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Purification, cDNA cloning and modification of a defensin from the coconut rhinoceros beetle, *Oryctes rhinoceros*

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A novel member of the insect defensins, a family of antibacterial peptides, was purified from larvae of the coconut rhinoceros beetle, *Oryctes rhinoceros*, immunized with *Escherichia coli*. A full-size cDNA was cloned by combining reverse-transcription PCR (RT-PCR), and 5′- and 3′-rapid amplification of cDNA ends (RACE). Analysis of the *O. rhinoceros* defensin gene expression showed it to be expressed in the fat body and hemocyte, midgut and Malpighian tubules. *O. rhinoceros* defensin showed strong antibacterial activity against *Staphylococcus aureus*. A 9-mer peptide amidated at its C-terminus, AHCLAICRK-NH_2 (Ala22–Lys30-NH_2), was synthesized based on the deduced amino-acid sequence, assumed to be an active site sequence by analogy with the sequence of a defensin isolated from larvae of the beetle *Allomyrina dichotoma*. This peptide showed antibacterial activity against *S. aureus*, methicillin-resistant *S. aureus*, *E. coli* and *Pseudomonas aeruginosa*. We further modified this oligopeptide and synthesized five 9-mer peptides, ALRLAIRKR-NH_2, ALLLAIRKR-NH_2, AWLLAIRKR-NH_2, ALYLAIRKR-NH_2 and ALWLAIRKR-NH_2. These oligopeptides showed strong antibacterial activity against Gram-negative and Gram-positive bacteria. The antibacterial effect of Ala22–Lys30-NH_2 analogues was due to its interaction with bacterial membranes, judging from the leakage of liposome-entrapped glucose. These Ala22–Lys30-NH_2 analogues did not show haemolytic activity and did not inhibit the growth of murine fibroblast cells or macrophages, except for AWLLAIRKR-NH_2.

Keywords: *Oryctes rhinoceros*; antibacterial peptide; cDNA cloning; gene expression; synthetic oligopeptides.

Insect antibacterial peptides have unique properties that disrupt bacterial membranes via peptide–lipid interactions [1], and some are known to be effective against antibiotic-resistant pathogenic bacteria such as methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* [2–4], suggesting their potential use as therapeutic agents. To develop novel antibiotics, many trials have been conducted using insect antibacterial peptides. Shortened cecropin A–mellitin hybrid 15-mer peptides, for example, have shown strong antibacterial activity [5]. Truncated antibacterial peptides have also been synthesized, and some of them were effective against bacteria [6–8]. Amphipathic α-helical regions have been identified as active sites of antibacterial peptides [9–11]. Modifications of these peptides can lead to greater and broader antibacterial activity than the original peptide [12,13]. The C-terminal β-sheet domain of an antibacterial peptide was found to be an active site and showed activity against fungi and Gram-positive and Gram-negative bacteria [14].

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Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; RT-PCR, reverse-transcription polymerase chain reaction; Fmoc, 9-fluorenylmethoxycarbonyl; PEG-PS, poly(ethylene glycol)-polystyrene; CL, cardiolipin; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine.

Note: the nucleotide sequence data reported here have been submitted to GSDB/SSBJ/EMBL/NCBI nucleotide sequence databases and are available under accession number AB011245.

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We have designed and synthesized oligopeptides based on the amino-acid sequence of *Allomyrina dichotoma* defensin [15]. These oligopeptides (8- to 12-mer peptides) were effective against both Gram-positive and Gram-negative bacteria including MRSA and *P. aeruginosa* isolated from patients. The antibacterial effect was due to their interaction with bacterial membranes. Oligopeptides showed no haemolytic activity and did not inhibit the growth of murine fibroblast cells.

In this work, we have purified and characterized a defensin from *O. rhinoceros* larvae and cloned its cDNA to synthesize short novel peptides that have strong antibacterial activity and a wide antibacterial spectrum against bacteria, including pathogenic bacteria. For this, we modified a putative active site of *O. rhinoceros* defensin and synthesized five novel 9-mer peptides. These synthetic oligopeptides indicated strong antibacterial activity and were demonstrated to interact with liposomal membranes of *S. aureus* and *E. coli* types. Peptides did not show haemolytic activity. Although one of the oligopeptides inhibited the growth of murine macrophages, other peptides did not suppress their growth or that of fibroblast cells.

MATERIALS AND METHODS

Experimental insects

O. rhinoceros larvae were collected in the field on Okinawa and Ishigaki Islands, Japan. Third instar larvae were used to purify antibacterial peptides and to analyse gene expression.

Immunization and collection of haemolymph

O. rhinoceros larvae were cooled on ice and injected with 50 μL of *E. coli* JM109 (2×10^5 cells per larva) suspended in

physiological saline (150 mM NaCl/5 mM KCl). Larvae were kept at 25 °C for 24 h. The haemolymph was collected by cutting off a leg in an ice-cooled tube containing 1 tablet of protease inhibitor cocktail, COMPLETE (Boehringer). After centrifugation at 4 °C for 50 min at 39 000 ***g*** to remove hemocytes, the clear supernatant was heated in a boiling water bath for 10 min and cooled on ice. The heated solution was centrifuged under the same conditions as above.

Assay for antibacterial activity

Throughout purification, antibacterial activity was analysed by measuring the bacterial growth inhibition zone [16] on thin agarose plates containing bacteria. Briefly, melted agar (20 mL) containing 1×10^6 logarithmic-phase cells of *S. aureus* was poured into sterile Petri dishes (8.4 cm diameter). Wells (2 mm diameter) were cut in the freshly poured plates after agar solidified. Each well held a 5-μL aliquot of the fraction. Plates were incubated overnight at 37 °C and the diameters of clear zones were recorded (after subtracting the well diameter). To determine the effect of purified defensin, different doses of the antibacterial peptide were added to a bacterial culture (50 μL) of *S. aureus*. Logarithmic-phase cells of the bacterium cultured in Müller and Hinton broth (Difco) were first suspended in 30 mM phosphate buffer, pH 7.0 containing 60 mM NaCl, and 10 μL of this suspension (1.5×10^5 cells) was then added to 40 μL of the fresh broth and cultured for 20 h at 37 °C with shaking. The culture was chilled and the bacterial concentration determined by measuring absorbance at 550 nm using a spectrophotometer (Beckman, DU-650). The minimal inhibitory concentration of peptides was observed 20 h after incubation at 37 °C.

Purification of *O. rhinoceros* defensin

A haemolymph sample (20 mL) was acidified with 0.1% trifluoroacetic acid and applied to a Sep-Pak Vac C_{18} cartridge (Waters Associates), previously equilibrated with 0.1% trifluoroacetic acid. The adsorbed materials were eluted stepwise with 10, 20, 30, 40, 50 and 100% acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing antibacterial activity were dried under a vacuum. Dried materials were dissolved in 0.05% heptafluorobuthanoic acid and applied to reverse-phase HPLC with a Resource RPC column (1 mL, Pharmacia), equilibrated with 0.1% heptafluorobuthanoic acid connected to an ÄKTA system (Pharmacia), equilibrated with 0.05% heptafluorobutyric acid. Adsorbed materials were eluted with a linear gradient of acetonitrile/0.05% heptafluorobuthanoic acid (0–20%) for 5 min followed by a second gradient (20–40%) for 40 min at 1 mL·min^{-1}. Fractions having antibacterial activity were pooled and dried under a vacuum. Dried materials were dissolved in 0.05% trifluoroacetic acid and applied to reverse-phase HPLC with a μRPC 3.2/3 column (3.2 × 30 mm, Pharmacia) equilibrated with 0.05% trifluoroacetic acid, connected to a SMART system (Pharmacia). The column was washed with 0.05% trifluoroacetic acid and adsorbed materials were eluted with a linear gradient of acetonitrile/0.05% trifluoroacetic acid (0–20%) for 5 min followed by a second gradient (25–35%) for 40 min at 0.2 mL·min^{-1}. Fractions showing antibacterial activity were again purified by reverse-phase HPLC with a μRPC 2.1/10 column at 0.1 mL·min^{-1} under the same conditions described above.

Molecular mass measurement

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS was conducted on a Voyager RP Biospectrometry System (PerSeptive Biosystems) using α-cyano-4-hydrocinnamic acid (Aldrich) as a matrix.

Amino-acid sequence analysis

The amino-acid sequence was determined using a Beckman LF-3400 gas phase sequencer.

cDNA cloning and nucleotide sequencing

mRNAs were isolated from fat bodies of third instar larvae 10 h after immunization with *E. coli* JM109 (2×10^5 cells) using a Quick Prep mRNA Purification Kit (Pharmacia). First-strand cDNA was synthesized using a First Strand cDNA Synthesis Kit (Pharmacia). Sixty nanograms of first-strand DNA was used for reverse-transcription (RT)-PCR conducted with the following degenerate primers: 5′-TGT/CGAT/CC/TTA/C/G/TT/CA/C/G/TT/AC/GA/C/G/TTTT/CGA-3′ for the forward primer and 5′-A/GCAA/G/TATA/GCAA/C/G/TACA/C/G/TCCA/C/G/TCG/TT/CT-3′ for the reverse primer. The forward primer was based on the amino-acid sequence of *O. rhinoceros* defensin and the reverse primer was designed based on the amino-acid sequence of the C-terminal conserved region of coleopteran defensins. PCR reactions were carried out with a step-down protocol [17], i.e. 5 min preheating at 94 °C, five cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s), five cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s), five cycles (94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s), 10 cycles (94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s) and a 7-min elongation at 72 °C. A 120-bp fragment obtained in this step was subcloned into a pCR2.1 vector using the Original TA Cloning Kit (Invitrogen). The nucleotide sequence of this fragment was determined by dye terminator cycle sequencing using a DNA sequencer (ABI 373 A). 3′-RACE was carried out by step-down PCR as descibed above using a first-strand cDNA as a template, an *O. rhinoceros* defensin specific primer (5′-AGC-TAAGGGTTTTGCTGCCA-3′) and the adaptor primer (5′-AACTGGAAGAATTCGCGGC-3′). Nested PCR was carried out using the PCR product as a template, the nested primer (5′-ATCACAGCCTGTGCGCTG-3′) and the adaptor primer under the same conditions as described above. 5′-RACE was carried out using a 5′-RACE kit (Gibco) with *O. rhinoceros* defensin-specific antisense primers, 5′-GATCTATCT-TCTGCACACA-3′, 5′-CAAACCCCATTTTGACAAGCA-3′ and an anchor primer, 5′-GGCCACGCGTCGACTAGTACGG-GIIGGGIIGGGIIG-3′. Finally, a full-size sequence encoding *O. rhinoceros* defensin was amplified using the primer 5′-TGACTCATACAACAAATCGCA-3′ and an adaptor primer under the following conditions: preheating for 5 min, 35 cycles (94 °C for 30 s, 48 °C for 30 s and 72 °C for 30 s) and elongation at 72 °C for 7 min. A 316-bp PCR product obtained in the final step was subcloned and nucleotide sequencing was conducted as described above.

Gene expression analysis in different tissues

RT-PCR was carried out to analyse gene expression of *O. rhinoceros* defensin in different tissues. Third instar larvae were immunized with *E. coli*. Fat body, hemocyte, midgut and Malpighian tubules were excised from larvae 10 h after immunization. As a control, the same tissues were excised from

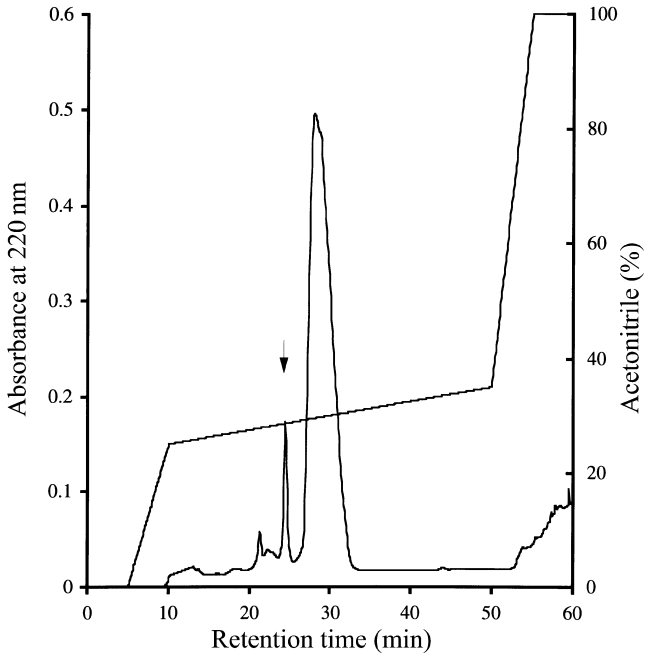

Fig. 1. Final purification profile by reverse-phase HPLC of *O. rhinoceros* defensin. Reverse-phase HPLC was conducted with an μRPC 2.1/10 column connected to a Smart system (Pharmacia). Protein adsorbed on the column was eluted with a gradient of 0–20% acetonitrile/0.05% trifluoracetic acid followed by the gradient (25–35%). Protein content was monitored by measuring the UV adsorbance at 220 nm. Antibacterial activity was examined in the plate-growth-inhibition assay using *S. aureus*. An arrow indicates a peak fraction having antibacterial activity. The acetonitrile concentration (%) is shown.

nonimmunized larvae. mRNA isolation and cDNA synthesis were as described above. Sixty nanograms first-strand DNA was used for RT-PCR with the following primers: 5′-TGACTCATACAACAAATCGCA-3′(forward primer) and 5′-CAAACCCCATTTTGACAAGCA-3′ (reverse primer). As an internal marker, the following insect actin primers were synthesized and used [18]: 5′-AGCAGGAGATGGCCACC-3′ (forward primer) and 5′-TCCACATCTGCTGGAAGG-3′ (reverse primer). Five RT-PCR cycles (25, 30, 35, 40 and 45) were conducted under the following conditions: 94 °C (30 s), 50 °C (30 s) and 72 °C (30 s). RT-PCR products were subjected to agarose-gel elecrophoresis (2%) and stained with ethidum bromide. A 100-bp ladder (Pharmacia Biotech) was used as DNA size markers.

Peptide synthesis

9-Fluorenylmethoxycarbonyl (Fmoc) L-amino acids and Fmoc-tris(alkoxy)benzylamide-poly(ethylene glycol)-polystyrene resins were purchased from PerSeptive Biosystems. The 9-mer peptides, AHCLAIGRK, ALRLAIRKR, ALLLAIRKR, AWLLAIRKR, ALYLAIRKR and ALWLAIRKR with a free amide group at their C-termini, were synthesized by a 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 C_2/C_{18} PC3.2/3 or of Resource RPC (1 mL) (Pharmacia). The column of μRPC C_2/C_{18} PC 3.2/3 was eluted in 50 min at 0.1 mL·min^{-1}, using a linear gradient from 0 to 30% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid. The 1-mL Resource RPC column was eluted in 20 min at 2 mL·min^{-1}, using a linear gradient from 0 to 25% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid.

Assay for liposomal membrane permeability

Liposomes containing trapped glucose was prepared under the method of Yamada and Natori [19]. Phosphatidylglycerol (PtdGro) from egg yolk lectin, cardiolipin (CL) from bovine heart and phosphatidylethanolamine (PtdEtn) from *E. coli* were purchased from Sigma. Two types of liposomes were prepared: the *S. aureus* type contained a PtdGro/CL molar ratio of 3 : 1 and the *E. coli* type contained a PtdGro/CL/PtdEtn molar ratio of 2 : 1 : 7. Two micromoles of the phospholipid mixture in chloroform was dried under nitrogen gas. After the addition of 0.3 M glucose (0.2 mL), a multilamellar liposome was formed by vortex mixing. Liposomes thus prepared were washed three times with 1 mL of 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. An aliquot of the liposome suspension (10 μL) was added to incremental amounts of peptides dissolved in 10 μL of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl. These mixtures were incubated for 1 h at 25 °C. Phospholipids in liposomes were calculated by measuring the amount of ester groups [20]. Glucose released from liposomes was assayed using a Glucose CII-Test kit (Wako Chemical). α-Lactalbumin was used as a negative control.

Haemolytic activity

Erythrocytes were isolated from heparinized rabbit blood by centrifugation at 800 ***g*** for 10 min after washing three times with isotonic NaCl/P_i (130 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) and resuspended in the same buffer. Erythrocyte solution [0.5% (v/v); final volume 100 μL] was incubated with different concentrations of peptides for 30 min at 25 °C. Samples were centrifuged at 1000 ***g*** for 5 min and the absorbance of supernatants was measured at 540 nm.

Cytotoxic activity

The cytotoxic activity of peptides was examined in a sterile 96-well plate using the murine macrophage cell line JA-4 [21] and the fibroblast cell line L929 [22]. Cells were cultured at 37 °C in RPMI 1640 medium (Nissui) containing 10% (v/v) fetal bovine serum, 100 μg·mL^{-1} streptomycin and 100 U·mL^{-1} penicillin in a 5% CO_2 humidified atmosphere. Cultured cells were suspended in RPM1 medium (10^5 cells·mL^{-1}) and 100 μL of cell suspension was transferred to culture plates. The mixture of NaCl/P_i (10 μL) containing different concentrations of peptides and Alamar Blue (10 μL) (Iwaki) 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

Purification of *O. rhinoceros* defensin

O. rhinoceros defensin was purified from 20 mL of the immunized haemolymph. In an initial purification through a

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TGACTCATACAACAAATCGCAGAGCTTACGACAAGATGTCGAGGTTTATCGTATTTGCTT   25
                                    M  S  R  F  I  V  F  A  F

TCATCGTAGCCATGTGCATTGCACACAGTTTAGCTGCGCCAGCACCAGAAGCGCTTGAAG   85
 I  V  A  M  C  I  A  H  S  L  A  A  P  A  P  E  A  L  E  A

CTAGCGTCATAAGACAAAAGAGACTGACGTGCGATCTTCTGAGTTTCGAAGCTAAGGGTT  145
 S  V  I  R  Q  K  R [L] T  C  D  L  L  S  F  E  A  K  G  F

TTGCTGCCAATCACAGCCTGTGCGCTGCTCATTGCCTAGCTATTGGACGCAAAGGTGGTG  205
 A  A  N  H  S  L  C  A  A  H  C  L  A  I  G  R  K  G  G  A

CTTGTCAAAATGGGGTTTGTGTGTGCAGAAGATAGATCTGTTATAGTTTTTTTTTAATAG  265
 C  Q  N  G  V  C  V  C  R  R  ***

ATCATTTTTTTTATTTAAAAAAAAAAAAAAAAAA                             299
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Fig. 2. Nucleotide and deduced amino-acid sequence of a cDNA encoding *O. rhinoceros* defensin. The number of nucleotides starting from the methionine codon is given at the right side of each line. Deduced amino acids are expressed by the one-letter code. The amino-acid residue of *O. rhinoceros* defensin N-terminus is boxed. The potential recognition sequence for the cleavage site within the constitutive secretory pathway (Arg-Xaa-Lys/Arg-Arg) [36] is double-underlined. Asterisks indicate the termination codon.

Sep-pak C_{18} cartridge, antibacterial activity against *S. aureus* was found in the 10, 30 and 40% acetonitrile fractions. Of these three fractions, 30 and 40% acetonitrile contained stronger antibacterial activity than the 10%. After Sep-Pak treatment of the haemolymph, combined 30 and 40% fractions were further purified by reverse-phase HPLC with a linear gradient of acetonitrile (20–40%). Antibacterial activity against *S. aureus* was eluted with 38–40% acetonitrile. The combined samples having antibacterial activity were subjected to a final reverse-phase HPLC. Several protein peaks were eluted with a linear gradient of acetonitrile (25–35%) and a sharp peak was shown to be the only fraction containing antibacterial activity against *S. aureus* (Fig. 1). This peak fraction was analysed by MALDI-TOF-MS and confirmed to be a pure substance having a relative molecular mass of 4466 (data not shown). Four micrograms of antibacterial material was obtained from 20 mL of haemolymph.

Determination of partial amino-acid sequence

The amino-acid sequence of the N-terminal region was analysed by automated Edman degradation, and a sequence of 15 amino acid residues (LTXDLLXFEAKGFAA, where X is unidentified amino acid residue) was obtained. Comparison of this sequence with those of reported proteins by a computer-aided homology search indicated that this partial amino-acid sequence has 80% similarity to that of *A. dichotoma* defensin [3] and 47% similarity to that of tenesin 1, a defensin from the

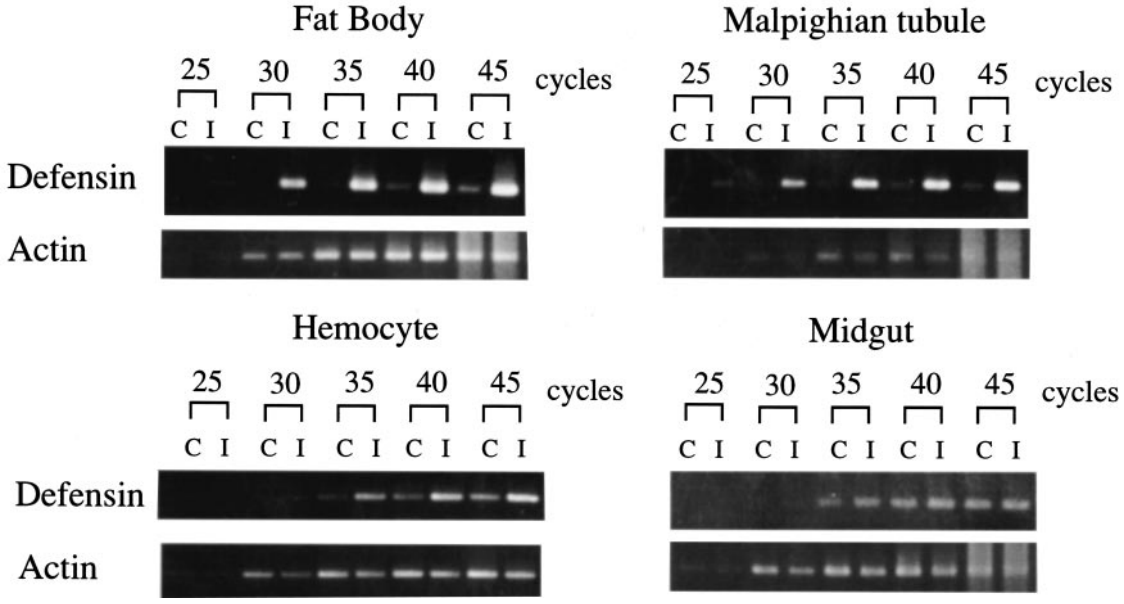

Fig. 3. Gene expression of *O. rhinoceros* defensin in different tissues. mRNA samples were extracted from the fat body, Malpighian tubules, hemocytes and midgut of nonimmunized third instar larvae (C) and those immunized with *E. coli* (I). RT-PCR products obtained after 25, 30, 35, 40 and 45 cycle reactions were electorophoresed on a 2% agarose gel. As an internal marker, actin primers were used for RT-PCR.

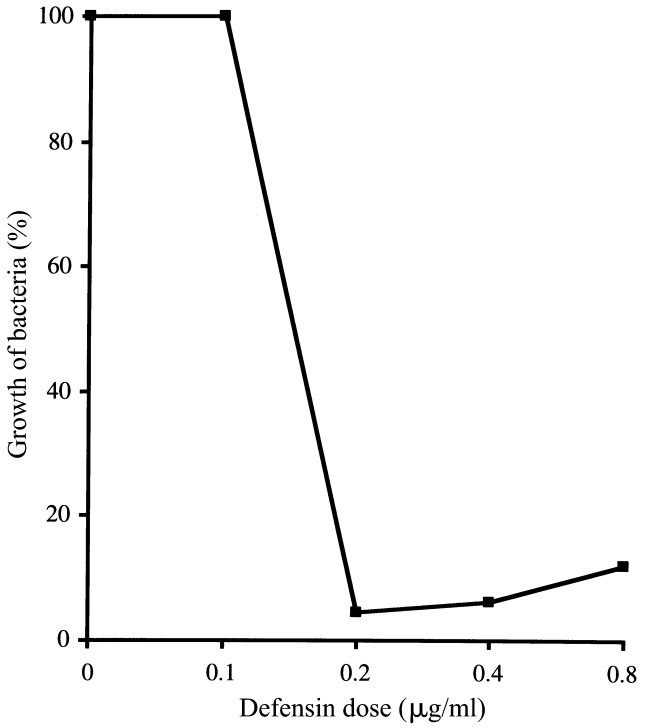

Fig. 4. Effect of *O. rhinoceros* defensin dosage on the growth of *S. aureus*. Growth rate of *S. aureus* was determined in the presence of different concentrations of *O. rhinoceros* defensin (0, 0.1, 0.2, 0.4 and 0.8 $\mu g \cdot mL^{-1}$). Bacterial growth rate without *O. rhinoceros* defensin was expressed as 100%.

beetle *Tenebrio molitor* [23], suggesting that the purified substance is an insect defensin.

cDNA cloning and nucleotide sequencing

Molecular cloning of the cDNA by RT-PCR was conducted to obtain the complete amino-acid sequence of the antibacterial peptide. A degenerate primer based on the amino-acid sequence of the N-terminal portion was synthesized for this purpose. The nucleotide sequence of a 120-bp fragment obtained by the first RT-PCR was sequenced and it was confirmed that this encoded the amino-acid sequence expected. 5′- and 3′-RACE was carried out to obtain a full-size cDNA. The nucleotide sequencing of this cDNA showed that it encodes 79 amino acids, whose mature portion was assumed to consist of 43 amino-acid residues (Fig. 2). Comparison of the overall amino-acid sequence of *O. rhinoceros* defensin with that of the *A. dichotoma* defensin indicated that the antibacterial peptides have 81% similarity (data not shown). The position of six cysteine residues of *O. rhinoceros* defensin was perfectly conserved among 16 insect defensins from *A. dichotoma* [3], *T. molitor* [23], *Zophobas atratus* [24], *Apis mellifera* [25], *Pyrrhocoris apterus* [26], *Sarcophaga peregrina* [2,27], *Drosophila melanogaster* [28], *Eristalis tenax* [29] and *Aedes aegypti* [30]. Gene expression of *O. rhinoceros* defensin was analysed with RT-PCR and showed that this gene is expressed in the midgut, Malphigian tubules, fat body and hemocyte (Fig. 3). In the latter three tissues, gene expression of this antibacterial peptide was induced by bacteria, whereas constitutive expression was seen in the midgut. The same gene expression pattern was observed in the case of rhinocerosin, another antibacterial peptide from *O. rhinoceros* larvae [31]. Midguts of *O. rhinoceros* larvae may be constantly infected by bacteria, because they feed on dung of cattles and grow in compost. Another possible explanation is that this gene is constitutively expressed in the midgut at a low level, as is the case with lyzozymes of *Hyalophora cecropia* [32] and *Bombyx mori* [33]. Mechanisms of differential gene expression among tissues remain to be an open question for the future.

Effect of *O. rhinoceros* defensin dossage on the growth of *S. aureus*

To determine the effect of *O. rhinoceros* defensin dossage, *S. aureus* was cultured in the presence of different concentrations of the antibacterial peptide. The growth of *S. aureus* was strongly inhibited by a low dose (0.2 $\mu g \cdot mL^{-1}$) as observed for *A. dichotoma* defensin [3], suggesting its potential use as a therapeutic agent (Fig. 4).

Effect of the synthetic Ala22–Lys30-NH_2 fragment and its analogues on the growth of bacteria

We first synthesized a peptide fragment, AHCLAIGRK-NH_2 (Ala22–Lys30-NH_2), assuming that this fragment contains antibacterial activity by comparison of the amino-acid sequence with that of *A. dichotoma* defensin [15]. The active site, LCAAHCLAIGRR-NH_2 of *A. dichotoma* defensin has an identical amino-acid sequence, except for the last amino acid residue, to that of residues 19–30 of *O. rhinoceros* defensin. We examined the antibacterial activity of the Ala22–Lys30-NH_2 fragment of *O. rhinoceros* defensin against *S. aureus*, MRSA, *E. coli* and *P. aeruginosa*. As we expected, this fragment showed antibacterial acitivity against these bacteria (Table 1). We then synthesized five 9-mer peptides modified based on the amino-acid sequence of the Ala22–Lys30-NH_2 and determined their minimal inhibitory concentration against

Table 1. Antibacterial activity of synthetic Ala22–Lys30-NH_2 fragment and analogues. MRSA and *P. aeruginosa* were isolated from infected patients. MIC, minimal inhibitory concentration.

	MIC ($\mu g \cdot mL^{-1}$)			
Sample	*S. aureus*	MRSA	*E. coli*	*P. aeruginosa*
AHCLAIGRK-NH_2 (Ala22–Lys30-NH_2)	25	> 50	25	> 50
ALRLAIRKR-NH_2	5	> 48	4	15
ALLLAIRKR-NH_2	4	40	6	4
AWLLAIRKR-NH_2	3	25	5	4
ALYLAIRKR-NH_2	3	30	4	4
ALWLAIRKR-NH_2	4	40	10	10

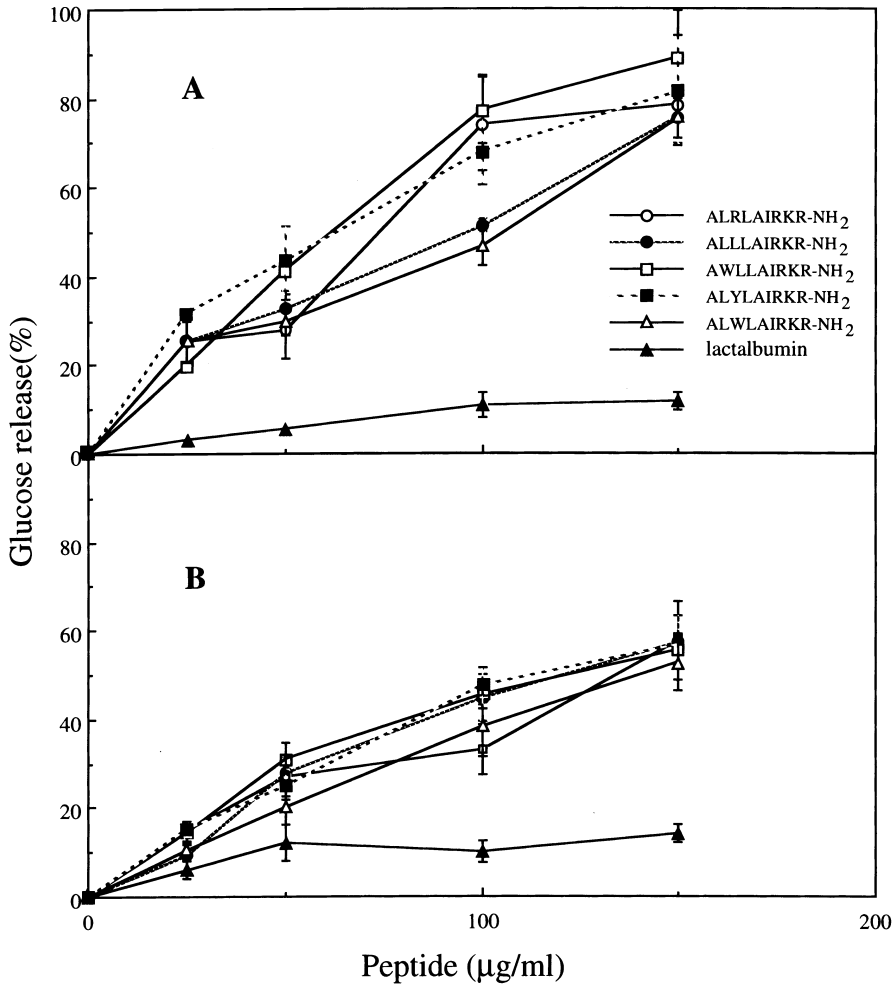

Fig. 5. Effect of the Ala22–Lys30-NH_2 analogues on glucose release from liposomes. *S. aureus* (PtdGro/CL, 3 : 1, mol/mol) (A) and *E. coli* (PtdEtn/PtdGro/CL, 7 : 2 : 1) (B) types of liposomes with entrapped glucose were prepared. The amount of glucose released by 0.3% Triton X-100 was defined as 100%. The amount of glucose entrapped in liposomes was 1.2 mol of glucose per mol of *S. aureus*-membrane-type phospholipids and 0.9 mol of glucose per mol of *E. coli*-membrane-type was phospholipids. The phospholipid concentration of the reaction mixture for the *S. aureus*-membrane-type was 275 μM and that for the *E. coli*-membrane type was 240 μM.

bacteria. Modified peptides showed strong antibacterial activity against Gram-positive and Gram-negative bacteria (Table 1).

Effect of synthetic peptides on liposomal membrane permeability

We prepared *S. aureus* and *E. coli* type liposomes with entrapped glucose to examine the direct effect of five synthetic Ala22–Lys30-NH_2 analogues on membrane permeability. The change in permeability was assayed by measuring glucose leaking into the buffer solution. Results indicated that liposomal membrane permeability increased proportionally to the increment of the peptide concentration (Fig. 5). Glucose leakage from the *S. aureus*-membrane-type was greater than that from the *E. coli*-membrane-type and parallel to the antibacterial activity of analogues (Table 1).

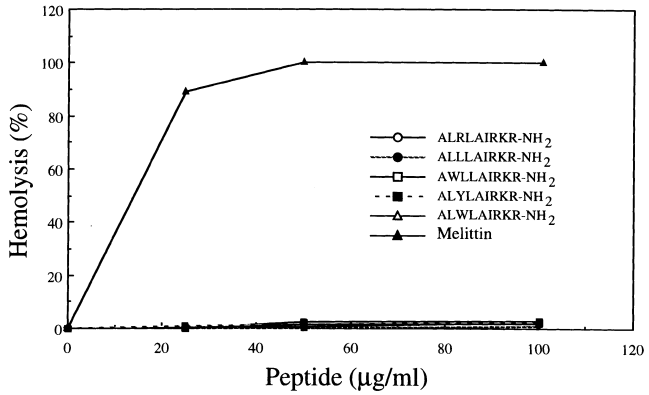

Fig. 6. Haemolytic activity of Ala22–Lys30-NH_2 analogues. Rabbit erythrocytes were incubated with different concentrations of the Ala22–Lys30-NH_2 analogues described in the figure. As a positive control, melittin was used. For details of experimental conditions, see Materials and methods.

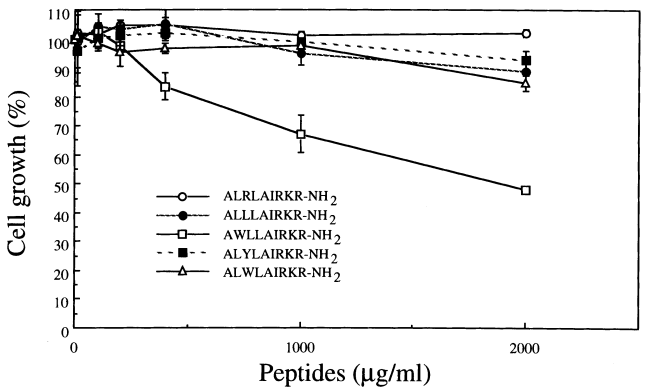

Fig. 7. Cytotoxic activity of Ala22–Lys30-NH_2 analogues on murine macrophages. The murine macrophage cell line JA-4 was used to observe growth inhibition by the Ala22–Lys30-NH_2 analogues described in the figure. Details of experimental conditions are given in Materials and methods.

Haemolytic and cytotoxic activity of Ala22–Lys30-NH_2 analogues

The effect of Ala22–Lys30-NH_2 analogues on haemolytic activity against rabbit erythrocytes was examined. None of the five analogues had haemolytic activity, whereas a bee venom, melittin, as a positive control showed strong activity (Fig. 6).

The cytotoxic activity of analogues against murine macrophages and fibroblast cells was also examined and we found that none had cytotoxic activity towards fibroblast cells (< 2000 $\mu g \cdot mL^{-1}$ peptide) (data not shown). For macrophages, four analogues showed no cytotoxic activity, whereas one peptide, AWLLAIRKR-NH_2, suppressed the growth strongly (34% inhibition at 1000 $\mu g \cdot mL^{-1}$ and 52% inhibition at 2000 $\mu g \cdot mL^{-1}$) (Fig. 7).

DISCUSSION

The main problems of using peptide antibiotics for direct therapeutic treatment are their antigenicity and cytotoxicity. Considerable attention has been paid to reducing the size and haemolytic and cytotoxic activity of antibacterial peptides [5–8,34]. In line with this approach, we synthesized novel short peptides that were modifications of *O. rhinoceros* defensin. Antibacterial activity and gene expression of *O. rhinoceros* defensin suggest that this antibacterial peptide is, in principle, similar to other insect defensins (Figs 1–4). We noted that the amino-acid sequence deduced from the nucleotide sequence of *O. rhinoceros* defensin cDNA was highly similar (86.0%) to that of *A. dichotoma* defensin [3]. Taking advantage of this result, we deduced its active site, as we had previously determined the active site of *A. dichotoma* defensin [15]. The active site of *A. dichotoma* defensin, Leu19–Arg30-NH_2 fragment, was highly similar (91.7%) to an amino-acid sequence of *O. rhinoceros* defensin. A shorter peptide of *A. dichotoma* defensin, Ala22–Arg30-NH_2, was still contained significant antibacterial activity, thus we synthesized a Ala22–Lys30-NH_2 fragment and confirmed its antibacterial activity (Table 1). Amidation at the C-terminus of *A. dichotoma* defensin was found to be indispensable for antibacterial activity [15].

Previous results suggested that the C-terminal region of the Ala22–Arg30-NH_2 fragment, i.e. RR-NH_2, is important in antibacterial activity [15], so we synthesized five novel Ala22–Lys30-NH_2 analogues, whose C-terminal portions were KR-NH_2, based on Ala22–Lys30-NH_2, and confirmed that all five peptides showed strong antibacterial activity (Table 1). Results suggest that the first arginine of the RR-NH_2 can be replaced with another basic amino acid residue, lysine, and that basic amino acid residues may play an important role in interacting with acidic phospholipids in bacterial membranes. Of the five synthetic peptides, AWLLAIRKR-NH_2 and ALYLAIRKR-NH_2 showed especially strong antibacterial activity, including activity against pathogenic species, and its minimal inhibitory concentration was similar to that of AWLLAIRRR-NH_2 and ALYLAIRRR-NH_2, which were synthesized based on *A. dichotoma* Ala22–Arg30-NH_2 [15]. Interestingly, AWLLAIRKR-NH_2 from *O. rhinoceros* defensin and AWLLAIRRR-NH_2 from *A. dichotoma* defensin exhibited similar cytotoxic activity towards murine macrophages but not towards fibroblast cells, although other synthetic peptides from *O. rhinoceros* (ALRLAIRKR-NH_2, ALLLAIRKR-NH_2, ALYLAIRKR-NH_2 and ALWLAIRKR-NH_2) and from *A. dichotoma* (ALRLAIRRR-NH_2, ALLLAIRRR-NH_2, ALYLAIRKR-NH_2 and ALWLAIRRR-NH_2) [15] did not have this activity. The evidence strongly suggests that the second amino-acid residue from the N-terminus, tryptophan, causes synthetic oligopeptides cytotoxicity towards murine macrophages. These data also support our previous assumption that no 9-mer analogues of an *A. dichotoma* defensin active site lyse eukaryotic cells, whose membranes contain cholesterol, and whose outer leaflet consists of zwitterionic and sphingomyelin phopholipids [35]. Although insect defensins are generally active against Gram-positive but not Gram-negative bacteria, our modified Ala22–Lys30-NH_2 was active against Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, and Gram-positive bacteria. This wide antibacterial spectrum was confirmed by the interaction of these peptides with *S. aureus* type and *E. coli* type liposomal membranes (Fig. 5). Results suggest that the target of Ala22–Lys30-NH_2 analogues is the bacterial membrane and that bacterial cell wall components, such as lipopolysaccharide present only in Gram-negative bacteria and teichoic acid present only in Gram-positive bacteria, do not interfere with peptide–lipid interaction. Results of glucose leakage tests from liposomes showed that *S. aureus*-membrane-type liposomes were more sensitive than *E. coli*-membrane-type liposomes to Ala22–Lys30-NH_2 analogues. The results were consistent with our previous observation using a 22A-30R-NH_2 fragment [15]. These 9-mer peptides thus have a higher affinity for a bacterial membrane with a high content of acidic phospholipids seen in Gram-positive bacteria. *O. rhinoceros* defensin Ala22–Lys30-NH_2 analogues were effective against drug-resistant pathogenic bacteria isolated from patients (Table 1), suggesting that the unique characteristics described above are very important in developing new antibiotics effective against drug-resistant bacteria. We think that these synthetic peptides and Ala22–Arg30-NH_2 analogues from *A. dichotoma* defensin will be able to serve as lead peptides in developing antibiotics effective against infectious diseases caused by drug-resistant bacteria, for example, in cystic fibrosis patients.

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