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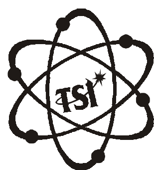

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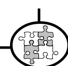
Isolation And Characterization Of Antibacterial Peptide From Indian Cobra *(Naja Naja)*

Karuna Kumar
Department of Studies in Biochemistry
University of Mysore, Manasagangothri,
Mysore – 570 006, Karnataka, (INDIA).
Phone: +91 – 98862 - 44818 (Mobile)
+91-821-2598034
E-mail: mkkmou@yahoo.co.in



M.K.Sachidananda., D.Channe Gowda.
Department of Studies in Biochemistry,
University of Mysore,
Mysore- 570006, (INDIA).

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ABSTRACT

Due to the development of antibiotic resistant in microorganisms, antimicrobial peptides from natural source have attracted attention in recent times. In this regard several antimicrobial peptides have been isolated from a wide range of animal source in general and snake venom in particular. Naja naja venom showed antibacterial, direct and indirect hemolytic activities. Antimicrobial peptide has been purified through gel permeation and ion exchange chromatography. The molecular mass was found to be 2491 Da by MALDI-TOF mass spectrometry and the amino acids sequence of the N-terminus was found to be DEQSTHGAYVWKL. The purified peptide showed potent antibacterial activities against gram-negative and gram-positive bacterial strains like *E.coli*,*Pseudomonas aeruginosa*, *Vibrio cholarae* and *Staphylococcus aureus*, *Streptococcus faecialis*, *Streptococcus pnemoniae*, *Streptococcus pyrogenes*, *Bacillus subtillis* respectively, comparatively most potent activity was towards gram-negative bacteria. Activity was retained at concentrations as low as 100 μg/mL. However, the peptide did not possess direct or indirect hemolytic activity. © 2007 Trade Science Inc. - INDIA

KEYWORDS

Indian cobra;
Naja naja;
Snake venom;
Antimicrobial peptide;
Antibacterial peptide;
Antibiotic resistant;
Hemolytic activity.

INTRODUCTION

Antimicrobial peptides (AMPs) are an extremely diverse group of small proteins that are considered together because of their native antimicrobial activity. The existence of AMPs has been known for several decades, but only recently has it been recognized that their function is essential to the animal immune response. They participate primarily in the innate immune system and are used as a first line of immune defense by many organisms, including plants, insects, bacteria and vertebrates[9]. These molecules are peptides with a high level of basic and hydrophobic amino acids. They present a broad antimicrobial spectrum against bacteria, fungi or parasites, by acting through insertion into the cell membrane or binding to receptors. Therefore these molecules are promising for development of antibiotics, especially for treatment of multiresistant microorganisms[7,20]. For this reason there has been a lot of commercial interest and effort in developing cationic peptides as potential antimicrobial therapeutics. It is becoming clear through recent studies that the antimicrobial peptides are an important component of the innate defenses of all species of life[5].

More than 700 AMPs have already been identified in the living species like bacteria, fungi, amphibians, insects, reptiles and mammals[10]. In the last years, several AMPs have been found in different venoms from different animals and these are traditionally linked to defense mechanisms[6]. Snake venoms are rich sources of pharmacologically active polypeptides and proteins. Snake venom peptides are of biological interest as a potential source of active compounds. These molecules could act as (or be used as a prototype for) (i) therapeutic agents; (ii) research tools for use in the diagnosis of several diseases; (iii) in basic research for understanding the physiological and pathological processes[13].

Snake venom has been established to show bactericidal activity[8] and its action of venom proteins on E coli has been studied extensively[23]. Further, the AMPs of snake venom on clinical bacterial strains have also been reported[24]. Venoms from 30 different snake species were tested in a disc diffusion assay for antibacterial activity[22]. In this study, we report on the isolation and characterization of a peptide from N.naja venom and the inhibitory activity of this peptide against different bacterial strains.

Abbreviations: °C-degree Celsius; μg-microgram; mL-milliliter; M-molar; μl- micro liter; % percentage; AMPs- antimicrobial peptides; NAP-Naja antibacterial peptide; ATCC-American type cell culture; NCTC: National cell type cell culture; CFU-Colony forming unites; E coli - Escherichia coli; OD - Optical density: nm-nanometer, MIC- minimal inhibitory concentration.

MATERIAL AND METHODS

Materials

Indian cobra (Naja naja) venom was purchased from Irulla Snake catchers association, Chennai, Tamilnadu, India. CM-Sephadex C-25, Sephadex G-25, Sephadex G-75 and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St. Louis, MO, USA. The bacterial strains *E.coli* ATCC 25922, *E.coli* ATCC 476, *Staphylococcus aureus* NCTC 6570, *Staphylococcous aureus* NCTC 6571, *Pseudomonas aeruginosa* ATCC 26519, *Pseudomonas aeruginosa* NCTC 10662, *Vibrio cholarae* Wild strain, *Streptococcus faecialis* MTCC 459, *Streptococcus faecialis* MTCC 439, *Streptococcus pnemoniae* MTCC 497, *Streptococcus pnemoniae* MTCC 7978, *Streptococcus pyrogenes* NCTC 7465, *Streptococcus pyrogenes* NCTC 7978, *Bacillus subtillis* NCTC 1040 and *Bacillus subtillis* NCTC 8236 were purchased from American Type cell culture Institute ,USA and Institute of Microbial Technology, Chandighar, India. Fresh human blood samples were collected from healthy volunteers from the Department of Biochemistry, University of Mysore, Mysore. All other chemicals used were of analytical grade. All the solvents were redistilled before use.

Protein estimation

Protein concentration was determined according to the method of Lowery et al[17] used BSA as standard.

Sephadex G -75 column chromatography

Lyophilized *N.naja* venom (300 mg in 1 mL) was dissolved in 10 mM potassium phosphate buffer pH 7.4; centrifuged at 5000 g for 5 min. The superna-

tant was applied to a column (0.8 cm X 120 cm) of Sephadex G-75 equilibrated and eluted with the same buffer at 20°C. The fractions from column were eluted at a flow rate of 20 mL / hr and 2 mL fractions are collected. Protein elution was monitored at 280 nm using a Shimadzu spectrophotometer (1601A). Alternate tubes were assayed for antibacterial activity. Fractions having activity were pooled individually, desalted, lyophilized and stored at - 4°C.

CM-Sephadex C-25 column chromatography

The IIIrd peak (90 mg in 3 mL of equilibrating buffer) from Sephadex G-75 column were loaded onto a CM- Sephadex C-25 column (1.2 cm X 40 cm) equilibrated in 10 mM potassium phosphate buffer pH 7.4. The column was eluted by a stepwise gradient of potassium phosphate buffers and NaCl with respective pH as indicated in figure 2. Fractions were carried out at 20°C at a flow rate of 25 mL / hr and 2.5 mL fractions were collected. Protein elution was monitored at 280 nm using a Shimadzu spectrophotometer. Fractions having antibacterial activity were pooled, desalted, lyophilized and stored at - 4°C.

Sephadex G-25 column chromatography

The antibacterial activity of peak IVth (36 mg in 1 mL of equilibrating buffer) from CM-Sephadex C-25 column were loaded on to a Sephadex G-25 column chromatography (0.75x 60 cm) equilibrated with 10 mM potassium phosphate buffer pH 7.4. The fractions from column were eluted at a flow rate of 20 mL / hr and 2 mL fractions are collected. Protein elution was monitored at 280 nm using a Shimadzu spectrophotometer (1601A). Alternate tubes were assayed for antibacterial activity. Fractions having activity were pooled individually, desalted, lyophilized and stored at - 4°C.

High performance liquid chromatography

Purified NAP was subjected to RP-HPLC on Vydac-C_{18} (5 µm, 0.21 X 25 cm) column. The column was first equilibrated with Solvent A (0.1 % TFA) till the base line monitored at 220 nm was stable. The peptide was then injected into the column. Elution was carried out with a linear gradient of 0 to 100 % Solvent B (70 % Acetonitrile in 0.1 % TFA) peptide elution was monitored at 220 nm.

Mass spectrometry

The molecular mass of NAP was determined by matrix-assisted laser desorption ionization time of flight (MALDI -TOF) mass spectrometry (Voyager Spec # 1 MC) in the positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

N-terminal sequencing

The terminal sequencing of NAP was carried out in a fully automated Shimadzu protein sequencer (PSQ-1) system that employs Edman's degradation reaction for sequential separation of N-terminal amino acids.

Hemolytic activity

Direct and indirect hemolytic activities were assayed as described by Bowman and Kalletta[4]. The substrate for direct hemolytic activity was prepared by suspending 1mL of packed fresh human RBC in 9 mL of phosphate buffered saline (PBS). For indirect hemolytic activity 1mL of fresh hen's egg yolk was included in the above suspension. One mL of the suspension was incubated with different concentrations of NAP for 45 min at 37°C. The reaction was stopped by adding 9 mL of ice cold PBS. The suspensions were centrifuged at 2000 g for 20 min and the released hemoglobin was read at 530 nm. The activity was expressed in % of hemolysis.

Evaluation of antibacterial activity

Antibacterial assay was described by Linzxing Zhong et al.,[16]. The microorganisms were grown in the Muller-Hinton broth. After incubation for 16 to 18 hr at 37°C, the bacteria were harvested by centrifugation (2000 g for 10 min), washed twice with 10 mM sodium phosphate buffer pH 6.0 by centrifugation and finally resuspended in 10 mL of buffer. Its density was determined by a measuring the absorbance at A_{600}. MIC of peptide was determined by a micro dilution susceptibility test in sterile 96 well micro dilution plates. Microorganisms (1 X 10^4 to 2 X 10^4 CFU/50 µL) were pipette into the wells, which consists of 20-300 µg of peptide/mL. Assays were performed in the duplicate for peptide with each

bacterium. After 24 hr of incubation at the optimal growing temperature, the optical density at 600 nm was read on an absorption micro titer plate reader (Biotek Instruments INC.,). Percentage inhibition was calculated as {1-(a\b) x100} where a= OD 600 nm of the bacteria with peptide and b = OD 600 nm of the control well containing only buffer, bacteria and media. The MIC evaluation was defined as 100 % inhibition. Control was run by replacing the peptide solution with buffer solution. Respective antibiotics were used as standard drugs replacing peptide solution.

Statistics

For all experiments, results were expressed as the mean ± SEM of at least 3 independent experiments.

RESULTS

Purification of NAP

When N. naja venom (300 mg) subjected to gel permeation chromatography on a Sephadex G-75 column resolved into three protein peaks. The antibacterial activity of the whole venom and its fraction is shown in TABLE 1. When all the peaks were screened for antibacterial activity, peak III rd showed activity (Figure 1). The III rd peak from the Sephadex G-75 fraction was pooled, concentrated and desalted using Sephadex G-10 column. The pooled III rd peak fraction was further resolved into four peaks on CM-Sephadex C-25 column by applying NaCl gradient (Figure 2). Only the IV th peak exhibits potent antibacterial activity and contributed to 73.98 % of the total activity loaded. 4.5 % of the protein loaded on CM-Sephadex C-25 column was recovered in this antibacterial fraction. The antibacterial activity of this fraction was increased by 6 fold compared to the whole venom activity. Further, IV th peak from the CM-Sephadex C-25 fraction was pooled, concentrated and loaded onto Sephadex G-25 column. On fractionation the peptide components resolved into two peaks, which are designated as peak A and peak B (Figure 3). Peak B showed significant antibacterial activity and increased by 10 fold compared to the whole venom activity.

The homogeneity of the antibacterial peptide was examined by RP- HPLC using C18 column. The elution buffer contained 0.1 % TFA and was eluted with acetonitrile gradient. NAP eluted as single symmetrical sharp peak with a retention time of 43.6 min

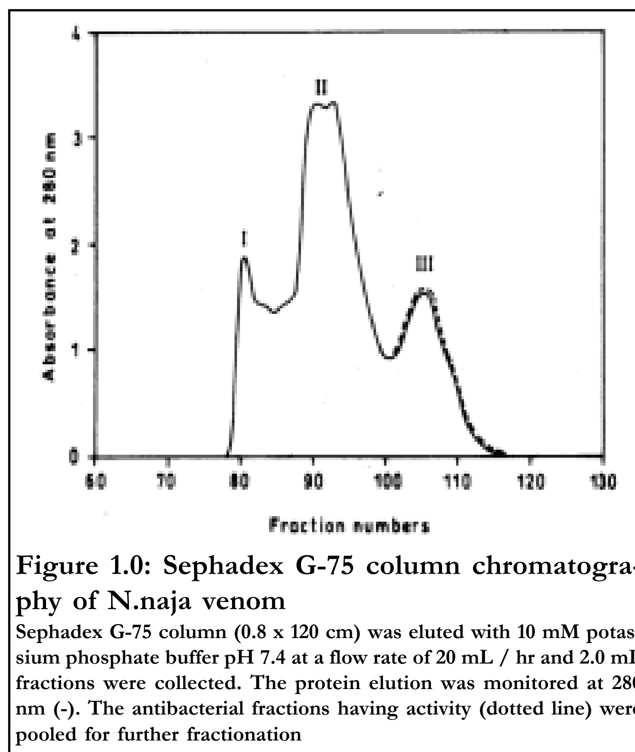

Figure 1.0: Sephadex G-75 column chromatography of N.naja venom

Sephadex G-75 column (0.8 x 120 cm) was eluted with 10 mM potassium phosphate buffer pH 7.4 at a flow rate of 20 mL / hr and 2.0 mL fractions were collected. The protein elution was monitored at 280 nm (-). The antibacterial fractions having activity (dotted line) were pooled for further fractionation

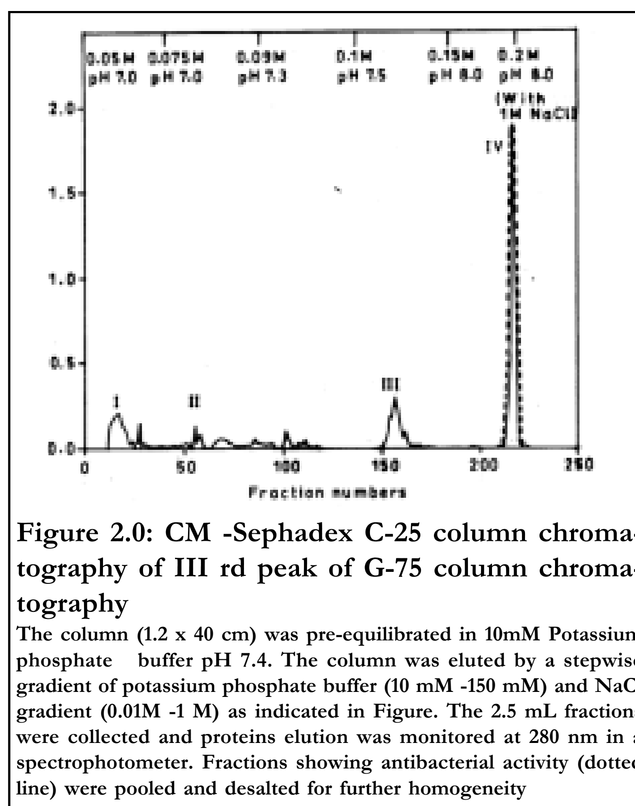

Figure 2.0: CM -Sephadex C-25 column chromatography of III rd peak of G-75 column chromatography

The column (1.2 x 40 cm) was pre-equilibrated in 10mM Potassium phosphate buffer pH 7.4. The column was eluted by a stepwise gradient of potassium phosphate buffer (10 mM -150 mM) and NaCl gradient (0.01M -1 M) as indicated in Figure. The 2.5 mL fractions were collected and proteins elution was monitored at 280 nm in a spectrophotometer. Fractions showing antibacterial activity (dotted line) were pooled and desalted for further homogeneity

TABLE 1: Minimum Inhibitory concentration (MIC) of each peak fractions

MIC in μg of each peak fractions of chromatographic effluent against Gram positive and Gram-negative bacterial strains was shown.

Microorganism	MIC (μg) Whole venom	MIC (μg) Sephadex G-75 column			MIC (μg) CM Sephadex C – 25 column				MIC (μg) Sephadex G- 25 column		MIC (μg) HPLC
		I	II	III	I	II	III	IV	P(A)	P(B)	NAP
Gram + ve *Staphylococcus aureus*	2850	1700	1780	630	600	680	580	500	800	400	350
Gram – ve *E.coli*	2700	1600	1680	400	750	700	650	400	550	200	135

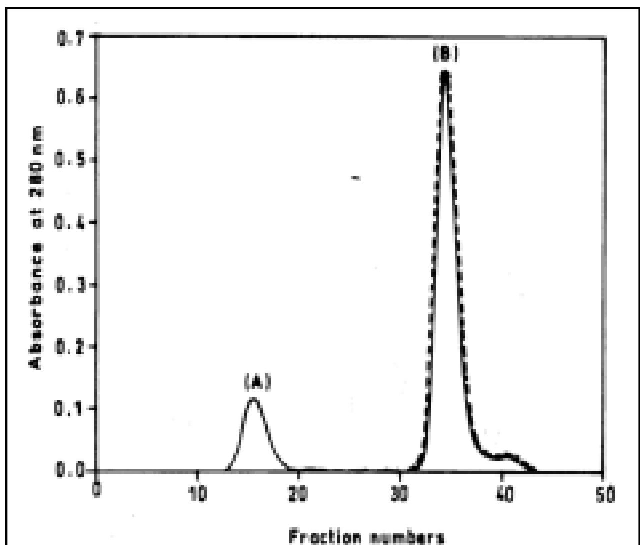

Figure 3.0: Sephadex G-25 column chromatography of IVth peak of CM-Sephadex C-25 column chromatography

Sephadex G-25 column (0.75 x 60 cm) was eluted with 10 mM potassium phosphate buffer pH 7.4 at a flow rate of 20 mL / hr and 2.0 mL fractions were collected. The protein elution was monitored at 280 nm (-). The antibacterial fractions having activity (dotted line) were pooled for further analysis.

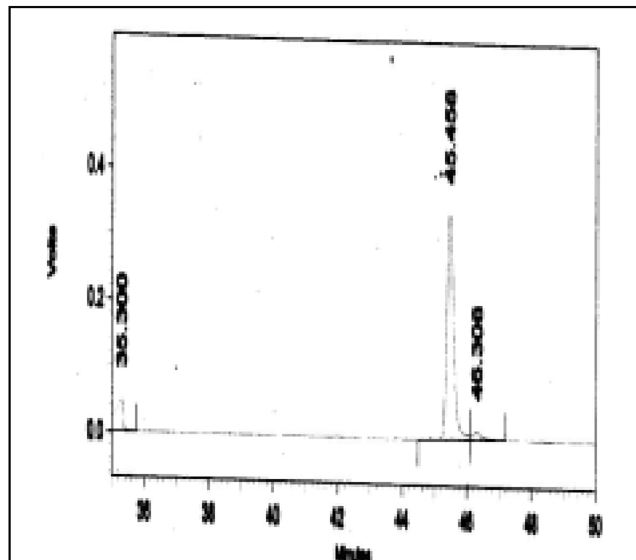

Figure 4.0: RP-HPLC elution profile of NAP

NAP was run on a Vydac C_{18} RP-HPLC column. Solvent A was 0.1 % TFA and Solvent B was 70 % acetonitrile in 0.1% TFA. A gradient of 0 -100 % solvent B was run from 0 to 60 min as indicated in the figure. Elution was monitored at 280nm.

(Figure 4). The molecular mass as determined by MALDI-TOF mass spectrometry was found to be 2491 Da (Figure 5). The N-terminal sequence analysis of the NAP gave the following 13 amino acid sequence; DEQSTHGAYVWKL. All these data clearly establishes that the antibacterial peptide is purified to homogeneity.

Antibacterial activity of NAP

The isolated peptide was tested against four species of gram-positive bacteria and three species of gram-negative bacterial strains. In addition, the NAP was also tested against a wide variety of gram positive and gram negative bacteria collected form immune suppressed patients, following disease or chemotherapy or from patients suffering form other chronic disease.

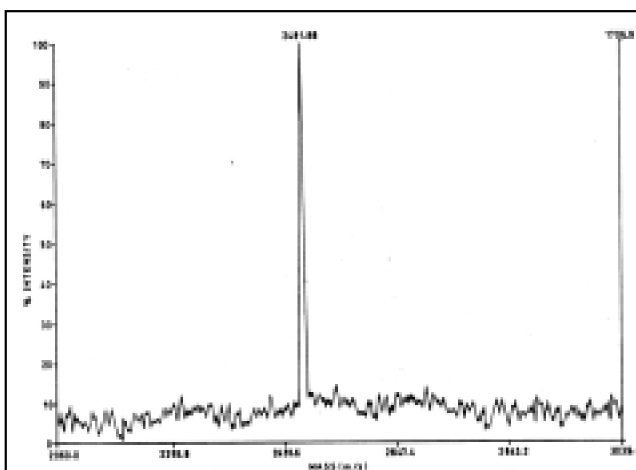

Figure 05 : MALDI-TOP mass spectrum of NAP

MALDI-TOF mass spectrometry of NAP in the positive ionization mode using α - Cyano- 4- hydroxycinnamic acid as MALDI matrix.

NAP exhibited antibacterial activity against a variety of bacterial clinical isolates; this was evaluated by determining the MIC values. The difference in activity of NAP between gram-positive and gram-negative is well marked. NAP was more effective among gram negative bacteria in general, and not all species were susceptible (TABLE 2). The *Staphylococcus aureus*, *Staphylococcus faecalies*, *Streptococcus pnemoniae* and *Streptococcus pyrogene* showed MIC values > 300 μg/mL .On the other hand, *Ecoli*, *Pseudomonous aurginosa*, *Vibriocho lare*, *Klibsilla pnomoniae species*, were found to be more susceptible to NAP. Hemolytic activity of whole venom exhibited direct and indirect lytic activity on human RBC causing hemolysis of 83 %. Similarly, NAP was incubated with washed RBC at 37°C for 10 min and increased in dose dependent manner. The NAP did not show direct or indirect hemolytic activity even at higher concentration.

TABLE 2 : MIC in μg of NAP and known antibiotic against Gram positive and Gram-negative bacteria:

Microorganism	Strains	Peptide MIC (μg)	Antibiotic	MIC (μg)
Gram-negative bacteria				
E.coli	ATCC 25922	130	Ciprofloxacin	80
	ATCC 476	120		100
Pseudomonas aeruginosa	ATCC 25619	100	Gentamicin	80
	NCTC 10662	120		80
Vibrio cholarae	Wild strain	140	Tetacycline	50
Gram-positive bacteria				
Staphylococcous aureus	NCTC 6570	>200	Cloxacillin	120
	NCTC 6571	>250		150
Strptococcus faecalis	MTCC 459	>220	Erythromycin	80
	MTCC 439	>250		100
Streptococcus pneumoniae	MTCC 497	>220	Ceftriaxone	120
	MTCC 7978	>250		120
Streptococcus pyogenes	NCTC 7465	>300	Amoxicillin	100
	NCTC 7978	>280		120
Bacillus subtillis	NCTC 1040	>200	Penicillin G	60
	NCTC 8236	>220		80

DISCUSSION

Several AMPs have been found in different venoms from different animals and these are traditionally linked to defense mechanisms[6]. AMPs have an ability to kill or neutralize gram-negative, gram-positive bacteria, fungi (including yeasts), parasites (including planaria and nematodes), cancer cells, even enveloped viruses like HIV and herpes simplex virus[11]. In this study, the low molecular weight peptide from snake venom, referred as NAP. For the first time the purification and N-terminal sequence of a new potent antibacterial peptide from Naja naja snake venom is reported here. Peptide was isolated from N.naja whole venom by subjecting it to gel permeation, Ion exchange chromatography resulted in 10 fold purification of NAP. RP-HPLC, MALDI-TOF and N-terminal sequencing analysis confirmed the homogeneity of NAP. Based on the primary N-terminal sequence Blast search of antibacterial peptide, the peptide is known to be distinctly different from known antibacterial peptide so far.

It is generally accepted that different venoms have several thousand proteins with different properties. However, in the recent years, more than 700 cationic peptides have been isolated from mammals, amphibians, reptiles, arthropods, plants, bacteria and viruses[3,7,20]. Some of the first reports about antibacterial activity in snake venoms were in 1948 and in 1968, involving Elapidae and Viperidae family venoms[8,25].Viperidae family venoms were described as having antimicrobial against the Sarcina species, while in the Elapidae family, a lytic factor or cytotoxin composed of a basic, low molecular weight protein was found in Naja species and H.haema chatus was shown to possess antibacterial activity. They were able to disrupt *Staphylococcus aureus* and *E.coli* phospholipid membranes respectively[23,25]. In our study, the NAP peptide displayed good inhibitory activity against gram-negative bacteria like *E.*

coli, *Pseudomonas aeruginosa*, *Vibrio cholarae*, than comparatively towards gram positive bacteria like *staphylococcus aureus*, *strptococcus facalis*, *Strp tococcus pnemoniae*, *Strptococcus pyrogenes* and *Bacillus subtills*.

The peptide dissolves the divalent cations that are essential for outer membrane and consequently distorts the outer membrane bilayer[21]. This allows access to the cytoplasmic membrane where peptides channel formation has been proposed to occur[14]. It is an intermediate step in the uptake of peptides into the cytoplasm, where it inhibits an essential function by binding to polyanionic DNA[19,26]. It has been argued that antimicrobial peptides provides an organisms with molecules that are rapidly synthesized because of small size, less costly to synthesize than antibodies or specific phagocyte's cells, and can be stored if necessary as processed biologically active components which are rapidly available for host defense[2]. The ability of the antibacterial peptide to lyse cells is the result of a complex interrelationship of factors involving conformation, charge, hydrophobic and amphipathicity. The cationic residues in an antimicrobial peptide are considered to be important in the initial binding to the negatively charged phospholipids in the cell membranes of microorganisms[26]. It has been suggested that increasing the hydrophobic moment of an antimicrobial peptide has a relatively modest effect on the ability to permeabilize the negatively charged cell membrane of microorganisms but not a marked effect on the more zwitterionic phospholipid membrane of the erythrocyte[26]. In general, although it is accepted that a polypeptide chain of at least 20 amino acids is necessary to span the lipid bilayer of membrane to effect the formation of ion channels[14], shorter cationic α helical amphipathic peptides of 8-12 residues can also form ion channels, presumably through head to tail dimerization of the peptides[1].

In a conventional assay on the human RBC, whole venom cause significant hemolysis, but NAP did not cause a significant hemolysis. It was known that appearance of numerous contiguous apolar residues in a helix is necessary for a significant hemolysis to occur[18]. Like other antimicrobial peptide, the polar residues in the NAP might be well interspersed among the hydrophobic residues, interrupting the contiguity of hydrophobicity, which gives the potential to form an amphipathic helix. For this reason, NAP probably exhibits little hemolytic activity like many other antimicrobial activity. The widespread use of antibiotics has caused numerous antibiotic resistant strains to develop, resulting in the continuous need for new antibiotics. Studies directed towards understanding the relationships between the secondary structure and biological activities of these natural peptides indicate that the amphipatic alpha helical conformation plays an important role in their biological activities[12]. In conclusion, our study as *N.naja* venom suggests the presence of a potent antibacterial peptide. Further, studies on this peptide is interesting, the clinical isolates were investigated can cause infections at sites where treatment with this type of peptide would probably help in the development as a potential therapeutic agent applicable for clinical isolates.

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