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An Antimicrobial Activity of a Peptidic Molecule from the Centipede, *Scolopendra subspinipes mutilans* L. Koch

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Abstract – An antimicrobial molecule was purified from centipede, *Scolopendra subspinipes mutilans* L. Koch, by reverse phase-HPLC. Its molecular weight was determined to be 1208.5493 by using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Total amino acid composition analysis revealed that it consists of E, G, P, V, L, F, and W. It exhibited a broad antimicrobial spectrum against not only Gram-negative, but also Gram-positive bacteria. Furthermore, it was found to have an antimicrobial activity against vancomycin resistant enterococci (VRE). It may be a useful molecule for a new antibiotic development, especially against drug-resistant bacteria. We suggest that it may play a role in the defense system of this animal. This is the first report of a peptidic antimicrobial substance from centipede.

Keywords □ Peptidic antimicrobial substance, antibacterial activity, vancomycin resistant enterococci, *Scolopendra subspinipes mutilans* L. Koch.

INTRODUCTION

VRE (Vancomycin resistant enterococci) has emerged as nosocomial pathogens in the past 10 years, causing epidemiological controversy (Schaberg *et al.*, 1991; Emori *et al.*, 1993). Their emergence in the past two decades is in many respects attributable to their resistance to many commonly used antimicrobial agents (aminoglycosides, aztreonam, cephalosporins, clindamycin, the semi-synthetic penicillins nafcillin and oxacillin and trimethoprim-sulfamethoxazole) (Murray *et al.*, 1990). Considering the spread of antibiotic-resistant bacteria and resistant genes, the emergence of VRE has emphasized the non-existence boundaries between hospitals, between people and animals, between countries. Therefore, it is very important to develop an effective therapeutics against antibiotic-resistant bacteria such as VRE, VISA (vancomycin intermediate *Staphylococcus aureus*), and MRSA (methicilline resistant *Staphylococcus aureus*).

Invertebrates are known to synthesize a battery of defense molecules in response to foreign invader challenge or body injury (Boman *et al.*, 1972; Boman & Hultmark, 1987; Natori, 1994). These include hemagglutinins and antimicrobial proteins showing broad spectra of activity against bacteria and fungi. They are encoded by independent genes, which are selectively activated when insects become infected (Kanai *et al.*, 1989; Kylsten *et al.*, 1990). Toll-like receptor and the *Rel* family of transcription factors including molecules such as NF-κB, participate in signal transduction and the activation of these defense protein genes (Sun *et al.*, 1992; Kobayashi *et al.*, 1993; Zasloff, 2002). The use of novel bioactive substances from natural sources for drug development is a promising field of current research. Invertebrate constitute the largest group of extent organisms. Therefore, they are suggested to be a useful source of bioactive substances. Recently, antimicrobial peptides have become a potential probe of new antibiotics to combat the increasing emergence of drug-resistant bacteria (Hancock *et al.*, 2000; Zasloff, 2002).

As mentioned above, lots of antimicrobial proteins have been reported from invertebrates. However the molecular masses of these compounds are usually more than 3,000. Therefore, we have to focus on a low molecule, since it is difficult to develop these large molecules as drug for human therapy. There are few reports about low molecular weighted substance from invertebrate. A peptidic antibacterial substance 5-S-GAD was reported from the extract of fresh fly *Sarcophaga* (Leem *et al.*, 1996). This is a low molecule with 573 of molecular weight and also defined as an inhibitor against protein tyrosine kinase (Hijikata *et al.*, 1997; Leem *et al.*, 1998). *P-*

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hydroxy cinnamaldehyde was isolated from saw fly *Acantholyda parki* (Leem *et al.*, 1999). We tried to isolate an antibacterial compound including the counterpart to 5-S-GAD from centipede, *Scolopendra subspinipes mutilans* L. Koch. It belongs to arthropod in invertebrate and has been used as a traditional medicine for many clinical purposes in Asia. Dried centipedes in combination with various herbal materials are usually used for sudden heart attack, infantile convulsion, tetanus, apoplectic hemiplegia, chronic rheumatic or rheumatoid arthritis, sores, lymphadenitis, venomous snake-bite, and cancer in the ancient oriental medicine (Pharmacopoeia Commission of the Ministry of Public Health, P. R. China 1995; Mohamed *et al.*, 1980; Gomes *et al.*, 1983). There has been no report about an antimicrobial peptide from this animal, however, three quinoline compounds, jineol, 8-hydroxy-1*H*-2-benzopyran-1-one, and scolopendrine have been recently reported as bioactive substances from the centipede (Moon *et al.*, 1996; Kim *et al.*, 1998; Noda *et al.*, 2001). In the present study, we purified an antibacterial substance among several active fractions and characterized its antibacterial activity, although we were unable to detect a 5-S-GAD-like substance.

MATERIALS AND METHOD

Preparation of crude extracts of *Scolopendra subspinipes mutilans*

Dried centipede *Scolopendra subspinipes mutilans* L. Koch, Jeju-island originated was purchased. Two hundred centipedes were homogenized in 10 volumes of 0.1% (v/v) trifluoroacetic acid (TFA) containing 10 μg/ml aprotinin using a ULTRA-TURRAX T25 homogenizer (IKA-Labortechnik, St. Augustin, Germany). The resulting homogenates were centrifuged for 30 min at 35,000 × *g*, and the supernatants were filtered through Millipore AA filters (pore size 0.22 μm). The filtrate was used as a crude extract.

Purification of the antibacterial substance

The crude extract of centipede was loaded onto ODS-AM AM12S50 cartridge (YMC, Kyoto, Japan). After washing the cartridge with 0.05% trifluoroacetic acid (TFA), the absorbed material was eluted with 10% (v/v) acetonitrile containing 0.05% trifluoroacetic acid. Each fraction was concentrated under vacuum to remove the acetonitrile and trifluoroacetic acid, and the residue was dissolved in autocalved-distilled water for measurement of the antibacterial activity. Fractions showing antibacterial activity were pooled and applied to a reverse-phase HPLC C18 column (YMC-Pack ODS-A, S-5, 120A, 250 × 4.6 mm; YMC, Kyoto, Japan) equilibrated with 0.1% trifluoroacetic acid. After washing the column with 0.1% trifluoroacetic acid, the absorbed material was eluted using a linear gradient of 0-20% acetonitrile in 0.1% trifluoroacetic acid. Fractions showing antibacterial activity were pooled and subjected to TSK gel Carbon-500 column (150 × 4.6 mm; Tosoh, Tokyo, Japan) and adsorbed material was eluted using a linear gradient of 0-30% acetonitrile in 0.1% trifluoroacetic acid. At this stage, one peak containing antibacterial activity was eluted from the column. Fractions containing high antibacterial activity were pooled and again subjected to reverse-phase HPLC using the same type of C18 column. Adsorbed material was eluted using a linear gradient of 6-25% acetonitrile in 0.1% trifluoroacetic acid for 40 min. At this stage, the antibacterial substance eluted as a single symmetrical peak, as detected by UV absorbance at 220 nm. All the HPLC analysis was performed by Waters HPLC system (Waters, MA, USA).

Bacterial strains, media, and assay of antibacterial activity

Antibacterial activity was measured in liquid medium or by agar well diffusion test using *Escherichia coli* K12 594 of the indicator bacterium as described previously (Leem *et al.*, 1996). *E. coli* was grown in antibiotic medium (Difco, MI, USA). At the exponential phase of growth, cells were collected and suspended in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl, 0.2% (w/v) bovine serum albumin, at a density of 2.5×10^8 cells/ml, and the suspension was then diluted 300-fold with antibiotic medium. The sample solution in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl, 0.05% (w/v) bovine serum albumin (10 ml) was mixed with 100ml of the bacterial suspension in each well of a 96-well microplate and incubated at 37°C for 5 h. The absorbance at 650 nm was measured to assess bacterial growth using Model 680 Microplate Reader (Bio-Rad, CA, USA). For the determination of the antibacterial spectrum of antimicrobial molecule, we used *Enterococcus faecium*, which is VRE (MIC 128 μg/ml) and various strains as follows; *Bacillus subtilis* KCTC 3069, *Micrococcus luteus* KCTC 1056, *Staphylococcus aureus* KCTC 1928, *Pseudomonas aeruginosa* KCTC 2004, *Escherichia coli* K12 594, *Escherichia coli* O157 H7 993, *Klebsiella pneumoniae* KCTC 2208, *Salmonella enteritidis* ACTC 13076, *Shigella flexneri* KCTC 2008, and *Candida albicans* KCTC 1940. Its MICs (minimum inhibitory concentrations) against VRE were determined by the microdilution method in 0.1 ml volumes of cation-adjusted Mueller-Hinton broth (National Com-

mittee for Clinical Laboratory Standards. 2000). Incubation was performed for 20 h at 35°C. Linezolid and vancomycin were used as control at various concentrations.

Characterization of an antibacterial substance

UV spectrum was analyzed using SPECTRONIC 3000 ARRAY (Milton Roi, NY, USA) spectrophotometer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer were measured using a Voyager DE-STR mass spectrometer (Jeol, Tokyo, Japan) in reflectron mode, using α-cyano-4-hydroxy cinnamic acid (CHCA) as a matrix. Total amino acid composition was analyzed by Waters HPLC system with Waters 996 photodiode array detector. MALDI-TOF mass spectrometry and Total amino acid composition analysis were performed at Korean institute of basic sciences (Taejon, Korea).

RESULTS

Purification of a peptidic antimicrobial molecule from *S. subspinipes mutilans*

We have tried to isolate *S. subspinipes mutilans* counterpart of 5-S-GAD, a low-molecular-weight antibacterial substance of *Sarcophaga*, from centipede using a method based on that described previously (Leem *et al.*, 1996). One major and one minor fraction of antibacterial activities were eluted with 10% acetonitrile in 0.05% trifluoroacetic acid during of the ODS-AM 12S50 cartridge column chromatography. We focused on the major fraction, and tried to purify this substance further by repeating of reverse-phase HPLC. Although we did not detect a *S. subspinipes mutilans* counterpart to 5-S-GAD, we were able to purify a molecule, with an antimicrobial activity, to homogeneity on the reverse-phase HPLC analysis (Fig. 1). We applied this active fraction to characterize its structure and antimicrobial activity.

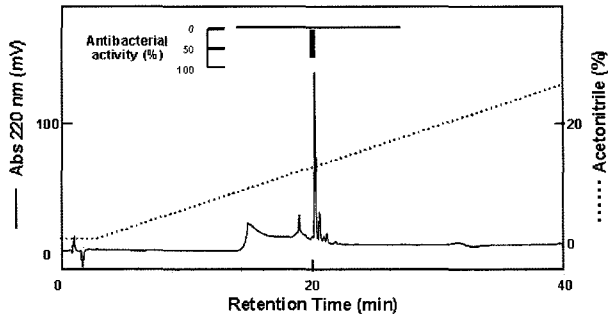

Fig. 1. Reverse-phase HPLC profile of an antimicrobial substance purified from *Scolopendra subspinipes mutilans* L. Koch. The UV absorption at 220 nm was monitored (solid line). Fractions were concentrated and assayed for antibacterial activity (inset). Chromatographic conditions were: column, YMC-Pack ODS-A, S-5, 120 Å, C18 250 ×4.6 mm; solution A, 0.1% trifluoroacetic acid; solution B, 0.1% trifluoroacetic acid in 30% acetonitrile; linear gradient 20-83% solution B in solution A (dotted line) for 40min; flow rate, 1 ml/min.

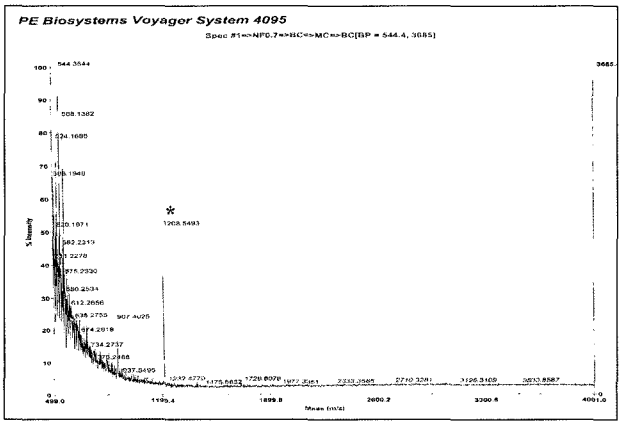

Fig. 2. MALDI-TOF mass spectrum. Its molecular weight was determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry in reflectron mode, using CHCA as a matrix. Mass peak was detected as shown by asterisk (found: m/z 1208.5493).

Molecular characterization of antimicrobial substance

The maximum UV absorption of this substance was at 215 and 285 nm (data not shown). As shown in Fig. 2, its molecular weight was determined by means of matrix-assisted laser desorption/ionization-time of flight mass spectrometry in reflectron mode, using CHCA as a matrix (found: m/z 1208.5493). The amino acid composition analysis of revealed that it consists of glutamic acid, valine, phenylalanine, trypthophane, glycine and proline, although we could not determine the amino acid sequence of this antibacterial substance by the means of automatic protein sequencer (data not shown). We were able to determine the amount of this molecule using BCA protein assay kit, which is useful for protein quantification (Pierce, IL, USA).

Antimicrobial activity on VRE

We found that it has a strong antibacterial activity against VRE, which is isolated from patient as shown in Fig. 3. Its antimicrobial activity is stronger than that of linezolid, which is clinically treated for VRE-infected patients. Antimicrobial substance from *S. subspinipes mutilans* was applied on VRE

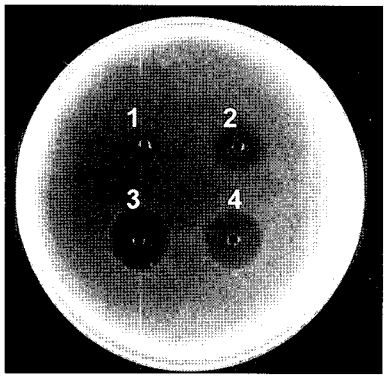

Fig. 3. Antibacterial activity on VRE. Its antibacterial activity on vancomycin-resistant enterococci was accessed by agar well diffusion test. An antimicrobial substance from *S. subspinipes mutilans* was applied on VRE spread plate at a various concentration with negative control and positive control as following; 1, 50 μg/ml of vancomycin; 2, 1 μM of antibacterial substance from *S. subspinipes mutilans*; 3, 2 μM of antibacterial substance from *S. subspinipes mutilans*; 4, 5 μM of Linezolid.

spread plate at a various concentration as following; 1, 50 μg/ml of vancomycin; 2, 1 μM of antibacterial substance from *S. subspinipes mutilans*; 3, 2 μM of antibacterial substance from *S. subspinipes mutilans*; 4, 5 μM of Linezolid. The antimicrobial activity of 2 μM of antibacterial substance from *S. subspinipes mutilans* is equal to that of 5 μM of Linezolid.

Antibacterial spectrum of antimicrobial molecule

We found that this molecule was effective against both Gram-negative and Gram-positive bacteria, with similar IC50 values (1-50 μM) for *E. coli* K12 594, *Salmonella enteritidis* ACTC 13076, *E. coli* O157 H7 993, *Shigella flexneri* KCTC 2008, *Klebsiella pneumoniae* KCTC 2208, *Bacillus subtilis* KCTC 3069, *Staphylococcus aureus* KCTC 1928, and *Micrococcus luteus* KCTC 1056 under the assay conditions we employed. The substance showed very weak activity against *Candida albicans* KCTC 1940 with an IC50 value of 300 μM. The antimicrobial activity of this substance is summarized in Table I.

Table I. Antimicrobial activity against various strains.

Microbe	IC50 (μM)
Bacillus subtilis KCTC 3069	40
Micrococcus luteus KCTC 1056	20
Staphylococcus aureus KCTC 1928	50
Pseudomonas aeruginosa KCTC 2004	100
Escherichia coli K12 594	2
Escherichia coli O157 H7 993	2
Klebsiella pneumoniae KCTC 2208	20
Salmonella enteritidis ACTC 13076	2
Shigella flexneri KCTC 2008	10
Candida albicans KCTC 1940	300

DISCUSSION

Many antimicrobial peptides have been discovered in animal kingdom ranging from insects to humans. These peptides were known to represent components of the system of the host defense called innate immunity (Zasloff, 1992; Boman, 1995; Oren *et al.*, 1998; Ganz & Lehrer, 1999; Lehrer & Ganz, 1999; Hancock, 1999). These peptides are usually cationic and possess broad-spectrum activities against bacteria and fungi. They are known to have an amphipathic structure with clusters of hydrophobic and positively charged region (Oren *et al.*, 1998; Andreu *et al.*, 1998; Tossi *et al.*, 2000; Hancock *et al.*, 2000). This structural property appears to be closely related to their antimicrobial activity.

Based on our result, the antimicrobial molecule from centipede includes at least 5 hydrophobic amino acids, phenylalanine, leucine, trypthophan, valine, and proline. We speculate that these hydrophobic amino acids contribute to its antimicrobial activity. It may consist of other hydrophilic amino acids including glycine and glutamic acid or glutamine, which were detected by total amino acid composition assay. It may result in amphipathic α-helical structure as the antimicrobial peptides purified from other animals, although further studies are needed to clarify its characterization. This molecule showed a broad antimicrobial spectrum against not only Gram-negative, but also Gram-positive bacteria. Furthermore, it was found to have an antimicrobial activity against VRE at a low concentration. These results suggested that it is potentially valuable for antimicrobial chemotherapy in the treatment of VRE infection.

Nearly 30 years after vancomycin was clinically introduced, VRE were first reported in 1986. More than 95% of VRE recovered are *E. faecium*; virtually all are resistant to high levels of ampicillin. The phenotypic association of ampicillin and vancomycin resistance is in some instances due to genetic linkage. Vancomycin resistance is conferred by one of two functionally similar operons, VanA or VanB (Arthur *et al.*, 1996). These are highly sophisticated resistance determinants, which are suggested that they evolved in other species and were acquired by enterococci. However, in specific patient populations, notably in liver transplant patients and patients with hematologic malignancies, VRE cause serious and often fatal disease (Linden *et al.*, 1996; Roghmann *et al.*, 1997). Fre-

quently identified risk factors for VRE colonization and infection include prolonged hospital stays, exposure to intensive care units, transplants, hematologic malignancies, and exposure to antibiotics (Edmond *et al.*, 1995). The epidemiology of VRE spread in the hospital involves both person-to-person transmission and selective antibiotic pressure. Therefore, it is very important to develop an effective therapeutics against antibiotic-resistant bacteria such as VRE, VISA (vancomycin intermediate *Staphylococcus aureus*), and MRSA (methicilline resistant *Staphylococcus aureus*). Recent studies have reported the isolation of linezolid resistant VRE and linezolid-resistant *S. aureus* in USA (Gonzales *et al.*, 2001; Tsiodras *et al.*, 2001). This indicates the possible spread of linezolid-resistant organisms by increasing the usage frequency and dosage.

Several antimicrobial peptides such as magainin, a 23-residue antimicrobial peptide, have been successful in pharmaceutical and commercial development (Zasloff, 2002). We suggested that this antimicrobial peptide from centipede may be a useful molecule for new antibiotic development against multidrug-resistant bacteria, since it is very low molecule, is expected to exhibit a unique mechanism such as membrane pore formation or increasing membrane permeability. As general antimicrobial peptides isolated from invertebrate to vertebrate were known to represent components of the system of innate immunity, this substance may play a role in the defense system of this animal. This is the first report of a peptidic antimicrobial substance from centipede.

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