

Molecular cloning and biological characterization of novel antimicrobial peptides, pilosulin 3 and pilosulin 4, from a species of the Australian ant genus *Myrmecia*

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Abstract

Venom of an Australian ant species of the *Myrmecia pilosula* species complex (mss. name *Myrmecia banksi* Taylor) contains two major allergenic peptides, pilosulin 1 and pilosulin 2. To obtain novel cDNA clones that encode the pilosulin-related bioactive peptides, mRNA of another *Myrmecia* species was subjected to RT-PCR in which the forward primer corresponds to a nucleotide sequence in the leader sequences of pilosulin 1 and pilosulin 2. As a result, we isolated cDNA clones encoding the novel antimicrobial peptides pilosulin 3 and pilosulin 4. The nucleotide and the amino acid sequences of all four pilosulins have high homology except for the mature peptide coding regions. Synthetic pilosulin 3 and pilosulin 4 peptides displayed antimicrobial activity with histamine-releasing and low hemolytic activities.

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Compared to non-social insects, ants have developed elaborate defensive systems to protect their colonies from pathogens related to their high population densities and exposure to soil bacteria [1]. For example, most ant species spray the nest with secretions from the metapleural gland on the thorax that exhibit broad-spectrum antimicrobial activity to inhibit the proliferation of microorganisms [2]. In some specialized ant species, the mandibular and venom gland secretions play an important role in sterilizing prey [3]. Antimicrobial peptides are included in these secretions. More than 170 antimicrobial peptides have been characterized from various insects [4] since Boman described the first antimicrobial peptide, cecropin, from the moth

Hyalophora cecropia [5]. These peptides generally have low molecular weights and are rich in positive and hydrophobic amino acids. In ants, however, only three species, *Myrmecia gulosa* [6], *Formica rufa* [7], and *Pachycondyla goeldii* [1], are known to produce antimicrobial peptides.

Australian ants of the *Myrmecia pilosula* species complex are about 10 mm long, and black with yellow mandibles. They have a powerful sting that is painful to humans. The sting potency is due to a venom that is believed to include various bioactive peptides as well as the enzymes phospholipase, hyaluronidase, and phosphatase [8]. The major allergenic peptides of the ant venom, pilosulin 1 [9] and pilosulin 2 [10], have been cloned by immunoscreening of a *M. pilosula* cDNA library using sera collected from individuals allergic to ant venom. Although pilosulin 1 has strong hemolytic

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and cytotoxic activities, the biological functions of these pilosulins are unknown [11].

In this study, we used a PCR-based method to isolate novel cDNA clones that encode pilosulin-related bioactive peptides from the RNA of a previously unexamined species of *Myrmecia*. We isolated and identified cDNA clones encoding novel antimicrobial peptides, which we named pilosulin 3 and pilosulin 4. We also analyzed the antimicrobial, histamine-releasing, and hemolytic activities of the pilosulin 3 and pilosulin 4 peptides.

Materials and methods

Ants

One colony of a member of the *M. pilosula* complex was collected in Nowra, New South Wales, Australia, in 1999, and was maintained at National Institute of Genetics, Mishima, Japan. The species was identified by morphology and karyotype as *Myrmecia banksi* [12,13].

Construction of cDNA library

Ninety-five adult worker ants were immersed in liquid nitrogen and homogenized. Total RNA was extracted by Trizol solution (Invitrogen, Carlsbad, CA) using the procedure described by the supplier. Poly(A)⁺ RNA was purified by Oligotex-dT (Takara, Otsu, Japan) and the first-strand cDNAs were synthesized by ReverTra Ace (Toyobo, Osaka, Japan) and Superscript II (Invitrogen) with an NXho(dT)₃₀ primer (5'-AAGCAGTGGTAAC AACGCAGAGTCTCGAG (T)₃₀ (GAC) (GTAC)-3').

The second strands were synthesized with DNA polymerase I, RNase H, and *Escherichia coli* DNA ligase (Takara). After both ends of the double-stranded cDNAs were filled with a DNA blunting kit (Takara), *EcoRI* adapters (Clontech, Palo Alto, CA) were added to the cDNAs. The cDNAs were then digested with *XhoI* and fractionated by agarose gel electrophoresis. DNA fragments with lengths of 0.8–2.0 kbp were electroeluted using a Centrilotor (Millipore, Bedford, MA) and concentrated using a Centricon 100 centrifugal filter (Millipore). The resulting DNA fragments were cloned into *EcoRI* and *XhoI* restriction sites of pSD64TR_{ER}, which was constructed by deleting an original *EcoRI* site and by inserting an *EcoRI* site into an *EcoRV* site of pSD64TR [14].

cDNA cloning and sequencing

Poly(A)⁺ RNA isolated from the ants was reverse transcribed to cDNA and subjected to PCR amplification with Ex Taq (Takara). The oligonucleotide primers used were T17ADP (5'-GAGTCGACTCGAGAA (T)₁₇-3')

and Myr1 (5'-GTCGGTTTTGCCGATGCA-3') corresponding to the nucleotide (nt)¹ 138–155 of pilosulin 1 (X70256). The PCR protocol consisted of 35 cycles of denaturation (94 °C, 30 s), annealing (48 °C, 30 s), and elongation (72 °C, 1 min). To isolate the 5' side of the cDNAs, PCR was carried out using the ant cDNA library as a template, an SP6 vector primer (Promega, Madison, WI) as a forward primer, and gene-specific primers Pilosulin 3-FL (5'-TCTTTACAAGTACACACG-3') corresponding to nt 176–193 of pilosulin 3, or Pilosulin 4-Bam (5'-CGCGGATCCACCCTTCGAGATCACTTT-3': the *Bam*HI site and flanking sequence are underlined) corresponding to nt 193–211 of pilosulin 4 as a reverse primer. The amplified products corresponding to the original and 5' side of the cDNAs were cloned into pCR 2.1-TOPO (Invitrogen). The full-length nucleotide sequences of the pilosulin 3 and pilosulin 4 cDNAs were identified by combining the nucleotide sequences of the 5' side and the original clones. We also confirmed the sequences of the full-length cDNAs that cover the entire open reading frames using PCR. The oligonucleotide primers used were an SP6 vector primer as a forward primer, and gene-specific primers Pilosulin 3-Bam (5'-CGCGGATCCCTCATCTTCATATTATCC-3': the *Bam*HI site and flanking sequence are underlined; the complementary sequence of the pilosulin 3 stop codon is double underlined) corresponding to nt 220–237 of pilosulin 3, or Pilosulin 4-BamFL (5'-CGCGGATCCATCC TTATATTATTCTTG-3': *Bam*HI site and flanking sequence are underlined; the complementary sequence of pilosulin 4 stop codon is double underlined) corresponding to nt 247–264 of pilosulin 4 as a reverse primer. The amplified products were cloned into pSD64TR_{ER}. All the inserts were sequenced using a Model 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Computer-assisted sequence analysis

BLAST and SSEARCH database searches were performed via the on-line server of the National Institute of Genetics, Mishima, Japan (<http://www.ddbj.nig.ac.jp>). Alignments were carried out using the ClustalW program by the MegAlign software component of LaserGene version 5.0. Leader sequences were predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP>). Synonymous versus non-synonymous substitution rates were analyzed by the modified Nei–Gojobori method using MEGA version 2.0.

Synthesis and purification protocol of peptides

Pilosulin peptides were prepared by American Peptide Company (APC, Sunnyvale, CA) using Fmoc

¹ Abbreviations used: nt, nucleotide; MIC, minimal inhibitory concentration; LDH, lactate dehydrogenase.

chemistry. The peptides were oxidized to form a disulfide bond and then purified using RP-HPLC with a preparative C18 column. The purity and molecular weight of the final peptides were verified by APC using HPLC and MS, respectively. The purities of pilosulins 3 and 4 peptides were 90 and 81%, respectively.

Antimicrobial assay

Escherichia coli (IFO 14237), *Staphylococcus aureus* (IFO 12732), *Pseudomonas aeruginosa* (IFO 12582), *Bacillus subtilis* (IFO 3037), *Candida albicans* (IFO 1594), and *Saccharomyces cerevisiae* (IFO 10217) were purchased from the Institute of Fermentation Osaka (IFO, Osaka, Japan). (These microorganisms have recently been transferred to the Biological Resource Center (BRC, Kisarazu, Japan), National Institute of Technology and Evaluation.) *Lactococcus garvieae* (ATCC 49156) was purchased from American Type Culture Collection (ATCC, Manassas, VA).

The antimicrobial assay was carried out essentially as described previously [15]. Four antimicrobial peptides, pilosulin 3, pilosulin 4, melittin (Sigma–Aldrich, St. Louis, MO), and magainin 1 (Peptide Institute, Osaka, Japan), were added to individual wells of a microplate at final concentrations of 50, 25, 12.5, 6.25, and 3.1 μM . As a control, LTM solution (100 mM NaCl, 1% tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD), 2 mM Na_2HPO_4 , and 8 mM NaH_2PO_4 , pH 6.5) was added to the wells with 4×10^5 cells/ml of microbial cell suspension. Bacterial cell concentrations were estimated from $f \times A_{600}$, where f is a conversion factor and A_{600} is the absorbance at 600 nm [15]. The conversion factors for *C. albicans* and *S. cerevisiae*, determined by the Neuroscience Research Institute laboratory, were 3.0×10^7 and 5×10^6 cells/ml, respectively. After incubating the microbial cell suspension at 24 °C (*C. albicans* and *S. cerevisiae*), 30 °C (*B. subtilis* and *P. aeruginosa*), or 37 °C (*E. coli*, *S. aureus*, and *L. garvieae*) for 3 h, $2 \times$ TSB was added to each well to give a final concentration of $1 \times$ TSB. For the culture medium of *C. albicans* and *S. cerevisiae*, Sabouraud dextrose broth (Becton, Dickinson and Co.) was used instead of TSB in all steps of the antimicrobial assay. The suspension was reincubated for 16–18 h. The A_{600} of the microbial cell suspension in each well was measured by a Spectra MAX 190 microplate reader (Molecular Devices, Sunnyvale, CA) as an index of the antimicrobial activity. The lowest concentration that did not result in any detectable growth was considered the minimal inhibitory concentration (MIC).

Hemolytic assay

Heparinized human whole blood was washed twice in PBS (100 mM NaCl, 7.5 mM Na_2HPO_4 , and 2.5 mM NaH_2PO_4 , pH 7.4) by centrifugation at 900g and sus-

pended in PBS to a concentration of 0.5% (v/v). The hemolytic assay was carried out essentially as described previously [11]. Pilosulin 3, pilosulin 4, and melittin were added to individual wells at final concentrations of 50, 25, 12.5, 6.25, and 3.1 μM . PBS and PBS containing 0.2% Triton X-100 were used for controls. After overnight incubation at room temperature, erythrocytes were removed by centrifuging the suspension at 900g for 10 min. The absorbance at 541 nm (A_{541}) of the supernatant was measured as an index of hemolysis.

Histamine release and lactate dehydrogenase release assays

The histamine-releasing activity of pilosulin 3, pilosulin 4, and melittin was determined with rat peritoneal mast cells essentially as described previously [16]. PS-glucose (145 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 1 mM CaCl_2 , and 5 mM Hepes, pH 7.4) and PS-glucose containing 0.5 $\mu\text{g}/\text{ml}$ of Compound 48/80 (Sigma–Aldrich) were used for controls. The data were expressed as a percentage release of total cellular histamine release. Spontaneous release in the absence of peptides was $8.65 \pm 1.9\%$.

Lactate dehydrogenase (LDH) release assay was carried out essentially as described previously [17]. LDH release was measured in both the supernatants and cell pellets of 1×10^6 cells and was expressed as a percentage of total LDH, which was 435.87 ± 47.5 Wroblewski units.

Results and discussion

Molecular characterization of pilosulins 3 and 4

Members of a certain family of antimicrobial peptides tend to have conserved N-terminal leader sequences, as far as the antimicrobial peptides belong to the same family. For example, cathelicidins share one conserved leader sequence [18] and dermaseptins share another [19]. The C-terminal residues of the leader sequence tend to have especially high homologies within each family.

Pilosulins 1 and 2, which were isolated from *M. pilosula* sensu stricto, have almost identical protein leader sequences [10]. Because this species is closely related to the subject species of this study (*M. banksi*), we postulated that our species has some bioactive peptides that have leader sequences similar to those of pilosulins 1 and 2. Thus, we designed an 18-base primer corresponding to the amino acid sequence 37–42 (residues VGFADA). These regions are highly conserved among pilosulins 1 and 2 in both nucleotide and amino acid sequences. Using this primer and the T17ADP poly(T) primer for RT-PCR, we detected several approximately 300-bp DNA fragments among the amplified products.

After sequencing, we found four clones encoding peptides related to pilosulin: pilosulin 1, pilosulin 2, and two novel but related pilosulins. After construction of the ant cDNA library, we isolated full-length cDNAs of each pilosulin-related gene. The novel peptides encoded by pilosulin-related genes were termed pilosulin 3 and pilosulin 4.

The cDNAs of pilosulin 3 and pilosulin 4 contain open reading frames encoding 74-amino acid and 84-amino acid peptides, respectively. The mature forms of the peptides, predicted from the sequences of the mature forms of pilosulins 1 and 2, have 24 and 36 amino acids with calculated molecular weights of 2.52 and 4.01 kDa, respectively (Figs. 1A and B). The amino acid sequences of leader peptides among the four pilosulins are more than 84.0% identical (Fig. 2A). In addition, it is noteworthy that the nucleotide sequences of the four pilosulin cDNAs show sequence identities higher than 89.7 and 73.3% in the 5'- and the 3'-untranslated regions,

respectively (Figs. 2B and C). The deduced amino acid sequences of the four mature pilosulin peptides have low identities with each other (13.2–33.3%; Fig. 2A).

We determined non-synonymous versus synonymous substitution rates (D_n/D_s), which were analyzed using the MEGA program. For the leader sequences and the mature peptides, the D_n/D_s ratios were 0.359 and 1.718, respectively. D_n/D_s ratios greater than 1 are thought to result from accelerated evolutionary change via positive selection. Since D_n/D_s ratios around 0.3 are usual, it is tempting to assume that the hyper variabilities of mature peptides indicate accelerated evolution.

The amino acid compositions for the mature peptides of pilosulins 2, 3, and 4 are similar in that each has two cysteine residues, and each is rich in hydrophobic and basic amino acids. The calculated pIs of the mature peptides of pilosulins 3 and 4 are 10.41 and 10.34, respectively. In addition, the C-terminal amino acid residue in the deduced amino acid sequences of pilosulins 2

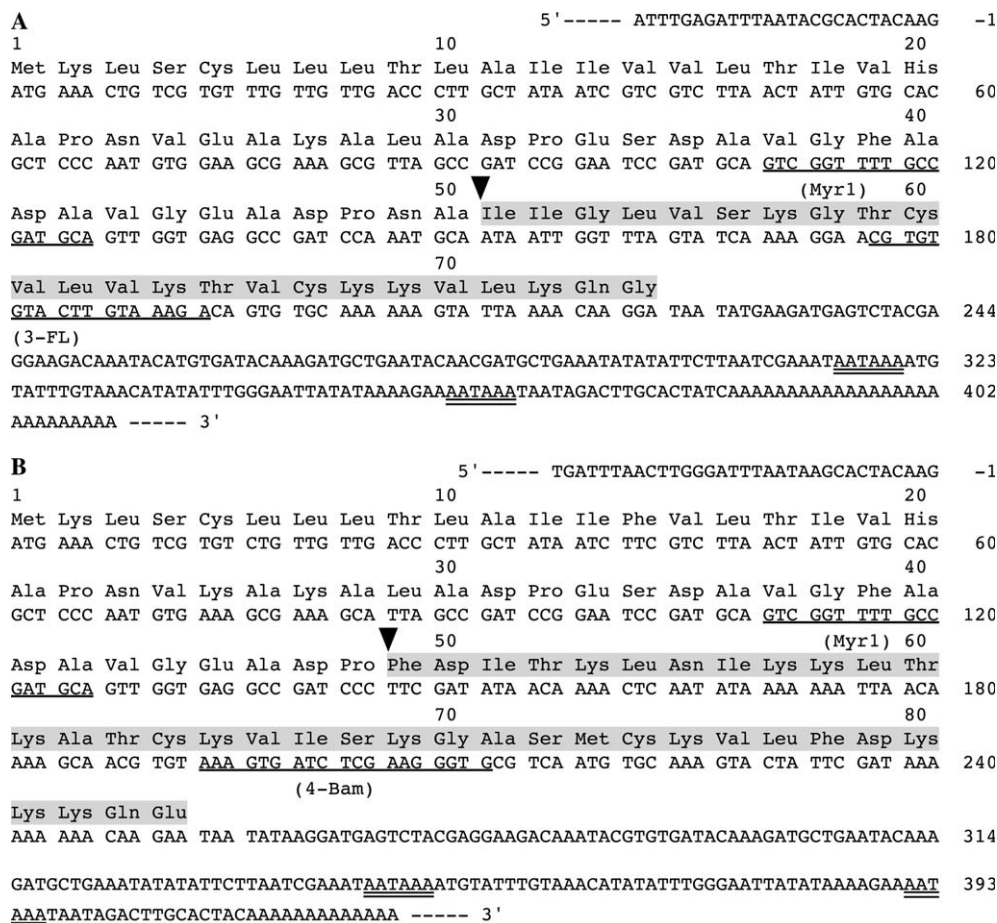


Fig. 1. Nucleotide and deduced amino acid sequences of pilosulins 3 (A) and 4 (B). The putative mature peptide is highlighted in grey. Primer sequences (Myr1, 3-FL, and 3-Bam in A and Myr1, 4-Bam, and 4-BamFL in B) are underlined. Arrowheads indicate the predicted N-termini of pilosulin 3 and 4 mature peptides. Polyadenylation signals are indicated by double underlines. The pilosulin 3 and 4 precursor peptides contain 74 and 84 amino acid residues, respectively. Their nucleotide sequences have been assigned DDBJ/EMBL/GenBank Accession Nos. AB128064 and AB128065, respectively. According to the convention recommended by the International Union of Immunological Societies (IUIS), we termed these peptides *Myr b* III and *Myr b* IV as another name for pilosulin 3 and pilosulin 4, respectively.

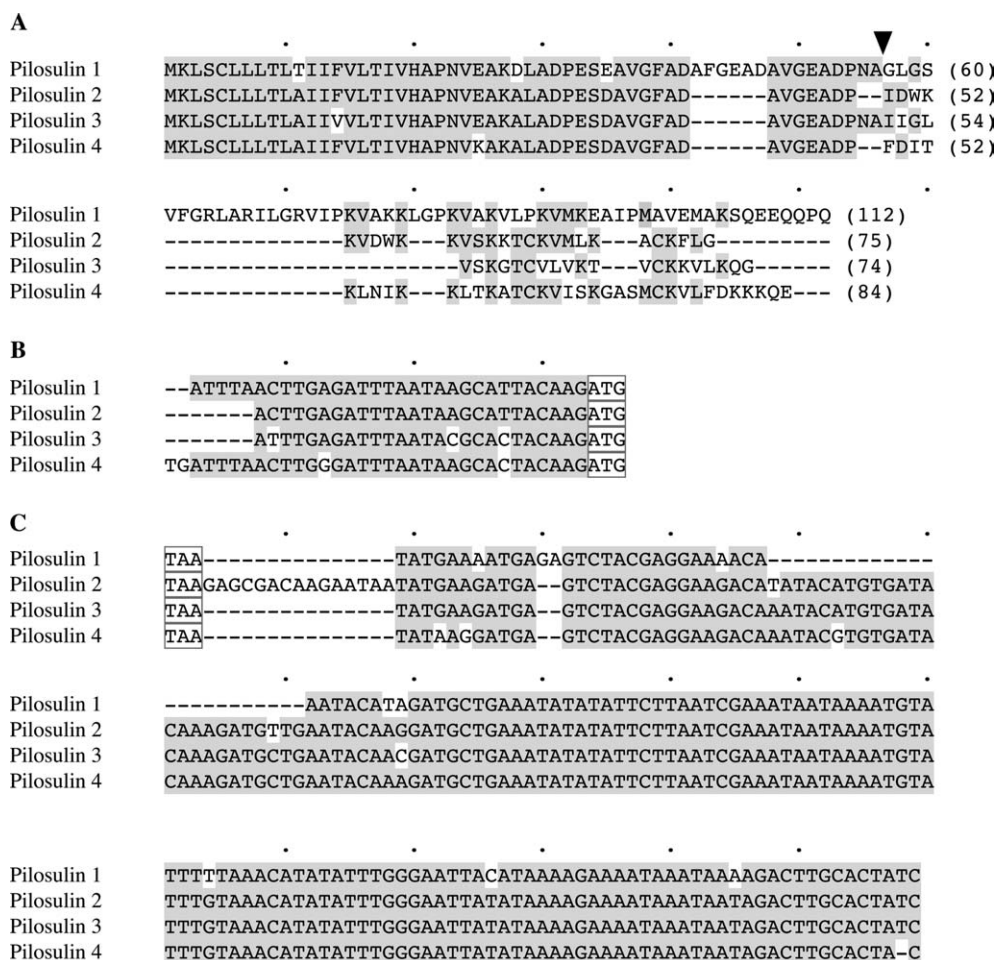


Fig. 2. Multiple alignments of the amino acid sequences of pilosulin precursor peptides (A), multiple alignments of the nucleotide sequences of the 5'- and 3'-untranslated regions of pilosulin cDNAs (B and C, respectively). Using the ClustalW program, the amino acid sequences and the nucleotide sequences of the 5'- and 3'-untranslated regions of four pilosulins were aligned. Identical amino acid and nucleotide sequences are highlighted in grey. Intervals of 10 amino acids and nucleotides are indicated by dots. The amino acid and the 3' nucleotide sequences of pilosulin 1 were reported previously (X70256). The alignments of N-termini of pilosulin mature peptides were refined manually in (A). The number of amino acid residues at the right end of each line is given in parentheses. An arrowhead indicates the predicted processing sites (A). The *EcoRI* adapter sequence of pilosulin 2 cDNA (S81785) and poly(A)⁺ sequences of four pilosulin cDNAs were removed. The initiation and termination codons for translation are indicated by boxes. The 5' nucleotide sequence of pilosulin 1, which was identified in this study, has been assigned DDBJ/EMBL/GenBank Accession No. AB159686 (B and C).

and 3 is a glycine. Because the C-terminal glycine residue in some other bioactive peptides is modified to an amide, this might also be the case for the mature peptides of pilosulins 2 and 3 [20].

A BLASTX search for peptides homologous to pilosulins 3 and 4 was unsuccessful. We then tried the SSEARCH program (Smith–Waterman), which is better able to find sequences with low homologies, because SSEARCH is based on a local dynamic programming algorithm. Pilosulins 3 and 4 were found to have some similarities to *Apis mellifera* melittin (GenBank Accession No. P01501) (18.6 and 17.1% identities, respectively) and an unknown peptide of *Caenorhabditis elegans* (U80031) (17.9 and 22.4% identities, respectively).

Since we identified the precursor forms of pilosulins 3 and 4 by PCR-mediated methods, the mature forms of the peptides were predicted in this study by analogy with pilosulin 1. Previous studies have identified the N-terminus of the pilosulin 1 mature peptide by Edman degradation [9]. The mature peptide of pilosulin 1 begins at the amino acid position 57 of the precursor, indicating that the precursor peptide undergoes processing to produce the mature peptide. By using the SignalP V2.0 program [21], we found a potential cleavage site beyond amino acid position 21 (closed triangle in Fig. 3), but not after 56 (open triangle in Fig. 3), and a typical leader peptide at the amino acid positions 1–21 in the pilosulin 1 precursor. Comparing this cleavage site with the N-terminus identified by Edman degradation, there are 35

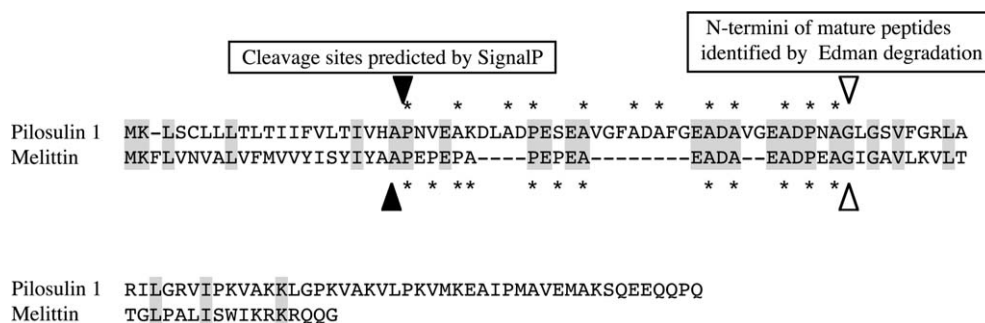


Fig. 3. Alignment of the amino acid sequences of pilosulin 1 and melittin. Asterisks indicate proline and alanine residues in amino acid positions 22–56 of pilosulin 1 and 21–44 of melittin. Closed triangles indicate the processing sites predicted by the SignalP V2.0 program and open triangles indicate the identified N-termini of mature peptide of melittin and pilosulin 1 by Edman degradation. Identical amino acid sequences are highlighted in grey.

additional amino acids at the N-terminus of the mature peptide of pilosulin 1. However, we noted that all four pilosulin precursors contain a repeated sequence in which proline or alanine locates at every second or fourth position (asterisks in Fig. 3). This repeated sequence is found in the pilosulin 1 precursor at the amino acid positions 22–56. The melittin precursor also contains the same repeated sequence (highlighted in Fig. 3), which is processed by dipeptidylpeptidase IV of the bee venom gland [22]. Because dipeptidylpeptidase IV cleaves after proline and alanine residues in a stepwise fashion to release dipeptides, we assume that pilosulin precursors are first deprived of their N-terminal sequences by a putative endopeptidase at amino acid position 21, and then processed by an exopeptidase such as dipeptidylpeptidase to convert the peptide into the active form.

Biological expression and chemical synthesis of pilosulins 3 and 4

To obtain sufficient quantities of pilosulins 3 and 4 mature peptides, these peptides were expressed in *E. coli* as GST fusion proteins. After cleavage of the fusion proteins, the recombinant mature peptides were purified. However, they did not show any antimicrobial activities (data not shown). The mature peptides of pilosulins 3 and 4 were then synthesized chemically by the Fmoc method. Since the antimicrobial peptides gomesin [23] and tachyplesin [24] become biologically active through the oxidation of cysteine residues to form a disulfide bridge, pilosulins 3 and 4 were oxidized after synthesis.

Many bioactive peptides that are amidated at the C-terminus, such as gomesin [23], have higher activities than antimicrobial peptides that have a glycine residue at the C-terminus. Amidation of the C-terminus increases the electropositivity of the peptide, which is thought to increase its affinity for negatively charged bacterial membranes. Thus, the C-terminus of pilosulin

3 was amidated. However, we do not know whether the C-terminus of the native pilosulin 3 peptide is amidated.

Antimicrobial activity

Pilosulin 3 showed moderate activity against *E. coli* and *S. aureus* (MIC < 25 μ M), slight activity against *B. subtilis* (MIC < 50 μ M), and no activity against *L. garvieae*, *P. aeruginosa*, *C. albicans*, and *S. cerevisiae*. Pilosulin 4 was more effective than pilosulin 3 against *E. coli* and *S. aureus* (MIC < 6.25 μ M), was moderately effective against *P. aeruginosa* (MIC < 25 μ M), weakly effective against *B. subtilis* (MIC < 50 μ M), and had no effect against *L. garvieae*, *C. albicans*, and *S. cerevisiae* (Fig. 4). The bacteria species that were sensitive to pilosulins 3 and 4 were not limited to Gram-negative bacteria nor to Gram-positive bacteria. Both pilosulins 3 and 4 showed no activity against yeast species, which were sensitive to pilosulin 2 (data not shown).

For comparison, magainin 1 had MICs of 6.25 μ M against *E. coli* and more than 12.5 μ M against *S. aureus* (Figs. 4A and B), and melittin had MICs of 3.125 μ M against *E. coli* and 3.125 μ M against *S. aureus*. These values were very similar to those previously reported [25,26] even though the previous studies used a different assay system. Furthermore, the antimicrobial activities of pilosulin 4 against *E. coli* and *S. aureus* are comparable to, or even higher than, those of magainin 1.

Hemolytic activity

Whole venom of *M. pilosula* was shown previously to have hemolytic activity at concentrations as low as 25 μ g/ml. Pilosulin 1 showed some hemolytic activity even at a concentration of 1.25 μ M, while pilosulin 2 did not show any obvious hemolytic activity at concentrations up to 80 μ M in the previous study [11].

As shown in Fig. 5, melittin, a hemolytic toxin, showed hemolytic activity at concentrations as low as 3.75 μ M. Although pilosulins 3 and 4 exhibited antimi-

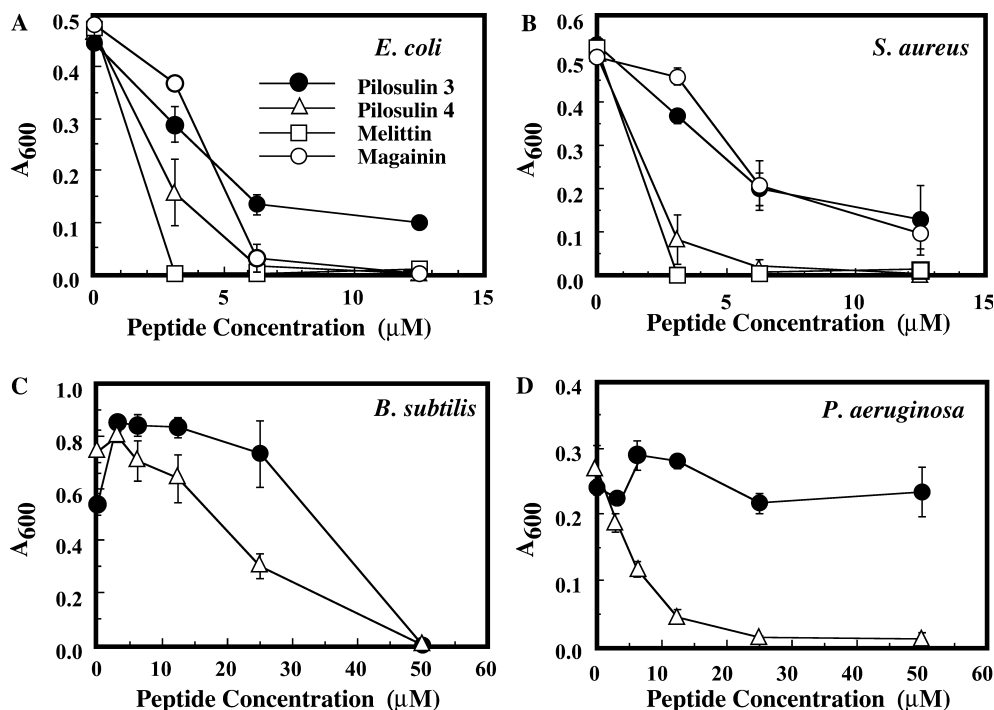


Fig. 4. Antimicrobial activities of pilosulin 3, pilosulin 4, melittin, and magainin. Ranges of concentrations of pilosulin 3 (●), pilosulin 4 (△), melittin (□), and magainin (○) peptides were tested for antimicrobial abilities against *E. coli* (A), *S. aureus* (B), *B. subtilis* (C), and *P. aeruginosa* (D). Three independent experiments were performed. Each data point represents the mean from a single experiment performed in triplicate. Error bars show the standard deviation from a single experiment.

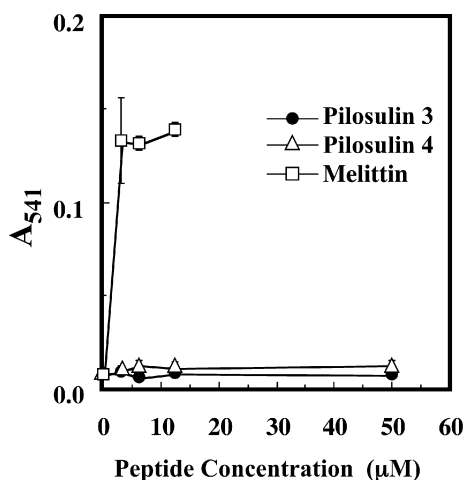


Fig. 5. Hemolytic activity of pilosulin 3, pilosulin 4, and melittin. Ranges of concentrations of pilosulin 3 (●), pilosulin 4 (△), and melittin (□) peptides were tested for hemolytic ability against human erythrocytes. Two independent experiments were performed. Each data point represents the mean from a single experiment performed in triplicate. Error bars show the standard deviation from a single experiment.

crobal activities at concentrations in the order of 50 μM , they did not show any obvious hemolytic activity in this concentration range. Thus, among four pilosulin peptides, only pilosulin 1 is partly involved in the hemolytic activity of the whole venom.

Histamine-releasing activity

Bioactive peptides isolated from bee, wasp, and frog have been shown to activate the release of histamines from rat peritoneal mast cells [27]. Pilosulins 3 and 4 caused a significant and dose-dependent histamine release: at a concentration of 5 μM , they induced the release of about 50% of the total cellular histamine. Comparison of the histamine-releasing activity of pilosulins 3 and 4 with the other mast cell-activating peptide melittin showed that pilosulins 3 and 4 were less effective than melittin (Fig. 6A). However, we noted that pilosulins 3 and 4 are the first examples of peptides from the ant that have histamine-releasing activity.

We also examined the release of β -D-glucosaminidase, which co-localizes with histamine [28], from RBL-2H3 cells by pilosulins 3 and 4. RBL-2H3 cells, a rat basophilic leukemia mast cell line, are homologous with rat mucosal mast cells and different from the connective tissue-type mast cell such as rat peritoneal mast cells. Pilosulins 3 and 4 did not induce the release of β -D-glucosaminidase at concentrations up to 50 μM (data not shown). This might indicate a difference in response between the mucosal and the connective tissue-type mast cells to pilosulins 3 and 4 [29].

To determine whether the histamine-releasing activity of pilosulins 3 and 4 is cytolytic or non-cytolytic, LDH release was measured. Melittin, whose action is cyto-

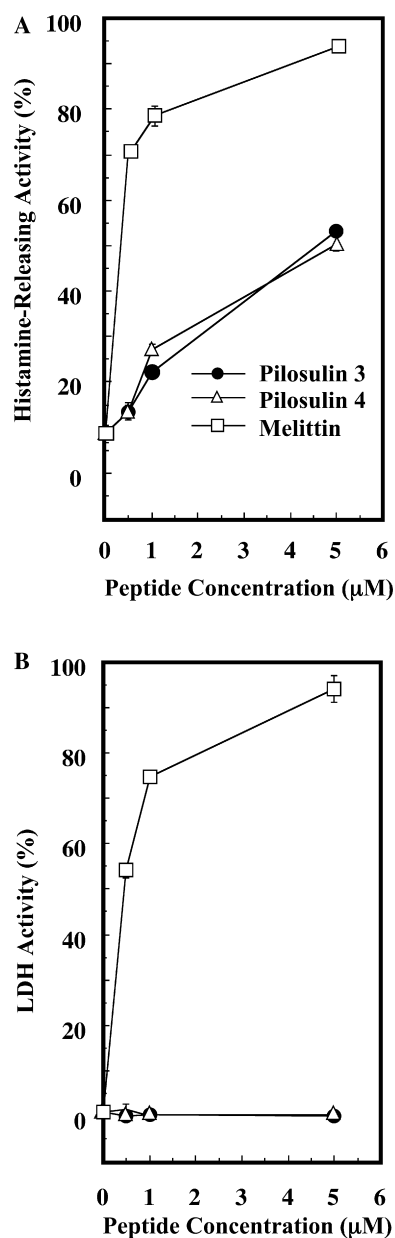


Fig. 6. Histamine (A) and LDH (B) release from rat peritoneal mast cells induced by pilosulin 3, pilosulin 4, and melittin. Ranges of concentrations of pilosulin 3 (●), pilosulin 4 (△), and melittin (□) peptides were tested for histamine-releasing or LDH activity from rat peritoneal mast cells. Two independent experiments were performed. Each data point represents the mean from a single experiment performed in triplicate. Error bars show the standard deviation from a single experiment.

lytic, released more than 90% of the LDH from mast cells at 5 μM. On the other hand, pilosulins 3 and 4 were devoid of LDH-releasing ability at all concentrations tested (0–5 μM) (Fig. 6B). Thus, pilosulins 3 and 4 might have a non-cytolytic mode of activation. Considering low hemolytic and non-cytolytic activities of pilosulins 3 and 4, these peptides may influence the signal transduction of mast cells to release histamine. Pilosulins 3

and 4 might activate this mechanism through a non-IgE-mediated pathway, because the histamine release assay was performed without IgE in our experiments.

Conclusions

We identified two novel antibacterial peptides, pilosulin 3 and pilosulin 4, from a species of the Australian ant genus *Myrmecia* in this study. Synthetic pilosulin 3 and pilosulin 4 peptides caused a significant and dose-dependent histamine release without releasing LDH from rat peritoneal mast cells at concentrations as low as 5 μM. Pilosulins 3 and 4 displayed antimicrobial activity against some bacteria species, although they did not show any hemolytic activities or antimicrobial activities against two yeast species at concentrations up to 50 μM. These antimicrobial activities of pilosulins 3 and 4 may partly explain the elaborate defensive systems of this ant species. Because we identified pilosulins 3 and 4 by RT-PCR, direct examination of the ant venom by LC/MS or immunological methods will be required in future study to further assess the physiological significance of pilosulins 3 and 4.

Many antimicrobial peptides, such as magainin, protegrin, and histatin analogues, have been examined in clinical trials [30]. To be clinically useful, they must be effective at a concentration lower than that toxic to humans. Pilosulin 4 showed antimicrobial activity comparable to that of magainin with low hemolytic activity. Pilosulins 3 and 4, however, may not be suitable for clinical use because of their histamine-releasing activity. The possibility that derivatives of pilosulins 3 and 4 might have clinically useful properties has not yet been excluded. Further studies are needed for the utilization.

Note added in proof

While this paper was in preparation, pilosulin 3 was identified by LC-MS and MS/MS analyses. Isoleucine at the N-termini of pilosulin 3 mature peptide is leucine in [31].

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