J. Biochem. **135**, 297–304 (2004)
DOI: 10.1093/jb/mvh036

Perinerin, a Novel Antimicrobial Peptide Purified from the Clamworm *Perinereis aibuhitensis* Grube and Its Partial Characterization

Weidong Pan[1], Xianghui Liu[1], Feng Ge[*,1], Jie Han[2] and Tao Zheng[3]

[1]State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, P.R. China; [2]Metallorganic complex Laboratory, Institute of Chengdu Organic Chemistry, Chinese Academy of Sciences, Chengdu, P.R. China; and [3]Bioelectromagnetic Laboratory, Institute of Electrical Engineering, Chinese Academy of Sciences, Beijing, P.R. China

Received October 31, 2003; accepted December 15, 2003

A novel antimicrobial peptide was isolated and partially characterized from the homogenate of an Asian marine clamworm, *Perinereis aibuhitensis* Grube. This novel peptide, named Perinerin, was purified to homogeneity by heparin-affinity column and reverse-phase HPLC, and biologically tested with a MTS-PMS colorimetric assay. Perinerin consists of 51 amino acid residues and structurally appears to be highly basic and hydrophobic. It shows marked activity *in vitro* against both Gram-negative and Gram-positive bacteria and fungi, which indicates a bactericidal effect as well. Perinerin appears to be constitutively present and its sequence is novel among all other known antimicrobial peptides. These results suggest that Perinerin has the potential to serve as a convenient "evaluation marker" for studying alterations in the biochemistry of the host, particularly with respect to environmental changes. In addition, the MTS-PMS colorimetric assay examination of antimicrobial activity appears to be superior to existing methods and may offer more general application in the search for new antibiotic molecules.

Key words: antimicrobial peptide, host defence, MTS-PMS assay, *Perinereis aibuhitensis* Grube, perinerin.

Abbreviations: HPLC, high performance liquid chromatography; tricine-SDS-PAGE, tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI, matrix-associated laser desorption ionization; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PMS, phenazine methosulfate; MOPS, 3-*N*-morpho-linopropanesulfonic acid; DPBS, Dulbecco's phosphate buffered saline; MICs, minimal inhibitory concentrations.

A series of pioneering studies has established that antimicrobial peptides are important in the first line of the host defence system against invading microorganisms in many animal species (*1*, *2*). Many antimicrobial peptides show remarkable specificity for prokaryotes with low toxicity toward eukaryotic cells, and some of them may also have antiviral or antitumour properties (*3*, *4*). To date, more than 2,000 antimicrobial peptides have been identified, most of which have been isolated from a variety of invertebrate sources, including insects, ascidians, chelicerates, annelids and mollusks (*5–9*). This huge clade of animals, which contains more species than the rest of the living world together, is characterized by a far-reaching lack of acquired memory-type immunity based on clonally derived immunoglobulins or T lymphocyte subsets. Although these peptides show little similarity in sequence, they have, in general, some common features such as being highly cationic and tending to form an amphipathic α-helix structure that seems crucial for their functioning as membrane-active agents (*10*, *11*). In some cases, antimicrobial peptides exert their activities through interaction with DNA or RNA after penetrating into the cell membranes (*12*, *13*). Antimicrobial peptides rarely induce bacterial resistance, which represents a major problem with conventional antibiotics. This provides good reasons for the pursuit of prospective candidates for the development of new antibiotics.

Among invertebrates, annelids are an important group worthy of investigation due to their extensive inhabitation and enormous commercial significance. The coelomic fluid of the earthworm *Eisenia fetida andrei* has been found to exhibit a strong antimicrobial activity against Gram-negative and Gram-positive bacteria (*14*). This activity was reconfirmed by Pan *et al*. using a new colorimetric method on the same biological materials (*15*). Two small peptides with antimicrobial and antitumor activities were isolated and partially characterized from the earthworm *Eisenia fetida andrei* by Zhang *et al.* (*16*). The earthworm *Lumbricus rubellus* was investigated and found to contain a novel proline-rich antimicrobial peptide named lumbricin I (*8*). Although these findings are preliminary achievements to date, the urge for a broader understanding of the host defences of different species in this group of invertebrates at the biochemical and molecular levels appears more desirable than ever.

Another representative annelid species is the marine clamworm, which ecologically prevails from the upper tidal lands to the ocean as deep as 5,023 meters. Aside

[*]To whom correspondence should be addressed. Present address: P.O.Box 27, Institute of Zoology, 25 Beisihuan West Road, Beijing 100080, P.R.China. Tel: +86-10-62548093, Fax: +86-10-62565689, E-mail: gef@panda.ioz.ac.cn

from a particular interest in fibrinolysin-kinase, there are many other bioactive components identified from clamworm including collagens, glycerophospholipids, eicosapentaenoic acid and fibrinolytic enzymes, *etc.* (*17–19*). Previously, we investigated the antimicrobial and antitumor activities of the homogenate of *Marphysa sanguinea* (Polychaeta, Eunicidae) against a variety of bacteria and human liver cancer HepG2 cells (unpublished work). In contrast to insects, the activity identified in *Marphysa sanguinea* does not appear to require wound stimulation, bacterial injection, or exposure to bacterial cell wall components (*20*). So far, there has been no report on antimicrobial peptides in other clamworms. In the present study, we report a novel antimicrobial peptide purified from the homogenate of *Perinereis aibuhitensis* Grube, an Asian clamworm, which is used extensively as bait in fisheries and aquaculture. This peptide shows marked activity against both Gram-negative and Gram-positive bacteria and fungi, indicating a bactericidal effect as well. Importantly too, this constitutively specialized molecule may be used as an "evaluation marker" of the host defence vigour of invertebrates in future investigations.

MATERIALS AND METHODS

Peptide Purification—An antimicrobial peptide was purified from the marine clamworm, Perinereis aibuhitensis Grube (Polychaeta, Nereididae). Adult clamworms (~200 g), which were immersed in clean, mildly cold artificial sea water (2.7–3% marine salt) for 48 h to clear gastrointestinal metabolites and filth, were homogenized in 400 ml of 1% (v/v) trifluoroacetic acid, 1 M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and pepstatin A at 1 μg /ml using a domestic homogenizer. The homogenate was centrifuged at 100,000 ×*g* for 20min in a Himac CP70G (Hitachi, Tokyo) and the supernatant was collected. The peptides in the supernatant were then subjected to reverse-phase concentration using a Sep-Pak C_{18} cartridge (Waters Associates, Milford, USA), and the eluates were loaded onto a 1.6 × 2.5 cm Hitrap Heparin HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) for separation with a stepwise NaCl gradient of 0.5, 1 and 1.5 M. The active fractions were pooled and further purified by reverse-phase HPLC on a Delta Pak C_{18} column (Waters Associates, Milford, USA) with a linear gradient of 10–50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid over 90 min at a flow rate of 1 ml/min. The column effluent was monitored by its u.v. absorption at 225 nm, and the absorbance peaks were pooled and subjected to an antimicrobial activity assay after lyophilization. The purity of the antimicrobial peptide was analyzed by 16.5% tricine-SDS-PAGE according to Schägger and von Jagow (*21*) and matrix-associated laser desorption ionization (MALDI) mass spectroscopy (Kratos Kompact MALDI, Manchester, UK).

Test Microbes—Seven strains of Gram-negative bacteria, *Escherichia coli* K-12 (CGC6476), *Pseudomonas aeruginosa* (CGMCC 1.50), *Aeromonas hydrophila* (CGMCC 1.1814), *Proteus vulgaris* (ATCC 13315), *Psychrobacter immobilis* (NCIMB 308), *Salmonella typhimurium* (CGMCC 1.1190), and *Alcaligenes faecalis* (ATCC15554), and seven strains of Gram-positive bacteria, *Staphylococcus aureus* (CMCC (B) 26001), *Bacillus megaterium* (CGMCC 1.151), *Bacillus subtilis* (CMCC (B) 63003), *Arthrobacter* sp.(CGMCC 1.8), *Micrococcus luteus* (NCIMB 376), *Streptococcus pneumoniae* (CGMCC 1.1692), *Aerococcus viridans* (ATCC10040) and filamentous fungi *Paecilomyces heliothis* (CGMCC 3.3728), *Aspergillus nidulans* (CGMCC 3.1327) and *Neurospora crassa* (CGMCC 3.1598) were selected. Bacteria *E. coli* K-12 and *B. megaterium* and the fungus *P. heliothis* were used as the main test microbes. Other bacteria and fungi were used to determine the activity spectrum of the purified peptides. All bacteria were grown in sterile liquid nutrient broth (LNB) made of 10 g of pancreatic peptone, 3 g meat extract, and 5 g NaCl/liter of distilled water adjusted to pH = 7.0 and used during the exponential phase. The fungi were grown on potato dextrose agar (PDA) medium, and conidia were harvested as described previously (*22*). The bacterial (fungal) concentrations were finally modulated to an inoculum size of 1×10^6 to 5×10^6 CFU/ml in fresh sterile saline with 0.05% Tween 20. The inoculum size was confirmed by plating serial dilutions on corresponding medium plates.

Medium and MTS/PMS Preparation—RPMI 1640 medium (with L-glutamine; without phenol red and sodium bicarbonate) was purchased from GibcoBRL Life Technologies (Woerden, The Netherlands) and prepared according to Meletiadis *et al*. (*23*). RPMI 1640 medium buffered to pH 7.0 with 0.165 M 3-N-morpho-linopropanesulfonic acid (MOPS) was used throughout. The combined MTS/PMS solution was obtained with CellTiter 96®AQ_{ueous} MTS Reagent Powder (Promega Corporation, Madison, WI, USA) and phenazine methosulfate (PMS) (Sigma-Aldrich Chemie GmbH) dissolved in Dulbecco's phosphate buffered saline (DPBS) at a final concentration of 2.5 mg/ml for MTS and 312.5 μM for PMS. Further dilutions of MTS/PMS, if needed, were made in RPMI 1640 medium. Pancreatic peptone and meat extract for the preparation of liquid nutrient broth were obtained from Beijing Medium Manufacture Corporation (Beijing, China). All other reagents were of analytical grade.

Antimicrobial Assays and Determination of Minimal Inhibitory Concentrations (MICs)—Throughout the purification steps, antimicrobial activity was tested by a colorimetric assay according to Pan *et al*. (*15*), which highlights a novel tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), acting with the presence of an electron coupling reagent, phenazine methosulfate (PMS), on bacterial (fungal) suspensions in 96-well flat-bottom microtiter plates (Nunc F96 microtiter plates, Denmark). Lyophilized eluates were reconstituted in Dulbecco's PBS (pH 7.2) and adjusted to 20–50 μg/ml by dilution in RPMI 1640 medium. Each well of a 96-well microtiter plate was filled with 200 μl medium, 50 μl of a strain suspension diluted 1:50 in medium to obtain a final concentration of $1–2 \times 10^4$ CFU/ml, and 50 μl of eluate dilution or synthetic Cecropin A (50 μg/ml). The antimicrobial peptide of synthetic Cecropin A (50 μg/ml) was used as an activity reference (positive control) for the measurement of the relative activities of the purified peptides. The growth control consisted of 50 μl of the same Dulbecco's PBS dilutions in medium. Background

OD was determined with microbe-free wells processed in the same way as the bacteria containing wells. After 24 h incubation at 37°C with gentle stirring, 20 μl of MTS-PMS mixture was added to each well and the plates were incubated for a further 1 h. The OD at 490 nm of each well was then measured to quantify the antimicrobial activity of the isolated peptides. The relative ODs of each well based on measurements at 490 nm were calculated (as percent) using the following equation: (OD of antimicrobial dilution containing well – background OD)/(OD of growth control well – background OD of growth control well) × 100%, and the relative activity of each peptide was determined from the relative ODs of Cecropin A/relative ODs of the isolated peptides × 100%. The tests were performed in triplicate in three independent experiments for each microbe and data analysis was carried out using one-way ANOVA.

Minimal inhibitory concentrations (MICs) of the purified peptide against test microbes as described by Moore *et al*. (*24*) were improved. The lyophilized peptide was reconstituted in Dulbecco's PBS (pH 7.2), and the protein levels were determined using the method described by Bradford (*25*). Serial dilutions of the peptide were prepared in a 96-well microtiter plate, and the antimicrobial activity was estimated from the absorbance at 490 nm after incubation with the MTS-PMS mixture. The MIC values of the antimicrobial peptide were expressed according to Casteels *et al*. (*26*) as an interval, $a - b$, where a represents the highest concentration tested at which the bacteria (fungi) were still growing, and b is the lowest concentration that inhibits the cell growth.

Bactericidal Assay—The purified peptide (10 μl) at a concentration 10 times the MIC was incubated with 90 μl of an exponential-phase culture of *B. megaterium* or *P. aeruginosa* (10^4 CFU/ml each). Aliquots were removed at different times and plated on nutrient agar. The number of colony-forming units was counted after overnight incubation at 37°C. As a control, 10 μl of water was incubated with the bacterial culture.

Determination of the Primary Structure of the Antimicrobial Peptide—The molecular mass of the antimicrobial peptide was determined by matrix associated laser desorption (MALDI) mass spectroscopy (Kartos Kompact MALDI, England) according to the method of Park *et al*. (*27*). The lyophilized peptide was dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removing the solvent under warm air, the peptide adsorbed to the Pt probe was applied to a vacuum chamber and analyzed. Amino acid sequencing was performed by the automated Edman degradation method on a pulse liquid automatic sequencer (Applied Biosystems, model 473A). The composition of the peptide was determined by amino acid analysis. The lyophilized peptide was hydrolyzed with 6 M HCl at 110°C for 24 h and converted to its phenylthiocarbamyl derivative. The sample was then analyzed in a Beckman 121 MB amino acid analyzer (Fullerton, CA, USA).

Enzymatic Digestion of the Antimicrobial Peptide—Twenty micrograms of the antimicrobial peptide was digested with 1 μg *S. aureus* V8 protease (Roche Diagnostics, Switzerland) in 100 μl of 50 mM ammonium acetate (pH 4.0) containing 2 mM EDTA at 37°C for 18 h. The resulting fragments were separated directly by reverse-phase HPLC using the same column under identical conditions, and the antimicrobial activity of each fragment was examined.

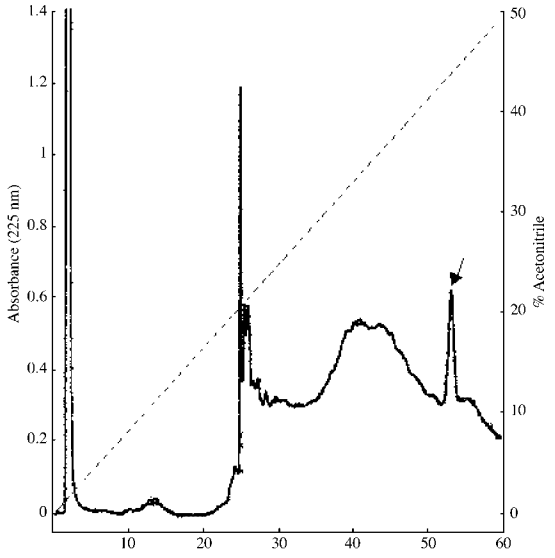

Fig. 1. **Purification of Perinerin by reverse-phase HPLC.** All experimental details are described in Materials and Methods. Briefly, active material from the homogenate was subjected to reverse-phase concentration, and the eluates were analyzed on a Hitrap Heparin HP column eluted with a stepwise NaCl gradient of 0.5, 1 and 1.5 M. The active fractions were pooled and further purified by reverse-phase HPLC with a linear gradient of 10–50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid over 90 min at a flow rate of 1 ml/min with u.v. detection at 225 nm. The peak containing Perinerin is indicated by the arrow.

Synthetic Peptide—A peptide corresponding to the antimicrobial peptide was chemically synthesized by standard fluoren-9-ylmethoxycarbony (Fmoc) chemistry on a Millipore 9050 Plus Pepsynthesizer with reagents supplied by the manufacturer. The product was purified by reverse-phase HPLC and its homogeneity was confirmed by tricine-SDS-PAGE, amino acid analysis and MALDI mass spectroscopy.

RESULTS

Purification of the Antimicrobial Peptide—Adult clamworms were homogenized and subjected to acidic extraction for antimicrobial peptides. After reverse-phase concentration and heparin-affinity fractionation, an active fraction was collected and further purified by reverse-phase HPLC (Fig. 1). Each peak was collected for antimicrobial activity assay and one peak that showed the strongest antimicrobial activity was pooled and lyophilized. The homogeneity of the purified peptide was assessed to be around 93% by tricine-SDS-PAGE (Fig. 2) and MALDI mass spectroscopy, and was named Perinerin. About 0.3 μg of the pure Perinerin was recovered from 1 g of clamworm. A 26-amino acid peptide and a 22-amino acid peptide were obtained from the 51-amino acid Perinerin after treatment with *S. aureus* V8 protease, and neither peptide showed detectable activity against test microbes.

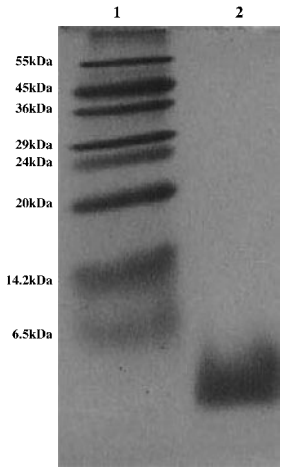

Fig. 2. **Tricine-SDS-PAGE of Perinerin.** Tricine-SDS-PAGE of purified Perinerin under reducing conditions was performed according to the method of Schagger and von Jagow (1987) using 4% stacking gels, 10% spacer gels and 16.5% separating gels. The gels were stained with Coomassie Brilliant Blue R-250. Lane 1 contains molecular mass marker proteins. Lane 2 shows Perinerin isolated by reverse-phase HPLC.

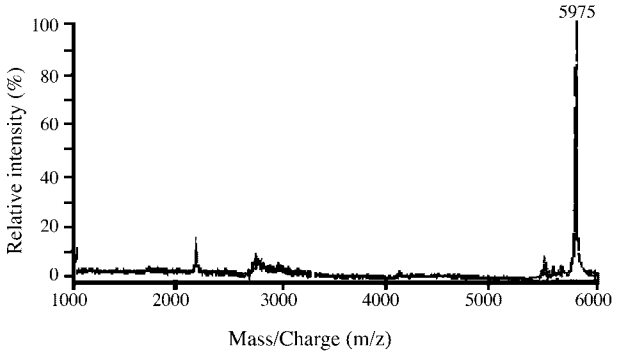

Fig. 3. **Mass spectroscopic analysis of Perinerin.** Mass of Perinerin (MH^+ = 5,975) as determined by MALDI mass spectroscopy.

Primary Structure Analysis—The molecular mass of Perinerin was determined by MALDI mass spectroscopy to be 5,975 (Fig. 3). After the amino acid composition of Perinerin was analyzed, the peptide was submitted to amino acid sequencing by the automated Edman degradation method on a pulse liquid automatic sequencer. A sequence of 24 amino acid residues was initially obtained. After enzymatic digestion of Perinerin with *S. aureus* V8 protease, the resultant fragments were purified directly by reverse-phase HPLC and their sequences were determined. The final complete sequence of Perinerin was determined to be Phe-Asn-Lys-Leu-Lys-Gln-Gly-Ser-Ser-Lys-Arg-Thr-Cys-Ala-Lys-Cys-Phe-Arg-Lys-Ile-Met-Pro-Ser-Val-His-Glu-Leu-Asp-Glu-Arg-Arg-Arg-Gly-Ala-Asn-Arg-Trp-Ala-Ala-Gly-Phe-Arg-Lys-Cys-Val-Ser-Ser-Ile-Cys-Arg-Tyr. Amino acid composition analysis showed Perinerin to have a highly charged sequence (Table 1). The molecular mass of the intact peptide, m/z = 5,975 from mass spectroscopy, is in good agreement with the average isotopic mass (MH^+) = 5,974.0 calculated from the sequence data, assuming that the four cysteines are engaged in two intramolecular disulfide bridges. The calculated pI of Perinerin is 10.45.

Table 1. **Whole protein composition analysis of Perinerin.**

Amino acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	23	51.94	45.10
Acidic (DE)	3	6.25	5.88
Basic (KR)	14	33.77	27.45
Polar (NCQSTY)	14	24.57	27.45
Hydrophobic (AILFWV)	14	26.15	27.45
A Ala	4	4.76	7.84
C Cys	4	6.90	7.84
D Asp	1	1.93	1.96
E Glu	2	4.32	3.92
F Phe	3	7.39	5.88
G Gly	3	2.86	5.88
H His	1	2.29	1.96
I Ile	2	3.79	3.92
K Lys	6	12.87	11.76
L Leu	2	3.79	3.92
M Met	1	2.20	1.96
N Asn	2	3.82	3.92
P Pro	1	1.62	1.96
Q Gln	1	2.14	1.96
R Arg	8	20.91	15.69
S Ser	5	7.28	9.80
T Thr	1	1.69	1.96
V Val	2	3.32	3.92
W Trp	1	3.12	1.96
Y Tyr	1	2.73	1.96
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
.Ter	0	0.00	0.00

Antimicrobial Activity and Mode of Action of the Purified Peptide and Synthetic Homologue—Antimicrobial activities of the purified peptide and synthetic Perinerin against test microbes were determined by colorimetric assay with a novel tetrazolium salt MTS plus the electron coupling reagent PMS on bacterial (fungal) suspensions in 96-well flat-bottom microtiter plates (Table 2). The results show that Perinerin has marked activity (MIC < 15 μg/ml) against *P. aeruginosa*, *B. megaterium*, and *A.viridans*, moderate activity against *E. coli* K-12, *S. aureus*, and *M. luteus*, and minor activity (MIC > 50 μg/ml) against *P. vulgaris*. Fungus *P. heliothis* was also killed at a MIC concentration of 25 μg/ml. Other test strains were not affected by Perinerin as the relative OD values exceeded 100%, indicating no antimicrobial activity available. The synthetic Perinerin showed activity similar to that of the native purified peptide except against *Arthrobacter* sp., for which minor activity (MIC > 50 μg/ml) was observed compared to that of the native Perinerin (Table 2).

To determine the mode of action of Perinerin, *B. megaterium* or *P. aeruginosa* (10^4 CFU/ml each) was incubated in the presence of Perinerin at a concentration 10 times higher than the MIC value (Table 3). Serial aliquots were incubated for 1, 3, and 10 min and 0.5, 1, 3, 10 h, after which the bacteria were plated on nutrient agar and the

Table 2. **Antimicrobial activity of purified and synthetic Perinerin against test microorganisms.**

Strains	Relative OD (%)[a]		Relative activity (%)[b]		Student-*t* test (*p* value)		MIC (μg/ml)[c]	
	P	S	P	S	P	S	P	S
Gram-negative								
E. coli K-12	25.34 ± 5.19	17.46 ± 5.28	127.83 ± 21.05	185.52 ± 23.69	0.003	0.011	12.5–25	12.5–25
P. aeruginosa	58.47 ± 6.21	71.05 ± 3.30	229.14 ± 37.40	188.57 ± 19.55	0.029	0.007	3.1–6.2	4.6–9.2
A. hydrophila	–[d]	–	–	–	–	–	–	–
P. vulgaris	89.63 ± 7.44	80.22 ± 13.57	86.25 ± 11.50	96.37 ± 8.43	0.046	0.028	50–100	50–100
P. immobili	–	–	–	–	–	–	–	–
S. typhimurium	–	–	–	–	–	–	–	–
A. faecalis	–	–	–	–	–	–	–	–
Gram-positive								
S. aureus	36.27 ± 14.02	28.15 ± 10.78	311.49 ± 42.33	401.34 ± 51.29	0.041	0.047	25–50	25–50
B. megaterium	67.34 ± 24.07	84.10 ± 16.65	148.22 ± 55.17	118.68 ± 41.02	0.012	0.032	1.5–3.1	2.5–5.0
B. subtilis	–	–	–	–	–	–	–	–
Arthrobacter sp.	–	94.82 ± 17.24	–	23.53 ± 8.55	–	0.007	–	50–100
M. luteus	90.56 ± 12.30	79.20 ± 17.32	305.39 ± 27.15	349.19 ± 21.76	0.032	0.014	25–50	25–50
S. pneumoniae	–	–	–	–	–	–	–	–
A. viridans	15.75 ± 1.43	16.09 ± 1.37	266.51 ± 18.27	260.88 ± 22.03	0.047	0.003	6.2–12.5	8.5–17
Fungi								
P. heliothis	65.42 ± 8.29	83.45 ± 19.21	370.11 ± 64.35	290.14 ± 44.87	0.007	0.031	12.5–25	12.5–25
A. nidulans	–	–	–	–	–	–	–	–
N. crassa	–	–	–	–	–	–	–	–

[a]Relative OD = (OD of Perinerin containing well – background OD)/(OD of Perinerin-free well – background OD of Perinerin-free well) × 100%. [b]Relative activity of Perinerin = Relative OD of Cecropin A/Relative OD of Perinerin × 100%. An antimicrobial peptide of synthetic Cecropin A (50 μg/ml, 50 μl/well) was used as an activity reference (positive control) for the measurement of the relative activity of Perinerin. See "MATERIALS AND METHODS" for details. [c]Minimal inhibitory concentrations were determined with serial dilutions of Perinerin in a 96-well microtiter plate, and are expressed as an interval, $a - b$, where a represents the highest concentration tested at which bacteria (fungi) still grow and b is the lowest concentration that inhibits the cell growth. (d) Relative OD ≥ 100% indicates no antimicrobial activity. "P" stands for purified Perinerin and "S" stands for synthetic Perinerin.

colony-forming units counted after 24 h. The results show that although less than 3 min contact with the peptide is sufficient to kill Gram-positive bacteria, it requirs 1 h to kill Gram-negative bacteria. Thus, Perinerin appears to be a potent and relatively rapid bactericidal compound.

Compared to synthetic Cecropin A, Perinerin displays more potency against a wide spectrum of microbes, as the relative activities were higher than 100% in most cases (except for *P. vulgaris*). At physiological concentrations, Cecropin A is active only against bacteria, whereas Perinerin is active against both bacteria and fungi, which may be inferred from the value of the relative activity against *P. heliothis* (370.11).

DISCUSSION

This paper confirms the presence of a novel type of antimicrobial peptide in the homogenate of Asian marine clamworm *Perinereis aibuhitensis* Grube that acts against Gram-positive and Gram-negative bacteria and fungi. To our knowledge, this is one of the first reports on antimicrobial molecules from marine annelid sources. This peptide, like other antimicrobial peptides, is highly basic and hydrophobic, and higher basicity tends to correlate with higher antimicrobial activity (*28*, *29*). The peptide has an unusual sequence that appears to be novel among all known antimicrobial peptides listed in the Swiss-Prot and TrEMBL databases. We propose the name "Perinerin" for this novel peptide.

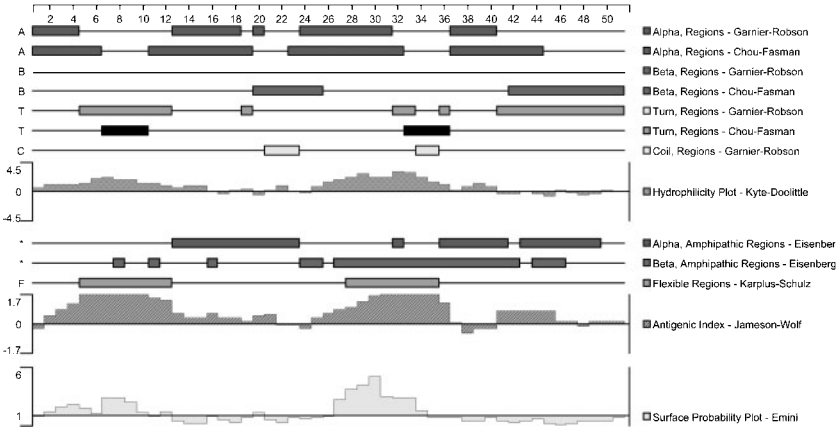

Fig. 4. **Secondary structural analysis of Perinerin.** Secondary structural analysis of Perinerin was predicted by the Protean module of the Lasergene sequence analysis software suite. Diverse structure matches were aligned according to each individual prediction model.

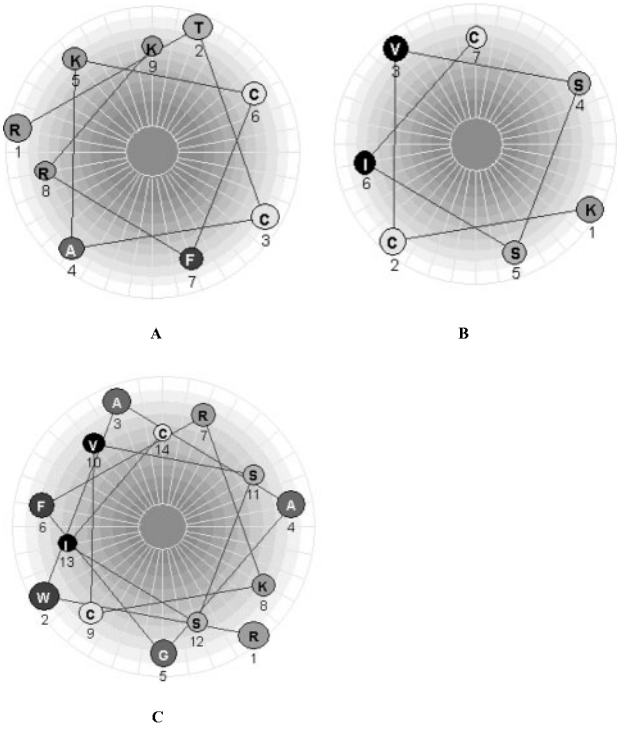

Fig. 5. **Helical wheel projection of predicted amphipathic segments of Perinerin.** Helical wheel projection of the segments of Perinerin residues was analyzed by the software module of Protean. (A) Residues 11–19 of Perinerin. (B) Residues 43–49 of Perinerin. (C) Residues 36–49 of Perinerin.

It seems that the whole Perinerin sequence is needed for its antimicrobial activity because the 26-amino acid peptide and 22-amino acid peptide fragments produced from Perinerin after treatment with *S. aureus* V8 protease were not as active against test microbes as the

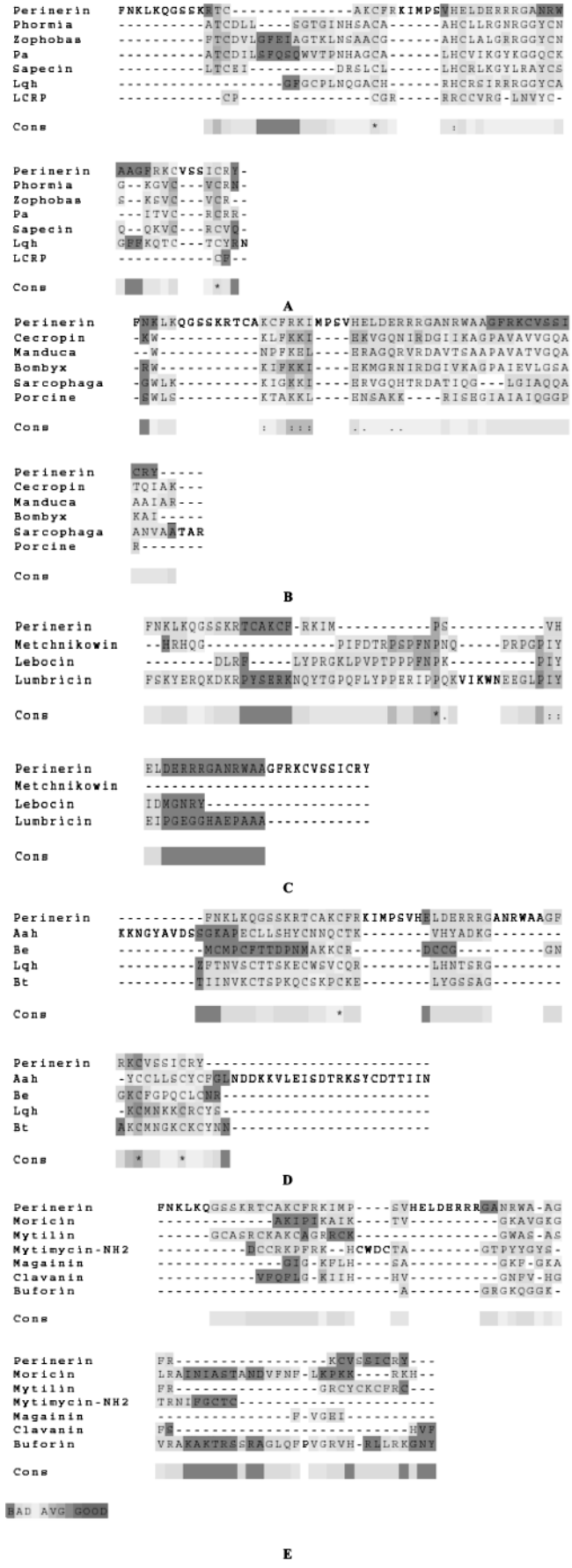

Fig. 6. **Multiple sequence alignments of Perinerin with antimicrobial peptides of diverse invertebrate orgins.** Multiple sequence alignments between Perinerin and diverse antimicrobial peptides of invertebrate orgin were produced using T-Coffee 1.41 with default parameters and requesting the score_pdf output option. The color scale goes from blue (CORE = 0, bad) to red (CORE = 9, good), indicating a gradual increase in consistency score. (A) Alignment of Perinerin with defensins from the dipteran *Phormia terranovae* (Phormia) and *Sarcophaga peregrina* (Sapecin), the coleopteran *Zophobas atratus* (Zophobas), the hemipteran *Pyrrhocoris apterus* (Pa), the scorpion *Leiurus quinquestriatus hebroeus* (Lqh) and the lamprey *Petromyzon marinus* (LCRP). (B) Alignment of Perinerin with cecropin sequences from the lepidopteran *Hyalophora cecropia* (Cecropin), *Manduca sexta* (Manduca) and *Bombyx mori* (Bombyx), the dipteran *Sarcophaga peregrina* (Sarcophaga) and pig intestine (Porcine). (C) Alignment of Perinerin with proline-rich peptides from the dipteran *Drosophila melanogaster* (Metchnikowin), the silkworm *Bombyx mori* (Lebocin) and the earthworm *Lumbricus rubellus* (Lumbricin). (D) Alignment of Perinerin with the scorpion toxins acting on Na^+ channels including *Androctonus australis* insectoxin (Aah) and *Buthus eupeus* toxin I5A (Be) and the scorpion toxins acting on K^+ channels including *Leiurus quinquestriatus hebroeus* charybdotoxin (Lqh ch) and *Butus tamulus* iberiotoxin (Bt). (E) Alignment of Perinerin with other types of antimicrobial peptides from the silkworm *Bombyx mori* (Moricin), the mollusc *Mytilus edulis* (Mytilin and Mytimycin-NH_2), the skin of *Xenopus laevis* (Magainin), the tunicate *Styela clava* (Clavanin) and the stomach of the Asian toad *Bufo bufo gargarizans* (Buforin).

Table 3. **Bactericidal effects of Perinerin on *B. megaterium* and *P. aeruginosa*.**

Time	CFU (*B. megaterium*)		Time	
	30 μg/ml	Control	60 μg/ml	Control
1 min	25	25	44	45
3 min	0	26	41	51
10 min	0	31	37	46
0.5 h	0	54	15	68
1 h	0	76	0	77
3 h	0	93	0	109
10 h	0	112	0	134

Ninety microtiter aliquots of exponential-phase cultures of *B. megaterium* or *P. aeruginosa* (each 10^4 CFU/ml) were incubated with 10 μl Perinerin (or water as a control) at concentration 10 times the MICs (30 μg/ml or 60 μg/ml, respectively). Aliquots were removed at different time intervals and plated on nutrient agar. The number of colony-forming units was counted after overnight incubation at 37°C. Results are expressed as 10^6 CFU/ml.

intact Perinerin. Based on secondary structure analysis, Perinerin is predicted to contain three amphipathic structure motifs (residues 13–23, 36–41 and 43–49) based on the Eisenberg model (Fig. 4). The α–helical wheel projection of amino acid residues 11–19 and 43–49 of Perinerin, shown in Fig. 5, A and B, results in a distinct arrangement of hydrophobic and hydrophilic faces. According to the method of Schiffer and Edmundson (*30*), protein segments may have thermodynamic stability by themselves, and at the same time have the capability to form interhelical interactions in the protein when a cluster of at least three hydrophobic residues, called a stabilization arc, is on the same side of the helical wheel and located in the n, $n \pm 3$, $n \pm 4$ type positions. In the case of residues 11–19 and 43–49 of Perinerin, Thr, Cys, Cys, Phe and Ala (positions 2, 6, 3, 7 and 4 on the wheel) and Cys, Ile, Val, and Cys (positions 2, 6, 3, and 7 on the wheel), respectively, fall on perfect hydrophobic arcs. This potential for helicity, combined with distinct polar arcs, suggests the existence of amphipathic a-helices in these regions. Moreover, residues 36–49 of Perinerin as a whole segment still assume an amphipathic arrangement (Fig. 5C), except that the helical wheel projection shows a breakdown in amphipathicity at Ala-39 (position 4 on the wheel), which argues for a deviation from an ideal helical conformation at this residue.

The overall impression obtained from examining the sequence composition is that Perinerin has no particular amino acid(s) richness in contrast with proline-rich metchnikowin, apidaecins, drosocin, lumbricin I or cysteine-rich insect defensins such as *Phormia* A, *Zophobas* B and sapecin B. We were intrigued by this situation, and in the course of a broad range phylogenetic studies on antimicrobial peptides in invertebrates, multiple sequence alignments between Perinerin and several representative species of invertebrate origin, such as defensins, cecropin sequences, and proline-line sequences, *etc.*, were performed using T-Coffee (version 1.41) (Fig. 6) and ClustalW (version 1.83) (Table 4) programs. Our data strongly argue that the average level of identity measured between Perinerin and other antimicrobial peptides is less than 30% (Table 4), and the two closest pair-wise sequences of Perinerin *vs* LCRP (lamprey corticosatin-related peptide) are only 21% identical. Although only four cysteine residues exist in the sequence of Perinerin, which were assumed to constitute two intramolecular disulfide bridges, these cysteines and even adjoining residues, as shown in Fig. 6, A and D, are generally conserved well at the sequence level. Interestingly too, Perinerin shares two KC motifs with the sequences of scorpion toxins, which act on Na^+ or K^+ channels (Fig. 6D); this may enable deeper exploration of the correlations between the two kinds of channels.

We had used bacterial challenge or the injection of bacterial surface molecules, such as lipopolysaccharide (LPS) and peptidoglycan (PG), to elicit immune reactions in clamworm *Perinereis aibuhitensis* Grube. Nevertheless, we have no indication that immunizing clamworms by bacterial challenge or exposure to bacterial cell wall components has modified the spectrum or the concentration of the native Perinerin. As Perinerin was isolated and purified directly from the homogenate of the clamworm *Perinereis aibuhitensis* Grube without such preinduction processes as live bacterial injection, it appears to be, unlike its counterparts in insects, constitutively present. It is well known that insect defensins are effective against Gram-positive bacteria and cecropins are bactericidal ionophores that act upon the bacterial plasma membrane (*31*). Proline-rich antimicrobial peptides, such as apidaecins, bactenecins and PR-39, kill only Gram-negative bacteria. Drosocin kills both Gram-positive and Gram-negative bacteria, but is inactive against fungi. Metchnikowin is active against Gram-positive bacteria and fungi, but inactive against Gram-negative bacteria. In the case of Perinerin, its exact action mode is not understood at present, however, its broad activity spectrum and its consistent presence at higher concentrations suggests that it has the potential to serve as a convenient "evaluation marker" for studying alterations in the biochemistry of the host, particularly with respect to environmental changes.

In conclusion, our study indicates that the host defense of marine clamworms, like that of arthropods, involves the synthesis of both antibacterial and antifungal, small sized, cationic peptides. Even though we did not confirm the actual biological role of Perinerin in *vivo*, it is tempting to propose that its constitutive presence as an antimicrobial peptide is an ancient situation in invertebrate host defense. Moreover, the antimicrobial activity assay

Table 4. **Multiple sequence alignments of Perinerin and antimicrobial peptides of invertebrate origin.**

Percent identity	Defensins	Cecropins	Proline-rich sequences	Scorpion toxins	Other types
Average percent identity	26	25	15	19	13
Maximum percent identity	55	68	23	48	30
Minimum percent identity	6	5	3	5	3

The multiple sequence alignments of Perinerin with other known antimicrobial peptides of invertebrate origin were performed by the ClustalW (1.83) program.

employed in this study is believed to be superior to existing methods and may have more general application in the search for new antibiotic molecules. Future works will focus on elucidating the cellular localization and biological role of Perinerin *in vivo*.

We are grateful to Dr. Li Du for providing valuable information on bioinformatics. We also thank Drs. Liping Qi and Yan Song for providing valuable help in performing tricine-SDS-PAGE. The technical assistance of Dr. Rui Wang in demonstrating reverse-phase HPLC is also appreciated. This work was supported by the Chinese Academy of Sciences Innovation Program "No.KSCX3-IOZ-04 and No.KSCX2-SW-103" through State Key Laboratory of Integrated Management of Pest Insects & Rodents at the Institute of Zoology, Chinese Academy of Sciences. The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL databases under the accession number P83551 for Perinerin in *Perinereis aibuhitensis* Grube (Clamworm).

REFERENCES

1. Boman, H.G. (2003) Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.* **254**, 197–215
2. Boman, H.G. (2000) Innate immunity and the normal microflora. *Immunol. Rev.* **173**, 5–16
3. Zhang, L.Q., Yu, W.J., He, T., Yu, J., Caffrey, R.E., Dalmasso, E.A., Fu, S.Y., Pham, T., Mei, J.F., Ho, J.J., Zhang, W.Y., Lopez, P., and Ho, D.D. (2002) Contribution of human a-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* **298**, 995–1000
4. Chen, Y.X., Xu, X.M., Hong, S.G., Chen, J.G., Liu, N.F., Underhill, C.B., Creswell, K., and Zhang, L.R. (2001) RGD-Tachyplesin inhibits tumor growth. *Cancer Res.* **61**, 2434–2438
5. Otvos, L. Jr. (2000) Antibacterial peptides isolated from insects. *J. Pept. Sci.* **6**, 497–511
6. Jang, W.S., Kim, K.N., Lee, Y.S., Nam, M.H., and Lee, I.H. (2002) Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. *FEBS Lett.* **521**, 81–86
7. Schnapp, D., Kemp, G.D., and Smith, V.J. (1996) Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* **240**, 532–539
8. Cho, J.H., Park, C.B., Yoon, Y.G., and Kim, S.C. (1998) Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. *Biochim. Biophys. Acta* **1408**, 67–76
9. Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A., and Bulet, P. (1996) Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *J. Biol. Chem.* **271**, 21808–21813
10. Bechinger, B., Zasloff, M., and Opella, S.J. (1992) Structure and interactions of magainin antibiotic peptides in lipid bilayers: a solid-state nuclear magnetic resonance investigation. *Biophys. J.* **62**, 12–14
11. Mor, A. and Nicolas, P. (1994) The NH2-terminal alpha-helical domain 1–18 of dermaseptin is responsible for antimicrobial activity. *J. Biol. Chem.* **269**, 1934–1939
12. Park, C.B., Kim, H.S., and Kim, S.C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* **244**, 253–257
13. Boman, H.G., Agerberth, B., and Boman, A. (1993) Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect. Immun.* **61**, 2978–2984
14. Lassègues, M., Roch, P., and Valembois, P. (1989) Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: Evidence, induction, and animal protection. *J. Invertebr. Pathol.* **53**, 1–6
15. Pan, W.D., Liu, X.H., Ge, F., and Zheng, T. (2003) Reconfirmation of antimicrobial activity in the coelomic fluid of the earthworm *Eisenia fetida andrei* by colorimetric assay. *J. Biosci.* **28**, 723–731
16. Zhang, X.C., Sun, Z.J., Zhuo, R.P., Hou, Q.M., and Lin, G.Q. (2002) Purification and characterization of two antibacterial peptides from *Eisenia fetida* (in Chinese). *Prog. Biochem. Biophys.* **29**, 955–960
17. Kimura, S. (1972) Studies on marine invertebrate collagens. 5. The neutral sugar compositions and glycosylated hydroxylysine contents of several collagens. *Bull. Jap. Soc. Sci. Fish.* **38**, 1153–1161
18. Tanaka, R., Hashimoto, A., Noda, N., and Miyahara, K. (1995) Two 1-alkyl-2-acyl choline glycerophospholipids having an arachidonoyl or eicosapentaenoyl group, from the clamworm (*Marphysa sanguinea*). *Chem. Pharm. Bull. (Tokyo)* **43**, 156–158
19. Liu, X.H., Ge, F., and Pan, W.D. (2002) Analysis of nutrient composition from tissues of *Perinereis abibuhitensis* (in Chinese). *Chin. J. Mar. Drugs* **21**, 35–37, 41
20. Chisholm, J.R. and Smith, V.J. (1995) Comparison of antibacterial activity in the hemocytes of different crustacean species. *Comp. Biochem. Physiol. A Physiol.* **110**, 39–45
21. Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379
22. Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Vanderleyden, J. (1990) An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* **69**, 55–60
23. Meletiadis, J., Mouton, J.W., Meis, J.F.G.M., Bouman, B.A., Donnelly, J.P., Verweij, P.E., and Eurofung Network. (2001) Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. *J. Clin. Microbiol.* **39**, 3402–3408
24. Moore, K.S., Bevins, C.L., Brasseur, M.M., Tomassini, N., Turner, K., Eck, H., and Zasloff, M. (1991) Antimicrobial peptides in the stomach of *Xenopus laevis*. *J. Biol. Chem.* **266**, 19851–19857
25. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
26. Casteels, P., Ampe, C., Jacobs, F., and Tempst, P. (1993) Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J. Biol. Chem.* **268**, 7044–7054
27. Park, C.B., Kim, M.S., and Kim, S.C. (1996) A novel antimicrobial peptide from *Bufo bufo gargarizans*. *Biochem. Biophys. Res. Commun.* **218**, 408–413
28. Hoffmann, J.A. and Hetru, C. (1992) Insect defensins: inducible antibacterial peptides. *Immunol. Today* **13**, 411–415
29. Gabay, J.E. (1994) Ubiquitous natural antibiotics. *Science* **264**, 373–374
30. Schiffer, M. and Edmundson, A.B. (1967) Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. *Biophys. J.* **7**, 121–135
31. Christensen, B., Fink, J., Merrifield, R.B., and Mauzerall, D. (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc. Natl Acad. Sci. USA* **85**, 5072–5076