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Isolation, cDNA cloning and gene expression of an antibacterial protein from larvae of the coconut rhinoceros beetle, *Oryctes rhinoceros*

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An antibacterial protein, designated rhinocerosin, was purified to homogeneity from larvae of the coconut rhinoceros beetle, *Oryctes rhinoceros* immunized with *Escherichia coli*. Based on the amino acid sequence of the N-terminal region, a degenerate primer was synthesized and reverse-transcriptase PCR was performed to clone rhinocerosin cDNA. As a result, a 279-bp fragment was obtained. The complete nucleotide sequence was determined by sequencing the extended rhinocerosin cDNA clone by 5′ rapid amplification of cDNA ends. The deduced amino acid sequence of the mature portion of rhinocerosin was composed of 72 amino acids without cystein residues and was shown to be rich in glycine (11.1%) and proline (11.1%) residues. Comparison of the deduced amino acid sequence of rhinocerosin with those of other antibacterial proteins indicated that it has 77.8% and 44.6% identity with holotricin 2 and coleoptrecin, respectively. Rhinocerosin had strong antibacterial activity against *E. coli*, *Streptococcus pyogenes*, *Staphylococcus aureus* but not against *Pseudomonas aeruginosa*. Results of reverse-transcriptase PCR analysis of gene expression in different tissues indicated that the rhinocerosin gene is strongly expressed in the fat body and the Malpighian tubule, and weakly expressed in hemocytes and midgut. In addition, gene expression was inducible by bacteria in the fat body, the Malpighian tubule and hemocyte but constitutive expression was observed in the midgut.

Keywords: antibacterial protein; *Oryctes rhinoceros*; insect immunity; cDNA cloning; gene expression.

Antibacterial proteins are important factors which can eliminate infectious bacteria in innate immune systems of animals (Dunn, 1986; Boman and Hultmark, 1987; Boman, 1991, 1995; Lehrer et al., 1991; Hoffmann, 1995). In insects, a large number of antibacterial proteins have been purified from six constituent orders; the Lepidoptera, the Diptera, the Coleoptera, the Hymenoptera, the Hemiptera and the Ephemeroptera (Hultmark, 1993; Kimbrel, 1991; Bullet et al., 1991). These antibacterial proteins are classified in five major groups (Hultmark, 1993); cecropin, insect-defensin, glycine-rich proteins, proline-rich proteins and lysozymes. As far as is known six antibacterial proteins have been isolated from coleopteran insects such *as Zophobas atratus*, *Tenebrio moliter*, *Holotrichia diomphalia and Allomyrina dichotoma*. These antibacterial proteins were divided into three groups, namely defensin-related proteins including *A. dichotoma* defensin (Miyanoshita et al., 1996), peptide B and C from *Z. atratus* (Bullet et al., 1991) and tenecin 1 from *T. molitor* (Moon et al. 1994), coleoptericin from *Z. atratus* (Bullet et al., 1991) and holotricin 2 from *H. diomphalia* (Lee et al., 1994).

Coleoptericin is a 74-residue glycine-rich protein containing no cystein residues, and is bactericidal against Gram-negative bacteria (Bullet et al., 1991). Holotricin 2 consists of 72 amino acid residues without cystein residues and shows antibacterial activity against Gram-negative bacteria (Lee et al., 1994). Both coleoptericin and holotricin 2 belong to the glycine-rich antibacterial proteins and share 39.2% sequence identity. Their N-terminal regions indicate significant similarity, suggesting that this common region is important for antibacterial activity.

In our efforts to search further for antibacterial proteins from larvae of coleopteran insects, we attempted to purify them using the coconut rhinoceros beetle, *Oryctes rhinoceros*. In this report, we describe the purification, cDNA cloning and gene expression of an antibacterial protein named rhinocerosin from this insect and the character of its antibacterial activity.

MATERIALS AND METHODS

Experimental animals. The coconut rhinoceros beetle, *O. rhinoceros* was collected in the field of the Ryukyu islands, Japan. Third-instar larvae were used to purify the antibacterial protein.

Immunization and collection of hemolymph. Third-instar larvae were cooled on ice and injected with 50 μl *Escherichia coli* K12 (2×10^6 cells) suspended in physiological saline (150 mM NaCl, 5 mM KCl). The larvae were kept at 25 °C for 24−48 h. Hemolymph was collected into ice-cooled tubes in the presence of aprotinin (Sigma, 50 μg/ml) by cutting off a leg. The hemolymph was centrifuged at $39\,000\times g$ for 50 min to remove hemocytes. The clear supernatant was filtrated through an 0.8-μm filter (Millex) and stored at −20 °C until use.

Assay of antibacterial activity. The plate-growth-inhibition assay was according to the methods described by Hultmark et al. (1982). Briefly, melted agar (20 ml) containing 1×10^6 loga-

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Abbreviations. RT-PCR, reverse-transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends.

Note. The novel nucleotide sequence data reported here have been submitted to the GSDB/DDBJ/EMBL/NCBI nucleotide sequence databases and are available under accession number AB010824.

rithmic-phase cells of a given bacterial strain was poured into sterile Petri dishes (8.4 cm diameter). Wells (2 mm diameter) were cut into the freshly poured plates after solidification of the agar. Each well received 2 µl fraction. The plates were incubated overnight at 37°C, and the diameters of the clear zones were recorded after subtraction of the well diameter. To investigate the effect of the purified rhinocerosin, different doses of the antibacterial protein were added to bacterial culture (50 µl) of *Staphylococcus aureus*, *Staphylococcus pyogenes*, *Pneumococcus aeruginosa* and *E. coli*. Logarithmic-phase cells of these bacteria, which were cultured in Müller and Hinton broth (Difco), were first suspended in 30 mM sodium phosphate, pH 7.0, containing 60 mM NaCl. 10 µl this suspension (1.5×10^5 cells) were added to 40 µl fresh broth and cultured for 20 h at 37°C with shaking. The culture was chilled and the bacterial concentration was determined by measuring the absorbance at 550 nm by a spectrophotometer (Beckman, DU-650).

Purification of antibacterial protein. 15 ml hemolymph was applied to a Sep-Pack C_{18} cartridge (Waters Associates), which was previously equilibrated with 20% acetonitrile/0.05% trifluoroacetic acid (solution A). After extensive washing of the cartridge with solution A, adsorbed materials were eluted stepwise with 30, 40, 50, 60 and 100% acetonitrile/0.05% trifluoroacetic acid and dried under vacuum. The antibacterial activity was recovered by elution with 60% acetonitrile/0.05% trifluoroacetic acid. The dried materials were dissolved in 0.05% trifluoroacetic acid and applied to reverse-phase HPLC with ProRPC HR5/10 C_8 column (5 mm×100 mm, Pharmacia) connected to a FPLC (Pharmacia), which was equilibrated with 0.05% trifluoroacetic acid. The column was washed with 0.05% trifluoroacetic acid, then adsorbed materials were eluted for 75 min with a linear gradient of 0−50% acetonitrile/0.05% trifluoroacetic acid. Fractions having antibacterial activity against both *E. coli* and *S. aureus* were pooled and dried under vacuum. The dried materials were dissolved in solution A and loaded onto the same HPLC column as described above, which was equilibrated with solution A. The column was washed with solution A and a linear gradient of 20−40% acetonitrile/0.05% trifluoroacetic acid was performed for 20 min. The antibacterial materials were separately eluted in two acetonitrile fractions. The latter fractions (≈140 µg proteins from 10 larvae) were combined, dissolved in 10 mM sodium phosphate, pH 6.0, containing 130 mM NaCl and heated at 100°C for 5 min. The heat-treated sample was centrifuged for 10 min at 39000×*g* to remove heat-denatured proteins. The supernatant was mixed with acetonitrile and trifluoroacetic acid (final concentration 30% and 0.05%, respectively). This sample was applied to reverse-phase HPLC with a µRPC C_2/C_{18} column (2.1 mm×100 mm, Pharmacia) connected to a Smart system (Pharmacia), which was equilibrated with 30% acetonitrile/0.05% trifluoroacetic acid (solution B). The column was extensively washed with solution B and adsorbed materials were eluted for 60 min with a one-step linear gradient of 30−40% acetonitrile/0.05% trifluoroacetic acid.

Tricine SDS/PAGE. Tricine SDS/PAGE was carried out according to the method of Schägger and von Jagow (1987). The separation slab gel contained 16.2% acrylamide. Protein samples were dissolved in 50 mM Tris/HCl, pH 6.8, containing 10% 2-mercaptoethanol, 20% SDS, 0.1% bromophenol blue and 10% glycerol, and heated for 5 min at 100°C. After electrophoresis, the gel was stained with Coomassie brilliant blue. Protein quantification was performed according to the method of Lowry et al. (1951).

Amino acid sequence analysis. The amino acid sequence was determined using an Applied Biosystems model 473A protein sequencer.

Cloning of cDNA and nucleotide sequencing. mRNAs were isolated from fat bodies of third-instar larvae 10 h after immunization with *E. coli* K12 (2×10^5 cells) using a Quick Prep mRNA Purification Kit (Pharmacia). The first strand cDNA was synthesized employing a First Strand cDNA Synthesis Kit (Pharmacia). 60 ng first strand DNA were used for reverse-transcription PCR (RT-PCR). RT-PCR was performed with the following degenerate primers: 5′-CC/ACGT/GG/ACGT/GC/ACGT/CC/ACGT/AA/CT/TT/CT/CC-3′ for forward primer and 5′-AACTGGAAGAATTCGCGGCCGCAGGA-3′ for reverse primer. The forward primer was based on the amino acid sequence of rhinocerosin (Fig. 3) and the reverse primer was a modification of the bifunctional primer Not I-d$(T)_{18}$ (Pharmacia). 35 cycles of RT-PCR were carried out under the following conditions: 94°C (30 s), 55°C (30 s) and 72°C (30 s). A 297-bp fragment obtained by RT-PCR was subcloned into pCR2.1 vector using an Original TA Cloning Kit (Invitrogen). The nucleotide sequence of this fragment was determined by a dye-terminator cycle sequencing method using a DNA sequencer (ABI 373A). 5′-rapid amplification of cDNA ends (5′-RACE) was performed using the rhinocerosin-specific antisense oligonucleotides: 5′-AGCTGCAGTATTGGGTCCCTGT-3′, 5′-TTTCAATATTAGAGGTGATAC-3′ and 5′-TGGTAGGTAACTGTGAACCAGG-3′. A 343-bp PCR product was subcloned and nucleotide sequencing was carried out as described above.

Analysis of gene expression in different tissues. RT-PCR was carried out to examine specific expression of the rhinocerosin gene in different tissues. The third-instar larvae were immunized with *E. coli*. Fat body, hemocyte, midgut and the Malpighian tubules were excised from third-instar larvae 10 h after immunization. As a control, the same tissues were excised from non-immunized larvae. mRNA isolation and cDNA synthesis were as described above. 60 ng first strand DNA were used for RT-PCR. RT-PCR was performed with the following primers: 5′-GATGATGAAGCTTTACATCG-3′ (forward primer) and 5′-ACCTGTAAGTTCCACCAATG-3′ (reverse primer). As an internal marker, the following insect actin primers were synthesized and used (Kasai et al., 1998): 5′-AGCAGGAGATGGCCACC-3′ (forward primer) and 5′-TCCACATCTGCTGGAAGG-3′ (reverse primer). Five different RT-PCR cycles (25, 30, 35, 40 and 45 cycles) were performed under the following conditions: 94°C (30 s), 50°C (30 s) and 72°C (30 s). The RT-PCR products were subjected to agarose-gel electrophoresis (2%) and stained with ethidium bromide. A 100-bp ladder (Pharmacia Biotech) was used as DNA size markers.

RESULTS

Purification of rhinocerosin. Rhinocerosin was purified from hemolymph of the third-instar larvae immunized with *E. coli*. After Sep-Pack treatment of the hemolymph, samples were first fractionated by reverse-phase HPLC with a linear gradient of acetonitrile (0−50%). The antibacterial activity of each fraction was separately monitored using *E. coli* and *S. aureus*. In this purification step, molecules having antibacterial activity against both bacteria were eluted with 26−31% acetonitrile. The pooled fractions containing antibacterial activity were further purified by the same HPLC with a relatively mild gradient of acetonitrile (20−40%). In this HPLC, two activity peaks were obtained at 22−24% and 24−27% acetonitrile fractions. The combined samples of the latter fractions were subjected to a final reverse-phase HPLC after heat treatment. A sharp protein peak was eluted with a linear gradient of acetonitrile (30−40%) and the antibacterial activity of the peak fractions was confirmed using *S. aureus* (Fig. 1). Tricine SDS/PAGE of this protein fraction

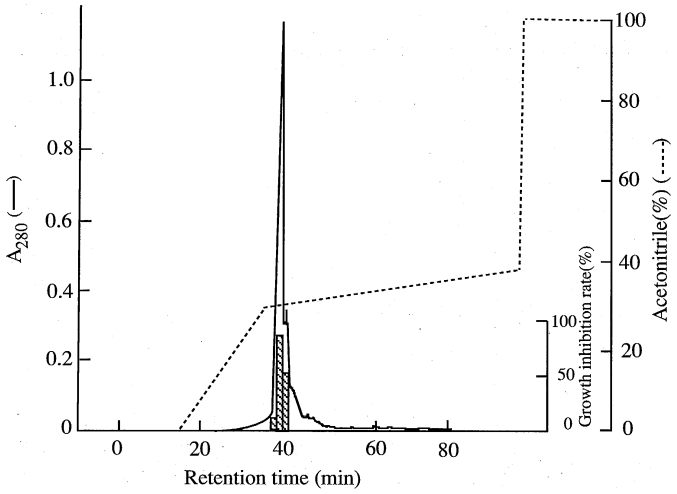

Fig. 1. Final purification by reverse-phase HPLC of rhinocerosin. Reverse-phase HPLC was performed with an mRPC C_2/C_{18} column connected to a Smart system (Pharmacia). Proteins adsorbed on the column were eluted with a gradient of 30−40% acetonitrile/0.05% trifluoroacetic acid. Protein content was monitored by measuring the ultraviolet adsorbance at 280 nm. Antibacterial activity was examined in the plate-growth-inhibition assay using *S. aureus*, and is expressed as a growth inhibition rate (%) (columns).

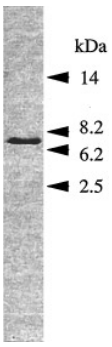

Fig. 2. Tricine SDS/PAGE of purified rhinocerosin. Electrophoresis was carried out with 2 μg purified protein under denaturing conditions. The following marker proteins (Pharmacia) were used to estimate the molecular mass of rhinocerosin: myoglobin I and II (14.4 kDa), myoglobin I (8.2 kDa), myoglobin II (6.2 kDa) and myoglobin III (2.5 kDa).

H_2N-SerLysGlnProGlyAlaProAsnPheProMetProGly

5'-CC/ACGT/GG/ACGT/GC/ACGT/CC/ACGT/AACT/TT/CT/CC-3'

Fig. 3. Amino acid sequence of rhinocerosin and degenerate primers. The amino acid sequence of the N-terminal region of rhinocerosin was determined by the Edman degradation method. Based on the amino acid sequence, degenerate primers were synthesized for RT-PCR.

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ACAGTTAAGTTTGTATCGTTAAACTGCGAACACATTGGTGAATATCTCATTTGCTGAACC -19
GACAAGCACCCTTTCAAGATGATGAAGCTTTACATCGTTTTCGGTTTTATCGCGTTTTCT  42
                  M  M  K  L  Y  I  V  F  G  F  I  A  F  S
GCTGCTTACGTCGTTCCAGAGGGTTACTACGAGCCCGAGTATTATCCAGCAGACGGATAC 102
 A  A  Y  V  V  P  E  G  Y  Y  E  P  E  Y  Y  P  A  D  G  Y
GAAAGTGAGCGCGTCGCGAGGGCTTCGCCTGCCGAATTGATTTTCGATGAAGACCTCGCC 162
 E  S  E  R  V  A  R  A  S  P  A  E  L  I  F  D  E  D  L  A
GATGAACCCGAAGTAGAGGAGCCGCAATATTATATCCGCACAAGAAGATCTCTCCAGCCA 222
 D  E  P  E  V  E  E  P  Q  Y  Y  I  R  T  R  R  S  L  Q  P
GGTGCTCCAAATTTTCCGATGCCTGGTTCACAGTTACCTACCAGTATCACCTCTAATATT 282
 G  A  P  N  F  P  M  P  G  S  Q  L  P  T  S  I  T  S  N  I
GAAAAACAGGGACCCAATACTGCAGCTACCATCAATGCCCAACATAAAACTGATAGGTAT 342
 E  K  Q  G  P  N  T  A  A  T  I  N  A  Q  H  K  T  D  R  Y
GACGTTGGAGCCACCTGGAGCAAGGTTATTCGTGGACCAGGAAGAAGTAAACCAAACTGG 402
 D  V  G  A  T  W  S  K  V  I  R  G  P  G  R  S  K  P  N  W
AGCATTGGTGGAACTTACAGGTGGTAACACCTGGACATGTTGTTAATTTTTTTTTTATTT 462
 S  I  G  G  T  Y  R  W  ***
TTTTTTACGAAATAAAGAGAACTTTTTAAACAAAAAAAAAAAAAAAAAAAAAAA       552
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Fig. 4. Nucleotide and deduced amino acid sequences of a cDNA encoding rhinocerosin. The numbers of nucleotides starting from the methionine codon is given at the right side of each line. Deduced amino acids are expressed by the one-letter code. The amino acid residue of rhinocerosin N-terminus is boxed. The potential Arg-Thr-Arg-Arg processing signal and the putative poly(A) addition signal, AATAAA, are underlined. Asterisks indicate the termination codon. Arrows denote the position of 5′-RACE primers.

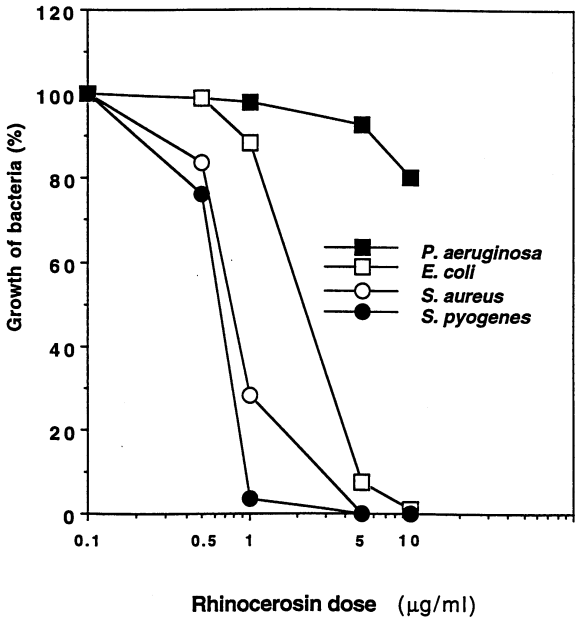

Fig. 5. Effect of rhinocerosin dosage on the growth of bacteria. Growth rate of bacteria (*P. aeruginosa*, *S. pyogens*, *S. aureus*, and *E. coli*) was determined in the presence of different concentrations of rhinocerosin (0, 0.5, 1.5 and 10 μg/ml). Bacterial growth rate without rhinocerosin was expressed as 100%. Rhinocerosin doses on the horizontal axis are expressed as logarithm. Experimental conditions are described in detail in Materials and Methods.

indicated a single band having molecular mass of 7300−7800 Da (Fig. 2).

Analysis of amino acid sequence. As the homogeneity of the antibacterial protein was confirmed by Tricine SDS/PAGE, the amino acid sequence of the N-terminal region was analyzed by the automated Edman degradation method. Amino acid sequence of 13 residues, starting with serine, was determined (Fig. 3). A computer-aided homology search of the partial amino acid sequence with those of reported proteins clarified that it is nearly identical with that of holotricin 2 isolated from the beetle, *H. diomphalia* (Lee et al., 1994).

cDNA cloning and nucleotide sequencing. To obtain the complete amino acid sequence of the purified antibacterial protein, molecular cloning of the cDNA by RT-PCR was carried out. For this, a degenerate primer based on the amino acid sequence of

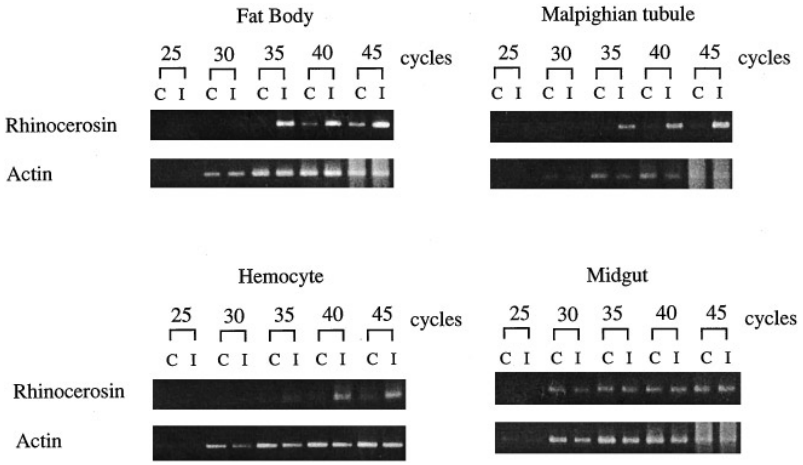

Fig. 6. Rhinocerosin gene expression in different tissues. mRNA samples were extracted from the fat body, the Malpighian tubules, hemocytes and midgut of third-star larvae non-immunized and immunized with *E. coli*. RT-PCR products after 25, 30, 35, 40 and 45 cycle reactions were electrophoresed on 2% agarose gel. As an internal marker, actin primers were used for RT-PCR. Details of experimental conditions are described in Materials and Methods.

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Rhinocerosin    SLQPGAPNFPMPGSQLPT-SITSNIEKQGP-NTAATINAQHKTDRYDVGA
Holotricin 2    SLQPGAPSFPMPGSQLPT-SVSGNVEKQGR-NTIATIDAQHKTDRYDVRG
Coleoptericin   SLQGGAPNFPQPSQQNGGWQVSPDLGRDDKGNTRGQIEIQNKGKDHDFNA

Rhinocerosin    TWSKVIRGPGRSKPNWSIGGTYRW
Holotricin 2    TWTKVVDGPGRSKPNFRIGGSYRW
Coleoptericin   GWGKVIRGPNKAKPTWHVGGTYRR
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Fig. 7. Comparison of amino acid sequence of rhinocerosin-related antibacterial proteins. Amino acid sequences of rhinocerosin, holotricin 2 and coleoptericin were compared. Identical amino acids are indicated by bold letters. Gaps were introduced to maximize the sequence alignment.

the N-terminal portion was synthesized (Fig. 3). A 297-bp cDNA fragment was obtained by RT-PCR. Based on the nucleotide sequence of this fragment, 5′-RACE was performed with three synthetic primers to determine the full-length nucleotide sequence of rhinocerosin cDNA (Fig. 4). The nucleotide sequence contained non-translation sequences at 5′ and 3′ regions and a typical poly(A) addition signal was located 15 bases upstream of the poly(A) site. The deduced amino acid sequence of rhinocerosin revealed that there is an open reading frame for 142 amino acid residues before the termination codon, TGA. It was characteristic that rhinocerosin contains no cysteine residues. Structural analysis of rhinocerosin indicated that it has a recognition sequence for the cleavage site within the constitutive secretory pathway (Arg-Xaa-Lys/Arg-Arg; Hosaka et al., 1992), suggesting that a mature portion (72 amino acid residues) is produced by cleavage of the signal peptide and propeptide from the 142-amino-acid precursor protein.

Antibacterial activity of rhinocerosin. To determine the effect of the rhinocerosin dosage on the growth of bacteria, two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and two Gram-positive bacteria (*S. aureus* and *S. pyogenes*) were tested.

Rhinocerosin strongly inhibited the growth of *E. coli*, *S. aureus* and *S. pyogenes* but not *P. aeruginosa* (Fig. 5). The two Gram-positive bacteria were more sensitive to rhinocerosin than the other two Gram-negative bacteria. In particular, the growth of *S. pyogenes* was totally suppressed by a low dose (1 μg/ml) of rhinocerosin (Fig. 5). This antibacterial specificity of rhinocerosin was in contrast to that of holotricin 2, which showed antibacterial activity against Gram-negative bacterial species including *E. coli*, *Shigella flexneri*, *P. aeruginosa* and *Proteus vulgaris* but not against Gram-positive bacteria such as *S. aureus*, *Micrococcus luteus* and *Corynebacterium diphtheria* (Lee et al., 1994).

Gene expression of rhinocerosin in different tissues. Gene expression of rhinocerosin was analyzed by RT-PCR. For this, mRNA samples were extracted from different tissues such as the fat body, the Malpighian tubules, hemocytes and the midgut of larvae non-immunized and immunized with *E. coli*. RT-PCR was carried out at various cycles. The rhinocerosin gene was, in principle, expressed in all tissues examined, the fat body and the Malpighian tubules being the main sites of expression (Fig. 6). In the fat body, Malpighian tubules and hemocytes, rhinocerosin gene expression was inducible by bacteria, whereas constitutive gene expression was seen in the midgut (Fig. 6).

DISCUSSION

Larvae of the coconut rhinoceros beetle, *O. rhinoceros* feed on dung of cattles and grow in compost. It was speculated that the larvae have developed strong immune systems against bacterial infection in such an environment. Thus, we tried to isolate antibacterial proteins from this insect. In the purification steps, at least three separated fractions were found containing antibacterial activity against *E. coli* and/or *S. aureus* (data not shown). In this study, we have purified an antibacterial protein, designated rhinocerosin, from a hemolymph fraction of the third-instar larvae, which were immunized with *E. coli*. Based on the amino acid sequence deduced from rhinocerosin cDNA, the pI value of the peptide was determined to be 10.67, suggesting that it is a basic protein. Rhinocerosin is rich in glycine (11.1%) and proline (11.1%) residues. Computer-aided search for sequence similarity of rhinocerosin to those of other reported proteins re-

sulted in matches with two antibacterial proteins from coleopteran insects, coleoptericin (Bullet et al., 1991) and holotricin 2 (Lee et al., 1994). Holotricin 2 and coleoptericin are also basic proteins and have high contents of glycine (12.5% and 17.6%, respectively) and proline residues (9.7% and 8.1%, respectively). The deduced amino acid sequence of the mature portion of rhinocerosin showed 77.8% and 44.6% identity to those of holotricin 2 and coleoptrecin, respectively.

Comparison of amino acid sequences of rhinocerosin, holotricin 2 and coleoptericin indicated that the N-terminal (15-amino-acid residues) and the C-terminal regions (24-amino-acid residues) of these proteins have high incidences of identical residues to those of rhinocerosin (67% and 46%, respectively), suggesting that N-terminal and C-terminal regions of these antibacterial proteins play important roles in expression of their antibacterial activity (Fig. 7). Furthermore, the distribution of basic amino acid residues was clustered within 24-amino-acid residues of the C-terminal region (56%, 45% and 54% for rhinocerosin, holotricin 2 and coleoptrecin, respectively, Fig. 7). In general, it is assumed that the charges of basic amino acid residues play essential roles in the interaction of antibacterial protein with bacteria (Christensen et al., 1988; Gabay, 1994). Thus, the results suggest that the positive charge at the C-terminal region of these antibacterial proteins contributes to adherence to the surface of bacteria, which has a negative charge. Results of computer-aided phylogenetic analysis of rhinocerosin, holotricin 2 and coleoptericin revealed that these antibacterial proteins have strong structural relationship, suggesting that they have been derived from a common ancestral gene (data not shown).

In order to analyze rhinocerosin gene expression, we employed a highly sensitive RT-PCR method. Various RT-PCR cycles were examined to avoid misjudgement of the results obtained under saturated conditions of amplified gene transcripts. Gene expression of rhinocerosin was confirmed to be inducible by bacteria in the fat body, the Malpighian tubules and hemocytes (Fig. 6). Results of RT-PCR, however, clearly indicated that the rhinocerosin gene is constitutively expressed in the midgut (Fig. 6). One possible explanation is that the rhinocerosin gene is constitutively expressed in the midgut at a low level, as is the case with lysozymes of *H. cecropia* and *B. mori*, regardless of the bacterial infection type (Sun et al., 1991; Morishima et al., 1995). Another possibility is that midguts of the larvae are constantly infected by bacteria, because of their living circumstances (as explained above). Sterile rearing conditions for this insect need to be developed to investigate gene expression of rhinocerosin in the midguts. Strong expression of the rhinocerosin gene in the Malpighian tubules is also unique as compared with expression patterns in different tissues of other insect antibacterial proteins (Kato et al., 1993; Sugiyama et al., 1995; Chowdhury et al., 1995). The biological significance of gene expression of rhinocerosin in the Malpighian tubules remains as a question for the future.

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