Novel synthetic antimicrobial peptides effective against methicillin-resistant *Staphylococcus aureus*

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Previously, we identified a core undecapeptide of sapecin B having antimicrobial activity. Based on the structure of this peptide, we systematically synthesized peptides consisting of terminal basic motifs and internal oligo-leucine sequences and examined their antimicrobial activities. Of these peptides, RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ were found to have potent microbicidal activity against *Staphylococcus*

aureus, *Escherichia coli*, methicillin-resistant *S. aureus* and *Candida albicans* in liquid medium. We also synthesized the D-enantiomer of KLKLLLLLKLK-NH₂. This enantiomer was resistant to tryptic digestion and persisted longer in the culture medium, showing greater antimicrobial activity than the original peptide.

INTRODUCTION

Many insects synthesize various antibacterial proteins in response to bacterial challenge or body injury [1–6]. These are inducible defence proteins and are thought to protect the insects from bacterial infection. Recently, we purified an antifungal protein from the larval haemolymph of *Sarcophaga peregrina* (flesh fly) [7]. Thus insect haemolymph is a good source of naturally occurring antimicrobial proteins. As most of these antimicrobial proteins have microbicidal activity, they are potentially interesting for the development of useful antimicrobial agents. However, even relatively small antibacterial proteins consist of 35–40 amino acids, so they are still too large to use as chemotherapeutic agents [6,8].

Previously, we demonstrated that an undecapeptide derived from sapecin B, an antibacterial protein of *Sarcophaga* that is effective against Gram-positive bacteria, showed almost the same antibacterial activity as the original sapecin B [9]. This finding indicated the possibility of fragmenting small insect antimicrobial proteins to identify their active cores. We modified the amino acid sequence of this undecapeptide of sapecin B to obtain peptides having high antibacterial activity. We found that some of them had more activity than the original peptide, and were effective not only against Gram-positive bacteria but also against Gram-negative bacteria and fungi [9].

In the present study, we further optimized the sequences of these peptides and finally obtained the following two peptides: RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂. These peptides have an RLK or KLK motif at both ends (basic region) with five internal leucines (hydrophobic region). As these peptides contain lysine and arginine residues that provide cleavage sites for various proteinases, they would probably be unstable when used as chemotherapeutic agents. Therefore, we synthesized the D-enantiomer of the latter peptide and examined its antibacterial activity and stability.

MATERIALS AND METHODS

Antimicrobial peptides

Peptides were synthesized by the solid-phase method in a peptide synthesizer (Shimadzu PSSM-8). Each peptide was purified to

homogeneity by h.p.l.c. on a reverse-phase column of a Syncropac RP-R (C₁₈) and its amino acid sequence was confirmed in a protein sequencer (Shimadzu PPSQ-10).

Assays of antimicrobial activity

The antibacterial activities of the peptides were measured using *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* K-12 594 and methicillin-resistant *S. aureus* SR1550 [10] as indicator bacteria. To determine the minimum inhibitory concentration (MIC), we used the micro-dilution broth method, essentially following the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, cells of each bacterial strain were collected in the logarithmic phase of growth, suspended in 30 mM phosphate buffer (pH 7.0) containing 60 mM NaCl, and the absorbance at 650 nm (*A*₆₅₀) of the suspension was adjusted to 0.3 arbitrary unit (2.5×10^8 cells/ml). Peptide samples were dissolved in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl and 0.2% (w/v) BSA and diluted serially. The sample solution (100 µl) was mixed with Mueller–Hinton medium (84 µl) and diluted bacterial suspension (16 µl). The mixture containing 1×10^5 bacteria was incubated for 20 h at 37 °C and the *A*₆₅₀ was determined. To examine the bactericidal activity, the mixture was diluted appropriately after incubation for 1 h at 37 °C and 100 µl of diluted mixture was plated on nutrient agar. The plates were incubated for 24 h at 37 °C and then numbers of colonies on test and control plates were compared.

Antifungal activity was assayed using *Candida albicans* ATCC 36232 as a target fungus. *Candida* cells in the logarithmic phase of growth were suspended in Sabouraud medium at a density of 2×10^6 cells/ml (*A*₆₅₀ is 0.33 arbitrary unit). A mixture of the sample solution (50 µl) and the fungal suspension (50 µl) was incubated for 2 h at 37 °C. Then the mixture was diluted appropriately, and samples of 100 µl of diluted mixture were spread on Nissui *Candida* GE agar plates. The plates were incubated for 48 h at 37 °C and the numbers of colonies on test and control plates were compared.

Preparation of liposomes with trapped glucose and assay of their release of glucose

Liposomes were prepared by the method of Kinsky et al. [11].

Abbreviations used: MIC, minimum inhibitory concentration; ED₅₀ and ED₉₀, effective dose for 50% and 90% microbicidal effect.

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Briefly, a mixture of phospholipids was dissolved in chloroform, and an aliquot containing 1 μ mol of phospholipid was dried completely in a rotary evaporator under reduced pressure. Then 0.1 ml of 0.3 M glucose solution was added, and the lipids were dispersed with the aid of a Vortex mixer to obtain multilamellar liposomes containing glucose. These liposomes were washed twice with 0.4 ml of 5 mM veronal buffer, pH 7.5, containing 0.15 M NaCl (veronal-buffered saline) to remove untrapped glucose, and finally suspended in 0.5 ml of the same buffer.

In tests examining the sensitivity of liposomes to peptides, various concentrations of the peptides dissolved in 10 μ l of 10 mM phosphate buffer, pH 6.0, containing 130 mM NaCl were added to 10 μ l of the liposome suspension. The mixtures were incubated at 37 °C for 30 min and then the amount of glucose released from the liposomes was determined [12]. Glucose released from the liposomes was measured with a glucose C-test kit (Wako Chemical Co., Tokyo, Japan), consisting of phenol, 4-aminoantipyrine, mutarotase, glucose oxidase and peroxidase [13]. A red-coloured quinone derivative was formed with phenol and 4-aminoantipyrine in the presence of glucose, the amount of glucose in the reaction mixture was determined from the absorbance of this compound at 492 nm.

Assay of haemolytic activity of antimicrobial peptides

Bovine erythrocytes (1×10^6) were incubated with increasing amounts of each peptide for 30 min at 37 °C in 100 μ l of 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl and 1% (w/v) BSA. After centrifugation, the A_{405} values of the resulting supernatants were measured to detect released haemoglobin. Melittin (Sigma) was used as a positive control.

RESULTS

Antimicrobial activities of synthetic peptides

Previously, we reported that an undecapeptide, RSLCLHCRK-NH₂ (the 7R-17K fragment), which corresponds to position 7 to 17 of sapecin B, and its homologues, including RLKCLLLCRK-NH₂, showed significant antimicrobial activity [9]. We modified these peptides further and synthesized various peptides having basic motifs at both termini with between three and five internal leucines. Then we examined their antimicrobial activities. As summarized in Table 1, RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ showed almost the same MIC values against the test bacteria, but RLRLLLLLRLR-NH₂ was clearly less active. All these peptides were effective only in liquid medium, not being effective in agar medium even at a concentration of 100 μ g/ml.

For the activities of these peptides, terminal RLK or KLK motifs were found to be important, and could not be replaced by other basic motifs like HLH, KK or K. An octapeptide, KLKLLLLL-NH₂, lacking the C-terminal KLK motif also showed significant antibacterial activity against *S. aureus* and *E. coli*, but not against methicillin-resistant *S. aureus*.

As RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ showed lower MIC values than other peptides, we synthesized a deca- and a nano-peptide, RLKLLLLLRLK-NH₂ and RLKLLLRLK-NH₂, by decreasing the number of internal leucine residues of RLKLLLLLRLK-NH₂ to see how this hydrophobic region contributes to the antimicrobial activity. We determined the ED₅₀ and ED₉₀ values (effective doses giving 50% and 90% microbicidal activity) against *S. aureus*, *E. coli*, methicillin-resistant *S. aureus* and *C. albicans*, and compared

them with those of RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂. As summarized in Table 2, KLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ again showed almost the same ED₅₀ and ED₉₀ values against all of the micro-organisms tested. However, RLKLLLLLRLK-NH₂ and RLKLLLRLK-NH₂ were found to have no activity against methicillin-resistant *S. aureus* at concentrations below 10 μ g/ml and the activity of RLKLLLRLK-NH₂ against *E. coli* was clearly less than those of the other peptides. Thus the number of internal leucine residues is critical for the bactericidal activity. We found that all of these peptides had fungicidal activity, and that their ED₅₀ and ED₉₀ values against *C. albicans* were almost the same, suggesting that the number of internal leucines is not so important for the fungicidal activity as for the bactericidal activity.

Interaction of antibacterial peptides with phospholipids

Previously, we showed that the 7R-17K fragment of sapecin B having antibacterial activity interacted with phospholipid vesicles with the same phospholipid compositions as those of the membranes of *S. aureus* and *E. coli* [9]. Therefore, we examined whether KLKLLLLLKLK-NH₂ and RLKLLLLLRLK-NH₂ interacted with phospholipid vesicles consisting of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in a molar ratio of 7:2:1, which resembles the phospholipid composition of *E. coli* membranes. As shown in Figure 1(a), glucose entrapped in the phospholipid vesicles was released concentration-dependently by the peptides, indicating that these peptides interact with phospholipid vesicles. Similar results were obtained with phospholipid vesicles consisting of phosphatidylglycerol

Table 1 MIC values of synthetic peptides against various bacteria

Peptide	MIC (μ g/ml)		
	<i>S. aureus</i>	<i>E. coli</i>	Methicillin-resistant <i>S. aureus</i>
RLKLLLLLRLK-NH ₂	1	20	30
RLKLLLLRLK-NH ₂	2	20	> 35
RLKLLLRLK-NH ₂	2	30	> 35
KLKLLLLLKLK-NH ₂	2	20	30
RLRLLLLLRLR-NH ₂	2	> 35	> 35
HLHLLLLLHLH-NH ₂	> 16	> 35	> 35
KKLLLLLKK-NH ₂	> 16	> 35	> 35
KLKLLLL-NH ₂	> 16	25	> 35
KLKLLLLL-NH ₂	8	20	> 35

Table 2 Effective doses of synthetic peptides for 50% and 90% microbicidal effects

Peptide	ED ₅₀ /ED ₉₀ (μ g/ml)			
	<i>E. coli</i>	<i>S. aureus</i>	Methicillin-resistant <i>S. aureus</i>	<i>C. albicans</i>
RLKLLLLLRLK-NH ₂	1/5	0.2/0.5	0.5/3	4/10
RLKLLLLRLK-NH ₂	1/5	0.3/0.5	> 10	8/10
RLKLLLRLK-NH ₂	4/8	0.3/0.5	> 10	5/10
KLKLLLLLKLK-NH ₂	1/5	0.3/0.5	1/3	5/10

Figure 1 (a) Release of glucose from liposomes in the presence of RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ and (b) their haemolytic activities

(a) Phospholipid vesicles with entrapped glucose were prepared from phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in a molar ratio of 7:2:1. Increasing amounts of RLKLLLLLRLK-NH₂ (●) or KLKLLLLLKLK-NH₂ (▲) were incubated with a fixed amount of liposomes for 1 h at 37 °C. Then the amount of glucose released from the liposomes was measured. (b) Bovine erythrocytes were incubated with increasing amounts of each peptide for 30 min at 37 °C. After centrifugation, the A₄₀₅ values of the resulting supernatants were measured. Total lysis was achieved by incubating the cells in distilled water. Key to symbols: ▲, RLKLLLLLRLK-NH₂; ○, KLKLLLLLKLK-NH₂; ▼, melittin (positive control).

and cardiolipin in a molar ratio of 3:1, which resembles the phospholipid composition of *S. aureus* membranes (results not shown).

As these peptides were found to interact with phospholipid vesicles, we examined whether they had haemolytic activity. As shown in Figure 1(b), no appreciable haemolysis of bovine erythrocytes was detected under conditions in which melittin caused 100% haemolysis. Thus these peptides probably do not

Figure 2 Antimicrobial effects of KLKLLLLLKLK-NH₂ and its D-enantiomer on methicillin-resistant *S. aureus*

Increasing amounts of peptide were added to the culture medium of methicillin-resistant *S. aureus* for 24 h at 37 °C, and growth of bacteria relative to the control (without peptide) was determined. Key to symbols: ●, KLKLLLLLKLK-NH₂; ▲, D-enantiomer of KLKLLLLLKLK-NH₂.

interact with mammalian cells appreciably, although they have potent antimicrobial activity.

D-Enantiomer of KLKLLLLLKLK-NH₂

Although the ED₅₀ values of RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂ for bactericidal activity against methicillin-resistant *S. aureus* were 3 μg/ml, their MIC values for the same organisms were 10 times higher. As we measured the MIC after incubation for 24 h, the peptides were probably degraded by proteinases produced by the bacteria during incubation, and the small population of surviving bacteria have multiplied again, increasing the turbidity. To examine this possibility, we synthesized the D-enantiomer of KLKLLLLLKLK-NH₂ (consisting entirely of D-amino acids) and examined its antibacterial activity, assuming that it would be resistant to proteolytic degradation.

The D-enantiomer was resistant to tryptic digestion under conditions in which no intact KLKLLLLLKLK-NH₂ remained in the reaction mixture (results not shown). Then we compared the antibacterial activity of this D-enantiomer with that of KLKLLLLLKLK-NH₂ using methicillin-resistant *S. aureus* as an indicator bacterium. The D-enantiomer showed higher activity than the original peptide: the MIC of KLKLLLLLKLK-NH₂ was 30 μg/ml, whereas that of the D-enantiomer was 15 μg/ml (Figure 2). No significant bacterial growth was detected above this concentration of D-enantiomer. We compared the bacterial growth in the presence of concentrations of 15 μg/ml of these peptides with that of the control. As is evident from Figure 3, more than 80% of the bacterial growth was inhibited after treatment with KLKLLLLLKLK-NH₂ for 12 h, but the growth was restored nearly 100% after 24 h. These results suggest that this peptide was degraded during the incubation period and that the surviving bacteria multiplied. On the other hand, no significant bacterial growth was detected in medium containing 15 μg/ml of the D-enantiomer even after 24 h. Probably, the D-

Figure 3 Time dependency of bacterial growth in the presence of antibacterial peptides

Methicillin-resistant *S. aureus* was cultured in medium containing KLKLLLLLKLK-NH₂ or its D-enantiomer, at a final concentration of 15 µg/ml. At the indicated times, bacterial growth relative to the control (without peptide) was determined. ○, KLKLLLLLKLK-NH₂; ▲, D-enantiomer of KLKLLLLLKLK-NH₂.

enantiomer persisted longer in the medium and killed the bacteria completely. Thus the D-enantiomer is clearly a better antibacterial peptide for methicillin-resistant *S. aureus* than the original peptide, in terms of its stability in the medium.

DISCUSSION

Insect antimicrobial proteins are naturally occurring chemotherapeutic agents, and many insects protect themselves from bacterial infections by transiently producing these antibacterial proteins [14].

We modified the sequence of the 7R-17K fragment systematically and obtained two peptides, RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂, with almost the same antimicrobial activities, which were higher than those of any other synthetic peptides so far tested [9]. We did not synthesize peptides with all the possible combinations of R and K in XLXLLLLLXLX-NH₂ (X = R or K), but we expect that they should all have significant antimicrobial activity. We found that for activity, a terminal KLK or RLK motif was important, and could not be replaced by HLH. We also found that KKLLLLLKK-NH₂ and KLLLLLKLK-NH₂ were not active. Therefore, a terminal KLK or RLK motif does not simply supply a positive charge, but is important structurally. ¹H-n.m.r. analysis showed that RLKLLLLLRLK-NH₂ has an α-helix structure (results not shown). It is known that all antibacterial proteins of insects so far analysed contain α-helix structures [15–18]. An α-helix structure is probably important for antibacterial activity [19], and a KLK or RLK motif contributes to the formation of the α-helix structure.

We found that both RLKLLLLLRLK-NH₂ and KLKLLLLLKLK-NH₂, at concentrations necessary for bac-

tericidal activity, interact with phospholipid vesicles having the same phospholipid compositions as bacterial membranes, but that they did not show haemolytic activity. These results suggest that the primary target of these peptides is the bacterial membrane, and the phospholipid composition of the membrane is crucial for their interaction with the membrane. More than 20 amino acid residues are needed for a peptide to span the lipid bilayer of the membrane [20]. So, as these peptides consist of only 11 amino acids, several molecules may assemble to form a channel in the bacterial membrane [21]. For this, these peptides must move freely in the medium. In fact, we found that these peptides are active only in liquid medium, showing no appreciable antibacterial activity in agar medium. Therefore, these peptides must be used in liquid medium for toxicity toward microorganisms.

We found that the D-enantiomer KLKLLLLLKLK-NH₂ has higher activity than the original peptide. D-Enantiomers of other antibacterial proteins (e.g. cecropin A, magainin 2 and melittin) have been synthesized and found to have the same activity as the original proteins [22]. Therefore, we think that the antibacterial activities of KLKLLLLLKLK-NH₂ and its D-enantiomer are essentially the same, but that as the D-enantiomer is resistant to proteolytic digestion by bacterial proteinases, it persists for longer in the culture medium than the original peptide and so represses bacterial growth continuously. As the D-enantiomer is effective for repressing the growth of methicillin-resistant *S. aureus*, it could be used as a lead for developing new antimicrobial agents against these organisms.

This work was supported by a Grant-in-Aid for Specifically Promoted Research from the Ministry of Education, Science and Culture of Japan, and by a Grant-in-Aid BMP 94-V-1-5-2 (Bio Media Programme) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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