Antimicrobial activity of a 13 amino acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides

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Abstract **It has long been speculated that porcine cathelin is an N-terminal fragment of a longer precursor protein which possesses antimicrobial activity. In an attempt to find such a precursor, a cDNA clone was recently isolated and sequenced by screening a cDNA library from porcine bone marrow. In order to identify the functional activity of the putative protein encoded by an open reading frame, we have synthesized various lengths of peptides that correspond to the C-terminal region of the protein and examined them for their antimicrobial activities. We found that a 13 amino acid tryptophan-rich region with the sequence of VRRFPWWWPFLRR had strong antimicrobial activity with a wide spectrum. It showed potency against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus epidermidis, Proteus mirabilis,* and *Streptococcus* group D as well as *Aspergillus fumigatus*. The action of this peptide is bactericidal rather than bacteriostatic and this activity is completely inhibited by 2 mM $MgCl_2$. Our results indicate that the previously identified putative precursor encoded by the isolated cDNA indeed possesses a potent antimicrobial activity and that this 13 amino acid synthetic peptide is considered to be a potentially effective drug against various infectious agents.**

Key words: Antimicrobial peptide; Bactericidal peptide; Cathelin

1. Introduction

The recent findings of a variety of antimicrobial peptides in various organ systems had a significant impact on the understanding of our immune system [1,2]. These peptides are widely distributed among living organisms and are considered to play crucial roles in the primary defense against intruding microorganisms. Although these peptides show a marked inter-species diversity in structure and spectrum of activity, the precursors of many antimicrobial peptides of porcine, bovine and rabbit origin contain a highly conserved preprotein region [3–5]. This region appears to be homologous to cathelin which is a putative proteinase inhibitor originally isolated from pig blood leukocytes. These peptides include Bac5 [6], indolicidin [7] and cyclic dodecapeptide from bovine [8] and CAP18 [9]. The mRNA sequences of these peptide share a remarkably high homology in signal peptide and pro sequence coding regions. However, they have only limited similarity in the region encoding the C-terminal regions. Therefore, it has been suggested that cathelin is the N-terminal fragment of a porcine homolog of antimicrobial peptides.

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In an attempt to identify a precursor protein for cathelin and antimicrobial peptides, Pungercar et al. [10] recently screened the cDNA library from porcine bone marrow using a rabbit CAP18 cDNA as a probe and identified clones which encoded a potential precursor protein. An analysis of the nucleotide sequence of the cDNA revealed that it encoded a 228 amino acid residue protein and that the N-terminal 98 residue is similar to cathelin [10]. On the other hand, the C-terminal half of 101 residues is very basic, rich in proline and arginine which appears to be very similar to cationic antimicrobial peptide such as bac5 and PR39 [11]. Therefore, they speculated that the C-terminal portion of this putative peptide may possess antimicrobial activity. In this report, we synthesized various peptides that corresponded to this region and tested them for their functional activities on both bacteria and fungi.

2. Materials and methods

2.1. Peptide synthesis and bacterial and fungal strains

The peptides were synthesized by Fmoc solid-phase synthesis using Opfp or Odhbt active esters on a Milligen 9050 peptide synthesizer (Perseptive Biosystems, Framingham, MA). The peptides were cleaved with phenol/thiophenol/anisole/TFA (1:1:2:30) for 1.5 h. Crude peptides were filtered, dried, solubilized in dilute acetic acid and lyophilized. The peptides were purified by preparative HPLC on a 20×200 mm Vydac C18 column. Elution of the peptide (100 mg injected) was accomplished with a linear gradient from 0 to 80% acetonitrile containing 0.1% TGA over 30 min. Characterization was performed by electrospray mass spectrophotometry (Vestec, Houston TX). The homogeneity was confirmed to be more than 95% for all peptides. *Pseudomonas aeruginosa, Staphylococcus epidermidis, Klebsiella pneumoniae, Proteus mirabilis,* Group D *Streptococcus* and *Candida albicans* are all clinical isolates and identifications were carried out by the pathology department at Southern Illinois University. *Eschelichia coli* (DH5) and *Aspergillus fumigatus* strains are from our laboratory stock.

2.2. Radial diffusion assay

The radial diffusion assay was performed using double-layered agarose as described by Lehrer et al. [12] with minor modifications. Briefly, 2×10^5 cells of mid-log phase bacteria grown in TSB medium were mixed with 1.0% agarose in 10 mM sodium phosphate buffer (pH 7.4) containing 0.02% Tween 20 and 0.03% Tryptic soy broth (TSB). The mixture was then poured into a petri dish. When the agarose was solidified, wells with a 2.5 mm diameter were made in the agarose and samples of the peptides in various concentrations were placed in each well. The plate was incubated at 37°C for 2 h. Top agar containing 1.0% agarose in 10 mM sodium phosphate buffer (pH 7.4) and 6% TSB was poured over it, and the plate was further incubated at 37°C for 18 h. The diameters of the inhibitory zones were measured for the quantitation of inhibitory activities.

2.3. Bactericidal assay

Bacterial cells were grown in TSB medium until the OD_{600} reached 0.2. Cells were diluted with 10 mM sodium phosphate buffer (pH 7.2) and aliquots (2×10^5 cells) were mixed with various concentrations of

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the peptides in 20 μl of sodium phosphate buffer. The mixtures were incubated at 37°C and samples were withdrawn at 10-min intervals, diluted with sodium phosphate buffer, and plated on L-agar plates. After overnight incubation of the plates at 37°C, the number of colonies was counted.

2.4. MIC assay

To determine the MIC, fungal strains were grown in YG media until the fungi reached the logarithmic phase. The cells diluted in YG medium [13] were placed in a 96-well plate along with a series of 2-fold dilution of the peptides, and then the plate was incubated at 37°C for 24 h. MIC was defined as the lowest concentration at which there was no fungal growth.

3. Results

3.1. Antibacterial activity of the tryptophan-rich peptides

To synthesize and test the antimicrobial activity of the proline/arginine-rich peptide predicted from the cDNA sequence of porcine bone marrow, we performed hydrophobic moment analysis for the C-terminal region. The hydrophobic moment is a semi-empirical quantity based on computation and experimental measurements which describe the distribution of hydrophilic and hydrophobic residue groups in a protein. Therefore, the hydrophobic moment indicates asymmetric regions of hydrophobicity or amphilicity [14]. Among several alpha-amphipathic regions, we chose the longest strech of an amino acid sequence which contained 13 amino acids including three tryptophans in a row. It should be noted that the three consecutive tryptophans is a very unique sequence and our data bank revealed that it is the rarest sequence among three consecutive sequences of other amino acids. As shown in Table 1, peptide 1 with the sequence of VRRFPWWWPFLRR showed strong antibacterial effects on *E. coli* when tested by the radial diffusion assay. The result suggests that this 13 amino acid peptide is the functional domain of the predicted precursor protein which consists of 228 amino acid residues. We then synthesized several derivatives of this peptide, that were named peptides 2–4 as shown in Table 1. Peptide 2, which consists of a 12 amino acid sequence corresponding to part of peptide 1 and two additional arginines at both ends, showed slightly higher activity than the original peptide 1. Peptide 3, which shared 9 amino acids with peptide 1, showed detectable but significantly less activity than peptide 1. On the other hand, peptide 4, which shared 7 amino acid residues with peptide 1, had no detectable activity. Therefore, the 13 amino acid residues of peptide 1 are considered to be a minimum sequence for antimicrobial activity.

We have also tested the antimicrobial effects of the above peptides on other gram-positive and -negative bacteria including *Pseudomonas, Klebsiella, Staphylococcus, Proteus* and *Streptococcus*. As shown in Table 2, peptide 1 showed a broad range of antimicrobial activity on all organisms tested.

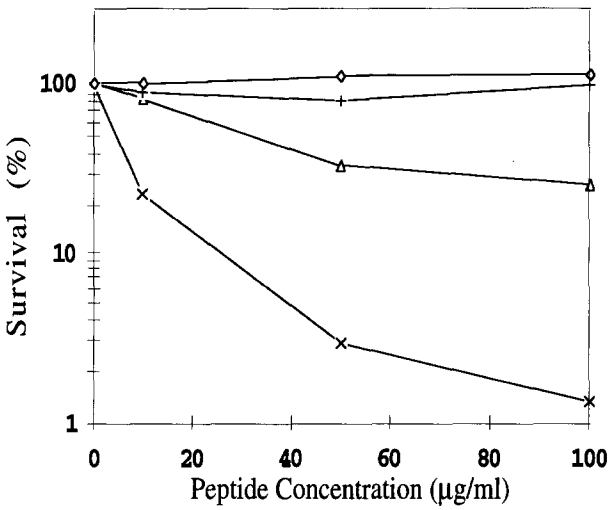

Fig. 1. Bactericidal activities of peptides. *E. coli* cells grown to logarithmic phase were mixed with various concentrations of peptides, 1 (×), 2 (△), 3 (+) and 4 (◇) in phosphate buffer. After incubation at 37°C for 10 min, aliquots of samples were withdrawn, diluted and plated on L-agar. The plates were incubated for 18 h and the number of colonies was counted.

Again, peptide 2 showed slightly higher activity on those same bacteria, while peptide 3 showed low but detectable activity. However, peptide 4 showed no detectable activity on any organisms.

3.2. Bactericidal activity of the peptide

In order to examine whether the antimicrobial activities of the peptides are bacteriostatic or bactericidal, we performed a bactericidal assay for all peptides using *E. coli*. Cells were incubated with various concentrations of peptides in sodium phosphate buffer. After incubation for 10 min, the number of surviving cells was counted. As shown in Fig. 1, peptide 1 showed strong bactericidal activity and 97% of cells were killed within 10 min at a concentration of 50 μg/ml. Peptide 2 showed significantly less bactericidal activity and peptides 3 and 4 showed minimum activity. These results suggest that the growth inhibitory effects of the peptides shown by the radial diffusion assay are due to bactericidal activities. However, it should be noted that the bactericidal activity of peptide 2 is significantly less than that of peptide 1 while the antimicrobial activity of peptide 2, assayed by the radial diffusion method, was stronger than that of peptide 1.

3.3. Antifungal activity of the peptide

Because peptide 1 showed a broad spectrum of antimicrobial activity, we also tested the effect of the peptide on two fungi, namely *A. fumigatus* and *C. albicans*. As shown in Ta-

Table 1
Zone inhibition assay for synthetic peptides

Peptide no.	Sequence	Inhibitory activity[a] at	
		50 μg/well	5 μg/well
Predicted from cDNA	...VRRFPWWWPFLRR...		
1	VRRFPWWWPFLRR	10	6
2	RRRFPWWWPFLRRR	11	7
3	RFPWWWPFLR	5	2
4	FPWWWPF	0	0

[a]Inhibitory activities are expressed as diameters of inhibited zones in mm.

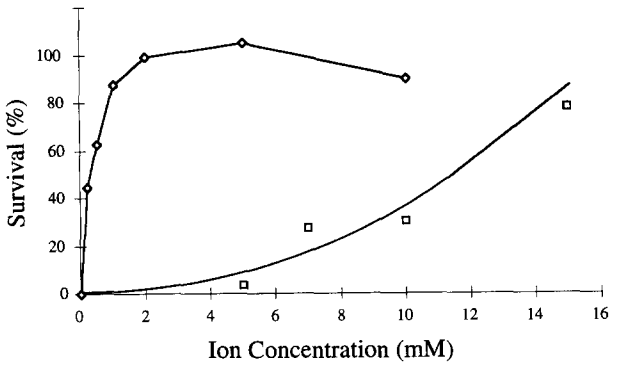

Fig. 2. Effects of Ca^{2+} and Mg^{2+} on bactericidal activity. Bactericidal assay was performed as described in the text. *E. coli* cells were mixed with peptide 1 (100 μg/ml) in the presence of indicated concentrations of $CaCl_2$ (◇) and $MgCl_2$ (□). The mixture was incubated at 37°C for 10 min and surviving cells were counted as described in the legend to Fig. 1.

ble 3, peptide 1 completely inhibited the growth of *Aspergillus* and *Candida* at concentrations of 250 and 1000 μg/ml, respectively. Peptide 2 also inhibited the growth of both fungal strains at similar concentrations. Peptides 3 and 4 also showed growth inhibitory effects on *A. fumigatus* at a concentration of 500 μg/ml but they had no inhibitory effect on *C. albicans*. These results suggest that peptide 1 could be an effective antifungal drug though it required much higher concentrations than that for bacteria.

3.4. Effects of cation on the bactericidal activity

We have investigated the effects of cations on the activity of peptide 1 since the activities of some antimicrobial peptides are known to be affected by cations [15]. *E. coli* cells were mixed with peptide 1 in the presence of various concentrations of $MgCl_2$ or $CaCl_2$ and examined for their bactericidal activities. As shown in Fig. 2, 15 mM $CaCl_2$ inhibited 80% of the antimicrobial activity. On the other hand, Mg^{2+} showed a significantly greater inhibitory effect on the bactericidal activity and only 2 mM $MgCl_2$ completely inhibited the activity of peptide 1.

4. Discussion

We have shown that the synthetic 13 amino acid peptide, wich corresponds to the C-terminal portion of a putative precursor for antimicrobial proteins, indeed has strong antimicrobial activities against various gram-positive and -negative bacteria and fungi. The original precursor protein predicted from the sequence of a cDNA clone consists of 228 amino acids, and the peptide with potential antimicrobial activity was speculated to reside within the 101 amino acid of the C-terminal [10]. We chose the longest stretch of amphipathic region among the 101 amino acid residues in an attempt to define the minimum sequence for the antimicrobial activity. This region of 13 amino acids contains three consecutive residues of tryptophan which is the least common sequence among the three consecutive identical amino acids found in Swisspro and PIR. Interestingly, Indolicidin which comprises 13 amino acids also contains 5 tryptophan residues [7]. Therefore, these unique multiple tryptophan residues may indeed play an important role in antimicrobial activities. The addition of two more arginine residues at both the N- and C-terminals to peptide 1 slightly increased the antimicrobial activity while deletion of two arginine residues from peptide 1 significantly reduced the activity. Therefore, consecutive arginine residues on both the N- and C-terminals of this peptide is considered to be an essential structure for the function. Because the native protein corresponding to the cDNA has not been isolated, we do not know whether the 13 amino acid peptide has full activity and, if so, what is the function of the remaining sequences. However, it is possible that this precursor protein could be further processed to be a smaller peptide which includes the 13 amino acids in order to show functional activity. Alternatively, these regions might possess some other biological functions.

Peptide 2, which has two additional arginine residues, showed stronger activity than peptide 1 using the zone inhibition assay while it demonstrated lower activity using the bactericidal assay. This may be due to the difference between the two assay systems. The zone inhibition assay is dependent on the diffusion of the peptide in agar, while the bactericidal assay is not because it uses a liquid medium. The net charge of peptides 1 and 2 are calculated to be +4 and +6, respectively. Therefore, these additional arginines may make the peptide more diffusible, hence, it shows higher activity using the zone inhibition assay.

Our results suggest that Mg^{2+} is a stronger inhibitor of the peptide than Ca^{2+}. The reason for the different effects of both ions is unknown at present. Mg^{2+} may directly affect the activity by changing the conformation of the peptide or it may affect cellular factors that augment the killing effects of the peptide. In this context, it is worth noting that some autolytic enzymes are strongly inhibited by Mg^{2+}. Kim et al. [16] reported that a suicide enzyme called autolysin, which is involved in the autolysis of bacteria, is strongly inhibited by Mg^{2+}. Therefore, the action of peptide 1 may involve such cellular autolytic enzymes which are inhibited by cations. More recently, Vescovi et al. [17] reported that Mg^{2+} is not merely a co-factor or a second messager, but that it directly activates a set of genes and that it functions as an external

Table 2
Zone inhibition assay with gram-positive and -negative bacteria

	Inhibitory activity[a]			
	Peptide 1	Peptide 2	Peptide 3	Peptide 4
E. coli	5	5	2	0
P. aeruginosa	3	4	1	0
K. pneumoniae	7	7	2	0
S. epidermidis	5	7	0	0
P. mirabilis	1	2	0	0
Streptococcus (Group D)	4	5	1	0

[a]Inhibitory activities are expressed as diameters of inhibited zones in mm.

Table 3
MIC for *Aspergillus fumigatus* and *Candida albicans*

Peptide no.	MIC (μg/ml)	
	A. fumigatus	*C. albicans*
1	250	1000
2	250	1000
3	500	>1000
4	500	>1000

signaling substrate. Therefore, it is tempting to assume that some of the gene products that are activated by cations act to protect bacterial cells from the killing effects of the antimicrobial peptide. Although we need further analysis of the ion effects, our results would provide a useful tool for understanding the mechanism of action of the peptide.

Our results indicate that peptide 1 has a wide spectrum of antimicrobial activity. The peptide is potent against both gram-positive and -negative bacteria as well as *Aspergillus*. This synthetic peptide is one of the shortest among all known antimicrobial peptides. Furthermore, it is easy to synthesize and quite soluble. Therefore, the peptide has potential as a therapeutic regent for a variety of infectious agents. Because this peptide contains three consecutive residues of tryptophan, we propose designating peptide 1 as Tritrpticin.

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