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PURIFICATION OF AN ANTIBACTERIAL PEPTIDE FROM CHICKEN HEART TISSUES BY RP-HPLC AGAINST *PSEUDOMONAS AERUGINOSA*

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ABSTRACT
Antibiotic resistance is an increasing problem worldwide. The emergence and spread of multidrug-resistant bacteria to the classical antibiotics leads to treatment failure associated with severe and chronic infectious diseases. So there is an urgent need to find out suitable alternatives to the classical antibiotics without developing resistance. The antimicrobial peptides of animal sources could form the suitable alternatives by their mode of action to combat this problem. The RP-HPLC purification of acid extracted crude proteins of chicken heart yielded four single peak 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)] displayed potent antibacterial activity against *P. aeruginosa* 25873 ATCC and was identified as active peptide fraction. The MIC of this active peptide was found to be 6 µg/ml. Further, this peptide did not cause hemolysis on any human hRBCs. The molecular weight of this active peptide was determined to be 13 kDa which is equal to the molecular weight of 'cathelin' domain of cathelicidin family peptides including human cathelicidin.

KEYWORDS: Chicken, antibacterial activity, RP-HPLC purification, active peptide fraction and human cathelicidin.

INTRODUCTION

Peptides of animal cells play a crucial role in many physiological processes, including actions as natural antibiotics. The peptide based antibiotics termed as antimicrobial peptides (AMP) found in a wide range of animal species, ranging from insect to lower vertebrates and mammals (Ganz et al. 1995) have been divided in to two major groups such as defensins and cathelicidins. Their primary role of these cationic peptides is defense against microbial attack and killing gram positive and gram negative bacteria, fungi, eukaryotic parasites and enveloped viruses (Zasloff 2002). A number of AMPs have been studied from different mammals. The α defensins like human neutrophil peptides 1-4 (HNP-1 to HNP-4) localized in azurophilic granules of neutrophils were reported (Ganz et al. 1985). The β-defensins of bovine known to be tracheal antimicrobial peptides (TAPs) (Diamond et al. 1991; Selsted et al. 1993) and human β-defensins like hBD-1 to -3 (Bensch et al. 1995; Harder et al. 1997, 2000; Schroder and Harder 1999) have been extensively studied. The antimicrobial peptides of cathelicidin family are distributed mainly in neutrophils and epithelial cells of mammals. About 30 different cathelicidins have been described in mammals (Xiao et al. 2006) including a single cathelicidin in human known as human cathelicidin (hCAP-18) with broad spectrum of 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 (Heilborn et al. 2003). Some cathelicidin peptides have also been studied in avian like chicken. Townes et al. (2004) have reported the induction of a cathelicidin peptide named chicken liver expressed antimicrobial peptide–2 (cLEAP-2) in response to *Salmonella enterica* infection. Further Xiao et al. (2006) have also identified three chicken cathelicidins (fowlicidin-1 to -3) displaying potent salt-independent activities against a range of gram-positive and gram-negative bacteria including antibiotic resistant strains.

Over the past decade, apart from the fowlicidins a few other antimicrobial peptides and proteins have also been reported from chicken. Cationic antimicrobial peptides homologous to β-defensins, known as gallinacins (Gal-1, -1α and -2) have been isolated and characterized from chicken heterophils (Evans et al. 1994; Harwig et al. 1994). Nile et al. (2006) purified and characterized a 3.5 kDa antimicrobial peptide from the mucosa and epithelial cells of chicken intestine, which was identified as the carboxy-terminal fragment of a novel 767 amino acid avian protein containing multiple domains with homology to protease inhibitory modules. Li et al. (2007) have also reported that two novel antimicrobial proteins and one antimicrobial polypeptide belonging to histone family were isolated from the chicken liver extract and these molecules exhibiting antimicrobial activity against both gram-positive and gram-negative bacteria. Thus a number of antimicrobial peptides have been investigated from different tissues of chicken. However, thus far there is no report on antimicrobial peptides from the heart tissues of chicken which is freely available for human consumption. In our previous paper we have described the antibacterial activities of chicken heart tissue extracts against certain human pathogenic bacteria (Sundaramoorthy and Saravanan 2010). In the present work, we have also tried to isolate and characterize the antibacterial peptides if any from the chicken heart which will form a suitable alternative to the conventional antibiotics in treatment regimes in order to focus our idea in the preparation of natural peptide-based antibiotics economically at large scale.

MATERIALS AND METHODS

Preparation of crude antimicrobial proteins

The crude antimicrobial proteins were prepared by acid extraction as described by Matutte et al. (2000). The

whole hearts of adult healthy chicken was obtained freshly from a local meat shop at Tiruchirappalli. The cleaned tissues of the ventricular portion were placed in liquid nitrogen, and after 24 hours, the frozen tissues were pulverized 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^{o}C. The supernatant containing the crude proteins was concentrated by lyophilization and stored at -20^{o}C until use.

Isolation and purification of antibacterial proteins

The acid extracted concentrated crude proteins were dissolved in 20 mM ammonium acetate (pH 5.0) and loaded on to a Carboxymethyl Cellulose (Sigma-Aldrich) cation-exchange column (3x15 cm) that was previously equilibrated with the same buffer. The proteins bound to the CMC were eluted using a gradient from 20-1500 mM ammonium acetate at a flow rate of 1 ml min^{-1}. Fractions were mixed and reconstituted in 0.01 % acetic acid after lyophilisation (Li et al. 2007). Exactly 10 µl of this partially purified protein sample 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. (2000). The single peak peptide 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^{o}C until use (Matutte et al. 2000).

Antibacterial assay

Seven bacterial isolates (*E. coli* ATCC 25922, *P. mirabilis*, *S. aureus*, *P. aeruginosa* ATCC 27853, *S. typhi*, *S. paratyphi* 'A', *S. typhimurium*) were collected from the clinical samples at the Microbiology Laboratory, KAPV Medical College, Tiruchirappalli. The isolates were also confirmed by gram staining and standard biochemical tests and maintained in the semisolid nutrient agar media until use.

Antibacterial activity was determined by the plate assay method (Diamond et al. 1991) with well diffusion as shown in Fig.2. The bacterial isolates were sub-cultured in to mid-logarithmic phase and suspended in 1% peptone water to adjust the turbidity to 2 X 10^{6} 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 NaF separately by using sterile swabs. The surface of the media with the lawn of the testing organisms was divided into four equal areas and a well was formed in each. Briefly the concentrated aliquotes of each peptide fraction (10 µl) was delivered into the respective wells. Then the plates were incubated overnight at 37^{o}C and the zone of inhibition was measured by measuring the width of zone formation using a divider and scale. The absence of zone formation indicates the absence of bactericidal activity of the peptide. The active peptide fraction was identified based on the size of the inhibitory zones (> 5 mm).

The active peptide fraction was then purified into required amounts by performing the appropriate number of RP-HPLC runs from the same samples setting the specific wave length at which the active fraction was purified (Matutte et al. 2000). The purified active fractions were then lyophilized into powders and dissolved in 200µl of PBS (pH-7) to yield the stock solution.

Minimum Inhibitory Concentration (MIC) of active peptide fraction (Microdilution method)

The MIC of the active peptide fraction, the 2nd fraction obtained at 214 nm [P_2(214 nm)] was determined adopting the microdilution method as described by Zhu et al. (2007) only against *P. aeruginosa* ATCC which responded well with this peptide. This test was performed in a 96 micro well plate. From the stock solution, two fold serial dilutions of the peptides were made with 1% peptone water so as to make the concentrations from 100 to 3µg/ml in the appropriate row of the micro well plate. Then aliquots of 100µl of the bacterial suspension were added in 100µl peptide solution. After 24 hours incubation at 37ºC, 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. (2007) the hemolytic assay for the active peptide fraction was carried out in a micro well plate. The human blood of each group (A+, B+ & O+) was collected from a private blood bank at Tiruchirappalli. The Red blood cells were sedimented by centrifugation at 3000 rpm and repeatedly washed with 0.9% Nacl. Then the hRBCs were converted into 4% with phosphate buffer solution (PBS pH.7). The wells of the respective rows (A, B and C) were filled with 100 µl of PBS in each. The peptide solution was serially diluted (two fold) 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) hemolysis 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 the transfer of 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 a significant haemolysis has occured. Finally the percentage of haemolysis is calculated by using the following formula.

$$\text{Percentage haemolysis} = 100 \times \{A_{sample} - A_{blank}\} / \{A_{triton} - A_{blank}\}$$

Determination of Molecular weight:

From the stock solution of the active peptide fraction, 20 µl aliquote was subjected to the molecular weight determination by SDS-PAGE on 15% mini gels according

to the standard protocols (Laemmeli 1970). The sample was heated for 3 minutes at 100 ^{o}C in sample buffer (25 % 1 M tris-Hcl, pH 6.8; 4% SDS; 2% β-mercaptoethanol and 5 % glycerol) and then it was run along with mid-range marker proteins. The gels were fixed and the bands were visualized after silver staining using UV lamp. Finally the molecular weights of the bands were documented using Gel.doc.system. Subsequently the crude proteins of the acid extract and the proteins partially purified by cationic exchange chromatography were also run along with samples to compare the bands.

RESULTS

Isolation and 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 by reverse-phase high performance liquid chromatography separation. As shown in the table 1 and Fig-1a two fractions were obtained at 214 nm. The first fraction (4.6µg of peptide) was eluted at 2.517 minutes of retention time whereas the second fraction (4.9µg of peptide) eluted at 3.450 minutes. Similarly at 281 nm, the fraction 1 (13.7µg of peptide) and fractions 2 (4.2 µg of peptide) were eluted at 1.258 and 3.125 minutes respectively.

TABLE 1. Antibacterial activity of chicken heart RP-HPLC peptide fractions against different human pathogenic bacteria

SNo	Bacterial isolates tested	Wavelength of the peptides fraction	Peptide fraction (peaks)	Zone of inhibition (mm)
1	*E.coli* ATCC 25922	214	P1	Nil
			P2	Nil
		281	P1	**2.0**
			P2	Nil
2	*P. mirabilis*	214	P1	Nil
			P2	Nil
		281	P1	Nil
			P2	Nil
3	*S. aureus*	214	P1	Nil
			P2	Nil
		281	P1	Nil
			P2	Nil
4	*P. aeruginosa*ATCC 27853	214	P1	Nil
			P2	**5.0***
		281	P1	Nil
			P2	Nil
5	*S. typhi*	214	P1	Nil
			P2	Nil
		281	P1	Nil
			P2	Nil
6	*S. paratyphi*'A'	214	P1	Nil
			P2	Nil
		281	P1	Nil
			P2	Nil
7	*S. typhimurium*	214	P1	Nil
			P2	Nil
		281	P1	Nil
			P2	Nil

Antibacterial activity

After the RP-HPLC purification, all the four fractions were lyophilized and subjected to antibacterial assays. The antibacterial activity of the purified peptide fractions fell in the range of 2.0 to 5.0 mm width of zone of inhibitions at both wave lengths as given in the tables 1. The width of 5 mm inhibitory zone produced by the 2nd fraction purified at 214 nm [P_2(214 nm)] against *P. aruginosa* ATCC strain was only considered as active fraction. The MIC of the active peptide fraction against *P. aeruginosa* ATCC was found to be 6.0 µg/ml.

Haemolytic activity

In order to test the cytotoxicity of the active peptide fraction to the human cells, the hemolytic assay was carried out with human ABO erythrocytes. The visual observations showed the absence of hemolysis for the active fraction against all the blood groups. The percentage of hemolysis caused by the peptide fraction was observed to be -3.5%, 2.7% and 3.5% for ABO hRBCs respectively (Fig-4). The hemolysis of all hRBCs has been occurred only at the highest concentration subjected (500 µg/ml) with the maximum of 3.5 % hemolysis. It is of interest to note that the concentration required for the antibacterial activity was only 6.0µg/ml which is very low to cause hemolysis on hRBCs.

Molecular weight dtermination

The SDS-PAGE analysis of the proteins after various stages of purification (acid extraction, cationic exchange chromatography and RP-HPLC purifification) showed the gradual reduction in the number of peptide bands and which indicates the stepwise purification. The purity of the RP-HPLC purified active peptide fraction was also confirmed by the single band formation (Fig-5). The electrophoretic pattern of the chicken heart tissue at different stages of purification showed some similar types of bands within the size range of antimicrobial peptides (1-30 kDa). The first two lanes showed two bands of 14 and 12 kDa in each. But the third lane showed a single 13 kDa band of RP-HPLC purified active peptide fraction.

FIGURE1a. RP-HPLC separation of antimicrobial peptides from chicken heart at 214 nm

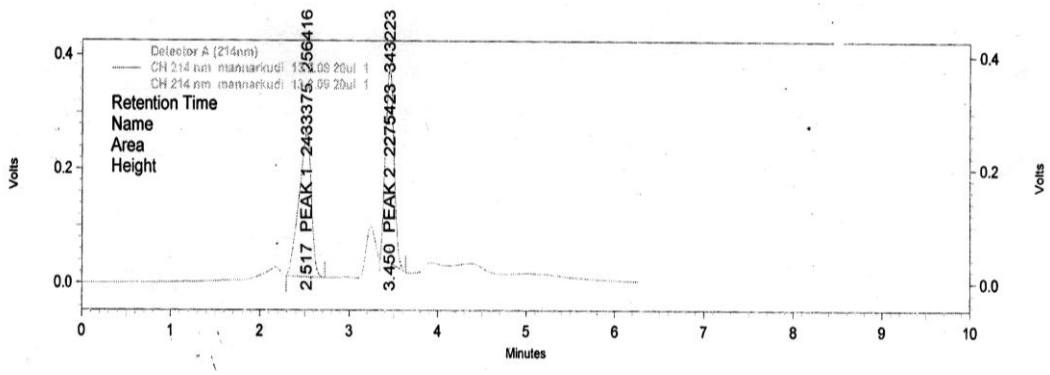


FIGURE1b. RP-HPLC separation of antimicrobial peptides from chicken heart at 281 nm

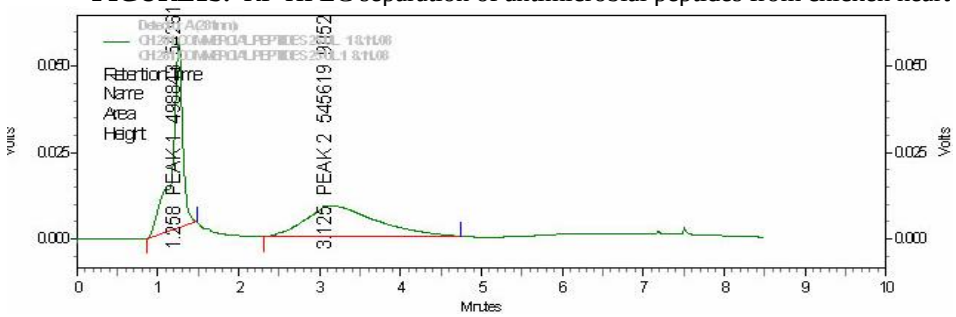
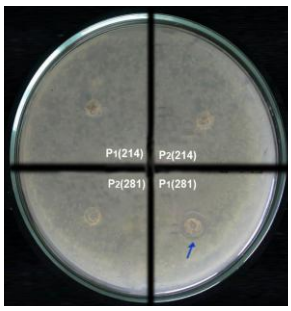
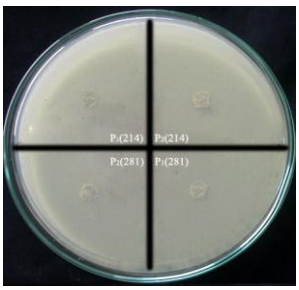


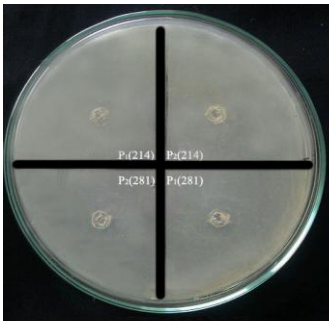
FIGURE 2. Antibacterial activities of chicken heart RP-HPLC peptide fractions against different human pathogenic bacteria. 5mm inhibitory zone is found only in *P. aeruginosa* ATCC plate



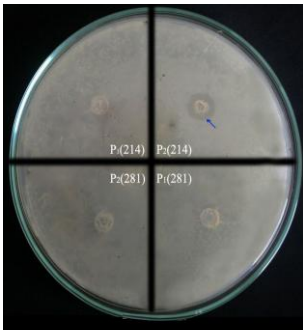
E. coli



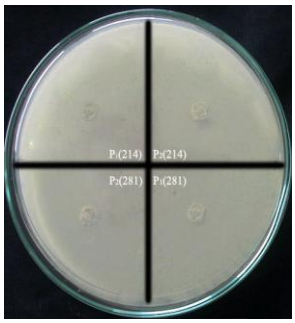
P. mirabilis



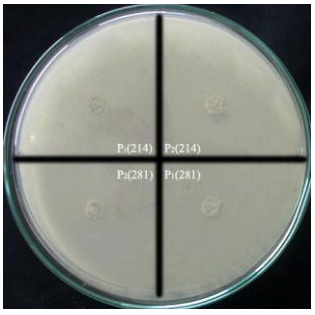
S. aureus



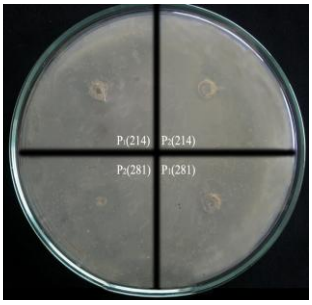
***P. aeruginosa* ATCC**



S. typhi



S. paratyphi 'A'



S. typhimurium

FIGURE 3. Minimum Hemolytic Concentrations of chicken heart active peptide fraction [P_2 (214)] for ABO hRBCs were found to be 500 µg /ml. each.

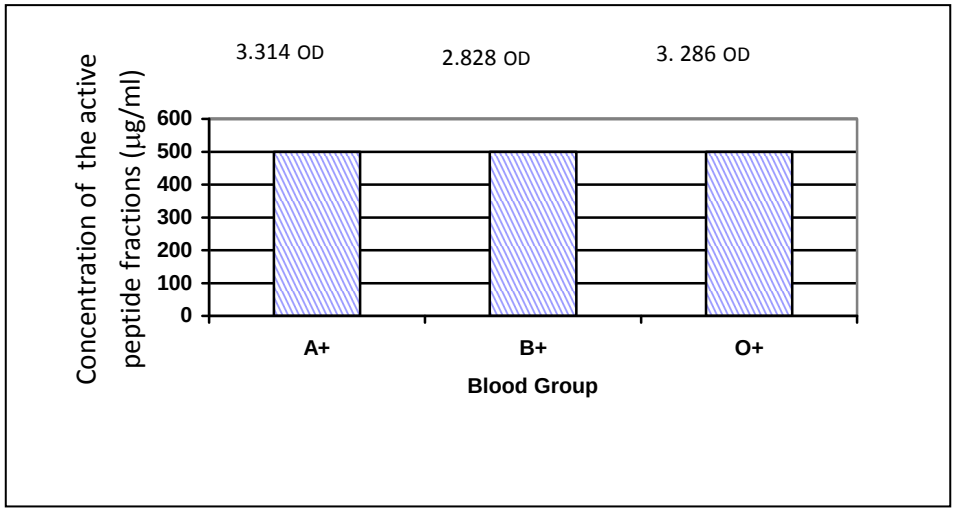


FIGURE 4. The percentage of haemolysis of ABO hRBCs by chicken heart active peptide fraction [P_2(214)] were calculated as -3.5%, 2.7%, 3.5% respectively**.**

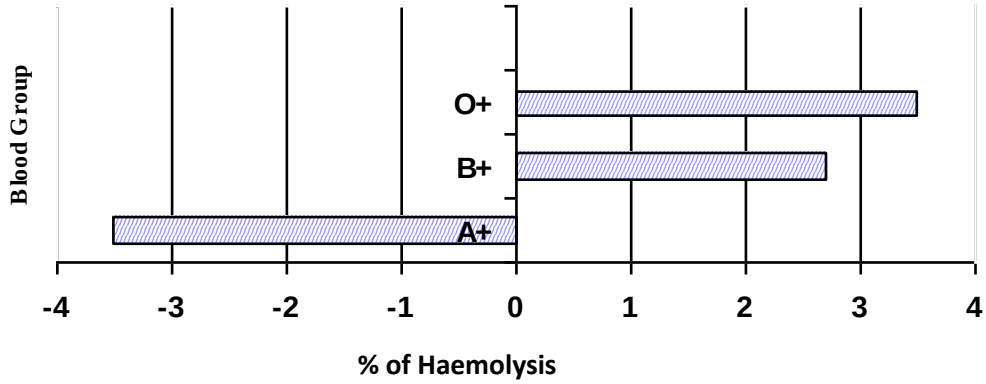
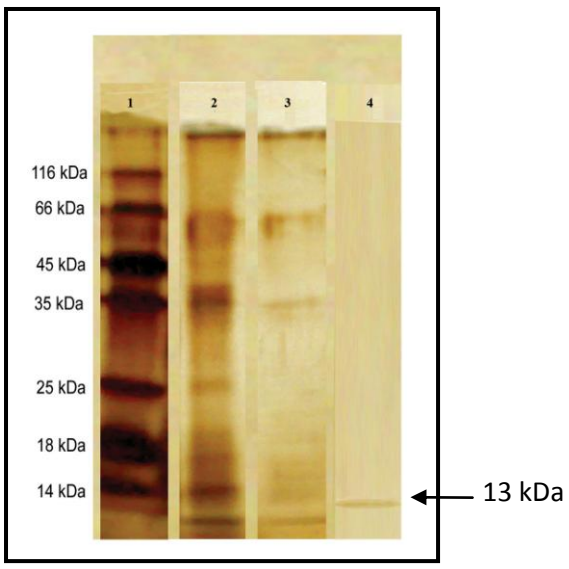


FIGURE 5. SDS – PAGE analysis of the chicken heart proteins at different stages of purifications



Lane 1. 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 (13 kDa).

DISCUSSION

In our previous work, we studied the antibacterial properties of the chicken heart buffer extract and its crude proteins (Sundaramoorthy and Saravanan 2010). In the present work we have isolated a 13 kDa antibacterial peptide [P_2(214 nm)] against *P. aeruginosa* from the acid extracted chicken heart crude proteins by ion-exchange chromatography and reverse phase-high performance liquid chromatography. It is important to note that *P. aeruginosa* is a human pathogen and is capable of producing resistance. Several research groups have demonstrated that a number of antimicrobial peptides/proteins have been isolated and studied extensively from different cells or tissues of avian including chicken (Harwig et al. 1994; Nile et al. 2006; Li et al. 2007).

The killing concentration of this peptide was found to be very minimum (MIC - 6.0 µg/ml). The microgram level inhibitory concentrations of some other antimicrobial peptides have also been reported in certain previous studies. The MIC range of TAP was observed to be 6-50 µg/ml for four bacterial strains (*E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus*) and a yeast *Candida albicans*

(Diamond et al. 1991). The Epididymis protein 2 (EP2) isoforms of human and rhesus monkey kill more than 99.9% of bacteria at 50-100 µg/ml (Avellar et al. 2004). Most of the cathelicidin peptides rapidly kill a wide range of microorganisms at micromolar and sub-micromolar concentrations, with a broad spectrum (Gennaro and Zanetti 2000). Xiao et al. (2006) have reported the synthesized chicken fowlicidins (1&2) had potent antimicrobial activity in a salt independent manner with MIC of 0.4 – 2.0µM.

Though some antimicrobial peptides are potential to act against different pathogens, their cytotoxic effects prevent the development of peptide-based natural antibiotics unless there are some modifications in the peptide sequences. For example, Fowlicidins (-1 and -2) of chicken displayed potent salt-independent activities against a range of gram-negative and gram-positive bacteria including antibiotic resistant strains with hemolytic property (Xiao et al. (2006)). The hemolytic activity of both fowlicidins was reduced by 2-4 fold in the presence of 10%PBS. The Trp/Pro-rich antimicrobial peptides tritrpticin and indolicidin are relatively short peptides consisting of only 13 residues, and containing high fractions of tryptophan and proline. Although both peptides have broad spectrum of antimicrobial activities against bacteria and fungi, their relatively high toxicity toward eukaryotic cells prevent their usage as antibiotics (Falla et al. 1996; Yang et al. 2002). Therefore much efforts has been taken in the past decade to decrease the cytotoxicity and to increase the cell selectivity of these two peptides (Yang et al. 2002). Furthermore Zhu et al. (2006) 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.

The maximum percentage of hemolysis occurred by this peptide was only 3.5% for 'O' group cells. The cells of 'A' and 'B' groups were also got hemolysis by less than 3.5%. The minimum hemolytic concentration (MHC) of this peptide determined was only 500 µg/ml for all hRBCs and which was the highest concentration of the peptide subjected (Fig-3). But the concentration of the peptide required for antibacterial activities was 6 µg/ml and which is the lowest concentration to cause hemolysis. Hence the purified active peptide can be considered as non-hemolytic potent antibacterial peptides and can be strongly recommended for treating the infectious disease especially respiratory diseases like cystic fibrosis at the micromolar concentrations. It is also interesting to note that the same molecular weight (13 kDa) antimicrobial peptide isolated from terrestrial Salamander (*P. cinereus*) caused 84% hemolysis (Fredericks and Dankert 2000).

The molecular weight of this peptide was found to be 13 kDa with single band formation in the SDS-PAGE which confirms the purity of this peptide (Fig-5). The 13 kDa size of this peptide is equivalent to the 12-14 kDa 'cathelin' domain which is the constant region of all cathelicidin family peptides including human cathelicidin (hCAP-18) as described by Ritonja et al. (1989) & Sorensen (2005). The 12 and 14 kDa bands were also found in the first two lanes (1^{st} and 2^{nd}) each of the first two stages of purification. A 16 and 18 kDa peptide bands were also found in the same two lanes each along with the 12 and 14 kDa bands. It is notable that the molecular weight of the human cathelicidin peptide (hCAP-18) is also 18 kDa. So, further studies in this direction could be definitely helpful, to develop natural antibiotics, which could fight against causative organisms of many dreadful diseases.

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