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Modification of the C Terminus of Cecropin Is Essential for Broad-Spectrum Antimicrobial Activity

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Cecropin A is a naturally occurring peptide with bactericidal activity against gram-negative and gram-positive bacteria. Production of large quantities of bactericidal peptides that are similar in structure and activity to cecropin A has been achieved by combining recombinant DNA techniques and chemical modification. Expression of the bactericidal peptide in *Escherichia coli* was accomplished through the formation of a fusion protein. The 5′ end of the L-ribulokinase gene was fused to a single copy of a synthetic gene encoding cecropin A. A methionine codon was engineered between the two genes, and a methionylglycine extension was introduced at the C terminus of cecropin A. Cyanogen bromide treatment of the fusion protein yielded cecropin A with a C-terminal homoserine. The recombinant cecropin A with a homoserine at the C terminus did not kill most gram-positive bacteria tested. However, recombinant cecropin A with a chemically modified C terminus has antimicrobial activity similar to that of cecropin produced by cecropia pupae.

Cecropins are a family of basic peptides which are produced in *Hyalophora cecropia* in response to bacterial infection (13). These peptides are induced as part of the humoral immune system and possess antimicrobial activity against a wide variety of both gram-positive [G(+)] and gram-negative [G(−)] bacteria. Three of these peptides, cecropins A, B, and D, have been identified and sequenced (12, 13, 23). Cecropin A consists of a single polypeptide chain of 37 amino acids with no sulfur-containing amino acid residues (4), and the N-terminal portion shares a high degree of sequence homology with other cecropin peptides. The C-terminal pentapeptides of different cecropins can be classified into two groups, with no sequence homology between the groups (4). However, the carboxyl termini of all naturally occurring cecropins isolated from insect larvae are blocked, and the blocking group R is a primary amide (2, 6, 9). Several experiments indicate that the amphipathic alpha-helix of the N terminus of cecropin A is required for activity against most, but not all, bacteria (2, 3, 10). A synthetic peptide of cecropin A, lacking the last four amino acids from the C terminus as well as the C-terminal amide group, exhibited a reduced activity against G(+) bacteria but retained activity against most G(−) bacteria (21).

Because cecropins consist of a single polypeptide chain of common L-amino acids and do not contain any disulfide bonds, this family of peptides is well suited for economical production through the application of recombinant DNA technology or by peptide synthesis. In this paper, we describe the production and characterization of cecropin A-like peptides by recombinant DNA technology and demonstrate that these cecropin A-like peptides with an unmodified C terminus lack activity against many G(+) bacteria. However, the introduction of ethylenediamine at the C terminus results in restoration of broad-spectrum activity.

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MATERIALS AND METHODS

Abbreviations and designations. The abbreviations and designations used in this study are as follows: AraB-CA, all the different cecropin A constructs fused with L-ribulokinase protein; CA-NH_2, authentic native cecropin A; CA-COOH, recombinant cecropin A without amidation at the C-terminal amino acid; CA-Hse-COOH, recombinant cecropin A with a homoserine extension; CA-Hse-lactone, recombinant cecropin A with a homoserine extension in the lactone form; CA-Hse-NH_2, recombinant cecropin A with an amidated homoserine at the C terminus; CA-Hse-NH-Et-NH_2, recombinant cecropin A with an ethylenediamine-modified homoserine at the C terminus; CA-EHPG-COOH, recombinant cecropin A with a Glu-His-Pro-Gly tetrapeptide extension at the C terminus; and CA-P-COOH, recombinant cecropin A with a proline extension at the C terminus.

Chemicals. Cyanogen bromide (CNBr) was purchased from Pierce Chemical Co., Rockford, Ill.; trifluoroacetic acid (TFA) was purchased from Applied Biosystems; ethylenediamine was purchased from J. T. Baker; and dimethyl sulfoxide was purchased from Mallinckrodt, Inc.

Plasmid construction. The cecropin gene was chemically synthesized by standard dinucleotide methods (14). The gene was placed in a vector containing the *araBAD* promoter from *Salmonella typhimurium* (15). The cecropin A gene with a methionine at the N terminus was located, in reading frame, at the 3′ end of a truncated *araB* gene coding for the first 490 amino acids of L-ribulokinase. This plasmid is referred to as pCA3D (Fig. 1) and can be used to express a fusion protein that upon cleavage with CNBr will produce a cecropin-like peptide. The primary sequence of this peptide is identical to that of cecropin A, but it lacks the C-terminal amide present in the naturally occurring peptide (CA-COOH). For the production of a cecropin-like peptide with a reactive C terminus (for C-terminal modification), a primer-directed mutagenesis technique (22) was used to add nucleotides coding for a methionylglycine dipeptide extension at the C terminus of the cecropin-like peptide. The resulting plasmid was referred to as pCAMG. Cleavage of the fusion protein encoded by pCAMG with CNBr produces a ce-

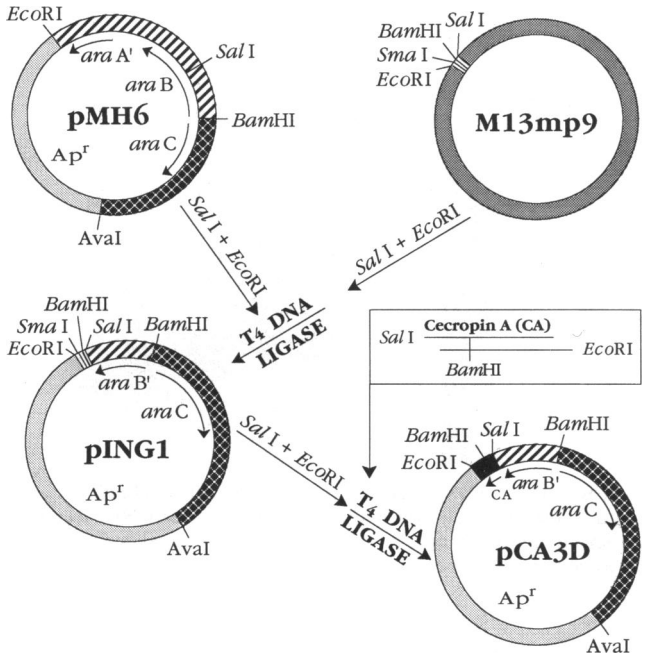

FIG. 1. Plasmid construction. A cecropin A gene with an ATG codon plus a *Sal*I site sequence at the 5′ end and a stop codon plus an *Eco*RI site sequence at the 3′ end was synthesized. The purified DNA fragment was ligated with *Sal*I-*Bam*HI-digested plasmid pING1.

cropin-like peptide (cecropin A) with a homoserine extension (CA-Hse-COOH), which has the same primary sequence as cecropin A but contains a homoserine residue at the C terminus.

Purification of the cecropin-like peptide. *Escherichia coli* MC1061 (7) harboring plasmid pCAMG was grown in TYE medium (15 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) by using a 10-liter Chemap fermentor vessel with an optical density at 600 nm of 10. The culture was then induced with 5 g of L-arabinose per liter, grown for an additional 7 to 10 h, and harvested by centrifugation. The cells were broken in a Gaulin homogenizer, and the suspension was centrifuged at 14,000 × *g* for 30 min to obtain the inclusion bodies present in the pellet. With the use of a blender, the pellet was resuspended in 50 mM phosphate–1 mM EDTA, pH 7.5, and centrifuged as before. The pellet was washed three or four times by this procedure and then dissolved in 70% formic acid (2 liters/50 g [dry weight] of inclusion bodies). Ten 1-ml aliquots of 1 g of CNBr per ml in acetonitrile were added over a 6-h period, and the reaction was allowed to continue for a minimum of 16 h at room temperature in a chemical hood, with constant stirring. After the 16-h reaction period, solvent was removed from the reaction mixture by rotary evaporation at 37°C. The residual material was washed several times with water, again with rotary evaporation to remove excess CNBr and most of the formic acid. The pellet thus obtained was extracted in a blender four times with 500 ml of water. After each extraction, the solution was centrifuged as before, and the supernatants from each step were combined.

The pH of the combined supernatant was adjusted to 4.0 by adding 10 N NaOH with vigorous stirring, and then the solution was centrifuged to remove material which had precipitated during the pH change. The pH of the supernatant was adjusted to 8.0, and the additional precipitate which formed was again removed by centrifugation. (When the pH was raised to 8.0 directly, the solution became cloudy and could not be clarified by centrifugation.) The pH 8.0 supernatant solution was concentrated and desalted by using an Amicon concentrator with a YM-2 membrane. The sample was purified by applying the material to a cation-exchange column equilibrated with 10 mM phosphate buffer at pH 8.0. A linear salt gradient (0 to 0.5 M NaCl) in the same phosphate buffer was used to elute the peptides. Fractions were analyzed for antimicrobial activity against *E. coli* D21 cells, and they were also analyzed with acetate-urea gels (15% acrylamide, pH 4.5) and then concentrated and desalted with an Amicon concentrator. This material (CA-Hse-COOH) was lyophilized and stored at −20°C. CA-COOH was prepared by the same procedure, except that pCA3D plasmid-containing *E. coli* was used instead of *E. coli* harboring pCAMG.

Modification of the C terminus of the cecropin-like peptide. The cecropin-homoserine-ethylenediamine derivative (CA-Hse-NH-Et-NH_2) was made by first converting CA-Hse-COOH to the homoserine lactone form (CA-Hse-lactone) as an intermediate. The lyophilized powder of CA-Hse-COOH-containing material was treated with concentrated TFA (20 to 50 mg/ml for 1 h at room temperature) to convert the C-terminal homoserine into the lactone form (11, 16). Excess TFA was removed under a vacuum, and the residual material was solubilized in dimethyl sulfoxide (dried over molecular sieves) to a protein concentration of 1 mg/ml. Triethylamine was added to make the solution basic (as determined by pH paper), and then ethylenediamine was added to 10% (vol/vol). The mixture was sealed and incubated at room temperature with constant agitation for 3 h (11). The reaction was stopped by evaporation of the triethylamine and ethylenediamine from the mixture under a vacuum, and the protein was allowed to precipitate overnight at −20°C by adding ethyl ether to 50% (vol/vol). The precipitate was harvested via centrifugation for 40 min at 23,000 × *g* and then dissolved in 10 mM phosphate buffer (pH 8.0) and applied to a cation-exchange column as described above.

The formation of the cecropin-homoserineamide (CA-Hse-NH_2) derivative was achieved by a similar procedure, except that 30% NH_4OH was used to bring the final ammonia concentration to 10% (vol/vol), and the reaction mixture was sealed and incubated with constant stirring for 24 h. Excess ammonia was removed by a nitrogen stream, and the sample was precipitated and purified as described above. The purification and chemical modification scheme for producing cecropin-like peptides is summarized in Fig. 2.

Characterization of the cecropin-like peptides. Gel electrophoresis and reverse-phase high-performance liquid chromatography (HPLC) were used to characterize the purity of each peptide. Gel electrophoresis was performed either with an acetate-urea-polyacrylamide gel electrophoresis (acetate-urea-PAGE) system at pH 4.5 (24) with 15% acrylamide–0.4% bisacrylamide or with a sodium dodecyl sulfate (SDS)-PAGE system at pH 8.8 (17) utilizing a 17 to 33% acrylamide linear gradient with 0.65% bisacrylamide. Bands were visualized by staining with 0.1% Coomassie blue. The antimicrobial activity could be identified by overlaying the unstained acetate-urea gel on the TYE soft agar medium as described previously (8).

Reverse-phase HPLC was also used to analyze the purity of the peptides. After applying the purified samples, the C_{18} column was brought to 34% acetonitrile in 8 min. Cecropin-like peptides were eluted from a 34 to 40% acetonitrile

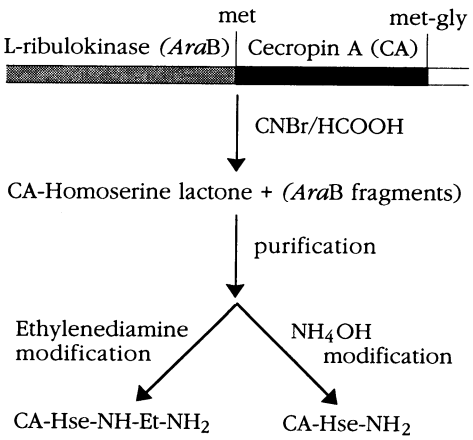

FIG. 2. Scheme for the isolation of the cecropin-like peptides from the fusion protein and the formation of C-terminal adducts.

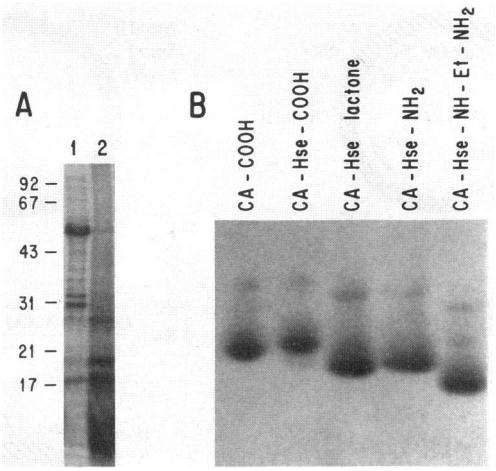

FIG. 3. (A) SDS-PAGE analysis of the inclusion bodies. Lanes 1 and 2, washed inclusion bodies before and after CNBr treatment, respectively. (B) Acetate-urea-PAGE (pH 4.3) analysis of the purified cecropin-like peptides.

gradient, which developed over 12 min, and then the column was brought to 100% acetonitrile in 2 min.

The C termini were characterized by carboxypeptidase Y digestion, which sequentially removes the C-terminal residue. Each derivative was incubated with enzyme for 30 min, and the released amino acids were identified by thin-layer chromatography.

N-terminal amino acid sequence and amino acid composition analysis. The amino acid sequences of CA-like peptides were determined by automated Edman degradation. Samples for amino acid analysis were hydrolyzed for 20 to 24 h in 6 N HCl at 110°C in vacuo. Amino acid composition analyses were done in triplicate with norleucine as the internal standard to normalize for losses due to sample handling and transfer.

Assay of bactericidal activity. The assay for antimicrobial activity was performed by mixing 10 ml of 0.8% agar (in TYE medium buffered with 50 mM phosphate; pH 7.4) with 10^6 to 10^7 viable cells of the test organisms and pouring the medium into an 85-mm-diameter petri dish. After the agar had hardened, 3-mm-diameter wells were punched into the agar. Various amounts of each of the modified cecropin-like peptides (in 10-μl volumes) were then added to individual wells so that each of the modified forms was present on a single plate. The diameters of the inhibition zones, or halos, around the wells were measured after overnight incubation at 25°C. Since each plate contained a uniform inoculum of bacteria, the area of the halo (the area of the well was subtracted from each area determination) was directly related to the number of bacteria lysed. The activities of the peptides could then be compared. The amount of each antimicrobial peptide applied was determined by amino acid analysis. MICs were determined as described elsewhere (1).

RESULTS

Purification of cecropin-like peptides. Induction of *E. coli* cells containing either the pCAMG or the pCA3D plasmid with L-arabinose resulted in the expression of high levels of insoluble L-ribulokinase-cecropin fusion protein. The cells were harvested and lysed, and the insoluble material was collected by centrifugation. The L-ribulokinase-cecropin fusion protein, with a molecular mass of approximately 55 kDa, was the major component of the insoluble fraction (Fig. 3A).

The cecropin-like peptide was excised from the L-ribulokinase-cecropin fusion protein during CNBr treatment and was then separated from the L-ribulokinase peptide fragments by cation-exchange chromatography as described in Materials and Methods. Cecropin-like peptides that differed at the C terminus could also be separated from one another by the same procedure. The majority of the CA-Hse-COOH or CA-COOH eluted at 0.1 M NaCl, while cecropin A with the homoserine extension modified to the ethylenediamine adduct (CA-Hse-NH-Et-NH_2) eluted at 0.15 to 0.2 M NaCl at pH 8.0.

Modification and characterization of cecropin-like peptides. The cleavage of the C-terminal methionylglycine linkage by CNBr resulted in the formation of CA-Hse-lactone. During extraction and chromatography, the CA-Hse-lactone slowly hydrolyzed to the acid form, CA-Hse-COOH. In order to perform the C-terminal modification, the lyophilized powder of CA-Hse-COOH was treated with anhydrous TFA for conversion back to the CA-Hse-lactone as an intermediate for preparing the ethylenediamine (CA-Hse-NH-Et-NH_2) and the amide (CA-Hse-NH_2) derivatives. Forty-six percent of the CA-Hse-COOH was converted to the more cationic CA-Hse-lactone within 1 h. However, incubation for up to 18 h failed to convert the remaining free acid to a lactone derivative. Reverse-phase HPLC analysis was used to characterize CA-Hse-COOH. Prior to modification, two peaks were identified and both of them had antimicrobial activity. N-terminal amino acid sequence data indicated that one peak contained the CA-Hse-COOH sequence, while the other peak had the cecropin sequence plus Met-Gly at the C terminus. This result indicated that the hydrolysis of the Met-Gly cleavage may be inefficient and accounts for the fact that the TFA reaction could convert only 46% of the free acid to the lactone form.

As described in Materials and Methods, CA-Hse-NH-Et-NH_2 was formed by reacting the lactone intermediate with ethylenediamine under basic conditions in dimethyl sulfoxide. The time course of the reaction was monitored by running samples taken at various time points on an acetate-urea gel. After a 1-h incubation, 52% of the lactone was converted to CA-Hse-NH-Et-NH_2 and by 3 h, the reaction was >85% complete. Incubation of CA-Hse-COOH with

TABLE 1. Production of cecropin-like peptides by recombinant DNA techniques

Cecropin-like peptide	Yield (mg) from 10 liters	Bactericidal activity against *E. coli* D21[a]
AraB-CA	4,000	−
CA-COOH	160	+
CA-Hse	120	+
CA-EHPG-COOH	150	+
CA-Hse-NH-Et-NH_2	40	++
CA-Hse-NH_2	30	++
CA-P-COOH	200	+

[a] +, bacterial strain is sensitive to the peptide; ++, greater sensitivity; −, the peptide failed to kill the bacterium.

ethylenediamine under the same reaction conditions did not result in the formation of an ethylenediamine adduct, as judged by ion-exchange chromatography, acetate-urea-PAGE, and reverse-phase HPLC (data not shown). Lactone formation was a requirement for production of the ethylenediamine adduct, demonstrating that adduct formation was specific for the C terminus.

The cecropin-like peptides with C-terminal modifications were purified by cation-exchange chromatography. The structure of each modified peptide was confirmed by amino acid composition analysis and carboxypeptidase Y digestion of the C termini. Following acid hydrolysis, all of the cecropin-like peptides had the predicted amino acid compositions. Each contained a single residue of homoserine (excluding the construct CA-COOH, which does not contain the methionylglycine extension), which confirmed that the modifications are acid labile. The C termini were also analyzed by carboxypeptidase Y digestion. Digestion of the purified CA-COOH yielded lysine (the C-terminal amino acid of cecropin A), while digestion of both CA-Hse-COOH and CA-Hse-lactone released homoserine. Lysineamide was released from the naturally occurring cecropin A (CA-NH_2). Carboxypeptidase Y cleavage of the crude amidation reaction mixture of CA-Hse-NH_2 resulted in the release of both homoserine and homoserineamide, indicating that the amide had been formed, but some CA-Hse-lactone remained. The ethylenediamine adduct (CA-Hse-NH-Et-NH_2) was resistant to carboxypeptidase Y treatment, even after a fourfold increase in enzyme levels and incubation time. The different kinds and yields of cecropin-like peptides generated are listed in Table 1.

The theory for protein separation by the acetate-urea gel system which we used to characterize these peptides is that a greater net positive charge-to-mass ratio results in greater mobility of the peptide. The cecropin-like peptides with a free carboxyl at the C terminus migrated more slowly than the other peptides (20). The CA-Hse-lactone migrated more rapidly than the free acid because of the absence of the negatively charged C terminus. The CA-Hse-NH-Et-NH_2 adduct migrated the most rapidly because of the introduction of the additional amine group (Fig. 3B). However, cecropin A with the homoserine extension in the lactone form (CA-Hse-lactone) and cecropin A with the homoserine extension modified to the amide adduct (CA-Hse-NH_2) comigrated on this gel system and also coeluted from the cation-exchange column. Therefore, formation of the amide was analyzed following a saponification reaction (0.1 N NaOH for 1 h at 55°C). This reaction converts the lactone to the free acid (i.e., it adds a negative charge to the unreacted starting material) but does not affect the amide, which is resistant to mild base hydrolysis. In this way, CA-Hse-NH_2 can be separated from the unreacted starting material (converted to CA-Hse-COOH) on a cation-exchange column, on which CA-Hse-COOH elutes first, followed by CA-Hse-NH_2.

TABLE 2. MICs of CA-Hse-NH-Et-NH_2 for different bacteria

Bacterium	MIC (μg/ml)
Escherichia coli	2
Shigella sonnei	0.06
Salmonella enteritidis	0.5
Pseudomonas aeruginosa	1–8[a]
Staphylococcus aureus 1	1–32[a]
Streptococcus pyogenes	0.25
Clostridium perfringens	32
Propionibacterium acnes	8

[a] Depends on strain tested.

Bactericidal activity of cecropin-like peptides. The MICs of CA-Hse-NH-Et-NH_2 for various microorganisms are presented in Table 2. The cecropin-like peptides described in this paper which possess a free C-terminal carboxyl group (CA-COOH and CA-Hse-COOH) have activity against G(−) bacteria similar to that of natural cecropin A. However, both show lower activity against *Bacillus megaterium* and undetectable activity against other G(+) bacteria tested (Table 3). Peptides with C-terminal amide groups (CA-Hse-NH_2 and CA-Hse-NH-Et-NH_2) possessed bactericidal activity against both G(+) and G(−) bacteria.

DISCUSSION

Production of cecropin-like peptides in *E. coli* was achieved, even though this organism is very sensitive to cecropin. This was accomplished through the formation of a fusion protein with a truncated portion of the L-ribulokinase gene product. The cecropin peptide was excised from the isolated fusion proteins by CNBr cleavage of a methionine, which was engineered into the junction between the truncated L-ribulokinase and the cecropin-like peptide. In order to facilitate modification of the C-terminal carboxyl group, a methionylglycine extension of the cecropin-like peptide was generated by site-directed mutagenesis at the 3′ end of the coding region. Treatment of the protein with CNBr resulted in release of the cecropin-like peptide with a C-terminal homoserine residue. The conversion of the homoserine free acid into the lactone provided a route for the preparation of cecropin-like peptides with modified C termini.

The C-terminal sequences of cecropins are highly variable (4), but all of the insect cecropins sequenced thus far contain an amidated C terminus (2, 6, 9). A cecropin-like peptide isolated from pig intestine was found to lack a C-terminal amide, but the spectrum of antibacterial activity was enhanced when a C-terminal amide was included (18). We extended the C terminus of CA-COOH with a proline (CA-P-COOH) or a tetrapeptide (Glu-His-Pro-Gly [CA-EHPG-COOH]), in each case without C-terminal modifying groups. The peptides exhibited a narrow spectrum of bactericidal activity similar to that of the unmodified peptide (CA-COOH). However, CA-Hse-NH_2 and CA-Hse-NH-Et-NH_2 possess a broad spectrum of bactericidal activity comparable to that of native cecropin A. This illustrates that simply extending the C terminus does not enhance bactericidal activity (4, 5, 20) and that the chemical nature of the C terminus is an important determinant in the bactericidal activity of cecropin A. The resistance of CA-Hse-NH-Et-

TABLE 3. Bactericidal activities of cecropin A and various derivatives

Bacterium type, species, and strain	Sensitivity to[a]:				
	CA-COOH, CA-EHPG-COOH, and CA-P-COOH	CA-Hse-COOH	CA-Hse-NH_2	CA-NH_2	CA-Hse-NH-Et-NH_2
G(−)					
Escherichia coli D21	+	+	++	++	++
Escherichia coli MC1061	+	+	++	++	+++
Enterobacter cloacae b11	+	+	++	++	+++
Salmonella typhimurium LT2	+	+	+++	+++	+++
Pseudomonas aeruginosa OT97	+	+	+	+	+
G(+)					
Bacillus megaterium Bm11	±	±	+	+	+
Bacillus subtilis 1A338	−	−	+	+	++
Micrococcus luteus Ml11	−	−	+	+	++++

[a] +, bacterial strain is sensitive to the peptide; ++, +++, and ++++, increasingly greater sensitivities; −, the peptide failed to kill the bacterium; ±, the peptide has very low activity against the bacterium.

NH_2 to carboxypeptidase Y digestion may suggest a correlation between the increased resistance of peptides to exopeptidase activity at the C terminus and increased bactericidal activity against certain G(+) organisms tested (Table 3).

The mechanism by which cecropins act is predicted to be interaction with the lipid portion of the cell membrane to form ion-permeable pores (4, 9, 29, 30, 31, 32). The formation of an amphipathic alpha-helix (2, 3, 10) by the N-terminal amino acids 1 through 11 is thought to be critical to the activity of cecropins. The requirement of a modified C terminus for activity against most G(+) [but not G(−)] bacteria indicates that different mechanisms may be involved in the bactericidal action of cecropins against G(+) and G(−) organisms.

Families of cationic peptides with bactericidal activity have been isolated from different sources (19, 25, 26, 28). One such family of peptides, isolated and characterized from leukocytes, are called defensins (19, 27). They are of sizes (32 to 34 amino acids) and charges (the more active defensins have 8 or 9 arginine residues) comparable to those of the cecropin family of peptides. Defensins also have amphiphilic structures which may serve the peptides' function of inserting into the lipid membranes of different organisms. The striking difference between these two families of antimicrobial peptides is that the defensins contain three internal disulfide bridges but do not contain C-terminal amides, while the cecropins contain no cysteine residues and are amidated at the C terminus.

In this paper, we demonstrated a method for the microbial production and subsequent chemical modification of a cecropin-like peptide (CA-Hse-NH-Et-NH_2) with bactericidal activity against a broad range of bacteria similar to that of natural cecropin A (CA-NH_2). For *Micrococcus luteus* and *Bacillus subtilis*, CA-Hse-NH-Et-NH_2 has greater bactericidal activity than CA-NH_2, while for *S. typhimurium*, similar activities were observed. The C-terminal modification through lactone formation allows the placement of a variety of chemical entities, enabling a detailed analysis of the effect of C-terminal modification on bactericidal activity against various bacterial strains to be performed.

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