibition, two parallel controls were set for each test extract. Bacterial growth was observed in the

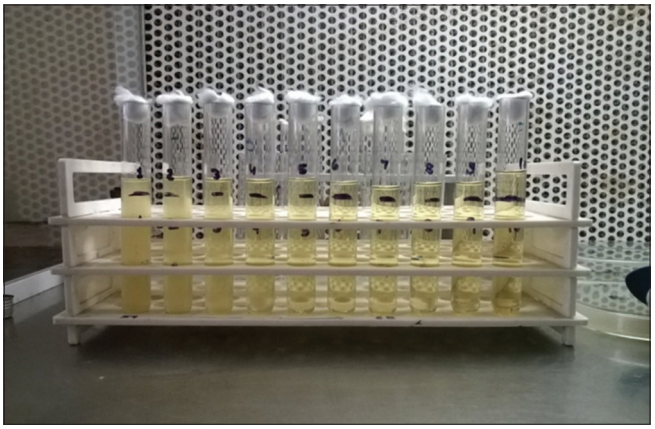

Figure 4: Minimum inhibitory concentration values for three infectious bacterial species by microdilution method

presence of different quantities of spider venom toxin as well as in its absence [Figure 5].

RESULTS

For determination of antimicrobial activity regular bacterial cultures were maintained at 27±05° C and sub cultured after 7 days in solid agar medium [Figure 1]. In the present investigation, Triton X-100 proved to be a good solubilizing agent for venom protein. Higher protein solubilization was observed in the supernatant than in the residue, except TCA [Figure 2]. The elution pattern of purified and homogenized toxin glands of spider exhibited two major peaks at 280 nm in the fraction no. 41–51 and fraction no. 81–91 [Figure 3a]. Further, concentration and fractionation of venom proteins again revealed two peaks at 640 nm, a minor one between the fraction no. 39 and 55 and a major peak between fractions 77 and 96 [Figure 3b]. Both peaks were eluted with 0.13 M NaCl PBS buffer (pH 6.9) and protein estimation was done for each fraction using Lowry's method. The total yield of protein was 56.21% and specific activity was determined in each fraction [Figure 3c]. The molecular weights of spider venom protein fractions were ranging from 6 to 64 kD [Figure 3d].

By agar disc diffusion method the diameter of inhibition zones in mm in presence of spider toxin at a concentration range 98.56-1.92µg/ml of investigation *E. coli*, *Salmonella typhi* and *Vibrio cholera* are 17.86±0.21mm, 16.16±0.21mm and 18.66±0.21mm respectively [Figure 4]. [Table 2]. Minimum inhibitory concentrations obtained in presence of different quantities of spider venom toxins were lower than broad spectrum drugs [Figure 5].

DISCUSSIONS

In the present study, antimicrobial susceptibility tests have clearly displayed that purified spider venom toxins are stronger bactericidal than synthetic antibiotics. It is supported

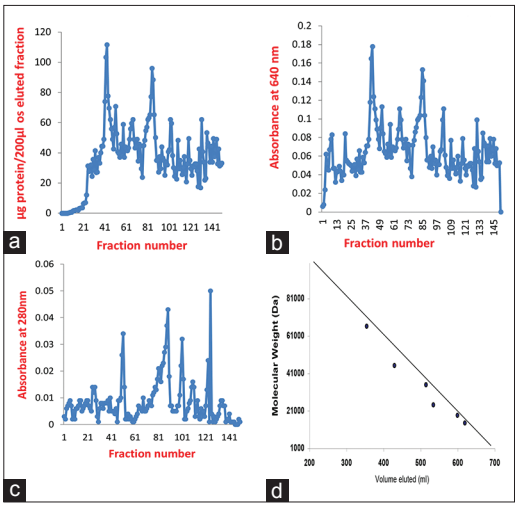

Figure 5: Elution pattern of phosphate-buffered saline extractable proteins of *Crossopriza lyoni* chromatographed on Sepharose CL-6B column (a) absorbance at 260 nm, (b) absorbance at 280 nm, (c) absorbance at 640, (d) µg protein/200 µl fraction, and (e) standard proteins chromatographed on Sepharose CL-6B 200 column for determining the molecular weights of venom proteins/peptides isolated from *C. lyoni.* Proteins used were bovine albumin mol. wt 66,000, egg albumin mol. wt. 45,000, pepsin mol. wt. 34,700, trypsinogen mol. wt. 24,000, beta-lactoglobulin mol. wt 18,400, and lysozyme mol. wt. 14,300. Elution volumes of unknown proteins were compared with log values on the X-axis for the estimation of molecular weights.

Table 1: Zone of inhibition of spider venom toxin isolated from *Crossopriza lyoni* on different microbes and their corresponding IZD

Name of species	Concentration of toxins (µg/ml)	Spider toxin (IZD in mm)	Negative	Positive 1	Positive 2	Positive 3
E. coli	98.56–1.92	17.86±0.21*	±	13.8±0.12	16.43±0.28	9.86±0.17
Salmonella typhi	98.56–1.92	16.16±0.21	±	11.3±0.25	13.60±0.23	12.00±0.24
Vibrio cholera	98.56–1.92	18.66±0.21	±	11.03±0.17	12.36±0.28	13.60±0.23

*Values are expressed as mean±SD (*n*=3) positive controls are 1 - Tetracycline, 2 - Ampicillin, 3 - Ciprofloxacin, Negative control is distilled water. *C. lyoni: Crossopriza lyoni*, SD: Standard deviation

Table 2: Antimicrobial activities of venom toxins isolated from spider *Crossopriza lyoni* on different microbes and their corresponding MIC

Name of species	Concentration of toxins (µg/ml)	Toxin MIC (test)	Negative	Positive 1	Positive 2	Positive 3
E. coli	60–0.127	7.5	_	12.5	27.5	25
S. typhi	60–0.234	3.75	_	6.75	6.87	12.5
V. cholera	60–0.234	3.75	_	6.75	6.87	12.5

Positive controls are 1 - Tetracycline, 2 - Ampicillin, 3 - Ciprofloxacin and test represent spider toxins, negative control is distilled water.
S. typhi: Salmonella typhi, V. cholera: Vibrio cholera, E. coli: Escherichia coli, MIC: Minimum inhibitory concentration

by significantly lower MIC values obtained in spider toxins in serial dilution and agar disc diffusion assays than the broad-spectrum antibiotics tetracycline, ampicillin, and ciprofloxacin, respectively. These were found 12.5–25 µg/ ml *E. coli* while it was 7.5 µg/ml for spider toxins, 6.75–12.5 µg/ml for *S. typhi,* and 6.75–12.5 µg/ml for *V. cholera* while it was 3.75 for spider toxins [Table 2]. Similar activity was reported in the spider venom peptide Lycosin-II[12] and latarcin against clinically isolated bacterial pathogens.[12,13] Similar MIC and MBC from 3.9 to 500 µg/mL were reported in *Aeromonas* sp., *Bacillus subtilis*, and *Micrococcus luteus* by Ferreira *et al*., 2016, in spider *Lasiodora* sp. Similarly, Lycosin-I peptide is isolated from the venom of the spider *Lycosa singorensis* shows much higher antipathogenic activity *in vitro* bioassays against multidrug-resistant *Acinetobacter baumannii*.[12,15,16] It is a cationic and amphiphilic peptide[17] which strongly acts on lipid membrane, and disintegrates its structure and cause lysis.[18,19] Similarly, LyeTx I was found active against bacteria *E. coli* and *S. aureus* when applied in dose-dependent manner. Most of the spider toxin peptides are alpha-helical amphipathic which directly interact with the cell membrane, perturb it and show bactericidal activity.[11] However, a single glycine to alanine substitution increases antimicrobial peptide interactions with lipid membrane as it is reported in latarcin 2a spider toxin peptide.[20] Similarly, L- to D-amino acid substitution affects deamidation activity and increases membrane interactions.[21] From the inhibition zone diameter assays, the IZDs obtained were larger in case of spider venom than antibiotics. By agar disc diffusion method, the diameter of inhibition zones in mm in the presence of spider toxin at a concentration range of 98.56–1.92 µg/ml of investigation *E. coli, S. typhi,* and *V. cholera* is 17.86 ± 0.21 mm, 16.16 ± 0.21 mm, and 18.66 ± 0.21 mm, respectively [Table 1]. These were much larger than that of tetracycline, ampicillin, and ciprofloxacin, i.e., 9.86 ± 0.17–13.8 ± 0.12, 11.3 ± 0.25–13.60 ± 0.23, and 11.03 ± 0.17–13.60 ± 0.23 found in three bacterial strains *E. coli, S. typhi,* and *V. cholera* [Table 1]. Triton X-100 (0.1%) proved to be a good solubilizing agent for toxin/proteins. Higher protein solubilization was observed in the supernatant than in the residue, except TCA [Figure 1].

Spider toxins show hemolytic[21] and strong cytolytic[22] anticancer activity against human cancer cells.[23] At very lower concentration, they exhibit much better broad, spectrum activity against bacteria.[24-26] As compared to previous reports, purified toxin peptide (6.7kDa mwt) from long tail spider *C. lyoni* inhibited the growth of microorganisms at low micromolar concentrations. These might also active against drug-resistant bacterial strains and could be used to combat bacterial infections mainly communicable pathogens.[27] Here, it can be concluded that spider toxins are potential antimicrobial therapeutic agents.[7] These could be used to develop novel antimicrobials can easily replace highly toxic, expensive, and put adverse effect on patients after their administration.[28] Hence, spider venom is good toxin resources that can be used to produce the next generation of newer safe, more effective antimicrobial drugs.[29]

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