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Cloning of mRNA Sequences for Two Antibacterial Peptides in a Hemipteran Insect, *Riptortus clavatus*

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ABSTRACT—*Escherichia coli* injection rapidly induced bactericidal activity in the hemolymph of a hemipteran insect, *Riptortus clavatus*. This activity reached its maximum at 9 hr after injection and thereafter declined slowly. Two types of cDNA clones involved in this response were isolated by differential screening. The predominant type encoded for an open reading frame of 678 amino acids, which consisted of fourteen tandem repeats. Each repeat was rich in charged residues and had a proline-rich region which had striking sequence similarities to proline-rich antibacterial peptides from other insect species, indicating these clones encode a multi-peptide precursor of antibacterial peptides. The other type encoded for a glycine-rich peptide similar to a known antibacterial peptide as well. Northern blot analyses revealed rapid induction of mRNAs corresponding to these clones after the injection. To our knowledge, this is the first report on the mRNA sequences of antibacterial peptides of hemimetabolous insects, and the second report on the occurrence of multi-peptide precursor structure in insect antibacterial peptides.

INTRODUCTION

Insects have both cellular and humoral self-defense systems to protect themselves against bacterial challenges. A well-characterized humoral defense is a rapid and transient synthesis of a battery of antibacterial peptides upon infection. Usually, these are small sized cationic peptides having bactericidal activity against a wide spectrum of bacteria, and their expressions are thought to be regulated at the transcriptional level (for review, see Boman *et al.*, 1991; Hultmark, 1993). Up to now several antibacterial peptides have been purified, and in some cases, characterized at the nucleic acid sequence level. These studies have been done almost exclusively in higher, holometabolous (showing complete metamorphosis) insects including Lepidoptera, Diptera, Hymenoptera and Coleoptera. Antibacterial peptides so far isolated from the higher insects can be classified as follows. i) The cecropins are about 4-kDa peptides and active against both gram-negative and gram-positive bacteria (Hultmark *et al.*, 1982; Qu *et al.*, 1982; Okuda and Natori, 1985; Dickinson *et al.*, 1988; Taniai *et al.*, 1992). ii) The insect defensins are 4-kDa in size, active against gram-positive bacteria and possess six cysteine residues engaged in three intramolecular disulfide bridges (Matsuyama and Natori, 1988; Lambert *et al.*, 1989; Fujiwara *et al.*, 1990; Bulet *et al.*, 1992). This class of peptides are absent in Lepidoptera. iii) Glycine-rich peptides such as attacins (Hultmark *et al.*, 1983), sarcotoxin II (Ando and Natori, 1988), dipterin (Dimarcq *et al.*, 1988)

and coleopterins (Bulet *et al.*, 1991) are active against a limited number of gram-negative bacteria. iv) Small sized proline-rich peptides, apidaecin (Casteels *et al.*, 1989) and abaecin (Casteels *et al.*, 1990) from honeybee and drosocin (Bulet *et al.*, 1993) from fruitfly are active against gram-negative bacteria. On the other hand, our knowledge about antibacterial peptides of hemimetabolous (showing incomplete metamorphosis) insects is quite limited. Recently Cociancich *et al.* (1994) have isolated and characterized three antibacterial peptides from a hemipteran insect, *Pyrrochoris apterus*: namely, the insect defensin of this species, a proline-rich peptide carrying an O-glycosylated substitution and a glycine-rich peptide. Experimentally, antibacterial peptides are induced rapidly in hemolymph following bacterial component injection or body wall injury. Increasing evidence suggests that this insect "immune" reaction is analogous to innate immunity in vertebrates, especially to the acute phase response. Recently, we have noticed that in the hemipteran insects, the bean bug, *Riptortus clavatus*, bactericidal activity can also be induced in the hemolymph by injection of formaldehyde-fixed *E. coli*. The present study was conducted in an attempt to directly identify genes involved in acute phase response of this species by a new approach, differential screening. In this paper, we describe cDNA cloning, sequence analysis and expression of two antibacterial peptide genes.

MATERIALS AND METHODS

Insects

The bean bugs, *Riptortus clavatus* were collected in Ishigaki Island, Okinawa pref. and reared at 25°C under a 16 hr light/8 hr

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dark cycle. F₁ and F₂ adult females were used in all experiments. Bugs were injected with 1 μ l of a suspension of formaldehyde-fixed *E. coli* LE392 (1.6×10^9 cells/ml in 0.9% NaCl) into the hemocoel while under CO₂ anesthetization. Bugs were kept at 25°C until dissection. Hereafter, insects treated as described above are designated as "immunized" insects. Hemolymph was collected and diluted 5-fold with 0.9% NaCl. Hemolymph samples were centrifuged at 12,000 rpm for 5 min, and the supernatant stored at -80°C until use.

Growth inhibition assay

Bactericidal activity in the hemolymph sample was estimated as follows. Five milliliters of 0.6% melted LB agarose (52°C) were mixed with 100 μ l of viable *E. coli* LE392 suspension (1.6×10^9 cells/ml), and poured into a 9 cm plastic dish. Wells of 4 mm in diameter were punched after the agarose layer was solidified. Five microliters each of hemolymph sample was applied into the wells, and incubated at 37°C overnight. The samples containing bactericidal activity produced clear zones where the bacterial growth was not seen (growth inhibition zone). The area of the growth inhibition zone was measured. Since a liner relationship was observed between areas of growth inhibition zone and relative quantity of immunized hemolymph (data not shown), we concluded that this assay was able to be used to estimate bactericidal activity.

Extraction of RNA and construction of cDNA library

Total RNA was extracted from the fat body of naive or immunized female adults by AGPC method (Chomczynski and Sacchi, 1987). RNA preparations were measured colorimetrically. A260/A280 and A260/A230 of RNA preparations were always above 1.7 and 2.0, respectively. In some cases, the total RNA fraction was further purified to poly(A)⁺ RNA by using oligo(dT)-latex (Japan Synthetic Rubber Co.). The cDNA library was constructed from poly(A)⁺ RNA isolated from the fat body of the female adults 9 hr after immunization using a cDNA Synthesis Kit (Pharmacia) according to the instructions of the manufacturer. Oligo(dT)-primed synthesis of the first cDNA strand was done using Molony murine leukemia virus reverse transcriptase. RNase H and DNA polymerase I were used for second strand cDNA synthesis. The cDNA preparation was ligated to *Eco*RI-digested λ gt10 arms (Stratagene) after the addition of *Eco*RI/*Not*I linkers and packaged *in vitro* using a commercial kit (Nippon Gene). *E. coli* NM514 cells were infected with recombinant phages, giving a titer of 1.72×10^7 pfu/ μ g RNA.

Differential screening

Single-stranded cDNA probes were prepared as described in Sambrook *et al.* (1989). One microgram each of fat body poly(A)⁺ RNA from naive or immunized (9 hr) female adults was reverse-transcribed by random priming. The reaction mixture had a total volume of 25 μ l of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 4 mM dithiothreitol, 0.8 mM each of dGTP, dATP and dTTP, 4.8 μ M dCTP, containing [α -³²P] dCTP (100 μ Ci, ICN), RNAase inhibitor (40 U, TOYOBO), random hexamer (9 μ g, BRL) and SuperScript reverse transcriptase (200 U, BRL). After 1 hr incubation at 37°C, the reaction was stopped by addition of EDTA (final conc. 20 mM) and sodium dodecyl sulfate (SDS, final conc. 0.8% w/v). The mixture was then incubated at 68°C for 30 min after the addition of 3 μ l of 3N NaOH. The solution was neutralized and extracted with phenol : chloroform (1:1). The single-stranded cDNA preparation was purified by gel filtration (Nick column, Pharmacia). *E. coli* NM514 cells were infected with phages at 37°C for 20 min. The infected cells were mixed with melted LB top agarose, plated on LB plates and incubated at 37°C for 9 hr. A set of duplicate plaque lifts was taken onto nylon membrane (Hybond N+, Amersham) for each plate. The lifts were prehybridized for 1 hr at 68°C in a solution containing 5 \times Denhardt's solution, 5 \times SSPE, 10 μ g/ml of denatured salmon sperm DNA, and 0.5% (w/v) SDS, and the subsequent hybridization step

was done in the same solution supplemented with radioactive probes prepared either from naive or immunized insects at 68°C overnight. The lifts were washed sequentially : twice in 2 \times SSPE at 68°C for 20 min; once in 1 \times SSPE at 68°C for 20 min. After washing the lifts were autoradiographed with Fuji HR-S X-ray films using intensifying screens at -80°C overnight. Plaques giving differentially expressed signals were picked up, plated and screened again in the same way. Plaques giving positive signals were purified through the two cycles of screening. Phage DNA was purified by using a commercial kit (QIAGEN), and the insert cut out by *Eco*RI. The size of the cDNA insert was determined by agarose gel electrophoresis.

Sequencing and analysis of sequence data

The cDNA inserts having *Eco*RI overhangs on both ends were subcloned into Bluescript II SK+ (Stratagene). Serial deletion mutants were made by exonuclease III followed by Mung bean nuclease using a commercial kit (Nippon Gene). Deletion mutants were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a *Taq* dye-deoxy terminator cycle sequencing kit and an Applied Biosystem Model 373A sequencer (ABI Inc.). Editing and analysis of cDNA sequence data were performed with a commercial program (GENETYX Ver. 8.0, SDC Software Development Co.). DNA and Amino acid sequence homology searches were done by a fasta program through an e-mail server of DNA Data Bank of Japan.

Northern blot hybridization

Radioactive cDNA probes were prepared from cDNA inserts by using a cDNA multi-primer labeling kit (Nippon Gene) and [α -³²P]-dCTP (ICN). Five micrograms per lane of total RNA preparation were run on a 1.2% agarose-formaldehyde gel, then transferred onto nylon membrane (Hybond N+, Amersham) under alkaline conditions. The membrane was hybridized with a cDNA probe at 42°C in a solution of 50% formamide (v/v), 5 \times SSPE, 5 \times Denhardt's solution, and 10 μ g/ml of denatured salmon sperm DNA (Sigma) following prehybridization. The membrane was washed twice at 68°C in 2 \times SSPE for 20 min, once in 1 \times SSPE for 20 min and finally in 0.1 \times SSPE for 20 min, then autoradiographed with Fuji HR-S X-ray films using intensifying screens at -80°C overnight.

RESULTS AND DISCUSSION

Figure 1 shows the time course of bactericidal activity induction in the hemolymph up to the first 48 hr after immunization. In the hemolymph of naive insects (0 hr), bactericidal activity was negligible while it was first recognized in hemolymph at about 1 hr after injection. Growth inhibition zone increased in size sharply up to 9 hr, reached its maximum, then decreased slowly. The bactericidal activity was thermo-stable up to 90°C and destroyed completely by proteinase K treatment (data not shown), suggesting that the activity could be attributed to rather small, proteinaceous factors. This immediate induction represents an acute phase response in this insect. We attempted to isolate cDNA clones associated with this response using differential screening.

The time course of bactericidal activity induction in immune hemolymph suggested that the acute phase-related mRNA would accumulate in relatively large quantities in insects around 9 hr after injection. A cDNA library was constructed from fat body poly(A)⁺ RNA of the insects 9 hr after injection. The library was screened differentially by using two sorts of single-stranded cDNA probes prepared

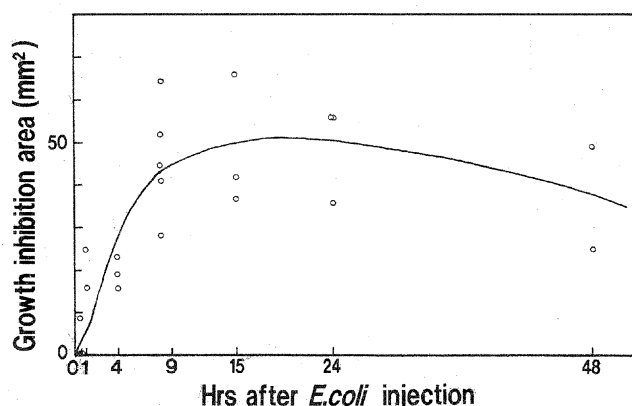


Fig. 1. Time course of induction of bactericidal activity following *E. coli* injection. The activity was determined by growth inhibition assay. The hemolymph sample equivalent to 1 μ l of original hemolymph was applied, and the areas of growth inhibition zones measured after incubation at 37°C. Open circles represent the results of individual insects.

from insects 9 hr after immunization and naive insects ((+) and (-) probes, respectively). Finally, 8 (+)-specific cDNA clones were isolated by screening approximately 1.44×10^4 clones of independent recombinant phage. These (+)-specific clones were categorized into two groups by restriction mapping and cross-hybridization. Group A consisted of seven clones which carried a cDNA insert of around 2000 bp. Restriction mapping of group A clones with *Kpn*I implied the existence of repeated sequences (data not shown). Group B consisted of only one clone carrying a cDNA insert of 600 bp in size. One representative clone each was selected from both groups: λ diff2 from group A; λ diff16 from group B. The cDNA inserts of the representatives were gel-purified and subcloned into Bluescript II SK+ for further characterization.

The nucleotide and deduced amino acid sequence for entire insert of λ diff2 clone is shown in Figure 2. λ diff2 had an insert of 2242 bp. The start codon (ATG) was located 24 bp downstream from the 5' end. First terminal codon (TAA) was at position 2058–2060. A recognition sequence for the addition of poly(A)⁺ tail (AATAAA) was located at position 2225–2230. Thus, an open reading frame extending 679 codons was deduced. This corresponds to a peptide with a molecular mass of 76367 daltons. As expected from the results of restriction mapping, the nucleotide sequence consisted of fifteen tandem repeats of a highly conserved short sequence, one of which, repeat #15 was located in the 3'-untranslated region. A typical repeat contained 141 bp encoding for 47 codons. The alignment of the fourteen-times repeated amino acid sequences is shown in Figure 3. The sequences were rich in charged residues and had a proline-rich core sequence. Nucleotide and deduced amino acid sequence of insert of λ diff16 is shown in Figure 4. The first start codon (ATG) was seen at position 32–34. The sequence consisted of 574 nucleotides and an open reading frame extended for 151 codons ($m = 16367.17$) including

the first terminal codon (TAA). The deduced amino acid sequence was rich in glycine residues (15.33%). The 3'-untranslated region contained polyadenylation signal (AATAAA) at position 553–555.

The time course of gene transcript accumulation was investigated for λ diff2 and λ diff16 clones by Northern blotting (Fig. 5). The estimated sizes of the transcripts (2.4 kb and 0.7 kb) corresponded well to the length of the λ diff2 and λ diff16 inserts. The bands of signals probed by the λ diff2 cDNA insert were rather broad and obscured by the less dense bands of rRNA. This may suggest the occurrence of λ diff2 variants having different numbers of repeats as reported in apidaecin (Casteels-Josson *et al.*, 1993). We did not investigate further this point. The λ diff2 transcript was detectable by 1 hr after immunization, increased sharply to its maximum at 9 hr, thereafter decreased gradually and returned to trace amounts at 48 hr. Although the level of the expression of λ diff16 was much lower than that of λ diff2 and its signal was very weak at 1 hr by overnight exposure, the induction profile of λ diff16 was appreciably similar to that of λ diff2: first detected at 1 hr, peaked at 9 hr, then slowly declined. This suggests that the regulation of the two genes shares a common mechanism. These profiles are well-coincident with that of bactericidal activity in immunized hemolymph (Fig. 1). Therefore, when the bactericidal activity is taken as an index of the final output of the acute phase response in this species, the two genes are apparently early genes involved in the acute phase response. From these results, these genes are thought to encode either antibacterial molecules themselves or molecules involved in transduction of bacterial infection signals. In order to elucidate the relationship between these genes and genes whose sequence information is available, DNA and protein data bases were searched by a fasta program of DNA Data Bank of Japan. The DNA data base search failed to establish similarity among published DNA sequences. On the other hand, the protein data base search revealed that λ diff16 shares appreciable homology to hemiptericin, a glycine-rich, attacin-like antibacterial peptide purified from other hemipteran species, *Pyrrhocoris apterus* (Cociancich *et al.*, 1994): 42.9% in 91 residues in the C-terminal half. This indicates that λ diff16 encodes a counterpart of hemiptericin in *R. clavatus*. Then, the sequence of the λ diff16 was aligned to published amino acid sequences of attacin-like antibacterial peptides. The amino acid sequence of λ diff16 showed homology to those of dipterin (24.6% in C-terminal 65 residues (Dimarcq *et al.*, 1988)) and attacin E (21.3% in C-terminal 89 residues (Hultmark *et al.*, 1983)).

As for λ diff2, a striking similarity was found between a proline-rich region of each repeat and pyrrhocoricin, a proline-rich antibacterial peptide isolated also from *Pyrrhocoris apterus* (Cociancich *et al.*, 1994): over 80% similarity for typical repeats #2 to #13. The core sequences of the λ diff2 repeat #1 and #2 were aligned to published amino acid sequences of proline-rich antibacterial peptides, drosocin from *Drosophila melanogaster* (Bulet *et al.*, 1993)

Fig. 2. Nucleotide and deduced amino acid sequences of λ diff2 cDNA clone. Fourteen tandem repeats of amino acid sequence are indicated with double-headed arrows. A putative polyadenylation signal is underlined. Asterisk represents the termination codon.

```

repeat #1  MRSP.VIHLACVIAYIVA.EA.DKPV.....IHP.LA.EVGWE..GQG.....E..L.....
repeat #2  --E-R-----RS---PVDKGG---YLPRPTPPRP-VYR-SRRDA-SLESE-LSPLSVAEVLPEVR
repeat #3  ---.-.-----.-.....-.....-.....-.....E.....
repeat #4  ---.-.-----.-.....-.....-.....-.....V.-.....E.....
repeat #5  ---.-.-----.-.....-.....-.....-.....EE.....
repeat #6  ---.-.-----G.-.....-.....-.....-.....D.....
repeat #7  ---.-.-----.-.....-.....-.....-.....E.....
repeat #8  ---.-.-----.-.....-.....-.....-.....E.....
repeat #9  ---.-.-----.-.....-.....-.....-.....E.....
repeat #10 ---.-.-----.-.....-.....-.....-.....E.....
repeat #11 ---.-.-----.-.....-.....-.....-T.....S.E.....
repeat #12 ---.-.-----.-.....-.....-.....-.....E.....
repeat #13 ---.-.-----.-.....-.....-.....-.....E..G.....
repeat #14 ---.-.-----.-..GQG..-----RT.-LC.-.....-N.DA.-Q..V.EGV.....
          *           *       * * * * * * * * * * * * * * * * * *

```

Fig. 3. Alignment of amino acid sequences of fourteen (#1–#14) tandem repeats of λ diff2. The repeats are aligned with repeat #2. Identical amino acid residues are shown by dots. Horizontal bars represent inserted gaps. Residues conserved in every repeat are indicated by asterisks.

```

TCAAAGACCTCTCGCAACTAAGAAAGGAAAAATGCATATTGCAAGATTTTGTCTCTCTTCGATGGCCGCTCTGGCTCTGTCACTGG  90
      M H I A R F C L L S S M A V L A L S A G      20

ATACGTATCTGGGCGAGTCATTGAGATCCCTGACGAAATTTGGACAGCGCCAGATTCACTTCTCTTATTAGATGGCCTCAGACAGAA  180
Y V S G A V I E I P D E I L D S A R F I S L Y S D G L R Q K  50

GCGTCAGTTAAATTTGAGCGGACCTGGATCAGAGCACGCAAGAACATAAGACTGGATGGGCAGAGGAACATCTTCGACAACGGTAGAAC  270
R Q L N L S G P G S E H A G T I R L D G Q R N I F D N G R T  80

CCGAGTAGATGGGACGGGATCCTACCACTGGACTATGCCCGTGAATGAAGCCTATTATGGAGCAGGCCTGGGTGCTGAGGTCAACCA  360
R V D G T G S Y Q L D Y A R G M K P I H G A G L G A E V N H  110

TAACATCTGGAGAGGTAGAGGAGGCCAGTCCCTGGATCTCTATGGTGGAGCAACCAGACAGTTCAACTTTGGAAATAGACCTAATGAGTG  450
N I W R G R G G Q S L D L Y G G A T R Q F N F G N R P N E W  140

GGGTGCTCATGGAGGCATCAGATATAACTTCTAAATCAAATAACGTAAGTATTAATATGTAATATGAATTGTAATATATTTTATACA  540
G A H G G I R Y N F *  150

TATATATTTGTAATAAAATTTTCTCTAAACA

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Fig. 4. Nucleotide and deduced amino acid sequences of cDNA insert of λ diff16 clone. A putative polyadenylation signal is underlined. Asterisk represents the termination codon.

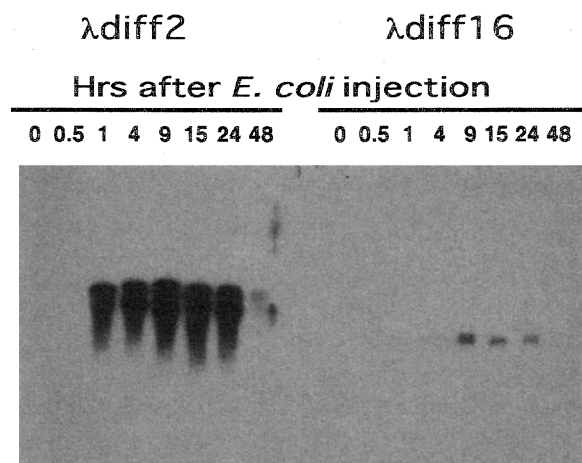


Fig. 5. Accumulation of λ diff2 (left) and λ diff16 (right) transcripts after *E. coli* injection. Five micrograms of total RNA prepared from the fat body at indicated time intervals were run. The probes for Northern blot hybridization were prepared from the λ diff2 and λ diff16 cDNA clones, respectively, and the probed blots exposed to X-ray films overnight at -80°C .

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λdiff2 repeat#1 core ...EAGDK--PVYLPRPT-PPRPIHPRLARE...
λdiff2 repeat#2 core ...SPVDK--GGYLPRPT-PPRPVY-RSRRD...
pyrrhocorin      VDK--GSYLPRPT-PPRPIYNRN
drosocin         G-KPRP-YSPRPTSHPRPT--RV
apidaecin        G-NNRPVYIPQ----PRPPHPRL

```

Fig. 6. Alignment of λ diff2 repeat #1 and #2 core sequences to three other established insect proline-rich antibacterial peptides. Conserved residues between λ diff2 repeats and other peptides are double-underlined. Horizontal bars represent inserted gaps. Sequences of pyrrhocorin, drosocin and apidaecin are from Cociancich *et al.* (1994), Bulet *et al.* (1993) and Casteels *et al.* (1989), respectively.

and apidaecin from the honeybee, *Apis mellifera* (Casteels *et al.*, 1989) in addition to pyrrhocorin (Fig. 6). Both repeat #1 and #2 were shown to have very high amino acid identity with pyrrhocorin and drosocin. The threonine-11 residues of both pyrrhocorin and drosocin are *O*-glycosylated and this substitution is reported to be necessary for the full bactericidal activity in the case of drosocin (Bulet *et al.*, 1993). This threonine residue was also conserved in every repeat of λ diff2 (Figs. 3 and 6). From these results the authors conclude that λ diff2 encodes for a multi-peptide precursor of proline-rich antibacterial peptides of *R. clavatus*. Assuming that the precursor peptide is processed to be biologically active molecules having the similar structure to pyrrhocorin, three types of variants could be generated (see Fig. 3). Among insect antibacterial peptides so far cloned, apidaecin was the only case to have multi-peptide precursor structure (Casteels-Josson *et al.*, 1993). Interestingly, the core region of the repeat #1 was very similar to apidaecin while the repeats #2–#14 were not, suggesting that the apidaecin genes might have evolved from the ancestral multi-peptide genes of Hemimetabola through selective exon amplifications and rearrangements.

Preliminary expression experiments were done using baculovirus vector and *Bombyx mori* culture cells to check the bactericidal activity of the product from λ diff2 clone. The results were negative: there was no detectable bactericidal activity in either culture medium or in cell lysate by growth inhibition assay (data not shown). These results possibly arose for the following reasons: 1) The activity of products were beneath threshold of the detection methods used because of their limited quantities. 2) The *B. mori* culture cells lacked enzymes necessary for proteolytic processing and/or an addition of sugar chains on the multi-peptide precursor of the λ diff2 product.

The induction profile of bactericidal activity in immunized hemolymph corresponds well to changes in transcript abundance of the λ diff2 and λ diff16 (Figs. 1 and 5), suggesting that products of the two genes are responsible for the bactericidal activity. However, it has not been evidenced whether the active peptides derived from the two genes occur *in vivo*. It awaits the analysis at protein level to clarify this point.

In the present study we have succeeded in direct isolation of cDNA clones of antibacterial peptides by using

differential screening without purification and analysis of corresponding peptides. This "direct" method is suitable especially for materials that are difficult to obtain in sufficient quantities. The PCR-based differential display method (Liang and Pardee, 1992; Asling *et al.*, 1995) may be a good alternative for differential screening. As far as the authors can determine, this is the first report of the mRNA sequences of antibacterial peptides in hemimetabolous insects, and the λ diff2 is the second case of multi-peptide precursor structure in insect antibacterial peptide genes.

SEQUENCE AVAILABILITY

The nucleotide sequence data of λ diff2 and λ diff16 reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession numbers D49415 and D49929, respectively.

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