A Natural Antibacterial Peptide from Goat Heart Tissues Against *S. typhi* and *P. aruginosa*

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Abstract: The emergence of multiple drug resistant bacteria has created alarming clinical situations in the treatment of infectious diseases. This has rekindled interest in the therapeutic use of certain natural products along with modern medicines. In the present scenario, the antimicrobial peptides, a diverse group of molecules abundantly present in the tissues and cells of all species of life are being in clinical trial to be used as natural antibiotics. A natural 16 kDa antibacterial peptide was isolated from the heart tissues of goat by RP-HPLC using C-18 column. The acid extract of this tissue yielded four fractions, two at 214 nm and other two at 281 nm wave lengths. Among the four fractions, the second fraction purified by 214 nm [P_2(214 nm)] showed potential antibacterial activity against *S. typhi* and *P. aruginosa* ATCC strains. The width of inhibitory zones was found to be 15 mm and 10 mm for *S. typhi* and *P. aeruginosa* ATCC respectively. The MIC of the peptide was also determined to be 0.3µg/ml for both isolates. Further it was also identified as a non-hemolytic peptide. The single band formation of this peptide in the gel of SDS-PAGE confirmed its purity.

Key words: Antibacterial activity · Antimicrobial peptide · Natural antibiotics · Inhibitory zone

INTRODUCTION

The continuous use of antibiotics has resulted in multi-resistant bacterial strains all over the world. The increasing trend of bacterial resistance to classical antibiotics is the major threat to the human society. This impending crisis has spurred the search for new therapeutic agents [1] without allowing the organisms to develop resistance. The antimicrobial peptides (AMPs) of vertebrate tissues show promise for becoming the next major group of natural antibiotics [2] to combat this problem. These are small, cationic, membrane-active peptides with broad spectrum of activity against bacteria, fungi and virus and may prove more difficult for microorganisms to develop resistance based on their mode of action. Instead of interacting with receptors or enzymes, which are more easily changed by mutations, these peptides interact with negatively charged phospholipids and lipopolysacharides (LPS) of the membrane [3]. The peptide-based antibiotics have been found in cells and tissue types of all animal species, ranging from insects to lower vertebrates and mammals, whereas they form part of the innate and possibly adaptive immune system [4-6]. Defensins and cathelicidins are the two major classes of antimicrobial peptides. To date hundreds of AMPs have been described from various cells and tissues of different vertebrates.

The α defensins like human neutrophil peptides 1-4 (HNP-1 to HNP-4) localized in azurophilic granules of neutrophils were studied by Ganz *et al.* [7]. Similarly the β-defensins such as human β-defensins-1-3 (hBD-1-3), porcine β-defensin-1 (pBD-1) and tracheal antimicrobial peptides (TAPs) of mammals have also been extensively studied [8-12].

About 30 different cathelicidins have been described in mammals. Bactenecins (Bac 5 and Bac 7) in bovine neutrophils, cathelin-related AMP (CRAMP) in mice, SMAP-29 in sheep myeloid cells are certain important examples for cathelicidin peptides [13- 15]. The 18 kDa human cathelicidin antimicrobial peptide (hCAP-18) is only the human cathelicidin, a component of the innate immune system and has broad antimicrobial activity conferred by its *C*-terminal fragment LL-37. It is constitutively produced in leukocytes and is induced in barrier organs upon inflammation and infection [16].

Thus, different AMPs have been reported in number of mammals. But, not much of work has been done on organs of goat which will be freely available from

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slaughter houses. In our recent study, we have screened the tissues like muscles, liver, kidney and heart of goat for their antibacterial activities. Among the four tissues, only the heart tissue showed bactericidal activities against certain human pathogenic bacteria [17]. Based on this, in the present work we have aimed to purify the low molecular weight antibacterial peptides from the goat heart tissues if any and to check their toxic effects to the human erythrocytes in order to prepare natural antibiotics suitably to the conventional antibiotics in treatment regimes.

MATERIALS AND METHODS

Preparation of Crude Antimicrobial Proteins: The crude antimicrobial proteins were prepared by acid extraction as described by Matutte *et al.* [18]. The whole hearts of three different adult healthy goats (*Capra hircus*) were obtained freshly from a meat shop located at Tiruchirappalli. The cleaned tissues of ventricular portion were cut and placed in liquid nitrogen and after 24 hours, the frozen heart tissues were pulverized separately with a mortar and pestle. The pulverized powder was placed in a boiling 10% (v/v) acetic acid for 10 minutes. The solution was allowed to cool to room temperature and centrifuged at 12,000 rpm for 30 minutes at 10°C. The supernatants containing the crude proteins were concentrated by lyophilization and stored at -20°C until use.

Purification of Antibacterial Proteins: The acid extracted crude proteins were dissolved in 20 mM ammonium acetate (pH 5.0) and loaded on to a carboxymethyl cellulose (CMC) (Sigma-Aldrich) cation-exchange column (3x15 cm) which was previously equilibrated with the same buffer. Proteins bound to the CMC column were eluted using a gradient from 20-1500 mM ammonium acetate at a flow rate of 1 ml min⁻¹. The fractions were mixed, lyophilized and reconstituted in 0.01 % acetic acid [19]. Exactly 10 µl of the fraction was injected on to a (25 X 1 cm) Shimadzu CLASS-VP V6.14 SP2 C-18 RP-HPLC column equilibrated with 0.1 % (v/v) trifluoroacetic acid/water at a flow rate of 2ml min⁻¹. The samples were injected separately monitoring two different wave lengths, 214 and 281 nm as described by Matutte *et al.* [18]. The single peak fractions were collected at their own retention times using the eluting solvent (Acetonitrile/Water in the ratio of 1:1 v/v). The collected fractions were freeze-dried and dissolved with 200 µl of autoclaved distilled water separately and then stored at -20°C [18] for antibacterial and hemolytic assays.

Antibacterial Activity: Seven bacterial isolates (*E. coli* ATCC 25922, *P. mirabilis, S. aureus, P. aeruginosa* ATCC 27853, *S. typhi, S. paratyphi* 'A', *S. typhimurium*) were procured from Microbiology Laboratory, KAPV Medical College, Tiruchirappalli.

Antibacterial activity was determined in triplicate by well diffusion plate assay method [8]. The bacterial isolates were sub-cultured in to mid-logarithmic phase and suspended in 1% peptone water to adjust the turbidity to 2 X 10⁶ Colony Forming Units (CFU). Then the suspended bacterial isolates were inoculated on to the entire surface of the plate containing Bacto tryptone (10 g/liter), Yeast extract (5 g/liter), 0.75 % Agarose, 25 mM Tris-Hcl (pH 7.4) and 50 mM Sodium fluoride separately. The surface of the media with the lawn of the test organisms was divided into four equal areas and a well with 5 mm diameter was formed in each. Briefly the concentrated aliquots of each fraction (10 µl) was delivered into the respective wells at sterile condition. Then the plates were incubated overnight at 37°C and the zone of inhibition was measured by a divider and scale.

Minimum Inhibitory Concentration (MIC): The MIC of the active fraction, the 2nd fraction obtained at 214 nm [P_2(214 nm)] was determined adopting the microdilution method in triplicate as described by Zhu *et al.* [20]. against *P. aeruginosa* ATCC and *S. typhi* which responded well with this fraction. This test was performed in 96 micro well plates. A stock peptide solution was prepared at a concentration of 400 µg / ml in PBS (pH-7). From this stock solution, serial dilutions of the peptides were made with 1% peptone water to make the concentration from 100 to 3µg/ml in the appropriate rows of micro well plate. Then aliquots of 100µl of the bacterial suspension were added in 100µl peptide solution. After 24 hours incubation at 37°C, the bacterial growth was determined by measuring the absorbance at 620nm using ELISA auto reader (RT2100C). A positive control (Growth inhibition with classical combined antibiotics; cephataxime and chloromphenicol at a concentration of 5 mg/ml, each) and a negative control (Bacterial growth without antibiotics or proteins) were also performed along with to set the base lines. The MIC is defined as the minimum peptide concentration that inhibits the bacterial growth.

Hemolytic Assay (Minimum Hemolytic Concentrations): According to Zhu *et al.* [20] the hemolytic assay of the active fraction was carried out in triplicate micro well plates. The human blood of each group (A+, B+ and O+) was collected from a private blood bank at Tanjore. The red blood cells were sedimented by centrifugation at

3000 rpm. The RBCs were repeatedly washed with 0.9% Sodium chloride. Then the concentrated hRBCs were converted into 4% with phosphate buffer solution (PBS pH.7). The wells of the first three rows (A, B and C) were filled with 100 µl of PBS in each. The peptide solution (prepared from the stock at the concentration of 2000µg/ml of PBS pH.7) was serially diluted from 500 to16 µg/ml. Then in the appropriate rows, 100 µl of the respective 4% hRBCs were added in all the wells. The 4 % hRBCs alone and 4% hRBCs in 1%Triton 100 X were used as 0% (-ve control/blank) and 100% (+ve control) haemolysis respectively. After 2 hours incubation, the button formations of RBCs were observed visually. The button formation indicated the absence of hemolysis. The percentage of hemolysis was also measured at 405nm wavelength using ELISA reader (RT2100C) after transferring the supernatants of the wells into other plates. The minimum haemolytic concentration (MHC) of the peptide is defined as the lowest concentration of peptide at which haemolysis occurs. The percentage of haemolysis was calculated by using the following formula.

Percentage haemolysis = 100 x {A_{sample} - A_{blank}}/ {A_{triton} - A_{blank}}

SDS-PAGE Analysis: From the stock solution of the peptide fraction, 20 µl aliquot was subjected to SDS-poly acrylamide gel electrophoresis on 15% mini gels according to the standard protocols [21]. The sample was heated for 3 minutes at 100°C in sample buffer (25 % 1 M tris-Hydrochloric acid, pH 6.8; 4% SDS; 2% β-mercaptoethanol and 5 % glycerol) and then were run along with mid-range marker proteins. The gel was fixed and the bands were visualized after silver staining using UV lamp. Finally the molecular weights of the bands were determined by gel documentation. Subsequently the crude proteins of the acid extract and the proteins partially separated by cationic exchange chromatography were also run along with sample to compare the bands.
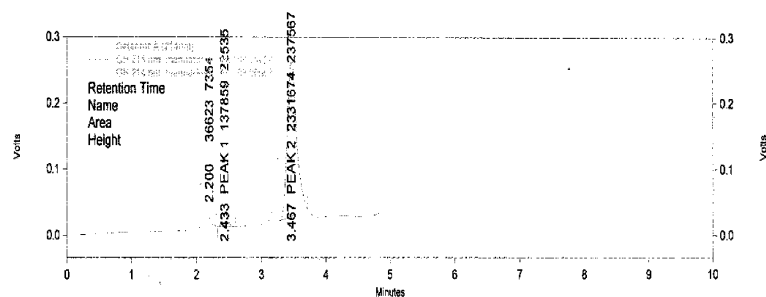
RESULTS

Purification of Antibacterial Proteins: The partially purified proteins of the heart tissue yielded totally four fractions; two at 214 nm and other two at 281 nm after reverse-phase high performance liquid chromatography separation. At 214 nm, the fraction 1 (1.076 µg of peptide) was eluted with 2.433 minutes of retention time where as

Table 1: Antibacterial activity of goat heart RP- HPLC peptide fractions against different human pathogenic bacteria

Bacterial isolates tested	Wavelength of the peptides fraction	Peptide fraction (peaks)	Zone of inhibition (mm)
Escherihia coli ATCC 25922	214	P1	2.0
		P2	Nil
	281	P1	Nil
		P2	Nil
Proteus mirabilis	214	P1	Nil
		P2	Nil
	281	P1	Nil
		P2	Nil
Staphylococcus aureus	214	P1	Nil
		P2	Nil
	281	P1	Nil
		P2	Nil
Pseudomonas aeruginosa ATCC 27853	214	P1	Nil
		P2	10.0*
	281	P1	Nil
		P2	Nil
Salmonella typhi	214	P1	Nil
		P2	15.0*
	281	P1	Nil
		P2	Nil
Salmonella paratyphi 'A'	214	P1	Nil
		P2	Nil
	281	P1	Nil
		P2	Nil
Salmonella typhimurium	214	P1	Nil
		P2	Nil
	281	P1	Nil
		P2	1.0

Note : * indicates the significant inhibitory zones (5 and above 5mm diameter)

Fig. 1a: Purification of antimicrobial peptides from goat heart tissues by RP-HPLC with a C-18 column. The elution was performed with 0.1 % (v/v) trifluoroacetic acid/water at a flow rate of 2.0 ml/min. Elution was monitored at 214 nm.

Fig. 1b: Purification of antimicrobial peptides from goat heart tissues by RP-HPLC with a C-18 column. The elution was performed with 0.1 % (v/v) trifluoroacetic acid/water at a flow rate of 2.0 ml/min. Elution was monitored at 281 nm.

Fig. 2: Antibacterial activities of goat heart RP-HPLC peptide fractions against different human pathogenic bacteria. 10mm and 15mm inhibitory zones were found in *P. aeruginosa* ATCC and *S. typhi* plates. The arrow marks indicate the inhibitory zones caused by the appropriate peptide fractions.

Fig. 3: Minimum Inhibitory Concentrations of goat heart active peptide fraction [P$_2$ (214)] against *P. aeruginosa ATCC* and *S. typhi.*The MICs were found to be 3µg /ml for both bacteria
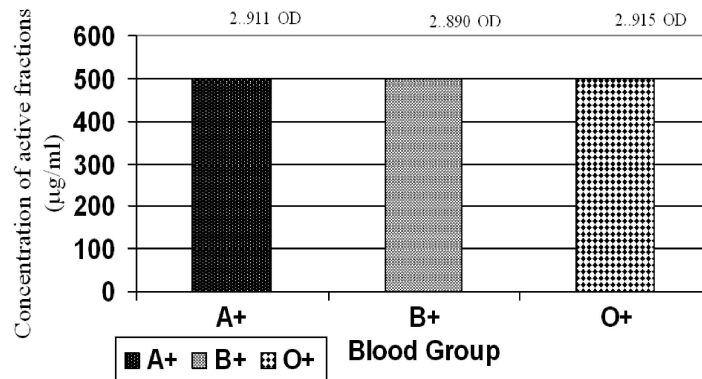

Fig. 4a: Minimum Hemolytic Concentrations of goat heart active peptide fraction [P$_2$ (214)] for ABO hRBCs were found to be 500 µg /ml. each.
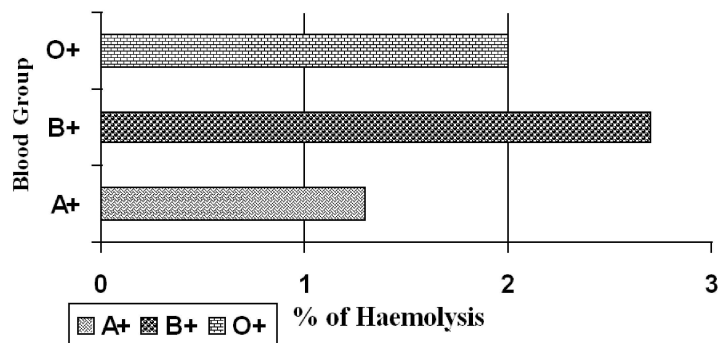

Fig. 4b: The percentage of haemolysis of ABO hRBCs by goat heart active peptide fraction [P$_2$(214)] were calculated as 1.3%, 2.7%, 2% respectively

the fraction 2 (3.538 µg of peptide) was eluted at 3.467 minutes. At 281 nm, the fractions 1 and 2 (4.461 and 2.301 µg of peptide) were separated respectively with 1.392 and 3.217 minutes of retention times.

Antibacterial Activity: After the RP-HPLC purification, all the fractions were lyophilized and subjected to antibacterial assays. The antibacterial activity of the purified peptide fractions fell in the range of 1.0 to 15 mm width of zone of inhibitions at both wave lengths. The fractions with 5 and above 5 mm width of inhibitory zones were only considered as active fractions. Among the RP-HPLC purified fractions, the second fraction purified by 214 nm [P$_2$(214 nm)] showed best activity to *S. typhi* and *P. aruginosa* ATCC strains with15and 10 mm width of inhibitory zones,, respectively.
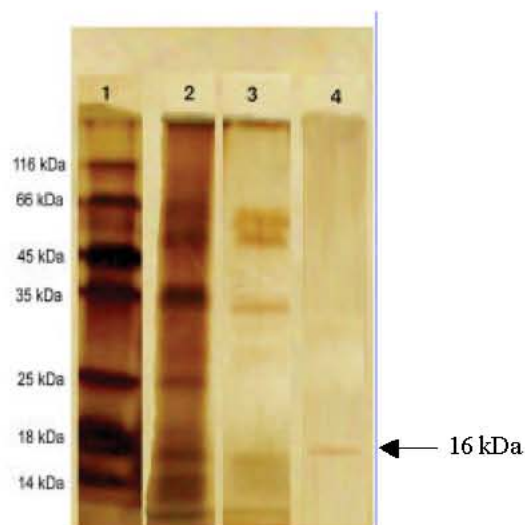
Fig. 5: SDS-PAGE analysis of the goat heart proteins at different stages of purification.
Lane 1. Molecular weight Markers; Lane 2. Acid Extracted crude proteins; Lane 3. proteins after cationic exchange chromatography; Lane 4. RP-HPLC Purified active peptide fraction (16 kDa).

Minimum Inhibitory Concentrations (MICs): The active peptide fraction, $[P_2(214\,nm)]$ was subjected to MIC for *S. typhi* and *P. aruginosa* ATCC and this peptide has inhibited the growth of both the organisms at very lowest concentration of 3 µg/ml. (Fig 3).

Haemolytic Activity: In order to test the cytotoxicity of the active peptide fraction to human cells, the haemolytic assay was performed with human ABO erythrocytes. The visual observations showed the absence of haemolysis of red cells of all the blood groups. The percentage of haemolysis was also calculated and found to be 1.3%, 2.7% and 2.0% for A, B and O hRBCs, respectively. The active fraction has caused only minimum haemolysis of all hRBCs only at the highest concentration subjected (500 µg/ml) with the maximum of 2.7%. It is of interest to note that the concentration required for the antibacterial activity was 3 µg/ml only and which was very low to cause haemolysis (Fig 4a and 4b).

SDS-PAGE Analysis: The SDS-PAGE after silver staining showed a single band of active peptide fraction with an approximate molecular weight of 16 kDa. The gradual reduction of the number of bands from the acid extracted crude proteins to HPLC purified active fraction indicated the stepwise purification. The purity of the RP-HPLC

purified fraction was also confirmed by the single band formation. The electrophoretic pattern of different stages of purification also showed some similar types of bands. The first and second lanes showed three bands of 16, 14 and 12 kDa peptides in each while the third lane revealed only the 16 kDa band which is the RP-HPLC purified active peptide (Fig.5).

DISCUSSION

The protein fraction eluted from CMC cationic exchange column was injected into RP-HPLC C-18 column using two different wave lengths 214 and 281 nm. To separate low molecular weight cationic peptides, normally these two wave lengths are used [18] The active peptide fraction $[P_2 (214\,nm)]$ of goat heart tissues achieved by 214 nm of RP-HPLC exhibited a potent antibacterial activity against *S. thphi* and *P. aeruginosa* ATCC. It is important to note that the both bacterial isolates are human pathogens and capable of producing resistance. Several research groups have investigated a number of antimicrobial peptides/proteins from different cells or tissues of aves and mammals [8, 9, 22].

The minimum inhibitory concentration of the purified active peptide fraction was found to be very low (3.0 µg/ml). This is in agreement with some previous similar works. According to Gennaro and Zanetti [23], most of the cathelicidin peptides rapidly kill a wide range of microorganisms at micromolar and sub-micromolar concentrations. Moreover, the MIC of this peptide was observed to be lesser than that of some other antimicrobial peptides reported previously. For instance, the MIC range of TAP was found to be 6-50 µg/ml for *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* and an yeast *Candida albicans* [8]. Similarly the epididymis protein 2 (EP2) isoforms of human and rhesus monkey kill more than 99.9% of bacteria at 50-100 µg/ml only [24].

Though many natural as well as synthetic antimicrobial peptides are potential to act against different pathogens, their cytotoxic effects prevent the development of peptide-based antibiotics unless there are some modifications in their sequences or solvents. SMAP-29 is a cathelicidin antimicrobial peptide of sheep myeloid cells displaying potent salt-independent activities against a range of gram-negative and gram-positive bacteria including antibiotic resistant strains with haemolytic effects [15]. The haemolytic activity of SMAP-29 was reduced by 2-4 folds in the presence of 10%PBS. Similarly, the Trp/Pro-rich antimicrobial peptides like tritrpticin and indolicidin are relatively short peptides consisting of 3 residues have also broad spectrum of

antimicrobial activities against bacteria and fungi. But their relatively high toxicity toward eukaryotic cells prevent their usage as antibiotics [25, 26]. Therefore much efforts have been taken in the past decade to decrease the cytotoxicity and to increase the cell selectivity of these peptides [26-28]. Furthermore Zhu *et al.* [29] have reported that the substitution of two proline residues of tritrpticin-amide (TP) with lysine peptoid residue (Nlys) was an effective method to decrease the cytotoxicity toward eukaryotic cells of TP while retaining strong antimicrobial activity.

In contrast, this antibacterial peptide described in this paper can be considered as safe, non-hemolytic potent antibacterial peptides for treating the infectious diseases caused by *S. thphi* and *P. aeruginosa* at the micro-molar concentrations. The percentage of hemolysis caused by this peptide was found to be very minimum (1.3% to 2.7%) which was also occurred only at the highest concentration (MHC-500µg/ml). But the concentration of the peptide fraction required for antibacterial activities was just 3 µg/ml or little more and this lowest concentration could not cause hemolysis. Thus, the purified antibacterial peptide overcome the problem of cytotoxicity to the human erythrocytes as it exhibit strong antibacterial effects without hemolytic effects.

The approximate molecular weight of this peptide was found to be 16 kDa by SDS-PAGE after the silver staining. The single band formation also confirmed the purity of this active peptide fraction (Fig 5). It is also notable that the size of the active peptide fraction (16 kDa) is some what nearest to 18 kDa human cathelicidin peptide (hCAP-18) [30]. With the above mentioned properties, the identified peptide is suggested to be a 16 kDa non-hemolytic antibacterial peptide. The molecular mass determination and sequencing of this peptide may further be helpful to develop it as a natural peptide antibiotic, which could fight against causative organisms of enteric fever and respiratory diseases.

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