Synthesis and characterization of bactericidal oligopeptides designed on the basis of an insect anti-bacterial peptide

Hisako SAIDO-SAKANAKA*, Jun ISHIBASHI*, Aki SAGISAKA*, Eiichi MOMOTANI† and Minoru YAMAKAWA*†

* Laboratory of Biological Defense, National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305–8634, Japan, and † Laboratory of Molecular Pathology, National Institute of Animal Health, Tsukuba, Ibaraki 305–0858, Japan

INTRODUCTION

Anti-bacterial peptides are one of the important factors in the innate immunity of vertebrates and invertebrates. They have been isolated from mammals [1], amphibians [2], insects [3] and chelicerates [4]. The search for novel anti-bacterial agents has become especially urgent because of the rapid spread of antibiotic-resistant pathogenic bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA) and multi-antibiotic-resistant Pseudomonas aeruginosa, which cause serious problems in hospitals, especially in Japan. Most anti-bacterial peptides have broad-spectrum activities and disrupt bacterial membranes via a peptide–lipid interaction, unlike the mechanism of action of antibiotics used currently [5]. Some anti-bacterial peptides have been shown to be effective against antibiotic-resistant bacteria [6–8], suggesting their potential use as therapeutic agents. The cytotoxicity and antigenicity of these peptides, however, make them unsuitable for direct therapeutic use. Thus considerable attention has been focused on modifying their size and anti-bacterial activity. d-Enantiomers of cecropin A, magainin and melittin are as active as their parent peptides [9]. Hybrid peptides of cecropin A and melittin displayed higher anti-bacterial activity and a broader spectrum than the parent cecropin A, and show no haemolytic activity [10]. Furthermore, shortened cecropin A–melittin hybrid peptides (e.g. a 15-mer peptide) were found to have strong anti-bacterial activity [11]. An analogue of indolicidin, in which five tryptophan residues were replaced with phenylalanines, showed strong anti-bacterial activity that was free of haemolytic activity [12]. Many other truncated anti-bacterial peptides have also been synthesized [13–15].

An understanding of the relationship between the structure and activity of anti-bacterial peptides is important in the development of new agents. In general, it is thought that the biological activity of amphipathic α-helical peptides results from their ability to form ion channels through membrane bilayers [16]. A study of the relationship between the secondary structure and biological activity of model short peptides (8–22-mer peptides) comprising leucine and lysine residues indicated that an amphipathic α-helical structure is an important condition for anti-bacterial activity [17]. The α-helical region of sapecin B, identified as the active site of this peptide, showed potent antimicrobial activity [18], and its modified analogue, an 11-mer peptide composed of leucine and lysine residues, showed greater activity against micro-organisms, including MRSA [19]. Synthetic histatin analogues with enhanced amphipathicity of the helical conformation were shown to have broad, improved antimicrobial activity [20].

We have previously isolated and characterized defensin from a beetle, Allomyrina dichotoma. This peptide is composed of 43 amino acid residues, and has been shown to be effective against MRSA [7]. In the present study, we determined the active centre of defensin by measuring the anti-bacterial activity of 64 overlapping 12-mer peptides derived from it, and investigated the mode of action of this fragment. We also synthesized truncated...
peptides and examined their anti-bacterial and cytotoxic activities. We found that some 9-mer analogues have greater anti-bacterial activity than the original peptide, but show no cytotoxic activity.

**MATERIALS AND METHODS**

**Materials**

9-Fluorenylmethoxycarbonyl (Fmoc)-t-amino acids and Fmoc-tris(alkoxy)benzylamide-PEG-PS-resins [where PEG-PS is poly-(ethylene glycol)–polystyrene] were purchased from PerSeptive Biosystems. Phosphatidylglycerol (PG) from egg yolk lecithin, cardiolipin (CL) from bovine heart and phosphatidylethanolamine (PE) from Escherichia coli were obtained from Sigma. All other reagents were special-grade. Water was drawn from a Milli-Q system.

**Peptide synthesis**

The 64 overlapping 12-mer peptides, each offset by one residue, spanning the sequence of *A. dichotoma* defensin (VTCDLLSF-EAKGFANHSLCAAHLCAIGRRGGSCERGVCICRR) with either a free carboxylate or a free amide group at their C-termini were synthesized by the multi-pin technique (Chiron Mimotopes, Clayton, Victoria, Australia) [21]. Other peptides were synthesized by the solid-phase method in a 9050 Plus peptide synthesizer (Millipore). Each peptide was purified to homogeneity by the SMART system or an ÄKTA Explorer using a reverse-phase column of µRPC C2/C18 PC 3.2/3 or of Resource RPC 1 ml (Pharmacia, Uppsala, Sweden). The column of µRPC C2/C18 PC 3.2/3 was eluted in 50 min at a flow rate of 0.1 ml/min, using a linear gradient from 0 to 30% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid. The column of Resource RPC 1 ml was eluted in 20 min at flow rate of 2 ml/min, using a linear gradient from 0 to 25% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid.

**Anti-bacterial activity**

Anti-bacterial activity assays were conducted in sterilized 96-well plates in a final volume of 100 µl as follows. Samples of 90 µl of suspensions of a mid-exponential-phase culture of bacteria in nutrient broth medium (Difco, Detroit, MI, U.S.A.) (suspensions of a mid-exponential-phase culture of bacteria in #1999 Biochemical Society using a cell path length of 1 mm. Spectra were recorded at 25°C. Anti-bacterial activity

**Liposome membrane permeability assay**

Liposomes containing trapped glucose were prepared as described by Yamada and Natori [18]. A sample of 2 µmol of phospholipids mixed in chloroform was dried under nitrogen gas, then 0.2 ml of 0.3 M glucose was added and multilamellar liposomes were prepared using a vortex mixer. Liposomes were washed three times with 10 mM sodium phosphate buffer (pH 7.0)/130 mM NaCl to remove untrapped glucose and finally suspended in 0.6 ml of the same buffer. The liposome suspension (15 µl) was added to different concentrations of peptides dissolved in 15 µl of the same buffer. Mixtures were incubated for 1 h at 25°C. Phospholipids in liposomes were calculated by measuring the amount of ester groups [27]. Glucose released from liposomes was assayed using a Glucose CII-Test kit (Wako Chemical, Osaka, Japan). z-Lactalbumin was used as a negative control.

**Cytotoxic activity**

The cytotoxic activity of peptides was examined in a sterile 96-well plate using the murine macrophage cell line J-4 [28] and the fibroblast cell line L929 [29]. Cells were cultured at 37°C in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin in a 5% CO₂ humidified atmosphere. Cultured cells were suspended in RPMI medium (106 cells/ml) and 100 µl of cell suspension was transferred to culture plates. The mixture of PBS (10 µl) containing different concentrations of peptides and Alamar Blue (10 µl) (Iwaki, Chiba, Japan) was added to the cell suspension, which was then incubated at 37°C for 24 h. Cell growth inhibition was determined by measuring fluorescence intensity on a Cytoflour 2300 (Millipore) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

**RESULTS**

**Anti-bacterial activity of synthetic peptides**

To determine the active site of defensin, we synthesized 64 overlapping 12-mer peptides spanning the sequence of defensin with either a free carboxylate or a free amide group at their C-termini, and studied the anti-bacterial activity of these peptides against *S. aureus*. Only one amidated peptide, corresponding to amino acid residues 19–30 of defensin, i.e. LCAAHCLAIGRR-NH₂ (19L–30R-NH₂), showed strong activity (100% growth inhibition). Other peptides had less or no anti-bacterial activity. For example, the amidated fragments comprising amino acid residues 18–29 and 20–31 of defensin did not inhibit growth. The
MIC of the 19L–30R-NH$_2$ fragment is shown in Table 1. The 19L–30R-NH$_2$ fragment showed anti-bacterial activity against Gram-positive bacteria (MIC of 18 $\mu$g/ml for S. aureus) and against Gram-negative bacteria (MIC of 48 $\mu$g/ml for E. coli). A 19L–30R fragment containing a free carboxylate group had no great activity against S. aureus (MIC of >50 $\mu$g/ml). The activity of these peptides was not affected by alkylation (results not shown). Anti-bacterial activity is not affected by either the free thiol groups in these peptides or by a disulphide bond.

**CD spectra**

The first nine amino acids of the 19L–30R-NH$_2$ fragment are identical with the C-terminus of the presumed $\alpha$-helical structure region, by sequence identity with some other insect defensins [7], suggesting that this fragment has the potential to form an $\alpha$-helical structure. CD spectra of the 19L–30R-NH$_2$ fragment were measured using a spectropolarimeter in the absence or presence of PG/CL (3:1, mol/mol) liposomes. This fragment formed an $\alpha$-helical structure in the lipidic environment, whereas it showed a random-coil structure in the absence of liposomes (Figure 1). The $\alpha$-helical content of the fragment was approx. 60%.

**Membrane permeability**

Most anti-bacterial peptides are known to interact with bacterial membranes [5]. We investigated the interaction of the 19L–30R-NH$_2$ fragment and its analogues with phospholipids. To do this, we prepared both S. aureus- and E. coli-membrane-type liposomes, i.e. the phospholipid composition for S. aureus was PE/PG/CL (7:2:1, by mol) (Figure 2). The 19L–30R-NH$_2$ fragment displayed activity against liposomes of both phospholipid compositions (Figure 2). The amount of glucose leakage from liposomes depended on the peptide concentration. Glucose leakage from the S. aureus-membrane-type liposomes was greater than that from the E. coli-membrane-type liposomes. These results are consistent with anti-bacterial activity of the peptide.

**Anti-bacterial activity of analogues of the 19L–30R-NH$_2$ fragment**

The anti-bacterial activities of the 19L–30R-NH$_2$ fragment and of its analogues are summarized in Table 1. The MICs of analogues against S. aureus were 1.8–3-fold higher than that of the 19L–30R-NH$_2$ fragment. The anti-bacterial activity of the analogues against E. coli was greater than or comparable with that of the 19L–30R-NH$_2$ fragment. Some indicated weak activity against MRSA.

The truncated peptides AAHCLAIGRR-NH$_2$, AHCLAIGRR-NH$_2$, and HCLAIGRR-NH$_2$, which show deletions of...
Table 2  Antibiogram of the synthetic 22A–30R-NH$_2$ fragment and its analogues

<table>
<thead>
<tr>
<th>Peptide</th>
<th>S. aureus</th>
<th>MRSA</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHCLAIGRR-NH$_2$ (22A–30R-NH$_2$)</td>
<td>6</td>
<td>&gt; 64</td>
<td>16</td>
<td>ND</td>
</tr>
<tr>
<td>ALLRAIRR-NH$_2$</td>
<td>2</td>
<td>&gt; 48</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>ALLLAIRR-NH$_2$</td>
<td>2</td>
<td>32</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AWLRAIRR-NH$_2$</td>
<td>2</td>
<td>20</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>ALYLAIRR-NH$_2$</td>
<td>1.5</td>
<td>24</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ALWLAIRR-NH$_2$</td>
<td>2</td>
<td>32</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Rabbit erythrocytes were incubated with different concentrations of the following synthetic peptides: ○, ALRAIRR-NH$_2$; ●, ALLLAIRR-NH$_2$; □, AWLRAIRR-NH$_2$; ▲, ALYLAIRR-NH$_2$; △, ALWLAIRR-NH$_2$; ▼, melittin. Details of experimental conditions are given in the Materials and methods section.

Figure 3 Haemolytic activity of 22A–30R-NH$_2$ fragment analogues

Figure 4 Cytotoxic activity of 22A–30R-NH$_2$ fragment analogues on macrophages

The murine macrophage cell line JA-4 was used to observe growth inhibition by the following 22A–30R-NH$_2$ analogues: ○, ALRAIRR-NH$_2$; ●, ALLLAIRR-NH$_2$; □, AWLRAIRR-NH$_2$; ▲, ALYLAIRR-NH$_2$; △, ALWLAIRR-NH$_2$. For other experimental conditions, see the Materials and methods section.

A. dichotoma defensin against MRSA was 20 μg/ml when examined 10 h after incubation of the peptide [7]. These analogues were also active against P. aeruginosa isolated from a patient.

Haemolytic and cytotoxic activities of defensin analogues

All 9-mer analogues were tested for haemolytic activity against rabbit erythrocytes. No haemolytic activity was observed (Figure 3). Similar results were obtained with 12-mer analogues (results not shown). The cytotoxic activity of 9-mer analogues against mouse macrophages and fibroblasts was examined, and results indicated that these analogues had no cytotoxic activity against fibroblasts (< 2000 μg/ml peptide). Although AWLRAIRR-NH$_2$ inhibited the growth of macrophages (20% inhibition at 400 μg/ml and 80% inhibition at 1 mg/ml), other 9-mer analogues did not show such inhibition (Figure 4).

DISCUSSION

In order to apply anti-bacterial peptides to the development of therapeutic agents, we looked for the active region of A. dichotoma defensin by measuring anti-bacterial activity against S. aureus. For this purpose, we synthesized 64 overlapping 12-mer peptides and examined their activity. Intact A. dichotoma defensin is known to be active against Gram-positive bacteria, but not against Gram-negative bacteria [7]. The 19L–30R-NH$_2$ fragment identified as an active region of defensin was effective against both Gram-positive and Gram-negative bacteria. The lipopolysaccharide of Gram-negative bacteria is reported to be a barrier for anti-bacterial peptides [31], so deletion of the other amino acid sequences of the peptide may enable the 19L–30R-NH$_2$ fragment to bind efficiently to the membranes of Gram-negative bacteria.

The results of glucose leakage tests from liposomes clearly indicated that the target of the 19L–30R-NH$_2$ fragment is the bacterial membrane. Many basic anti-bacterial peptides have been reported to interact with liposomes containing acidic phospholipids and to change bacterial membrane permeability [5]. Liposomes with the phospholipid composition of Gram-positive bacteria were more sensitive to the 19L–30R-NH$_2$ fragment than those mimicking Gram-negative bacteria (Figure 2). The content of acidic phospholipids is higher in Gram-positive compared with Gram-negative bacterial membranes.
tryptophan residue in AWLLAIRRR-NH₂ inhibited the growth of macrophages (Figure 4). The second sphingomyelin phospholipids [36]. In addition, cholesterol in the or fibroblasts significantly, suggesting an inability to lyse eukary-

bacteria. 

22A–30R-NH₂ with acidic phospholipids. All analogues derived from the bacterial activity. This region may be necessary for interaction

region of 19L–30R-NH₂. The mechanism of anti-bacterial activity of the 19L–30R-NH₂ fragment remains to be elucidated.

We synthesized various analogues of the 19L–30R-NH₂ fragment by replacing amino acids in order to increase anti-bacterial activity. The results indicated that the enhanced anti-bacterial activity of peptides such as LRAAHRLAIGRR-NH₂, LCAAHCLAIRR-NH₂ and LCAAALCLAIRR-NH₂ is due to their increased helical amphipathicity. Enhanced segmental amphipathicity seen in further modified peptides, such as LLAHAIGRR-NH₂ and LCAAHCIRRR-NH₂ induced strong activity. These two analogues may have detergent-like properties, due to enhanced hydrophobicity in their N-terminal region or enhanced hydrophilicity in their C-terminal region.

The results of the truncation indicated that the C-terminal region of 19L–30R-NH₂, i.e. RR-NH₂, is important for anti-
bacterial activity. This region may be necessary for interaction with acidic phospholipids. All analogues derived from the 22A–30R-NH₂ fragment had cationic C-terminal regions and were rich in hydrophobic amino acids in their N-terminal portions. The 9-mer analogues may also act as a detergent to kill bacteria.

No analogue tested damaged murine erythrocytes (Figure 3) or fibroblasts significantly, suggesting an inability to lyse eukaryotic cells, whose outer leaflet is composed of zwitterionic and sphingomyelin phospholipids [36]. In addition, cholesterol in the membranes of eukaryotic cells may prevent peptides from disrupting the membrane [37]. In one case, an analogue strongly inhibited the growth of macrophages (Figure 4). The second tryptophan residue in AWLLAIRRR-NH₂ may be the cause of its cytotoxicity towards macrophages, since ALLAIRRR-NH₂ and ALWLARRR-NH₂ have no cytotoxic activity.

In summary, in this study the 19L–30R-NH₂ fragment derived from A. dichotoma defensin was shown to exert anti-bacterial activity by permeabilizing the bacterial membrane. Some modifications of the 19L–30R-NH₂ fragment that enhanced helical or segmental amphipathicity resulted in increased anti-bacterial activity without haemolytic activity. These peptides were effective against pathogenic bacteria such as MRSA and P. aeruginosa isolated from patients, and could be used as lead peptides in the design of therapeutic agents. At present, the effect of a magainin analogue has been evaluated in clinical tests for the treatment of patients with diabetic ulcers [38]. Furthermore, analogues of sapecin B showed chemotherapeutic activity against MRSA in mice [39]. Finally, it is worth noting that the mechanisms of action of these peptides are totally different from those of any antibiotics used thus far. This difference enables insect anti-bacterial peptides to kill antibiotic-resistant pathogenic bacteria. We believe that a strategy to modify insect anti-bacterial peptides is an important approach to developing novel antibiotics.

This work was supported by the Enhancement of Center of Excellence, Special Coordination Funds for Promoting Science and Technology, Science and Technology Agency, Japan.

REFERENCES


Received 4 September 1998/30 October 1998; accepted 27 November 1998