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Isolation and Characterization of Psacotheasin, a Novel Knottin-Type Antimicrobial Peptide, from *Psacothea hilaris*

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We report the isolation and characterization of a novel knottin-type antimicrobial peptide from the yellow-spotted long-horned beetle *Psacothea hilaris*. A cDNA encoding a 56-mer knottin-type propeptide was identified and its predicted molecular mass and pI were 5.92 kDa and 8.28, respectively. A 34-mer mature peptide was also selected and named herein as psacotheasin. The antimicrobial activity of chemically synthesized psacotheasin against human bacterial pathogens was subsequently investigated. The results showed that psacotheasin exerted potent activities against both Gram-positive and Gram-negative bacterial strains. The present study suggests that psacotheasin can be applied to develop novel therapeutic agents.

Keywords: *Psacothea hilaris*, psacotheasin, knottin-type peptide, antimicrobial peptide

Insects possess self-defense systems such as passive structural barriers and cellular and humoral immune responses. Among the humoral immune responses, the synthesis and secretion of humoral factors like antimicrobial peptides can protect insects against invading pathogens [4, 7]. So far, more than 200 antimicrobial peptides have been identified in insects. In particular, the insect antimicrobial peptides are classified into three groups: linear alpha-helical peptides devoid of cysteine residues, peptides in which proline and/or glycine residues are over-represented, and cysteine-rich peptides with a cysteine-established α-β motif [2].

Recently, a new class of antifungal peptides was also isolated from the coleopteran *Acrocinus longimanus*. These peptides display sequence similarities with four other antifungal peptides isolated from plants. A distinct characteristic of these peptides is that they show a knottin-like cysteine motif in their sequence. Le Nguyen *et al*. [8] first recognized a unique three-dimensional fold that featured a cysteine–knotted triple-stranded β-sheet. Based on this "knot-like" feature, they introduced the term "knottins" for this molecular scaffold. Knottins can bind to a diverse range of molecular targets to perform distinct functional activities but share a common scaffold composed of a small triple-stranded antiparallel β-sheet and disulfide bond framework with a C···C···CC···C···C consensus sequence and a 1–4, 2–5, and 3–6 pairing pattern.

In this study, a new knottin-type peptide, named herein as psacotheasin, from the yellow-spotted long-horned beetle *Psacothea hilaris* was isolated and its potential as an antibacterial peptide was suggested.

Immunization

To induce antimicrobial peptides from *P. hilaris*, its larvae were cooled on ice and individually injected with 4 μl of lipopolysaccharide (LPS) (Sigma, 0.5 mg/ml), which was suspended in a saline buffer. The larvae were kept at 25°C for 24 h and were then ground in liquid nitrogen.

ACP-Based GeneFishing PCR

Differentially expressed genes (DEGs) were screened by annealing control primers (ACP) based on PCR methods using the GeneFishing DEGs kits (Seegene). After this procedure, which involved the total isolation of RNA from final-instar larvae, RT–PCR was conducted using oligo dT-ACP to synthesize first-strand cDNAs from normal and immunized *P. hilaris*. Second-strand cDNAs were then synthesized, and subsequent PCR amplification was conducted with an arbitrary ACP and dT-ACP2.

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Table 1. Differentially expressed genes in LPS-immunized larvae of *P. hilaris.*

DEG No.	Putative identification	Species	Score	Identity (%)
GP8	NI[a]	-	-	-
GP15	NI	-	-	-
GP24	NI	-	-	-
GP41	NI	-	-	-
GP43	NI	-	-	-
GP48	NI	-	-	-
GP72	NI	-	-	-
GP89	NADH dehydrogenase	*Aedes aegypti*	52.8	74
GP99	NI	-	-	-
GP101	NI	-	-	-
GP102	Similar to CREG1 protein precursor	*Tribolium castaneum*	88.6	52
GP104	Similar to 13 kDa hemolymph protein	*Tribolium castaneum*	58.5	39
GP116	NI	-	-	-
GP120	Antimicrobial peptide Alo-2	*Acrocinus longimanus*	55.8	61

[a]Not identified.

Nucleotide Sequencing and Data Analysis

The nucleotide sequence was determined by using a BigDye Terminator cycle sequencing kit and an automated DNA sequencer (Model 310 Genetic Analyzer, Perkin-Elmer Applied Biosystems). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). GenBank, EMBL, and Swiss-Prot databases were searched for sequence homology using a BLAST algorithm program. The CLUSTAL W program was used to align the amino acid sequences of the isolated peptide. SignalP analysis was also employed to investigate the cleavage site for determining the potential signal peptide and mature peptide.

Identification and Characterization of Psacotheasin

As noted earlier, to identify the genes that were differentially expressed, mRNA expression profiling was done on the *P. hilaris* larvae, which were untreated or treated with LPS. Thereafter, mRNAs were extracted from the larvae that had or did not have LPS treatment, and they were then subjected to ACP RT–PCR analysis using a combination of 120 arbitrary primers (ACP_1-ACP_{120}) and oligo(dT) primer (dT-ACP2) (data not shown). After synthesizing cloning DNA from 14 DEGs, sequencing homology analysis was performed by doing a BLAST search. As a result, 4 known genes and 10 unknown genes were revealed (Table 1). Among the 14 DEGs that were identified, a cDNA that exhibited high homology with previously reported knottin-type antifungal peptides was revealed (Table 1). Therefore, 5' RACE–PCR was performed to obtain the full cDNA sequence of the novel knottin-type peptide. The results showed that the full-length cDNA sequence of the knottin-type peptide was 387 bp in length, having a 5' untranslated region (UTR) of 102 bp, a 3' UTR of 114 bp, and an open reading frame (ORF) of 168 bp encoding a polypeptide. The DNAs encoded a deduced propeptide of 56 amino acid residues having a predicted molecular mass of 5.92 kDa and a pI of 8.28. Moreover, SignalP analysis revealed that the cleavage site for the potential signal peptide was

```
tagtgattcgcggatcctgacgctgcgtttgctggctttgatgaaaacttatttcaacaa   60
gccgatttatcaaataacctctttatctaccaactcatcaaaatgaaattcttcagtatt  120
                                          M  K  F  F  S  I      6
Ttcttcatggttgttcttgcacttcttggtctgcaagacgcaaccgcttgcattgctaaa  180
 F  F  M  V  V  L  A  L  L  G  L  Q  D  A  T  A  C  I  A  K    26
gtaatggctgccaacctagcggagttcaaggcaactgttgttcaggacactgtcacaag   240
 G  N  G  C  Q  P  S  G  V  Q  G  N  C  C  S  G  H  C  H  K    46
gaaccaggctgggtagctggttactgcaaatgatttacccaactggcttattggatttat  300
 E  P  G  W  V  A  G  Y  C  K  *                               56
gtacccatatgtttttgtctctctgttgtttatcatgaatatataaaaataataaaaaaa  360
 tgtaactgtaaaaaaaaaaaaaaaaaa                                   387
```

Fig. 1. Nucleotide and deduced amino acid sequence of the knottin-type peptide isolated from *P. hilaris.*
The predicted amino acid sequence (single-letter abbreviation) is shown below the nucleotide within the open reading frame. Codons for ignition, termination, polyadenylation, and poly(**A**) tail are in bold font. The asterisk denotes a stop codon.

```
Alo-1   CIKNGNGCQPDGSQGNCCSRYCHKEPGWVAGYCR-   79%
Alo-2   CIANRNGCQPDGSQGNCCSGYCHKEPGWVAGYCR-   82%
Alo-3   CIKNGNGCQPNGSQGNCCSGYCHKQPGWVAGYCRRK  79%
Ps      CIAKGNGCQPSGVQGNCCSGHCHKEPGWVAGYCK--  ref
Mj-AMP1 CIGNGGRCNENVGPPYCCSGFCLRQPGQGYGYCKNR  44%
Mj-AMP2 CIGNGGRCNENVGPPYCCSGFCLRQPNQGYGVCRNR  35%
PAFP-S  CIKNGGRCNASAGPPYCCSSYCFQIAGQSYGVCKNR  38%
Me-AMP1 CIKNGKGCREDQGPPFCCSGFCYRQVGWARGYCKNR  47%
```

Fig. 2. Comparison with the known knottin-type antifungal peptides.
The six observed Cys(C) residues make up the disulfide bridges. The GenBank accession numbers for the analyzed sequences are *A. longimanus* Alo-1 (P83651), Alo-2 (P83652), Alo-3 (P83653), *M. jalapa* AMP1 (P25403), AMP2 (P25404), *M. crystallinum* AMP (O81338), and *P. americana* PAFP-S (P81418).

Table 2. Antibacterial activity of psacotheasin.

Bacterial strains		MIC (μM)	
		Psacotheasin	Melittin
Gram-positive	*E. faecium* (ATCC 29212)	25	1.56
	P. acnes (ATCC 6919)	12.5	0.78
	S. aureus (ATCC 25923)	25	1.56
Gram-negative	*E. coli* O-157 (ATCC 43895)	12.5	0.78
	P. aeruginosa (ATCC 27853)	25	1.56–3.125

predicted to be between 22-Ala and 23-Cys. Moreover, the putative mature protein of the knottin-type peptide was composed of 34 amino acids. The amino acid sequence of the mature portion exhibited a 79%, 82%, and 79% similarity to those of Alo-1, Alo-2, and Alo-3 from *A. longimanus*, and a 44%, 35%, 38%, and 47% similarity to those of Mj-AMP1 and Mj-AMP2 from *M. jarapa*, PAFP-S from *P. americana*, and Me-AMP1 from *M. crystallum*, respectively. This 34-mer novel peptide was herein named psacotheasin and its potential as an antimicrobial peptide was subsequently examined.

Peptide Synthesis

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [11]. The crude peptides were repeatedly washed with diethylether, dried in a vacuum, and purified by using reversed-phase preparative HPLC on a Waters 15 μm Deltapak C_{18} column (19×30 cm). The purity of the peptides was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (4.6×25 cm; Beckman, U.S.A.). The molecular mass of the synthetic peptides was determined by using MALDI mass spectrometry [5, 6].

Antimicrobial Susceptibility Testing

Three Gram-positive bacterial strains [*Enterococcus faecium* (ATCC 29212), *Propionibacterium acnes* (ATCC 6919), and *Staphylococcus aureus* (ATCC 25923)] and 2 Gram-negative bacterial strains [*Escherichia coli* O-157 (ATCC 43895) and *Pseudomonas aeruginosa* (ATCC 27853)] were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Bacterial cells (2×10^7 cells/ml) were inoculated into Mueller–Hinton broth, and 0.1 ml/well was dispensed into 96-well microtiter plates [10]. The MIC (minimum inhibitory concentration) was determined by a serial 2-fold dilution of the peptides, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [3]. After 24 h of incubation at 37°C, the minimal concentration of peptide required to prevent the growth of the given test organisms was determined and was defined as MIC. The growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, CA, U.S.A.) by monitoring the absorption at 620 nm [12]. In this study, melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), derived from the venom of the European honey bee *Apis mellifera*, was used as a positive control. This peptide is one of the most well-known antimicrobial peptides containing powerful lytic activity against both bacterial and eukaryotic cells [1]. Previously, it was also suggested that melittin exerted potent antibacterial activities, with a membrane-active mechanism, against pathogenic bacteria including clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and antibiotics-resistant *Pseudomonas aeruginosa* [9]. The results showed that all bacterial strains tested were susceptible to psacotheasin, with MIC values in the 12.5 to 25 μM range (Table 2). Although melittin exhibited more potent activities than psacotheasin, the results indicated that psacotheasin contained significant antibacterial activities, and therefore, this peptide can be employed as a model peptide to design or develop novel potent antibacterial peptides.

In summary, a novel 34-mer knottin-type peptide, psacotheasin, was isolated from *P. hilaris* and its antibacterial activity was investigated. The results showed that psacotheasin contained remarkable antibacterial activities against Gram-positive and Gram-negative bacterial strains. Although its antibacterial properties and mechanism of action require further clarification, this is the first study that has been conducted regarding the isolation and characterization of psacotheasin and its potential as an antimicrobial peptide.

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