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Biochemical and Biophysical Research Communications 307 (2003) 261–266

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Scarabaecin, a novel cysteine-containing antifungal peptide from the rhinoceros beetle, *Oryctes rhinoceros*☆,☆☆

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Received 12 May 2003

Abstract

A novel antifungal peptide, scarabaecin (4080 Da), was isolated from the coconut rhinoceros beetle, *Oryctes rhinoceros*. Scarabaecin cDNA was cloned by reverse transcriptase-polymerase chain reactions (RT-PCR) using a primer based on the N-terminal amino acid sequence. The amino acid sequence deduced from scarabaecin cDNA showed no significant similarity to those of reported proteins. Chemically synthesized scarabaecin indicated antifungal activity against phytopathogenic fungi such as *Pyricularia oryzae*, *Rhizoctonia solani*, and *Botrytis cinerea*, but not against phytopathogenic bacteria. It showed weak activity against *Bauberia bassiana*, an insect pathogenic fungus, and *Staphylococcus aureus*, a pathogenic bacterium. Scarabaecin showed chitin binding property and its K_d was 1.315 μM. A comparison of putative chitin-binding domains among scarabaecin, invertebrate, and plant chitin-binding proteins suggests that scarabaecin is a new member of chitin-binding antimicrobial proteins.

Keywords: Insect immunity; Antifungal peptide; Purification; cDNA cloning; Chemical synthesis; Chitin binding

Insect immune systems contain a battery of potent antibacterial and antifungal peptides/polypeptides [1,2]. Although numerous antibacterial peptides have been identified [3,4], only a small number of antifungal peptides/polypeptides have been reported from insects. Insect immune peptides/polypeptides that show antifungal activity are drosomycin [5], metchinikowin [6], cecropin A and B [7], and andropin [8] from *Drosophila melanogaster*, heliomicin from *Heliothis virescens* [9], cecropin A from *Hyalophora cecropia* [7], thanatin from *Podisus maculiventris* [10], antifungal protein (AFP) from *Sarcophaga peregrina* [11], holotricin 3 from *Holotrichia diomphalia* [12], and termicin and spinigerin from *Pseudacanthotermes spiniger* [13]. Of these antifungal peptides/polypeptides, drosomycin, thanatin, heliomicin, and termicin are known to contain cysteine residues and form disulfide bridges.

In our study of insect antifungal peptides as genetic resources to establish transgenic plants, we isolated a novel cysteine-containing antifungal peptide from the coconut rhinoceros beetle, *Oryctes rhinoceros*. The antifungal peptide, designated scarabaecin, shows chitin-binding property seen in the chitin-binding proteins [14–21], suggesting that the peptide is a chitin-binding antimicrobial substance. Here we report the purification, chemical synthesis, characterization, and cDNA cloning of the novel antifungal peptide from an insect.

☆ *Abbreviations:* PD, potato-dextran; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; HFBA, heptafluorobutyric acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RACE, rapid amplification of cDNA ends; IC_{50}, 50% growth inhibition concentration; RT-PCR, reverse transcriptase-polymerase chain reactions.

☆☆ The sequence data reported in this paper will appear in the GSDB/DDBJ/EMBL/NCBI nucleotide sequence databases under Accession No. AB081620.

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doi:10.1016/S0006-291X(03)01162-8

Materials and methods

Insects. *O. rhinoceros* larvae were collected in the field on Okinawa and Miyako Islands, Japan. Third instar larvae were used for antifungal peptide purification.

Collection of immunized hemolymph. *O. rhinoceros* larvae were injected with *Escherichia coli* JM109 (10^6 cells/larva) suspended in physiological saline (150 mM NaCl/5 mM KCl). Larvae were kept at 25 °C for 15 h. Hemolymph collection was based on Ishibashi et al. [23].

Antifungal activity assay. Throughout scarabaecin purification, antifungal activity was analyzed by measuring the fungal growth inhibition zone. The phytopathogenic fungus *Rhizoctonia solani* from rice was used as an indicator. *R. solani* was cultured at 25 °C for 5 days in potato-dextran (PD) medium (Difco). Hyphae were cut with a homogenizer and clots were removed. Hyphae were collected by centrifugation for 10 min at 10,000*g* and adjusted with PD medium to 10^5 hyphae/ml. Five milliliters of PD agar medium containing 250 μl of the *R. solani* hyphae solution was poured into a petri dish (8.4 cm in diameter) and solidified. One-microliter sample was spotted onto the medium and incubated at 25 °C for 24 h and the growth inhibition zone was measured.

Purification of scarabaecin. Heat-treated hemolymph was acidified with 0.05% trifluoroacetic acid (TFA) and applied to a Sep-Pak Vac C_{18} cartridge (Waters), preequilibrated with 0.05% TFA. The column was washed with 0.05% TFA and adsorbed materials were eluted stepwise with 20%, 40%, and 60% acetonitrile containing 0.05% TFA. Acetonitrile in each fraction was evaporated in a vacuum and antifungal activity of eluted materials against *R. solani* from rice was analyzed. The 40% acetonitrile fraction showing the strongest activity was further subjected to reverse-phase high-performance liquid chromatography (HPLC) on a Resource RPC column (1 ml, Pharmacia), equilibrated with 20% acetonitrile containing 0.05% TFA connected to an ÄKTA Explorer (Pharmacia). Adsorbed materials were eluted with a linear gradient of acetonitrile/0.05% TFA (20–40%) for 20 min at a flow rate of 1 ml/min. Fractions eluted with 26–32% acetonitrile showing antifungal activity were pooled and dried in a vacuum. Eluates were dissolved in 0.05% TFA and applied to reverse-phase HPLC on a Senshupak VP-318 column (4.6 × 250 mm, Senshu Kagaku) connected to a 600E Multisolvent Delivery System (Waters). Adsorbed materials were eluted with a linear gradient of acetonitrile/0.05% TFA (20–26%) for 25 min at a flow rate of 1 ml/min. Fractions eluted with 22–23% acetonitrile showing antifungal activity were further purified by reverse-phase HPLC on a Senshupak VP-318 column (4.6 × 250 mm, Senshu Kagaku) connected to a 600E Multisolvent Delivery System. Adsorbed materials were eluted with a linear gradient of acetonitrile/0.05 heptafluorobutyric acid (HFBA) (30–40%) for 20 min at a flow rate of 1 ml/min. Fractions showing antifungal activity were pooled and dried in a vacuum. Dried materials were dissolved in 0.05% TFA and applied to reverse-phase HPLC on a Sephasil C8 SC 2.1/10 column (Pharmacia) connected to a SMART system (Pharmacia). The column was washed with 20% acetonitrile containing 0.05% TFA and adsorbed materials were eluted with a linear gradient of acetonitrile/0.05% TFA (20–26%) for 25 min at a flow rate of 0.1 ml/min.

Molecular mass measurement. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was conducted on a Voyager RP Biospectrometry System (PerSeptive Biosystems) using α-cyano-4-hydrocinnamic acid (Aldrich) as a matrix.

Amino acid sequence analysis. The amino acid sequence was determined using a 492cLC Protein Sequencer (PE Applied Biosystems). The C-terminal amino acid sequence was also analyzed using a Sequazyme C-Peptide Sequence Kit (PerSeptive Biosystems) and MALDI-TOF-MS.

cDNA cloning and nucleotide sequencing. mRNAs were isolated from fat bodies 6 h after immunization with *E. coli* JM109 (10^6 cells/larva) using ISOGEN (Nippon Gene). First-strand cDNA was synthesized using 3 μg mRNA with a *Not*I-(dT18) primer (Amersham Biosciences) using a First-Strand cDNA Synthesis Kit (Amersham Biosciences). 3′ Rapid amplification of cDNA ends (RACE) was conducted using a *Not*I-(dT18) primer and a degenerate primer based on amino acid sequences of scarabaecin. The nucleotide sequence of this primer was 5′-AAA/GC/TTA/T/C/GCCA/T/C/GGAC/TGAC/TAAA/GGT-3′ (P4080-1). Several DNA bands appeared. We screened an objective DNA band using the *Not*I-(dT18) primer and the following degenerate primer: 5′-CCA/C/G/TGAC/TGAC/TAAA/GGTA/C/G/TC/TTA/C/T/GAT-3′ (P4080-2). We also screened the DNA band using the *Not*I-(dT18) primer and the following primer: 5′-GAC/TGCA/C/G/TGAA/GC/TTA/T/G/CCCA/T/GCAA-3′ (P4080-3). In the screening a single DNA fragment (ca. 200 bp) was obtained in each case. Fragments were subcloned into a pCR2.1 vector using an Original TA Cloning Kit (Invitrogen). The nucleotide sequence of these fragments was determined by dye-terminator cycle sequencing using a DNA sequencer (ABI 373A). The nucleotide sequence was confirmed to encode the same amino acid sequence as purified scarabaecin. We next conducted 5′-RACE using a 5′-RACE System for Rapid Amplification of cDNA End Reagent Assembly, Ver. 2 (Gibco-BRL) with the following primer based on the nucleotide sequence obtained by 3′-RACE: 5′-CGATATACAAGCA TCAATGC-3′ (P4080-4). Because several DNA bands appeared, we screened an objective DNA band using a Universal Amplification Primer (Gibco-BRL) and one of the following primers: 5′-GCGAA CGGTGATTTACAGTCAAATCCATTC-3′ (P4080-5) and 5′-GC CTTTTGGACAGTTACTCCTACTTCTAAT-3′ (P4080-6). In each screening we obtained a 250-bp DNA fragment. The amplified fragment was confirmed to code the amino acid sequence obtained. PCR was conducted to obtain full-size cDNA covering the entire region of the purified peptide using *Not*I-(dT18) primer and the following primer based on the nucleotide sequence of the 5′-RACE fragment: 5′-AAGGGACGATCAAAATGAAA-3′ (P4080-7). The nucleotide sequence of the 310-bp PCR product was determined as detailed above.

Peptide synthesis. Two types of scarabaecin mature peptides, SP4080 and SP4266, deduced from scarabaecin cDNA were synthesized by the solid-phase method in a Plus Peptide Synthesizer (PerSeptive Biosystems) as described previously [22]. Amino acid sequences were as follows: NH_2-ELPKLPDDKVLIRSRSNCPKGKV WNGFDCKSPFAFS (SP4080), NH_2-DAELPKLPDDKVLIRSRSN CPKGKVWNGFDCKSPFAFS (SP4266). Thirty milligrams of each synthetic peptide was dissolved in 90 ml of 10 mM Tris–HCl buffer, pH 8.0, and 5 ml of 5 mM 2,2′-bispyridyl disulfide was added at room temperature for 10 min with stirring to form disulfide bonds. Resulting peptides were purified by reverse-phase HPLC. Peptide peaks monitored at 220 nm were fractionated and molecular masses were analyzed by MALDI-TOF-MS. Amino acid sequences of all synthetic peptides were analyzed with a 492cLC Protein Sequencer (PE Applied Biosystems) and confirmed to be the same as that deduced from the nucleotide sequence of scarabaecin cDNA.

Analysis of antifungal and antibacterial spectra. Antifungal and antibacterial spectra of synthetic scarabaecin against phytopathogenic fungi and bacteria were analyzed. For this a 50% growth inhibition concentration (IC_{50}) of scarabaecin against 7 phytopathogenic fungi and 9 phytopathogenic bacteria was determined. Fungi included *Pyricularia oryzae* from rice, *R. solani* from rice, *Septoria tritici* from wheat, *R. solani* from bentgrass, *Pythium aphanidermatum* from bentgrass, *Phytophora infestans* from potato, and *Botrytis cinerea* from cucumber. Bacteria included *Agrobacterium tumefaciens* N1001 from rose, *Clavibacter michiganense* pv. *michiganense* from tomato, *Erwinia carotoba* subsp. *carotoba* from Chinese cabbage, *Pseudomonas chichori* NL7630 from lettuce, *Pseudomonas glumae* N1169 from rice, *Pseudomonas syringae* pv. *mori* S6804 from mulberry, *P. syringae* pv. *japonica* from wheat, *Xanthomonas campestris* pv. *campestris* N1076 from Japanese radish, and *X. campestris* pv. *oryzae* N1086 from rice. Hyphae of *R. solani* from rice or bentgrass were prepared as detailed above. Spores from *P. oryzae*, *S. tritici*, and *B. cinerea*

were prepared using Potato Dextrose Agar plates (Difco) at 25 °C under a black light blue lamp. Spores of *P. infestance* were harvested from V8 juice agar (20% V8 juice, 0.3% $CaCO_3$) cultured at 20 °C. Spores of *P. aphanidermatum* were collected after fungi were grown in V8 juice medium at 25 °C for 14 days. The final spore concentration was 10^4/ml of 1/2 PD medium containing 30 μg/ml chloramphenicol. Similarly, the final hyphae concentration was 3.2×10^3 hyphae/ml of 1/2 PD medium containing 30 μg/ml chloramphenicol. Eighty microliters of culture medium containing spores or hyphae was transferred to a 96-well titer plate (Becton–Dickinson) and 20 μl of different concentrations of synthetic scarabaecin was added. For *P. infestance* V8 medium was used instead of PD medium. The titer plate was incubated at 25 °C for 48 h and absorbance of cultures at 595 nm was measured. As a positive control, amphotericin B (Wako) was used instead of scarabaecin.

Effect of synthetic scarabaecin on the growth of E. coli, Staphylococcus aureus, and Bauberia bassiana. E. coli and *S. aureus* were preincubated in nutrient broth (NB, Difco) at 37 °C for 12 h. The bacterial concentration was adjusted to an absorbance of 0.001 at 600 nm. Eighty microliters was transferred to a 96-well titer plate and 20 μl of NB containing 0, 0.4, 0.8, 1.6, or 3.2 μg of synthetic scarabaecin was added. The bacterial culture was incubated at 37 °C for 24 h and absorbance was measured at 595 nm. *B. bassiana* was cultured in PD medium at 25 °C for 5 days. Spores were collected by centrifugation at 10,000*g* for 10 min after the removal of hyphae clots. Culture was conducted in a 96-well titer plate as detailed above. The fungal culture was incubated at 25 °C for 45 h and absorbance was measured at 595 nm.

Chitin-binding assay. The chitin binding assay was mainly based after Osaki et al. [25]. Briefly, chitin (0.5 mg) was mixed with synthetic scarabaecin (SP4080) (0.5, 1, 2, 4, 8, 16, or 32 μM) in 200 μl of 10 mM sodium phosphate buffer, pH 7.15, containing 0.15 M NaCl, and then incubated at room temperature for 15 min in Ultrafree UFC30GV00 (Millipore, pore size 0.22 μm). Free scarabaecin was removed by centrifugation at 18,600*g*. Scarabaecin bound to chitin was washed twice with 200 μl of the same buffer above. The scarabaecin–chitin complex was incubated with 200 μl of 0.1 M HCl at room temperature for 15 min. Scarabaecin separated from chitin was collected by centrifugation at 18,600*g* and washed twice with 0.1 M HCl as detailed above. Quantitative analysis of chitin-bound scarabaecin was conducted as follows: scarabaecin was adsorbed on a Pegasil-ODS column (2×100 mm, Senshu Kagaku) equilibrated with 20% acetonitrile containing 0.05% TFA connected to an Agilent 1100 Series and eluted with a linear gradient of 20–30% acetonitrile. The area of the scarabaecin peak was measured.

Results and discussion

Purification of scarabaecin

Scarabaecin was purified from the hemolymph of third-instar larvae immunized with *E. coli* by monitoring antifungal activity against *R. solani* from rice. Because antifungal activity against the phytopathogenic fungus and antibacterial activity against *E. coli* were detected in the same hemolymph sample in the first screening, we used the hemolymph from *E. coli*-injected larvae to purify scarabaecin. After Sep-Pak treatment of the hemolymph, samples were subjected to four purification steps using reverse-phase HPLC. Results showed a single antifungal peak against *R. solani* (Fig. 1). In the final purification, 10 μg of scarabaecin was obtained from 180 larvae, indicating that each larva has about 55.6 ng of scarabaecin in about 3 ml of hemolymph.

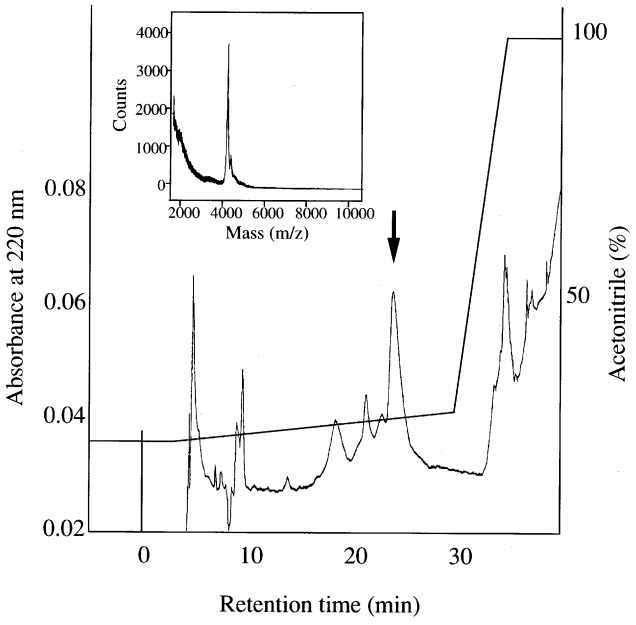

Fig. 1. Final scarabaecin purification by reverse-phase HPLC. Reverse-phase HPLC was conducted with a Sephasil C8 SC 2.1/10 column connected to a SMART System (Pharmacia). Proteins adsorbed on the column were eluted for 25 min with a linear gradient of 20–26% acetonitrile/0.05% TFA. Protein content was determined by measuring ultraviolet absorbance at 220 nm. Antifungal activity was determined using *R. solani* from rice. The arrow indicates the antifungal peak. The inset shows the MALDI-TOF-MS spectrum of the peak.

Molecular mass measurement and N-terminal amino acid sequencing

The molecular masses of scarabaecin analyzed by MALDI-TOF-MS were 4080 Da (major component) and 4266 Da (minor component) (Fig. 1). Despite attempts, these two components could not be separated. The amino acid sequences of the N-terminal region were therefore analyzed using mixed components. Results showed major and minor amino acid peaks with their molar ratio always being constant (data not shown). Amino acid sequences of these components were as follows: NH_2-ELPKLPDDKVLIRRSNXPK, where X is an unidentified amino acid residue (major component) and NH_2-DAELPKLPDD (minor component). These results indicate that the minor component has two additional amino acid residues at the N-terminus of the major component sequence. In addition, the difference in the molecular masses of these two components was equal to the added molecular mass of the two amino acid residues (D and A) of the minor component. We thus assumed that the two components have the same amino acid sequence except for two amino acid residues

at the N-terminus. We next digested mixed components with endoprotease Arg-C and separated digested fragments by HPLC. The amino acid sequences of the digested fragments were determined. The combined amino acid sequence of the major component was NH_2-ELP KLPDDKVLIRRSRSNXPKGKVWNGF.

cDNA cloning, nucleotide sequencing, and C-terminal amino acid sequencing

Molecular cloning of cDNA encoding scarabaecin was conducted to obtain the complete amino acid sequence. For this, 3′ and 5′ RACE were done using primers based on the amino acid sequence of the N-terminal region of scarabaecin. Nucleotide sequencing of scarabaecin cDNA showed that this clone contains nontranslation sequences at 5′ and 3′-regions and a putative poly(A) addition signal (Fig. 2). The deduced amino acid sequence was shown to contain an open reading frame of 66 amino acid residues including four cysteine residues before the translation termination codon, TGA. Results suggest that a mature portion (38 amino acid residues for the major component and 40 for the minor component) is produced by cleavage of the signal peptide from the 66 precursor peptide. The anticipated molecular masses of scarabaecin mature peptide from the deduced amino acid sequence, however, did not coincide with MALDI-TOF-MS measurements. The difference was 258 Da equal to the added mass of C-terminal amino acid residues, KK. We thus determined the C-terminal amino acid sequence of scarabaecin by digesting scarabaecin with an enzyme and analyzing it with MALDI-TOF-MS (data not shown). The results showed the sequence, HOOC-SFA, suggesting that the C-terminal amino acid sequence, KK, deduced from scarabaecin cDNA, is cleaved. We therefore conclude that scarabaecin contains two different N-terminal amino acid sequences and the C-terminal amino acid is serine.

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AAGGGACGATCAAAATGAAAACGTTAACGTTTTATACTCTATTACTATGTGCTGCTCTTT    60
            M  K  T  L  T  F  Y  T  L  L  L  C  A  A  L

ATAGTAATTTCTTCGATTGTAAAGCCGTTGCGGATGCGGAATTGCCGAAATTACCAGATG   120
Y  S  N  F  F  D  C  K  A  V  A  D  A  E  L  P  K  L  P  D
                                 ▲     △
ACAAAGTTTTGATTAGAAGTAGGAGTAACTGTCCAAAAGGC1AAAGTATGGAATGGATTTG  180
D  K  V  L  I  R  S  R  S  N  C  P  K  G  K  V  W  N  G  F

ACTGTAAATCACCGTTCGCATTTAGCAAAAAGTGAAGAATTTTTAGCATTGATGCTTGTA   240
D  C  K  S  P  F  A  F  S  K  K  ***

TATCGTATATGTAGTACGTATATAGGACGAAGCTAATAAATATTATGTAATTTCTAAAAA   300
AAAAAAAAAA                                                     310
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Fig. 2. Nucleotide and deduced amino acid sequences of scarabaecin cDNA. Deduced amino acids are denoted by a one-letter code. Amino acid residues of mature scarabaecin are denoted by bold type. Asterisks indicate the termination codon. The putative poly(A) addition signal, ATAAA, is underlined. Solid and open triangles show the N-terminal amino acid residues for the minor and major scarabaecin, respectively.

Antimicrobial spectra of synthetic scarabaecin

We chemically synthesized two types of scarabaecin and purified them after the formation of disulfide bonds. We tested first whether the major and minor scarabaecin show significant differences in biological activity and found no difference in antifungal and antibacterial activity between these two types (data not shown). We thus determined IC_{50} against several phytopathogenic fungi and bacteria using only major scarabaecin. The scarabaecin showed antifungal activity against *P. oryzae* and *R. solani* from rice, *S. tritici* from wheat and *R. solani* from bentgrass, and *B. cinerea* from cucumber but not against *P. aphanidermatum* from bentgrass and *P. infestance* from potato (Table 1). Scarabaecin showed very weak antibacterial activity against the phytopathogenic bacterium *P. syringae* pv. *mori* S6804 and showed no activity against other bacterial phytopathogens such as *A. tumefaciens* N1001, *C. michiganese* pv. *michiganese*, *E. carotovora* subsp. *carotovora*, *P. cichori* NL7630, *P. glumae* N1169, *P. solanacearum* N1023, *P. syringae* pv. *japonica*, *X. campestris* pv. *compestris* N1076, and *X. campestris* pv. *campestris* N1086 (Table 1). We tested the effects of synthetic scarabaecin against two bacteria, *E. coli* and *S. aureus*, and a natural

Table 1
Antimicrobial activity of synthetic scarabaecin against phytopathogenic fungi and bacteria

	IC_{50} (μM)	
	Scarabaecin	Amphotericin B
Fungi		
P. oryzae	16.0	20.6
R. solani[a]	32.0	18.2
S. tritici	16.0	1.4
R. solani[b]	128	6.7
P. aphanidermatum	>250	>250
P. infestance	>250	>250
B. cinerea	4.0	2.7
Bacteria		
A. tumefaciens N1001	>100	
C. michiganese pv. *michiganese*	>100	
E. carotovora subsp. *carotovora*	>100	
P. cichori NL7630	>100	
P. glumae N1169	>100	
P. syringae pv. *mori* S6804	25	
P. syringae pv. *japonica*	>100	
X. compestris pv. *compestris* N1076	>100	
X. campestris pv. *campestris* N1086	>100	

Amphotericin B, a fungicide, was used as a positive control.
[a] *Rhizoctonia solani* was isolated from rice.
[b] *Rhizoctonia solani* was isolated from bentgrass.

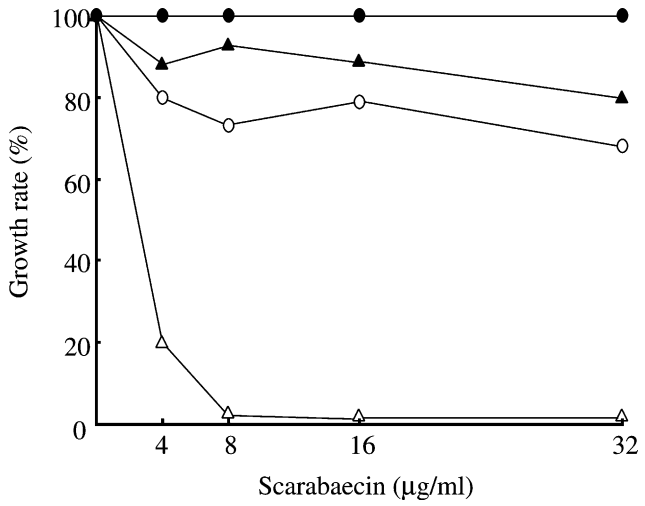

Fig. 3. Effect of synthetic scarabaecin on the growth of *E. coli*, *S. aureus*, and *B. bassiana*. *E. coli* (solid circle), *S. aureus* (solid triangle), and *B. bassiana* (open circle) were incubated in the presence of different synthetic scarabaecin concentrations. As a positive control, *B. mori* cecropin B [24] was incubated similarly with *E. coli* (open triangle). Growth of 100% denotes growth without scarabaecin or *B. mori* cecropin B. Data are means from three experiments. Experimental conditions are detailed in Materials and methods.

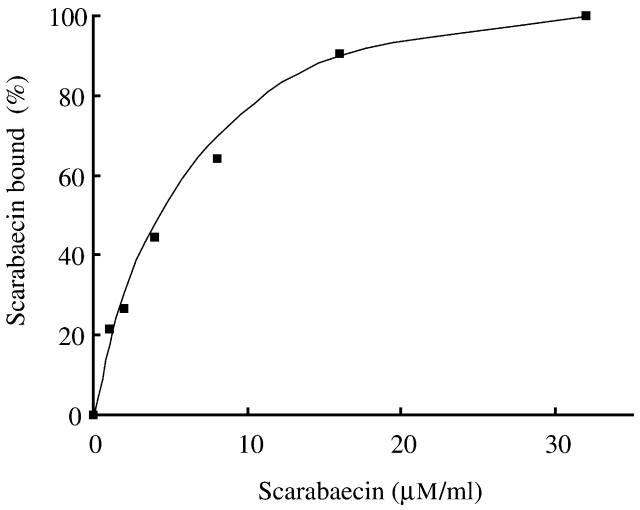

Fig. 4. Scarabaecin chitin binding. Chitin was mixed with the indicated amount of scarabaecin and incubated at room temperature for 15 min. Scarabaecin was measured as detailed in Materials and methods. One hundred percent denotes scarabaecin bound to chitin in 32 μM/ml of scarabaecin. Data are means from three experiments.

pathogenic fungus *B. bassiana* that can infect insects, and found no activity against *E. coli* and very weak activity against *S. aureus* and *B. bassiana*, whereas *Bombyx mori* cecropin B, a positive control, showed strong antibacterial activity against *E. coli* (Fig. 3).

Scarabaecin shows antifungal activity against some phytopathogenic fungi but not against the natural insect pathogenic fungus, *B. bassiana* (Table 1 and Fig. 3). This result raises the question of what is the real role of scarabaecin in insect immunity. At present, we cannot exclude the possibility that scarabaecin plays some other physiological role in hemolymph. Nevertheless, we believe that scarabaecin from *O. rhinoceros* is very useful for developing transgenic crops, especially rice plants, because of its antifungal activity against two major phytopathogenic fungi, *P. oryzae* causing rice blast and *R. solani* causing rice sheath blight, which we are now proceeding to study.

Chitin-binding property

Because scarabaecin showed antifungal activity against phytopathogenic fungi, we analyzed its chitin binding property using synthetic scarabaecin. Different amounts of scarabaecin were mixed with a constant amount of chitin and the amount of scarabaecin bound was quantitated. A progression curve of scarabaecin is shown in Fig. 4. Based on data K_d value was determined to be 1.315 μM. The results indicate that scarabaecin belongs to the family of chitin-binding antimicrobial substances.

As scarabaecin showed chitin-binding property, we compared the amino acid sequences focusing on putative chitin-binding domains among scarabaecin, tachycitin, an antimicrobial polypeptide from the

Invertebrate	
Scarabaecin (18-36)	CPKGKVWNGFD--CKS-PFA-FS
Tachycitin (40-60)	CPKGLHYNAYLKVCDW-PSK-AG
Ag-chit (501-521)	CPPGTLFDPALHICNW-ADQ-VK
Pj-chit1 (494-514)	CPAGTVWNQAIKACDW-PAN-VD
Ch-chit (465-483)	CPQGLCFNPANNYCDW-PSQ---
Peritrophin-44 (62-82)	CPDGYLYNNKLGICDS-PAN-VK
Tn-IM (453-473)	CPGNLHFSPATQSCES-PVT-AG
Plant	
Hevein (12-32)	CPNWLCCSQW-GWCGST-DEYCS
Ac-AMP2 (9-29)	CPSGMCCSQF-GYCGKGP-KYCG
WGA A (12-32)	CPNNLCCSQY-GYCGMGGD-YCG
WGA B (55-75)	CPNNHCCSQY-GHCGFGA-EYCG
WGA C (98-118)	CPNNLCCSQW-GFCGLG-SEFCG
WGA D (141-161)	CTNNYCCSQW-GSCGIGP-GYCG

Fig. 5. Sequence alignment of several chitin-binding peptides or polypeptides in invertebrates and plants for the region corresponding to Cys-18 to Ser-36 of scarabaecin. Chitin-binding peptides or polypeptides were isolated from the following invertebrates or plants: *O. rhinoceros* (Scarabaecin), *T. tridentatus* (Tachycitin) [14], *Anopheles gambiae* chitinase (Ag-chit) [15], *Penaeus japonica* chitinase 1 (Pj-chit1) [16], *Chelonus* sp. chitinase (ch-chit) [17], 44-kDa glycoprotein from *L. cuprina* (Peritrophin-44) [18], *T. ni* intestinal mucin (Tn-IM) [19], hevein from the rubber tree (Hevein) [20], *A. caudatus* antimicrobial protein 2 (AcAMP2) [21], and four homologous domains of wheat germ agglutinin (WGA A, -B, -C, and -D) [22]. Residue numbers for each segment are indicated in parentheses. Conserved amino acid residues and polar and hydrophobic residues significantly influencing structural constructions are highlighted.

horseshoe crab, *Tachypleus tridentatus* [14], three invertebrate chitinases [15–17], an invertebrate glycoprotein from *Lucia cuprina* [18], an insect intestinal mucin from *Trichoplusia ni* [19], hevein, a plant chitin-binding peptide from the rubber tree [20], Ac-AMP2, a plant antimicrobial protein from *Amaranthus caudatus* [21], and four wheat germ agglutinins [22] (Fig. 5). It appears that residues of cysteine, proline, and glycine, all of which may significantly influence structural constructions, are well conserved in chitin-binding proteins [14]. Conservation of polar and hydrophobic residues was further identified for putative chitin-binding residues (e.g., Asn-25 and Phe-27 for scarabaecin) [14]. These results suggest that scarabaecin belongs to the family of chitin-binding antimicrobial substances, although this peptide has no overall sequence similarity to other peptides/polypeptides.

Acknowledgments

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences of the Bio-oriented Technology Research Advancement Institution, Japan, and by a Grant-in-Aid (Insect Technology Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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