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Structure-Antifungal Activity Relationships of Cecropin A Hybrid Peptides against *Trichoderma* sp.

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The hybrid peptides, CA-ME, CA-MA and CA-BO, with the N-terminal sequence 1-8 of cecropin A and the N-terminal sequences 1-12 of melittin, magainin 2 and bombinin, respectively, have more improved antibacterial activities. CA-MA was found to have stronger antifungal activity against *Trichoderma* sp than other hybrid peptides and their parental peptides. In order to elucidate the relationships between the peptide structure and antifungal activity, several analogues of CA-MA or CA-BO were also designed and synthesized by the solid phase method. Antifungal activity was measured against *T. reesei* and *T. viride*, and hemolytic activity was measured by a solution method against human red blood cells. The residue 16 of CA-MA, Ser, was found to be important for antifungal activity. When the residue was substituted with Leu, antifungal activity was dramatically decreased. CA-MA, P1, P4 and P5 designed in this study showed powerful antifungal activity against *T. reesei* and *T. viride* with low hemolytic activity against human red blood cells. These hybrid peptides will be potentially useful model to further design peptides with powerful antifungal activity for the effective therapy of fungal infection and understand the mechanisms of antifungal actions of hybrid peptides.

Key words: Antifungal activity; Cecropin A hybrid peptide; Hemolytic activity; *Trichoderma* sp.

A number of antibacterial peptides have been discovered from mammalian, amphibian, and insect. Among them, cecropin A (CA), melittin (ME), bombinin (BO) and magainin-2 (MA) are found to possess potent antimicrobial activity with broader spectrum (1-4). These peptides are known to play important roles in insect immunity and host defense (5, 6). The unique amphipathic structures of these peptides are considered to be essential for the interaction with the outer phospholipid bilayer of bacteria (7-9). Recent studies suggest that the mechanism of antibacterial activity of these peptides involves in the formation of ion channels with consequent disruption of bacterial phospholipid bilayer leading to eventual cell death (8, 9).

CA, BO, and MA are not hemolytic, but ME, a bee venom, is highly hemolytic due probably to the highly basic region at C-terminus of ME (2, 10). A number of synthetic peptides have been designed to improve their antibacterial activity while to decrease the undesirable hemolytic activity (11-13).

Previous reports (10-12) suggested that CA-ME hybrid peptides consisting of N-terminal regions from CA and ME have more potent antibacterial activities than parental peptides. Among CA-ME hybrid peptides, CA-ME composed of CA(1-8) and ME (1-12) has highly powerful antibacterial activity without hemolytic activity in assays using the agarose hole method (12). Since the N-terminal sequences 1-12 of MA and BO are similar to ME(1-12), the hybrid peptides, CA-MA and CA-BO, were also designed, respectively. In our previous study, CA-MA and CA-BO were found to have more improved antibacterial activity than MA and BO, respectively (14).

In the present study, in order to elucidate the relationships between the peptide structure, antifungal and hemolytic activity, several analogues (P1-P5) from CA-ME, CA-MA and CA-BO were also designed. All peptides were synthesized by the solid phase method (15). Antifungal activity was investigated by a growth inhibition assay against *T. reesei* and *T. viride*. Hemolytic activity for the human red blood cells (h-RBCs) were also examined by the solution method (7).

Materials and Methods

Strains and human red blood cells (h-RBCs)

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Trichoderma reesei (KCTC 1285) and *Trichoderma viride* (KCTC 6047) were obtained from Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology, Korea. The fungi were grown at 30°C in the PG medium (2% glucose and 20% Potato extract). h-RBCs were purchased from the Blood Center of Korean Red Cross.

Reagents

Chemical reagents for solid phase peptide synthesis were purchased from Sigma Chemical Co. (USA). Fmoc (9-fluorophenylmethoxycarbonyl) -amino acids, Fmoc-Ser(tBu)-Wang-resin, Fmoc-Asn(Trt)-Wang-resin and Rink Amide MBHA(methyl benzhydrylamine)-resin were obtained from Nova Biochem (USA). Potato dextrose broth for the preparation of PG medium was from Difco Laboratories (USA).

Peptide synthesis

All peptides were synthesized manually by solid phase techniques. The solid support used was Rink amide MBHA-resin for the synthesis of the C-terminal amidated peptides except MA and BO. Fmoc-Ser(tBu)-Wang-resin and Fmoc-Asn(Trt)-Wang-resin were used as the starting materials for the synthesis of MA and BO, respectively (16). The chain elongations were manually performed using DCC (dicyclohexylcarbodiimide) and HOBt (N-hydroxybenzotriazole). The protected final peptide-resin were 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) HPLC on a C_{18} column (Delta Pak, 19×300 mm, Waters, Japan).

Antifungal activity assays

T. reesei and *T. viride* were inoculated at the concentration of 1×10^5 spores per tube containing 2.0 ml PG medium and 20 μg of synthetic peptides were added, respectively. After the tubes were incubated at 30°C for 48 h under constant shaking (140 rpm), the dry weight(DW)s of fungi were measured. Zero % inhibition (negative control) were determined in the absence of peptide. The % inhibition was calculated from the following formula. % inhibition=[DW in the absence of peptide -DW in the presence of peptide/DW in the absence of peptide]× 100

Hemolytic activity assays

h-RBCs were washed three times with phosphate buffered saline (PBS: 35 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) prior to assay. Each peptide was dissolved in 0.5 ml of PBS at the concentrations of 400 μg/ml and 100 μg/ml, and then 0.5 ml of a 4% solution of the h-RBCs suspended in PBS was added (The final peptide concentrations, 200 μg/ml and 50 μg/ml, respectively). The mixture was incubated for 1 h at 37°C, and then centrifuged for 5 min at 1,000× g. The absorbance of the supernatant was measured at 414 nm. Zero % hemolysis and 100% hemolysis were determined in PBS and 1% Triton-X 100, respectively. % hemolysis (% HL) in the peptide solution was calculated from the following formula. %HL=[(A_{414} in the peptide solution-A_{414} in PBS)/(A_{414} in 1% Triton-X 100-A_{414} in PBS)] × 100.

Results and Discussions

The purities of all the purified peptides were judged to be above 95% by an analytical RP-HPLC and the molecular weights and amino acid compositions of the peptides determined by MALDI spectra and amino acid analyses were consisted with the expected molecular weights and amino acid compositions, respectively (date not shown). The amino acid sequences of parental peptides, hybrid peptides and their analogues designed in this study are shown in Table 1. The hybrid peptides and their analogues were designed to have amphipathic-flexible-hydrophobic structure. Since an amphipathic α-helical structure is critical for antibiotic activity, the amphipathic sequence 1-8 of CA was placed at N-termini of the hybrid peptides. The flexible-hydrophobic sequences of CA-ME, CA-MA and CA-BO were derived from the sequence 1-12 of ME, MA and BO, respectively.

T. reesei and *T. viride* were used for the measurement of the antifungal activities of the peptide in this study. These fungi cause an occasional infection in the immunocompromised patients. The antifungal activities of the peptides against *T. reesei* and *T. viride* are shown in Table 2. Although both fungi are belong to the same genus, the antifungal effects of the peptides is much different. In the parental peptides, only ME which is known to be highly hemolytic showed higher antifungal activity against both fungi. The other parental peptides were not specific to *T. viride*, but showed various antifungal activity against *T. reesei*. Among them, MA showed the strongest antifungal activity against *T. reesei* (Table 2). As shown in Table 3, hemolytic activities of the parental peptides were consisted well with previous reports (16).

Among the hybrid peptides, only CA-MA inhibited completely the growth of both *T. reesei* and *T. viride* at a fixed concentration, 10 μg/ml. However, the other hybrid peptides, CA-ME and CA-BO, showed 0% and 3.2% growth inhibitions against *T. reesei*, and

Table 1. Amino acid sequences of antibacterial peptides and their hybrid peptides

Peptides	Sequences	Remarks
CA	KWKLFKKIEKVGQNIRDGIIKAGPAVAVV-GQATQIAK-NH_2	Native cecropin A
ME	GIGAVLKVLTTGLPALISWIKRKROO-NH_2	Native melittin
MA	GIGKFLHSAKKFGKAFVGEIMNS	Native magainin 2
BO	GIGGALLSAAKVGLKGLAKGLAEHFN	Native bombinin
CA-ME	KWKLFKKI GIGAVLKVLTTG-NH_2	CA:1-8 ME:1-12
CA-MA	KWKLFKKI GIGKFLHSAKKF-NH_2	CA:1-8 MA:1-12
CA-BO	KWKLFKKI GIGGALLSAAKV-NH_2	CA:1-8 BO:1-12
P1	KWKLFKKI GIGAFLHSAKKF-NH_2	CA-MA:[K^{12}]→[A^{12}]
P2	KWKLFKKI GIGKFLHLAKKF-NH_2	CA-MA:[S^{16}]→[L^{16}]
P3	KWKLFKKI GIGAFLHLAKKF-NH_2	CA-MA:[K^{12}. S^{16}]→[A^{12}, L^{16}]
P4	KWKLFKKI GIGKFLHSATTF-NH_2	CA-MA:[K^{18}, K^{19}]→[T^{18}, T^{19}]
P5	KWKLFKKI GIGALLSAAKVG-NH_2	CA:1-8 BO:1-3, 5-13

The underlined amino acid residues indicate the substituted ones from CA-MA or CA-ME.
The superscript numbers indicate the positions in CA-MA or CA-ME.
All peptides except MA and BO are the C-terminal amidated peptides.

Table 2. Antifungal activities of antibacterial peptides and their hybrid peptide against *Trichoderma viride* and *Trichoderma reesi*

Peptides	*Trichoderma viride*		*Trichoderma reesi*	
	Dry weight (mg)	Inhibition[a] %	Dry weight (mg)	Inhibition[a] %
CA	6.5	0	4.5	26.2
ME	0	100	1.1	82.0
MA	6.4	0	0	100
BO	6.6	0	3.4	43.3
CA-ME	6.5	0	4.3	30.0
CA-MA	0	100	0	100
CA-BO	6.1	3.2	1.7	72.1
P1	0	100	0.8	86.9
P2	4.9	22.2	1.1	82.0
P3	3.7	41.3	4.4	27.9
P4	0	100	2.4	60.7
P5	0	100	0.8	86.9
Control[b]	6.3	0	6.1	0

[a] Percent inhibition was determined by comparison of the dry weights of the fungus in the peptide treatment with no peptide treatment.
[b] Control indicates no peptide treatment.

Table 3. Hemolytic activities of antibacterial peptides and their hybrid peptide against human red blood cells

Peptides	% Hemolysis	
	50 μg/ml	200 μg/ml
CA	0	0.2
ME	100	100
MA	0	0.1
BO	0.7	1.5
CA-ME	5.4	34.5
CA-MA	0.2	1.1
CA-BO	0.2	0.5
P1	0.7	1.5
P2	0.7	3.5
P3	11.6	53.8
P4	0.25	0.95
P5	0.2	0.7

The hemolysis percentage of the peptides was measured at 50 μg/ml and 200 μg/ml, respectively.

30% and 72.1% growth inhibitions against *T. viride*, respectively. Since the N-terminal sequence 1-11 of the hybrid peptides are identical, their different effects could be considered to be derived from their C-terminal sequences. Among them, the residues at positions 12, 16, 18 and 19 are remarkably different. Hemolytic activities of the hybrid peptides, CA-ME, CA-MA and CA-BO, were 5.4, 0.2 and 0.2% at 50 μg/ml of the peptide, and 34.5, 1.1 and 0.5% at 200 μg/ml of the peptide. CA-ME was known not to be hemolytic when measured by the agarose hole method (12, 14), and our data also showed the same result in that method (14). We could not observed any clear zones up to 1000 μM of the peptide except ME, but found much different activity in the solution method. The results suggest that the solution method is more sensitive than the agarose hole method.

To investigate the effect of these residues of CA-ME and CA-MA on their antifungal and hemolytic activity, four analogues (P1-P4) substituted at positions 12, 16, 18 and 19 of CA-MA were designed (Table 1). The substitutions were focused to mimic CA-ME. The Ala substitution (P1) of Lys at position 12 of CA-MA did not show any significant effect on the antifungal and hemolytic activity. P2 in which Ser at position 16 was substituted with Leu displayed much less antifungal activity against *T. viride* than CA-MA, but the substitution was less effective against *T. reesei*. However, the double substitution (P3) with Ala and Leu of Lys and Ser at position 12 and 16 of CA-MA, respectively, induced a significant reduction in antifungal activity again-

st both *T. viride* and *T. reesei*, but a drastic increase in hemolytic activity. The substitution (P4) with Thr-Thr of position 18 and 19 of CA-MA caused slight reduction in antifungal activity against *T. reesei*, but did not alter antifungal activity against *T. viride* and hemolytic activity. These results suggest that Ser at position 16 of CA-MA may play an important role for antifungal activity against T. viride, and the effect of the residues 12, 16, 18 and 19 against *T. reesei* may be cumulative. Also, the residue 12 of the hybrid peptide is less related to antifungal activity, but considered to be correlated to hemolytic activity when the residue 16 is hydrophobic (Leu or Val).

Analogue 5, in which the fourth residue, Gly, of the BO segment of CA-BO is deleted and Gly at its C-terminus is added, had much more powerful antifungal activity than CA-BO against *T. viride* (Table 2). When remove the fourth Gly residue from Gly-Ile-Gly-Gly, the central flexible segment of CA-BO may considerably decrease and this effect may produce improvement in antifungal activity. The similar phenomenon was observed in antibacterial activity against both Gram-negative and Gram-positive bacteria (data not shown). Thus, the results suggest that the length of the central flexible region of the hybrid peptide molecule seem to be important for the antifungal and antibacterial activities.

The overall activities of the hybrid peptides against the fungi used were appeared in the order of CA-MA > CA-BO > CA-ME. Their antibacterial activities on both Gram-positive and Gram-negative bacterial strains of these peptides were observed in the order of CA-ME ≥ CA-MA > CA-BO (14). The differences between antibacterial and antifungal activity of the hybrid peptides could be considered to be related to the differences of the cell surface compositions in bacteria and fungi.

In conclusion, the hybrid peptides, CA-MA, P1, P4 and P5 showed more potent antifungal activity against *T. reesei* and *T. viride*, but were less hemolytic against h-RBCs. Therefore these hybrid peptides can be potentially used as model peptides to develope the powerful antifungal drug for treating the fungal infection, and be also used for understanding the structure-antifungal activity relationships.

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References

1. **Steiner, H.D., Hultmark, A., Engstrom, H., Bennich, H. and H.G. Boman.** 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**, 246-248.
2. **Habermann, E.** 1972, Bee and wasp vemon. *Science* **177**, 314-322.
3. **Simmaco, M., Barra, D., Chiarini, F., Noviello, L., Melchiorri, P., Kreil, G. and K. Richter.** 1991. A family of bombinin-related peptides from the skin of bombina variegata. *Eur. J. Biochem.* **199**, 217-222.
4. **Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA.*, **84**, 5449-5453.
5. **Boman, H.G.** 1991. Antibacterial peptides: Key components needed in immunity. *Cell* **65**, 205-207.
6. **Zasloff, M.** 1992. Antibiotic peptides as mediators of innate immunity. *Curr. Opin. Immunol.*, **4**, 3-7.
7. **Blondle, S.E. and R.A. Houghten.** 1992. Design of model amphipathic peptides having potent antibacterial activities. *Biochemistry* **31**, 12688-12694.
8. **Cruciani, R.A., Barker, J.L., Durell, S.R., Raghunathan, G., Guy, H.R., Zasloff, M. and E.F. Stanley.** 1992. Magainin 2, a natural antibiotic from skin, forms ion channels in lipid bilayer membranes. *Eur. J. Pharmacol.* **226**, 287-296.
9. **Matsuzaki, K., Murase, O., Fujii, N. and K. Miyajima.** 1995. Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a fore. *Biochemistry* **34**, 6521-6526.
10. **Blondelle, S.E. and R.A. Houghten.** 1991. Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin. *Biochemistry* **30**, 4671-4678.
11. **Boman, H.G., Wade, D., Boman, I.A., Wahlin, B. and R.B. Merrifield.** 1989. Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. *FEBS Lett.* **259**, 103-106.
12. **Andreu, D., Ubah, J., Boman, A., Wahlin, D., Wade, D., Merrifield, R.B. and H.G. Boman.** 1992. Shorted cecropin A-melittin hybrid: significant size reduction retains potent antibiotic activity. *FEBS Lett.* **296**, 190-194.
13. **Wade, D., Andreu, D., Mitchell, S.A., Silveira, A.M. V., Boman, A., Boman, H.G. and R.B. Merrifield.** 1992. Antibacterial peptides designed as analogs or hybrids of cecropins and melittin. *Int. J. Peptide Protein Res.*, **40**, 429-436.
14. **Shin, S.Y., Kang, J.H., Lee, M.K., and K.-S. Hahm.** 1996. Antibacterial activities of peptides designed as hybrids of antibacterial peptides. *J. Biochem. Mol. Biol.* **29**, 545-548.
15. **Merrifield, R.B.** 1963. Solid phase peptide synthesis I. The synthesis of tetrapeptide. *J. Am. Chem. Soc.* **85**, 2149-2154.
16. **Merrifield, R.B.** 1983. Solid phase synthesis. *Science* **232**, 341-347.
17. **Lee, M.K., Lee, D.G. Shin, S.Y., Lee, S.G., Kang, J.H. and K.-S. Hahm**. Antifungal activities of peptides with the sequence 10-17 of magainin 2 at the N-termini against Aspergillus fumigatus. *J. Microbiol.* **34**, 274-278.
18. **Tosteson, M.T., Holmes, S.J., Razin, M. and D.C. Tosteson.** 1985. Melittin lysis of red cells. *J. Membr. Biol.* **87**, 35-44.