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Antibacterial Activities of Peptides Designed as Hybrids of Antimicrobial Peptides

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Abstract: CA(1-8)ME(1-12), the CA-ME hybrid peptide of the amino terminal segments of cecropin A (CA) and melittin (ME), has been reported to have a broad spectrum and improved potency without a hemolytic property. In order to obtain new synthetic peptides with powerful antibacterial activity without hemolytic activity, several hybrid peptides were designed from the sequences of CA, ME, magainin 2, bombinin and lactoferricin. All hybrid peptides were constructed to form an amphipathically basic-flexible-hydrophobic structure and synthesized by the solid phase method. Their hemolytic activities against human red blood cells and antibacterial activities against both Gram-positive and Gram-negative bacteria were determined. CA(1-8)MA(1-12), CA(1-8)BO(1-12), MA(10-17)ME(1-12) and LF(20-29)ME(1-12) showed comparable activities with broad spectra against both Gram-positive and Gram-negative bacteria relative to CA(1-8)ME(1-12) but without hemolytic properties. These hybrid peptides, therefore, could be useful as model peptides to design a novel peptide with improved antibacterial activity and study on structure-activity relationships of antimicrobial peptides.

Key words: antibacterial activity, hemolytic activity, hybrid peptides.

Antimicrobial peptides are widely distributed in nature including insects, horseshoe crabs, frogs and mammals. These peptides have been known to play important roles in insect immunity and host defense (Boman, 1992; Boman, 1995). Most of them have a common feature of being highly basic due to multiple basic amino acid residues, and of forming amphipathic structures in phospholipid bilayers. Their basic natures are related to the interaction with negatively charged membranes of the target cells and their amphipathic character which allow them to be incorporated into the membrane, ultimately disrupting membrane potential and/or structure, and cell death (Cruciani *et al.*, 1991).

Cecropins isolated from cecropia moths are very active against Gram-negative bacteria, but less active against Gram-positive bacteria (Steiner *et al.*, 1981; Zasloff *et al.*, 1988). Melittin (ME), a bee venom, has good antibacterial potency against both Gram-negative and Gram-positive bacteria, but is also hemolytic for eukaroytic cells (Habermann, 1972).

Until now, many studies on structure-activity relationships of these antimicrobial peptides have been carried out in order to improve their antimicrobial potency without hemolytic activity (Chen *et al.*, 1988; Zasloff *et al.*, 1988; Boman *et al.*, 1989; Andreu *et al.*, 1992; Wade *et al.*, 1992). Hybrid peptides consisting of segments from two antimicrobial peptides have been used as one possible way to improve antimicrobial potency without hemolytic activity. Athough shorter in length than CA and ME, CA-ME hybrid peptides consisting of amino terminal segments from cecropin A(CA) and ME were reported to have a broad spectrum and more improved potency than CA and ME but without hemolytic property (Andreu *et al.*, 1992; Wade *et al.*, 1992). Magainin 2(MA), bombinin(BO) and lactoferricin(LF) were known to be antimicrobial peptides with potent antibacterial activities (Berkowitz *et al.*, 1990; Morishima *et al.*, 1990; Bellamy *et al.*, 1992).

In this study, we designed hybrid peptides where the N-terminal segments of MA or BO are linked with the N-terminal segment of CA, CA(1-8). Also, hybrid peptides incorporating the N-terminal segment of MA, BO or LF(20-29) and the N-terminal segment of ME, ME(1-12) were designed. All peptides were synthesized by the solid phase method (Merrifield, 1986; Shin *et al.*, 1995). These synthetic peptides were assayed for hemolytic activities against human red blood cells, and for antibacterial activities against two Gram-positive and two Gram-negative bacteria with the agarose hole meth-

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od (Hultmark *et al.*, 1983).

Materials and Methods

Antibacterial strains and human red blood cells

Escherichia coli (HB 101), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 65389) were obtained from Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology, Korea. Human red blood cells (hRBCs) were purchased from Korean Red Cross, Blood Center.

Cecropin A and chemical reagents

Cecropin A and chemicals for solid phase peptide synthesis were purchased from Sigma Chemical Co. (St. Louis, USA). Fmoc(Nα-fluorophenylmethoxycarbonyl)-amino acids and Rink Amide p-methoxybenzhydrylamine(MBHA)-Resin were obtained from Nova Biochem. (San Diego, USA).

Peptide synthesis and purification

All peptides except CA were synthesized by solid phase method with Rink Amide MBHA-Resin. The chain elongations were manually performed using the dicyclohexylcarbodiimide (DCC)-*N*-hydroxybenzotriazole (HOBt) method. The protected final peptide-resin was treated with Reagent K [TFA-phenol-thioanisole-H_2O-1, 2 ethanedithiol (85:5:5:5:2.5, v/v)]. The crude peptides were purified using a preparative reverse-phase (RP) liquid chromatography on C_{18} column (Delta Pak, 15 μ, 300 Å, 19×300 mm) with eluting gradient composed of acetonitrile and 0.05% trifluoroacetic acid. Peak integration by analytical RP-HPLC indicated >95% purity of the purified peptides. The molecular weights of peptides were determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (Hill *et al.*, 1991).

Antibacterial assays

Antibacterial lytic activity of synthetic peptides against bacterial strains was measured by an inhibition-zone assay on agarose plates (Hultmark *et al.*, 1983). Three μl of serially diluted peptides were placed in 3 mm wells of 0.7% agarose plates seeded with the respective test bacteria ($1\sim5\times10^6$/6 ml). Plates were incubated overnight at 37℃. Lethal concentration (LC; the lowest concentration that inhibits growth of bacteria) was calculated from the plot of square diameter of inhibition zone *vs.* peptide concentration.

Hemolytic assays

Hemolytic activity of synthetic peptides against human red blood cells (RBCs) were also measured by an inhibition-zone assay on agarose plates. Agarose plates containing 6 ml of medium with 1% agarose, 0.9% NaCl and 10 % RBC suspended in Alsevers solution were prepared. Serial dilutions of peptide were loaded in 3 mm wells, and the plates were incubated at 37℃ for 24 h. Hemolytic concentrations (HCs: the lowest concentration which cause hemolysis of human RBCs) were determined as described above.

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          1         10        20        30
CA: KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2

ME: GIGAVLKVLTTGLPALISWIKRKRQQ-NH2

BO: GIGGIALLSAAKVGLKGLAKGLAEHFN

MA: GIGKFLHSAKKFGKAFVGEIMNS

          20        30
LF: FKCRRWQWRMKKLGAPSITCVRRAF
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Fig. 1. Amino acid sequences of antimicrobial peptides.

Results and Discussion

CA has an amphipathically basic-flexible-hydrophobic structure and ME has a flexible-hydrophobic-basic structure (Fig. 1). The highly basic segment, C-terminal 6 residues (KRKRQQ) of ME are known to be essential for hemolytic activity (Kini and Evans, 1989; Rivett *et al.*, 1996).

A number of CA-ME hybrid peptides have been developed with the purpose of improving their antibacterial activity while decreasing the undesirable hemolytic property of ME. The CA-ME hybrid peptides, which are composed of the N-terminal amphipathically basic region of CA and N-terminal flexible-hydrophobic region of ME were reported to have powerful antibacterial activity without hemolytic activity (Boman *et al.*, 1989; Andreu *et al.*, 1992; Wade *et al.*, 1992).

Although MA and BO isolated from frog and moth, respectively are less active against Gram-negative bacteria than CA and ME, these peptides show relatively strong antibacterial activity against Gram-positive bacteria, comparable with ME. Like ME, their N-termini are composed of a hydrophobic segment with the flexible sequence GIG, and C-termini are basic (Fig. 1). LF, antimicrobial peptide which is obtained by pepsin digestion of lactoferrin is amphipathically basic (Bellamy *et al.*, 1992) (Fig. 1).

In this study, in order to obtain hybrid peptides with potent antibacterial activity comparable to or better than CA-ME hybrid peptides, several hybrid peptides were designed from the combination of the sequences of two peptides from CA, MA, BO, ME and LF(20-29). CA(1-8)ME(1-12), one of the CA-ME hybrid peptides

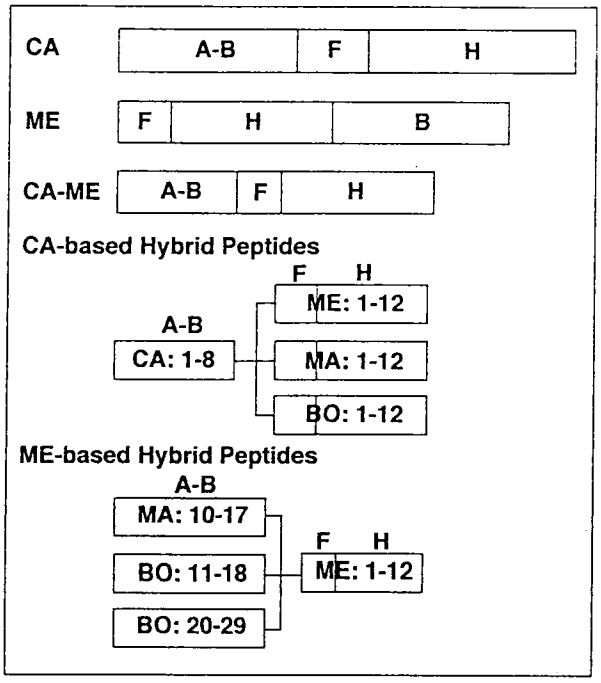

Fig. 2. Strategy for design of synthetic hybrid peptides. A-B: amphipathic basic region, F: flexible region, B: basic region, H: hydrophobic region.

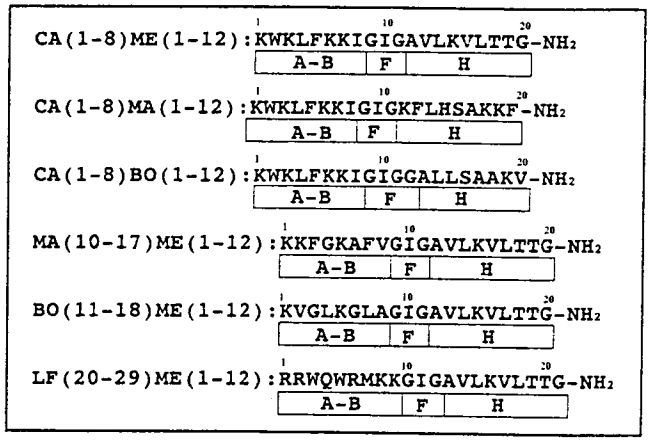

Fig. 3. Amino acid sequences of synthetic hybrid peptides. A-B: amphipathic basic region, F: flexible region, B: basic region, H: hydrophobic region.

was used as a model peptide for designing other hybrid peptides (Fig. 2).

CA(1-8)ME(1-12), CA(1-8)MA(1-12), and CA(1-8)BO (1-12) based on the N-terminal sequence of CA and MA(10-17)ME(1-12), BO(11-18)ME(1-12) and LF(20-29)ME(1-12) based on the N-terminal sequence of ME were synthesized by the solid phase method. The amino acid sequences of all synthetic peptides are shown in Fig. 3. Hybrid peptides were designed such that they could be easily synthesized because they are shorter (20~22 residues) in size than the parental peptides.

Table 1. The molecular weights of synthetic peptides determined by the MALDI mass spectra

Peptides	Calculated value	Observed value
ME	2847.4	2850.6
MA	2467.6	2469.6
BO	2493.8	2494.2
CA(1-8)ME(1-12)	2200.5	2200.8
CA(1-8)MA(1-12)	2404.7	2406.2
CA(1-8)BO(1-12)	2128.4	2129.1
MA(10-17)ME(1-12)	2034.2	2034.0
BO(11-18)ME(1-12)	1895.1	1895.8
LF(20-29)ME(1-12)	2598.0	2598.0

Table 2. Lethal and hemolytic concentration for parental and hybrid peptides

Peptides	Lethal cencentration (μM)			Hemolysis concentration (μM)
	E. coli	*P. aeruginosa*	*S. aureus*	
CA	1.5	2.3	156.1	>1000
ME	4.5	9.9	33.5	57.7
MA	51.0	59.9	46.5	>1000
BO	135.8	115.6	16.0	>1000
CA(1-8)ME(1-12)	4.4	5.3	12.8	>1000
CA(1-8)MA(1-12)	12.0	2.6	33.8	>1000
CA(1-8)BO(1-12)	7.5	3.7	60.5	>1000
MA(10-17)ME(1-12)	6.9	3.3	43.8	>1000
BO(11-18)ME(1-12)	63.4	13.3	110.2	>1000
LF(20-29)ME(1-12)	4.9	8.4	27.3	>1000

All synthetic peptides were confirmed to be homogeneous from an elution peak by analytical RP-HPLC (data not shown) and their correct molecular weights which were determined by Matrix Assisted Laser Desorption Ionization mass spectrometry (Table 1).

Lethal and hemolytic concentrations for antibacterial and hemolytic activities of parental and hybrid peptides are summarized in Table 2. ME showed hemolytic activity at 57.7 μM as expected, but all hybrid peptides had no hemolytic activities up to 1000 μM (Table 2).

CA(1-8)MA(1-12), CA(1-8)BO(1-12), MA(10-17)ME (1-12) and LF(20-29)ME(1-12) as well as CA(1-8)ME (1-12) showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria without cytotoxic effects. However, BO(10-17)ME(1-12) displayed lower antibacterial potency than other hybrid peptides. The overall activities of the hybrid peptides against the bacteria used are in the order of CA(1-8)ME(1-12) ≥ LF(20-29)ME(1-12) ≥ CA(1-8)MA(1-12)> MA(10-17) ME(1-12) > CA(1-8)BO(1-12) > BO(10-17)ME(1-12).

In conclusion, although hybrid peptides, CA-MA, CA-BO, MA-ME, LF-ME as well as CA-ME are shorter in size than their parental peptides, they have a broad

spectrum and potent antibacterial activities against both Gram-positive and Gram-negative bacteria without lytic properties against eukaryotic cells. The results also suggested that the amphipathically basic-flexible-hydrophobic structure of hybrid peptides seems to be an important structural feature which plays a critical role in antibacterial activities.

In particular, CA(1-8)MA(1-12), CA(1-8)BO(1-12), MA(10-17)ME(1-12) and LF(20-29)ME(1-12) as well as CA(1-8)ME(1-12) will be useful model peptides to further design synthetic peptides with more powerful antibacterial activity and to study the structure-activity relationships of antimicrobial peptides.

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