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Short Communication

Purification of a Novel Antibacterial Short Peptide in Earthworm *Eisenia foetida*

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Abstract A novel antimicrobial short peptide was purified from earthworm (*Eisenia foetida)* by a five-step protocol including ammonium sulfate precipitation, ultrafiltration, DE-52 ion exchange chromatography, Sephadex G-10 column chromatography, and C-18 reversed-phase HPLC techniques. The purified peptide was applied to the MALDI-TOP MS to determine the molecular mass and was also subjected to TOF MS-MS analysis to determine the amino acid sequence. As a result, a novel antibacterial peptide, named OEP3121, was obtained, with the molecular mass of 510.8 Da and the sequence being "ACSAG".

Key words antibacterial peptide; earthworm; purification

Earthworms live in an environment with abundant pathogens. These pathogens are, firstly, bacteria living in water or soil that are ingested during feeding or introduced into the body following injury. Parasites, particularly larval forms, which represent the dissemination phase, are another important group of potentially pathogenic agents. During the course of evolution, earthworms have developed defense strategies against these living pathogens [1,2]. Earthworms lack true antibodies and hence an adaptive immune response, and instead have efficient innate immune systems to defend themselves against invading foreign materials [3]. In living organisms, peptides are an important defense component. Many peptides were found in various living organisms [4–6]. Therefore, it can be supposed that earthworms living in the pathogen-abundant environment must have peptides against bacteria.

The purpose of this study was to find out some novel antibacterial peptides in earthworm in order to make foundation for the chemotherapy of inflammation disease and for the mechanism of earthworm immunity.

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Materials and Methods

Animals and bacterial strains

The experiments were done with earthworm *Eisenia foetida*. All the earthworm used herein came from our laboratory breeding and were nearly at the same age.

Bacteria strains used for determining antimicrobial activity included *E. coli*, *S. aureus*, and *Pseudomonas aeruginosa*, which were obtained from the microbial research laboratory, College of Veterinary Medicine, China Agricultural University. For these bacteria Luria-Bertani (LB) medium was used as growth medium.

DE-52 was purchased from Whatman, Sephadex G-10 from Amersham Pharmacia, and trifluoracetic acid made from Merck-Schuchardt. Chromatography purity methanol and all the other chemical purity chemicals were made in China.

Coelomic fluid extruding and tissue homogenation

Earthworms were washed, dried in soft paper, and then excited with a 5 V stimulation, which induced them to extrude coelomic fluid through epidermal dorsal pores. Then the stimulated earthworms were washed with pH 6.8 PBS (5 mM) twice and dried in soft paper. At last, the earthworms were homogenized with a tissue blender

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in 2 fold volume pH 6.8 PBS (5 mM) containing 1% EDTA (4 mM) and 1% 2-mercaptoethonal (5 mM). The homogenate was centrifuged at 10,000 r/min for 30 min in a centrifuge (Backman) at 4 °C and the supernatant was collected.

Ammonium sulfate precipitation and ultrafiltration

The supernatant was precipitated with ammonium sulfate at a final saturation of 85% in a cooling bath on top of a magnetic stir plate and kept in a refrigerator overnight at 4 °C followed by centrifugation at 10,000 r/min for 30 min at 4 °C. The precipitate was collected and dissolved in pH 6.8 PBS (5 mM), then ultrafiltrated by 1 kD and 10 kD molecular weight cut-off ultrafiltration membranes (Shanghai Atomic Nucleus Institute, Shanghai Institute of Applied Physics, CAS). The components with a molecular weight over 10 kD and with a molecular weight between 1–10 kD were freeze dried, and kept at –30 °C. What passed through the 1 kD membrane was also freeze dried and dissolved in methanol, then centrifuged to collect the supernatant. The peptides <1 kD were obtained by evaporating the methanol in a vacuum condition. At last, the antimicrobial activities of the three components were tested using disk method.

Antibacterial assays

Disk method Bacterial cells were grown overnight in Luria-Bertani media and inoculated into 5 ml of molten 0.6 g/L Luria-Bertani agar with a final concentration of 10^7 colony-forming units per ml, which was overlaid onto a 90-mm Petri dish containing 10 ml of 2 g/L Luria-Bertani solidified agar. After the top agar hardened, sterilized blotting paper (about 6 mm in diameter), free from any antibacterial activity, were impregnated with 20 μl of the fractions to be tested and placed on agar dishes inoculated with one bacterial strain. The dishes were incubated overnight at 37 °C. Control tests were performed with paper impregnated with PBS. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 6-mm papers.

Purification of the peptide

DE-52 ion exchange chromatography The most active fraction was loaded onto a DE-52 column (1.6 cm×30 cm) previously equilibrated with PBS (5 mM, pH 8.0) containing 10 μM EDTA. After washing with PBS (5 mM, pH 8.0) until the UV absorbance returned to baseline, the absorbed peptides were eluted with a linear gradient of NaCl (0–500 mM) in PBS (5 mM, pH 8.0) at a flow rate of 24 ml/h. The elution profile was monitored at 220 nm. The fractions from each peak were pooled and lyophilized to dry, then desalted with methanol as described above. The peptides in the methanol were dried with a vacuum pump, and stored at –30 °C. The antibacterial activity of the fractions both unabsorbed and eluted fractions by NaCl solution were evaluated using disk method.

Gel filtration Fractions with antibacterial activity after DE-52 ion exchange chromatography were further purified using a column of Sephadex G-10 (2 cm×100 cm). Dissolve 250 mg of the active fraction in 10 ml of 50% methanol (*V*/*V*). Load the active fraction gently onto the surface of column. After absorption, add 3 ml of 50% methanol (*V*/*V*) and gently wash the wall without disturbing the column bed. Then elute with 50% methanol at a flow rate of 18 ml/h, monitor the absorbance at 220 nm, and collect the fractions and lyophilize to dry. The antibacterial activity of the fractions was tested again using the disk method.

HPLC purification of the peptide The active fraction after the Sephadex G-10 gel filtration was further purified by high-performance liquid chromatography (HPLC) on a 3.9 mm×300 mm Delta Pak C18 column (Millipore) connected to an Agilent 1100 HPLC system with a simple linear gradient from 0.1% (*V*/*V*) trifluoracetic acid (TFA) to 70% acetonitrile + 0.1% TFA (*V*/*V*) at a flow rate of 0.8 ml/min at ambient temperature. The elution pattern was monitored at 220 nm. The biggest peak were collected, lyophilized to dry, and assayed for antibacterial activity using disk method.

Purity determination and molecular weight determination

The purity of the active fraction after HPLC was assessed by C18 reversed phase HPLC eluted with 70% methanol, 30% water, at a flow rate of 0.8 ml/min, detected at 220nm. The exact molecular mass of the purified peptide was determined by a matrix-associated laser adsorption ionization (MALDI) mass spectroscopy. Approximately 20 nmol of the lyophilized peptide was dissolved in 50% acetonitrile (*V*/*V*) containing 7% sinapinic acid (*W*/*V*) and mixed with a Pt probe. After removing the solvent in warm air, the peptide, adsorbed to the Pt probe, was applied to a vacuum chamber and analyzed.

Amino acid sequence determination of the isolated peptide

The amino acid sequencing of the purified peptide was performed by MS-MS at the Academy of Military Medical Sciences.

Results

In the first isolation step, after ultrafiltration by 1 kD and 10 kD molecular weight cut-off membranes, three components, >10 kD, 1–10 kD, and <1 kD in molecular mass, were obtained especially. The most active component was one with molecular mass less than 1 kD. This component was subjected to further fractionation in the DE-52 ion exchange chromatography. A chromatogram obtained was shown in Fig. 1. The most active component was in the third fraction, which was not absorbed by the column. The most active fraction eluted in the second peak during Sephadex G-10 gel filtration was subjected to C18 reversed-phase HPLC, yielding one main fraction with retention time of 3.7 and 4.1 min (Fig. 2). The purity

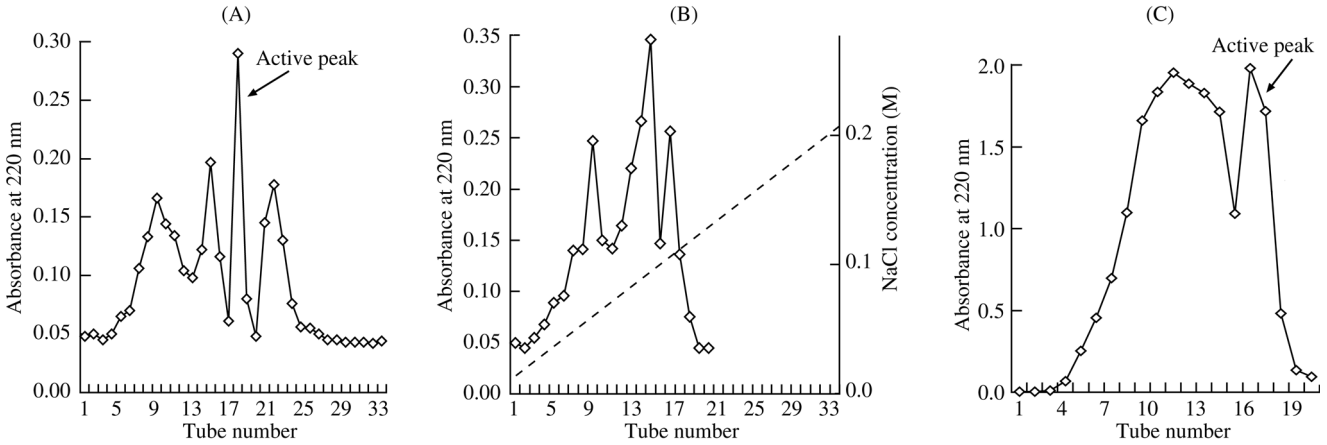

Fig. 1 Purification of the peptide from earthworm *Eisenia Foetida*

(A) The most active component under 1 kD was applied to a DE-52 column (1.6 cm×30 cm) was not absorbed by the column. (B) The most active component under 1 kD was applied to a DE-52 column (1.6 cm×30 cm) eluted with a linear gradient elution of 0.5 M NaCl. (C) Elution pattern of the active fraction after DE-52 was loaded on Sephadex G-10 gel filtration chromatography.

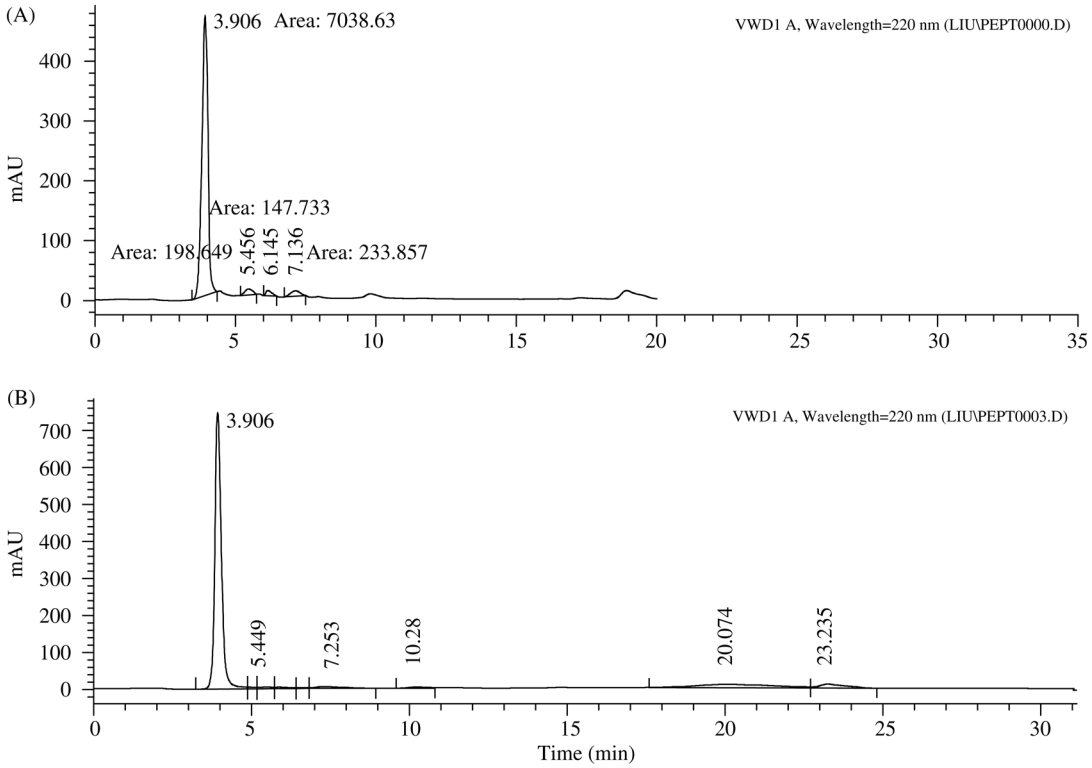

Fig. 2 Purification and purity detection of the peptide by C18 HPLC

of the peptide was determined by HPLC, and detected as a single peak with retention time of 3.7 and 4.1 min (Fig. 2).

The molecular mass of the purified peptide was 510.8 Da, as determined by MLADI-TOF MS (Fig. 3). The amino acid sequence of the purified peptide was ACSAG, as detected by MS-MS (Fig. 4). This novel antimicrobial peptide purified from earthworm (*Eisenia foetida*) is provisionally named OEP3121 according to original serial numbers.

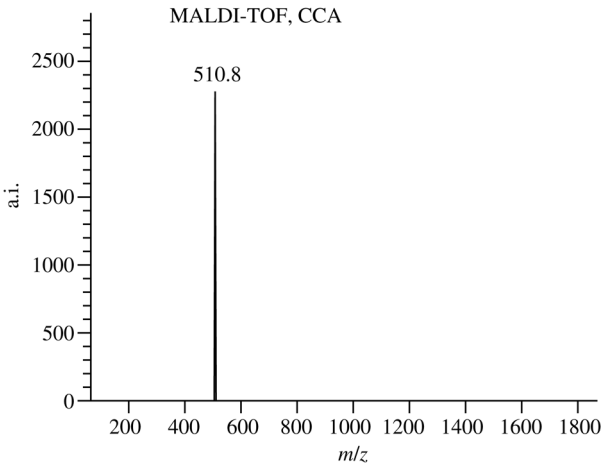

Fig. 3 MALDI-TOF mass spectrum of OEP3121

Discussion

Antimicrobial peptides constitute a very important component of the innate immune system in organisms across the evolutionary scale [7–15]. During the past 2 decades, living organisms of all types have been found to produce a large repertoire of gene-encoded antimicrobial peptides that play an important role in innate immunity to microbial invasion. More than 824 such peptides from a variety of eukaryotic species were listed on databases (see, for example, http://www.bbcm.univ.trieste.it/~tossi/), although a few have been described from earthworm.

The purification and partial characterization of a novel peptide, OEP3121, from earthworm (*Eisenia foetida*) have been described in this study. This peptide is composed of only 5 residues that do not have primary structural homology to any of the known antimicrobial peptides

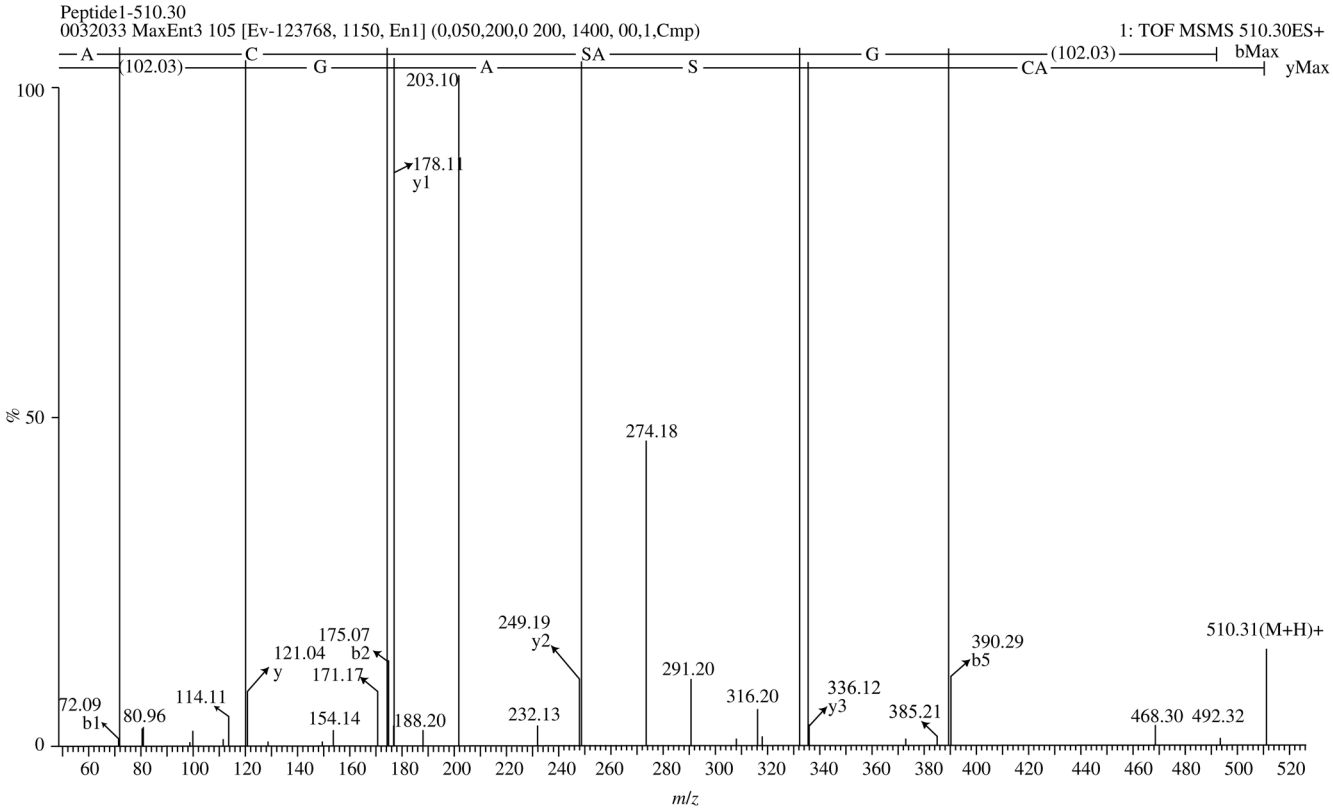

Fig. 4 Amino acid sequencing of OEP3121 by MS-MS

The y ions are the C-terminal ions from a peptide bond breakage, and the b ions are the N-terminal ions from a peptide bond.

Table 1 Sequence homology comparing of OEP3121 with other peptides found in earthworm

Peptide name	Amino acid sequence	Reference
LUMBRICIN 1	MSLCISDYLYLTLTFSKYERQKDKRPYSERKNQYTGPQFLYPPER-IPPQKVIKWNEEGLPIYEIPGEGGHAEPAA	Cho JH [16]
FETIDIN	MSSRAGIAEGYEQIEYDVVAVWKEGYVYENRGSTSVEQKIKITKG-MRNLNSETKTLTASHSIGSTISTGDIFEIATVDVSYSYSHEESQV-SMTETEVYESKEIEHTITIPPTSKFTRVQLNADVGGADIEYMYLI-DEVTPIGGTLSIPQVIKSRAKILVGREIYLGETEIRIKHADRKEY-MTVVSRKSWPAATLGHSKLYKFVLYEDMYGFRIKTLNTMYSGYEY-AYSSDQGGIYFDQGSDNPKQRWAINKSLPLRHGDVVTFMVKYFTR-SGLCYYDGPATDVYCLDKREDKWILEVVKP	Lassegues M [18]
ANTIMICROBIAL-LIKEPEPTIDEPP-1	MYSKYERQKDKRPYSERKDQYTGPQFLYPPDRIPPSKAIKWNEEG-LPMYEVLPDGAGAKTAVEAAAE	http://www.bbcm.univ.trieste.it/~tossi/
F-1	AMVSS	Zhang XC [18]
F-2	AMVGT	Zhang XC [18]
OEP3121	ACSAG	This study

derived from earthworm [16–18]. The primary structure of OEP3121 is compared with other earthworm peptides (Table 1), which evidently indicates no sequence homology. Furthermore, extensive homology searches from the protein databanks do not yield any other peptide homologous to this peptide.

Thus, this paper describes a short antimicrobial peptide in earthworm. As short peptides have the obvious advantage of easy chemical synthesis, the new structural motif observed in OEP3121 would be easily amendable for the synthesis of analogs with improved activity and conceivably useful against multidrug-resistant microbes. We are currently investigating other pharmacological activities of the peptide from earthworm.

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