Insect Immunity

ISOLATION FROM A COLEOPTERAN INSECT OF A NOVEL INDUCIBLE ANTIBACTERIAL PEPTIDE AND OF NEW MEMBERS OF THE INSECT DEFENSIN FAMILY*

(Received for publication, June 25, 1991)

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Insect immunity is at present a rapidly expanding field of research. A series of pioneering studies had established by 1930 that larvae of Lepidopteran insects are able to build up a potent humoral antibacterial response when challenged with low doses of bacteria (1-4). It however took nearly 50 years until the first molecules responsible for this inducible humoral antibacterial activity of Lepidopteran were isolated and their structures characterized. Studies in Hyalophora cecropia, Galeria mellonella, and Manduca sexta have since established that these insects synthesize, in response to a bacterial challenge or a septic injury, three major groups of antibacterial peptides: diptericins, 8-kDa glycine-rich molecules (17), and insect defensins (18). The latter are 4-kDa cysteine-rich molecules which exhibit some sequence similarity to mammalian defensins present in polymorphonuclear neutrophils (19, 20). Finally, in the immune blood of the honey bee, a hymenopteran insect, small proline-rich antibacterial peptides were identified (apidaecins, 2-kDa basic peptides (21), abeclin, 4-kDa basic peptides (22)).

Interestingly, within the vast class of the insects, inducible antibacterial peptides have so far been isolated only from species belonging to the endopterygote clade. This huge group of insects, which contains more species than the rest of the living world together, is characterized by a non-feeding and immobile last immature instar (the pupa), from which the adult organism emerges after far-reaching metamorphic events. The overwhelming species richness in the Endopterygota results from their diversification in only four of the constituent orders: the Coleoptera, the Hymenoptera, the Diptera, and the Lepidoptera.

Surprisingly, no inducible immune peptide had been characterized so far from the coleopteran branch of the endopterygote clade, although an antibacterial response can be elicited in this order (23, 24). In the present study we have used larvae of a large tenebrionid beetle, Zophobas atratus, for the isolation and structural characterization of antibacterial peptides which appear in the blood in response to injection of heat-killed bacteria. We report the identification of a novel 74-residue antibacterial peptide, which we propose to name coleopercin, and two isoforms of a new member of the insect defensin family of antibacterial peptides. We were unable to detect in the immune larvae molecules such as cecropins, attacins, diptericins, or apidaecins which participate in the antibacterial response in other orders of the Endopterygota.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains were gifts from the following colleagues: Escherichia coli D31 (streptomycin resistant) and Enterobacter cloacae J21 (naldixic acid resistant) from H. Boman (University of Stockholm); E. coli ATCC 7624 from Y. Plémont (University of Strasbourg); E. coli D22 from P. L. Bogert (Centre d’Etudes Nucleaires, Saclay); Bacillus megaterium and B. subtilis QB 935 from J. Millet, A. Klier (Pasteur Institute, Paris); Acinetobacter baumannii (= calcoaceticus), Pseudomonas aeruginosa, P. maltophilia, Alcaligenes faecalis, Klebsiella pneumoniae, Salmonella typhimurium, Proteus vulgaris, Staphyloccocus aureus, S. saprophyticus, Streptococcus pyogenes, Aerococcus viridans (= Gaffkya homarii), Listeria monocyto genes, Corynebacterium D2, Pediococcus acidilactici from H. Monteil (Centre Hospitalo-Universitaire, Strasbourg). Bacillus cereus 6452, B. thuringiensis S3157, Micrococcus luteus A270 were obtained from the Pasteur Institute collection, Paris. Erwinia carotovora, Agrobacterium...
The bacterial strains were grown in the following media: (a) L. monocytogenes; Corynebacterium D2, P. acidilactici; Columbia gelose medium (bioMérieux); (b) E. carotovora and A. tumefaciens; LPG medium (yeast extract 7%, bactopeptone 7%, glucose 7%, and agar 15%); (c) all other strains were grown in Bertani’s rich nutrient medium.

### Immunization

3rd instar larvae (6–8-month-old) of Z. atratus (average weight, 1 g/larva) received a 10-μl injection of overnight heat-killed cultures of M. luteus and E. coli D31 containing approximately 1 million cells of each germ. After various time intervals, the insects were chilled for 1 min in ice-cold water, and several drops (~30 μl/larva) of hemolymph were recovered by sectioning a metathoracic leg and gently squeezing the abdomen. The hemolymph was pooled in a precooled plastic tube in the presence of aprotinin (Sigma; final concentration, 10 μg/ml of hemolymph). After centrifugation at 70,000 × g for 50 min at 4 °C, the cell-free hemolymph was clarified through a Millex 0.8-μm filter.

### Antibacterial Assays

#### (a) Plate Growth Inhibition Assay—Assay conditions were essentially those described by Lambert et al. (18): sterile Petri dishes (9-cm diameter) received 7.5 ml of melted agar in buffered nutrient medium, pH 7.2, containing ~2 × 10^8 logarithmic-phase cells of a given bacterial strain. Wells (2-mm diameter) were cut into the freshly poured plates after the solidification of the agar. Each well received a 2-μl aliquot of the fraction suspected to contain antibiotic molecules. The plates were incubated overnight at 37 °C, and the diameters of the clear zones were recorded, after subtraction of the well diameter.

#### (b) Bactericidal Assay—250 pmol of purified peptide A in 10 μl of distilled water were incubated in microtiter plates in the presence of 100 μl of a logarithmic-phase culture of E. coli D22 at a starting OD_600 of 0.15. Aliquots were removed at different time intervals and plated on nutrient agar to determine, by an overnight culture at 37 °C, the number of colony forming units.

### Purification of Antibacterial Peptides

#### Step I: Sep-Pak Prepurification—The cell-free filtered hemolymph was acidified (0.05% trifluoroacetic acid) and applied on a Sep-Pak C_18 cartridge (Waters Associates). Stepwise elution was performed with increasing proportions of acetonitrile (up to 60%) in water acidified with 0.05% trifluoroacetic acid. Antibacterial activity was monitored on aliquots of the fractions that had been vacuum-dried to remove acetonitrile.

#### Step II: Reversed-phase HPLC—The active fractions from the Sep-Pak prepurification were vacuum-dried, and the residue was dissolved in 250 μl of 10% acetonitrile in acidified water (0.05% trifluoroacetic acid) and subjected to reversed-phase HPLC on an Aquapore RP 300 C_8 (250 × 4.6-mm) column (Brownlee Associates). Elution was performed with a linear gradient of 10–60% of acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min. Ultraviolet absorption was monitored at 225 nm. The eluted fractions were vacuum-dried and dissolved each in 50 μl of distilled water; the antibacterial activity was monitored on 2-μl aliquots.

#### Step III: Final Purification—Different methods were used for the final purification of the active compounds present in the three peaks referred to as A, B, and C under "Results" (Fig. 2): A, reversed-phase HPLC on an Aquapore RP 300 C_8 (250 × 4.6-mm) column with a relatively mild gradient elution of 18–38% acetonitrile in acidified water over 120 min; B, isocratic size exclusion chromatography on a Protein-Pak column (Waters Associates) with 30% acetonitrile in acidified water; C, reversed-phase HPLC on an Aquapore RP 300 C_8 (250 × 4.6-mm) column with an elution gradient of 20–40% acetonitrile in acidified water over 120 min. The antibacterial activity was monitored on 2-μl aliquots of the concentrated fractions as in step II.

### Amino Acid Sequence Analysis

Automated Edman degradation of peptides and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems, model 473).

### Mass Spectrometry

The mass spectra were recorded on a Bio-Ion 10 K plasma desorption mass spectrometer (Bio-Ion AB). Peptides were analyzed using nitrocellulose targets.

### Enzymatic Digestion

Endoproteinase Glu-C (Staphylococcus aureus V8 protease) was purchased from Pierce Chemical Co. Peptide A (10 μg) was dissolved in 100 μl of 25 mM HCO_3-NH_4, pH 4, and 750 ng of enzyme were added. Enzymatic reaction lasted for 18 h at 25 °C. The digestion peptides were recovered from the mixture by reversed-phase HPLC (conditions as above for (step II)).

### Protease Assay

100-μl samples of immune cell-free hemolymph (see above) were subjected to protease treatment during 18 h at 37 °C. The following enzymes were used: pepsin (EC 3.4.23.1, Sigma), protease from Streptomyces griseus (Sigma), trypsin (EC 3.4.21.4, Worthington) and a-chymotrypsin (EC 2.4.21.1, Worthington). The enzymes were diluted in appropriate buffers: 50 mM KCI-HCl, pH 2.0, for pepsin, and 50 mM NH_4Cl-NaOH, pH 8.5, for the other enzymes. Each enzyme was assayed at a concentration of 5, 10, and 20 IU/mg of protein (cell-free hemolymph). After enzymatic treatment, the samples were directly assayed against M. luteus or E. coli strains D22 and D31 in the plate growth inhibition assay. In parallel, the inocuity of the various enzymes on the bacterial growth was tested in strictly identical conditions (pH, concentration). Control experiments were run in the absence of enzymes.

### RESULTS

#### Appearance of Antibacterial Activity in the Hemolymph of Larvae of Z. atratus after Injection of Heat-Killed Bacteria (Immunization)—In a pilot experiment, nine groups of seven 3rd instar larvae received an injection of heat-killed bacteria, and their hemolymph was collected after various time intervals up to 4 weeks. The presence of antibacterial activity was monitored for each larva on an aliquot of cell-free hemolymph in the plate growth inhibition assay against the Gram-positive M. luteus. The results are presented in Fig. 1 and show that a strong antibacterial activity appeared between 6 and 12 h following the inoculation of bacteria. A high activity was monitored at 24 h and was maintained for at least 72 h. Significant activity was still detectable after 4 weeks. In contrast, untreated larvae were devoid of antibacterial sub-

![Fig. 1. Induction of anti-M. luteus activity in hemolymph of Z. atratus after injection of heat-killed bacteria.

Last instar larvae of Z. atratus received a 10-μl injection of 1 million cells of each of M. luteus and E. coli D31. Hemolymph was collected from individuals after various time intervals and freed from hemocytes by centrifugation. Anti-M. luteus activity was assayed on 2-μl aliquots by the plate growth inhibition assay. Antibacterial activity is expressed in diameter (mm) of growth inhibition zone. Values are means of seven individual measurements.](https://example.com/fig1.png)
stances in their cell-free hemolymph.

Essentially similar results were obtained when the antibacterial activity was tested on the Gram-negative *E. coli* strains D22 and D31 (data not shown).

When the cell-free hemolymph of challenged larvae was subjected to protease treatment (see "Materials and Methods"), no antibacterial activity could be evidenced, indicating that the molecules responsible for this activity are peptides.

Isolation of Three Antibacterial Peptides (Peptides A-C) from Immune Hemolymph of *Z. atratus*—150 third instar larvae of *Z. atratus* were immunized as above, and their hemolymph was collected in the cold after 48 h, yielding a total volume of 5 ml. The hemocytes were removed by centrifugation, and the supernatant was filtered through a Sep-Pak C18 cartridge. The antibacterial activity was recovered by elution with 60% acetonitrile in acidified water. The eluate was applied to a reversed-phase HPLC column and eluted with a linear gradient of acetonitrile in acidified water as shown in Fig. 2. Aliquots of the eluted fractions were tested in the plate growth inhibition assay on *M. luteus* and *E. coli* D31 and D22. Anti-*E. coli* activity was observed in absorption peak A (at 27.5% of acetonitrile) while two peaks, B and C (at 33 and 35% acetonitrile), contained anti-*M. luteus* activity. No other fraction was observed to contain antibacterial activity in our conditions. The active compounds were further purified by reversed-phase HPLC (A and C) and size exclusion chromatography (B). Apparently pure substances were recovered, as judged by UV monitoring at 225 nm (Fig. 3). The estimated purification yields were as follows: from 5 ml of hemolymph containing 150 mg/ml of total proteins, we recovered in pure form 40 μg of peptide A, 15 μg of peptide B, and 15 μg of peptide C. This extraction procedure was repeated several times to obtain sufficient pure material for the subsequent studies.

Primary Structure Determination of Peptides A, B, and C—The three peptides were sequenced by automated Edman degradation. The sequences are presented in Fig. 4 and show two distinct types of molecules: A is a 74-residue peptide devoid of cysteines, and B and C are isoforms of a 43-residue cysteine-rich peptide with sequence similarities to the insect defensins identified in the immune blood of the dipteran *Phormia terranovae* (18).

**Peptide A**—The sequence of the first 60 NH2-terminal residues was established on 350 pmol of pure material with a repetitive yield of 94%. The presence of a Glu in position 38 prompted the use of endoproteinase Glu-C to cleave the peptide. 1200 pmol were subjected to this enzyme and two

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**Fig. 2. Reversed-phase HPLC separation of immune hemolymph of *Z. atratus*.** After an intermediary step on a Sep-Pak C18 cartridge, the active material from the hemolymph of 150 larvae was fractionated on an Aquapore RP 300 C8 column (250 × 4.6 mm). Elution was performed over 90 min with a linear gradient (dotted line) from 10 to 60% acetonitrile in acidified water at a flow rate of 1 ml/min. The absorbance was monitored at 225 nm (full line). The antibacterial activity was tested on aliquots of each fraction by the plate growth inhibition assay against Gram-negative and -positive bacteria and is expressed in diameter (mm) of growth inhibition zone (columns). Full column, activity against *E. coli*; dotted columns, activity against *M. luteus*.

**Fig. 3. A-C, final purification of peptides A, B, and C (step III).** Different methods were used for the final purification of the active compounds A, B, and C. Peptide A was submitted to reversed-phase HPLC on an Aquapore RP 300 C8 (250 × 4.6 mm) column with a relatively mild gradient elution of 18–38% acetonitrile in acidified water (dotted line) over 120 min. Peptide B was submitted to an isocratic size exclusion chromatography on a Protein-Pak 125 column with 30% acetonitrile in acidified water. Peptide C was submitted to reversed-phase HPLC on an Aquapore RP 300 C8 (250 × 4.6 mm) column with an elution gradient of 20–40% acetonitrile in acidified water over 120 min. The absorbance was monitored at 225 nm (full line). Antibacterial activity was tested in the plate growth inhibition assay on aliquots of each fraction on *E. coli* (A) or *M. luteus* (B and C), and is expressed in diameter (mm) of growth inhibition zone (dotted columns).
fragments were separated by reversed-phase HPLC and sequenced by Edman degradation. One fragment of 38 residues corresponded to the 38 NH$_2$-terminal residues identified above, while the other fragment of 36 residues gave the COOH-terminal sequence of peptide A. Mass spectrometric measurement of the intact peptide yielded a molecular mass of m/z 8114.7 which is in good agreement with the calculated average isotopic mass (MH$^+$) of 8110.8. Peptide A is strikingly rich in Gly residues (18%) which are evenly distributed in the molecule. The NH$_2$-terminal third (up to residue 23) is devoid of charges. The central part of the molecule is highly charged, both positively and negatively, while the COOH terminus of the peptide has a marked basic character. The overall pl of the molecule is 10.9. The peptide contains no cysteines.

**Peptides B and C**—The sequence of 43 residues of peptide C was obtained by subjecting 400 pmol of pure material to Edman degradation. The repetitive yield of the sequencer was about 95%. No phenylthiohydantoin was present at positions 3, 20, 24, 34, 40, and 42; the intensities of the signals preceding and following these six blanks clearly indicated that they correspond to cysteines, as is the case in defensins from *Phormia*, allowing for a gap of 4 residues in the dipteran peptides (see Fig. 4 and “Discussion”). This assumption was corroborated by determination of the molecular mass which was found to be at m/z 4395. Indeed, the calculated monoisotopic molecular mass (MH$^+$) is 4396 when the six blanks are considered to correspond to cysteines engaged in three intrachain disulfide bridges.

For peptide B, 400 pmol of pure material were subjected to Edman degradation, and a sequence was obtained which was fully superposable on that of peptide C with a single replacement of Thr-30 in C by Arg-30 in B.

Both peptides are basic with a pl of 7.9 for C and 8.2 for B (the presence of an Arg in B in place of a Thr in C accounts for the difference in basicity of the two peptides).

**Activity Spectrum of Peptides A and C and Mode of Action of Peptide A from Immune Hemolymph of Z. atratus**—Purified peptide A and peptide C were tested in the plate growth inhibition assay against various bacterial strains as illustrated in Table I. Peptide A was found to be highly active against *E. coli* D31 and D22. Lower but significant activity was also observed against other Gram-negative strains, *A. baumanii* and *P. maltophilia*. *M. luteus* was the only Gram-positive cell affected by this peptide in our conditions. Peptide C was strongly active against *M. luteus*, *S. pyogenes*, and *Corynebacterium D2*. No Gram-negative cell was found to be sensitive to peptide C, except for the D22 strain of *E. coli*.

Pure peptide A was tested in the liquid growth inhibition assay at 2.5 μM against the highly sensitive strain D22 of *E. coli*. As shown in Fig. 5, a 0.5-h contact with the peptide was sufficient to kill growing cells of this strain. Pure peptides B and C yielded similar results when tested against *M. luteus* at the same concentration (data not shown). This result is in agreement with our previous observations on the bactericidal activity of insect defensins from *P. terranovae* (18).
orates earlier investigations in *Eleodes*, another coleopteran insect (23; see also Ref. 24). The time course of the appearance is roughly equivalent to that observed in representative species of Lepidoptera and Diptera. The activity persists for a longer time period, which may reflect the remarkably long duration (several months) of the 3rd larval instar of *Z. atratus*.

As shown in this report, the inducible antibacterial activity monitored by the plate growth inhibition assay in the cell-free hemolymph of challenged larvae of *Z. atratus* is essentially attributable to two types of molecules. Cecropins, attacins, dipterics, apidaecins, and lysozymes, which are active against one or all of the three routinely used test organisms (*M. luteus*, *E. coli* D22 and D31) were not observed although our working conditions were favorable for their detection.

Peptide A has no sequence similarity to other known peptides, and we propose therefore the name coleoptericin for this novel inducible antibacterial peptide. Coleoptericin is basic in character (pI 10.9) and is strikingly rich in glycine residues (15%). This is not uncommon among insect antibacterial peptides. The 8-kDa dipterics of *Phormia* and *Drosophila* contain 18 and 22%, respectively, of glycine residues and within the larger attacins (20–22 kDa, (9)) and the attacin-related sarcotocin II (27 kDa, (16)) 60-residue glycine-rich domains are observed which contain up to 20% glycine residues (see also "Discussion" in (25)). Coleoptericin, like diptericin, is bactericidal against Gram-negative bacteria.

The presence of insect defensins, peptides B and C, in Coleoptera is interesting in many respects. Insect defensins were initially discovered in Diptera (immune blood of *P. terranovae*; (18); cf. also sapecin, a homologous molecule secreted by an embryonic cell line of *Sarcophaga peregrina*, (26)). Their presence has not been reported so far in Lepidoptera, where they are probably absent, given the efforts which have been devoted to the isolation of antibacterial peptides in this order. However, a defensin-related 51-residue peptide is present in the royal jelly of the honey bee (referred to as royalisin, (27)). The presence in insects of antibacterial peptides with sequence similarities to the mammalian defensins has attracted attention and prompted the suggestion that defensins were ancestral antibacterial peptides (18, 20). Mammalian defensins form a relatively large family of variably cationic peptides comprised of 29–34 amino acid residues. They all contain a characteristic motif of 6 cysteines engaged in three intramolecular disulfide bridges. However, the spacing between the cysteine residues differs markedly between insect and mammalian defensins and the connectivity between the three disulfide bridges has been shown recently to be different (28–30), which casts some doubt on the proposed homology.

The sequence similarities between the defensins of *Z. atratus* and the three dipteran defensins (defensins A and B of *P. terranovae* and sapecin of *S. peregrina*) is especially high in the COOH-terminal part of the molecule. A detailed NMR analysis of recombinant *Phormia* defensin A produced in yeast (31) has recently shown that the COOH-terminal part of defensin forms a short α-helix followed by two antiparallel β-sheets. This arrangement is probably conserved between *Phormia* and *Z. atratus* given the 80% sequence similarity for this region. In contrast, the NH2-terminus of *Phormia* defensin forms a large loop and we observe that the amino acid sequence is only poorly conserved (40%) in this region between the two species with the exception of a stretch of 5 residues also "coteria" and comprising the first NH2-terminal cysteine (which in *Phormia* defensin anchors the loop to the β-sheet). In other words, if we accept the idea that the coleopteran defensin has a three-dimensional organization basically similar to that of dipteran defensin, the major change in sequence affects the NH2-terminal loop. Whether and how this change reflects on the mode of action or the spectrum of activity remains to be established. So far in our biological tests, both defensins were bactericidal against the same Gram-positive bacteria at similar concentrations (comprised between 0.5 and 2.5 μM).

Taken in conjunction with the results we obtained in Lepidoptera, Diptera, and Hymenoptera, our data show that insects of the four major orders of the endopterygote clade all respond to a bacterial challenge by the production of several groups of antibacterial peptides. The full characterization of these peptides has only been performed in a few species and frequently only at one stage of development, as is the case in this paper. It is therefore premature to draw firm conclusions as to the distribution of the various antibacterial peptide families within this insect clade. However, it is now clear that the four orders under investigation do not necessarily produce the same array of antibacterial peptides in contrast to earlier assumptions. Defensins probably play a paramount role in the anti-Gram-positive response of Coleoptera, Diptera, and possibly Hymenoptera while Lepidoptera rely on cecropins and lysyozyme. Gram-negative cells are also countered by cecropins in Lepidoptera and Diptera, and in addition, by particular, large-sized glycine-rich peptides in Coleoptera (coleoptericin) and Diptera (dipertics) and by small proline-rich peptides in Hymenoptera (abecains, apidaecins).

It is at present unclear whether insects belonging to other clades also synthesize antibacterial peptides in response to a bacterial challenge. In fact with the exception of lysyozyme, no antibacterial peptide has been chemically characterized so far from any insect species outside the Endopterygota. It will be interesting to extend these studies to other insect groups; it is obviously also a necessity to complete our information on the four orders of the endopterygote clade.

In conclusion, we have shown that within a few hours after an injection of heat-killed bacteria, three basic antibacterial peptides of relatively low molecular weights appear in the hemolymph of 3rd instar larvae of *Z. atratus*. These molecules certainly participate in the antibacterial response of the insect species against Gram-positive and Gram-negative bacteria. In particular, large proteins with antibacterial activity, such as some lectins (32), would not have been detected in the plate growth inhibition assay. Also the role of phagocytosis and capsule formation by hemocytes, as evidenced in other insect orders (33–34), remains to be investigated in Coleoptera. Clearly, we are only at the beginning of our understanding of the immune response in this group which outnumbers in terms of species the sum of all the other animal groups.

Acknowledgments—We are indebted to Dr. Ka Wan Li, Biological Laboratory, Vrije Universiteit, Amsterdam, and to Dr. P. Paroutaud, Applied Biosystems Industries, Roissy, for peptide sequencing. The larvae of *Z. atratus* were kindly provided by Dr. A. Quennedey, Laboratoire de Cytologie et de Physiologie des Arthropodes, Dijon. We would also like to thank Pr. M. Ptak, Centre de Biophysique Moléculaire, Orléans, for allowing us to quote unpublished results.

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