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Antibacterial peptides of the moth *Galleria mellonella*★✪

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The work describes purification and biochemical characterization of two inducible antimicrobial peptides from the hemolymph of *Galleria mellonella*. The peptides were isolated by a sequence of reversed-phase chromatography steps from the hemolymph of larvae immunized with viable bacteria. The first peptide is a member of the cecropin family while the second one is rich in proline residues and has a unique sequence.

The long and widespread use of antibiotics has resulted in a fast development of pathogenic bacteria that are resistant to classical antibiotics [1]. The recent years brought therefore intensive studies towards more effective antimicrobial drugs. Particularly interesting are antimicrobial peptides discovered as components of unspecific innate mechanisms of infection fighting in humans and animals [2]. This report describes the isolation and biochemical characterization of peptides isolated from the hemolymph of the moth *Galleria mellonella*, a common pest of apiary storehouses. Previous work has shown that after immunization the level of antimicrobial activity in the hemolymph increases significantly [3]. Here we demonstrate that this activity is related to the presence of two peptides that are able to kill bacteria. One has a sequence similar to the so called cecropin family

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Abbreviations: CFU, colony-forming unit; MIC, minimal inhibitory concentration; TFA, trifluoroacetic acid.

members – the peptides originally found in cecropia moth (*Hyalophora cecropia*) [4]. The second peptide has a unique sequence and is probably a representative of a novel family of antimicrobial peptides.

MATERIALS AND METHODS

Last-instar *Galleria mellonella* larvae were pricked with a needle dipped into a pellet of viable *Escherichia coli* D31 and *Micrococcus luteus* cells (1:1). After 24 h the hemolymph (about 20 μl per insect) was collected to an equal volume of an ice cold anticoagulant (0.041 M sodium citrate buffer, pH 4.5, containing 0.186 M NaCl, 0.017 M EDTA and 2 mM phenylmethanesulfonyl fluoride) and centrifuged at 200 $\times$ ***g*** for 5 min at 4°C. The collected supernatant was freeze-dried and kept at –20°C until use. The dry powder was dissolved in water, acidified with trifluoroacetic acid (TFA) to pH of 3.5, spun down at 16 000 $\times$ ***g*** for 10 min at 4°C and deprived of proteins on a C-18 SepPak Plus cartridge (Waters). After applying the samples (equivalent to 0.5 ml of hemolymph) the unretained material was washed out with 0.1% TFA in water (buffer A) and the adsorbed peptides were collected after elution with 80% acetonitrile, 0.07% TFA (buffer B). The obtained material was freeze-dried, dissolved in a small amount of 0.1% TFA in water and applied on a C-18 μBondapak 3.9 $\times$ 300 mm HPLC column (Waters) using buffers as above and a gradient from 0 to 70% B in 40 min. The obtained fractions were again freeze-dried, dissolved in water and subjected to SDS/PAGE [5] and to antibacterial assays. The antibacterial activity was determined by incubating different concentrations of the peptides at 37°C for 1 h with 10^5 CFU of mid-logarithmic phase *E. coli* cells. After incubation the bacteria were plated on agar, incubated for 24 h at 37°C and the colonies were counted. The active fractions of low-molecular mass peptides visible on SDS/PAGE were subjected to further HPLC purification on a C-18 ODP-50 4 $\times$ 150 mm column (Supelco) in 0.2 M ammonium hydroxide (buffer C) and 0.2 M ammonium hydroxide, 80% acetonitrile (buffer D). The gradient was from 15 to 60% D in 40 min. The collected fractions were rechromatographed on a C-18 μBondapak column using TFA-containing buffers and a gradient from 0 to 80% B in 15 min.

The obtained peptides were sequenced on Procise 491 automatic protein sequencer (Applied Biosystems) using the standard program. Sequence similarity searches were performed using the NCBI Protein BLAST program, ver. 2.2 (available on-line at http://www.ncbi.nlm.nih.gov/blast/index.html). The search was performed on NR database (PDB+SwissProt+PIR+PRF), updated 29 Jan., 2001.

Mass spectroscopy analysis on a Finnigan MAT 95 (Finnigan MAT) mass spectrometer equipped with electrospray source (ESI) confirmed the obtained sequences. The concentration of the peptides was determined by amino acid analysis [6].

RESULTS

The hemolymph of *Galleria mellonella* after reversed-phase HPLC separation in acid pH shows a profile rich in proteins of molecular masses below 30 kDa (Fig. 1). Almost each of the nine fractions was able to kill *E. coli* (not shown). To identify bactericidally active compounds all fractions of interest containing peptides with molecular masses below 6 kDa (fractions 4–8) were subjected individually to further purification using high pH conditions. The change of pH was very effective for obtaining almost pure peptides (Fig. 2), but in this pure form only two peptides (from fractions 5 and 8) exhibited antibacterial activity. The active peptides were purified to homogeneity by a third chromatography step performed in acidic conditions (not shown). The final preparations, peptides named 5.11.1 and

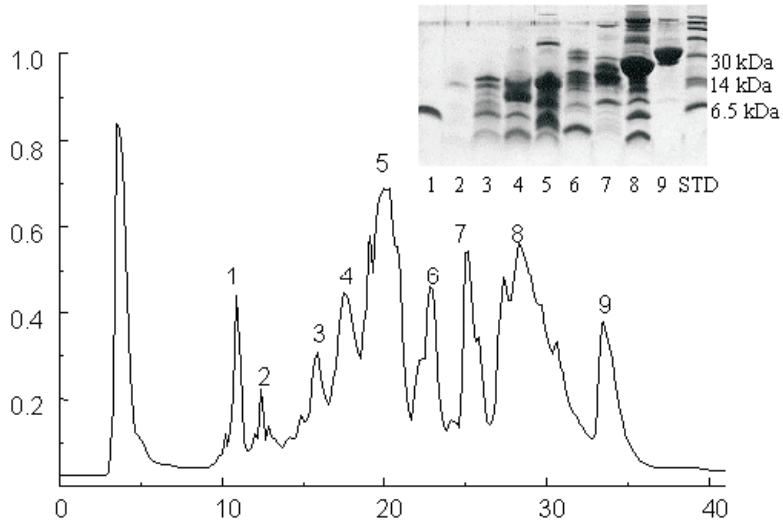

Figure 1. HPLC chromatography of *Galleria* hemolymph deprived of proteins.

Equivalent of 0.5 ml hemolymph was applied on C-18 column. SDS/PAGE image of marked fractions is also presented. See Materials and Methods for details.

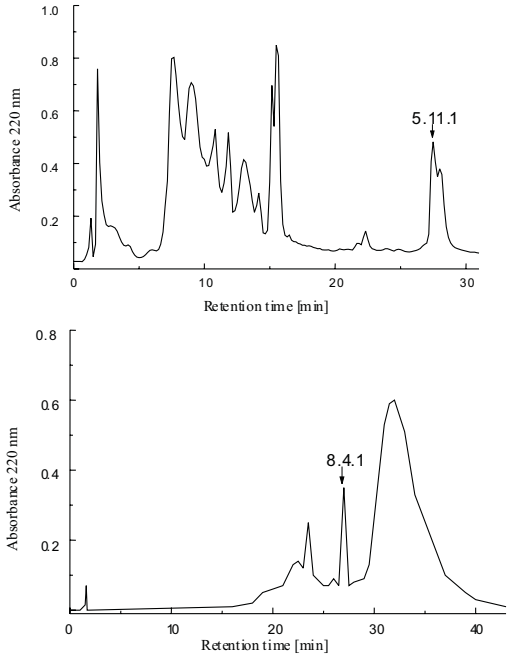

Figure 2. Chromatography of fractions 5 and 8 from C-18 column on ODP-50 column performed at basic pH.

Retention times of the antibacterial peptides are marked. See Materials and Methods for separation details.

8.4.1, were subjected to sequencing and gave single sequences counting 37 and 39 residues, respectively (Fig. 3). The calculated molecular masses of the isolated peptides were 4322.95 Da and 4255.84 Da, respectively. Mass spectrometry confirmed the obtained sequences giving experimental masses of 4322.0 Da and 4255.0 Da (Fig. 4), respectively. The single bands visible on SDS/PAGE, clear amino-acid sequence, single ion peaks on mass spectrometry, single peaks on HPLC chromatography (Fig. 5) as well as very high agreement of amino-acid analysis results (data not shown) with amino-acid composition from sequence data, all these methods confirmed high purity of obtained peptides.

The isolated compounds were subjected to minimal inhibitory concentration (MIC) determination toward *E. coli*. The peptide 5.11.1 gave the MIC value of 43 μM and the peptide 8.4.1 53 μM.

DISCUSSION

Insects are very resistant to pathogenic microbial infections. Early immunological studies conducted at the beginning of the 20th century were focused on the role of the morphotic elements of the hemolymph, but just at that time a strong, heat-stable hemolymph antibacterial activity induction after immunization of insects with low doses of virulent pathogens was observed. Late 1970s brought a series of

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                      1           11          21          31
Peptide 5.11.1        DIQIPGIKKP THRDIIIPNW NPNVRTQPWQ RFGGNKS

                      1           11          21          31
Peptide 8.4.1         ENFFKEIERA GQRIRDAIIS AAPAVETLAQ AQKIIKGGD
Cecropin D             ····DL·KM ···V···V·· ·····D···K ·KALGQ·
Bacteriocin B-4        ·P···L··· ···V····** *****A·VG· ·AA·AR·
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Figure 3. Amino-acid sequences of the newly isolated *Galleria* antibacterial peptides.

For comparison, two sequences most similar to the 8.4.1 peptide are also shown: cecropin D from *Bombyx mori* and bacteriocin B-4 from *Manducta sexta*. The dots represent residues identical to respective amino acids in 8.4.1.

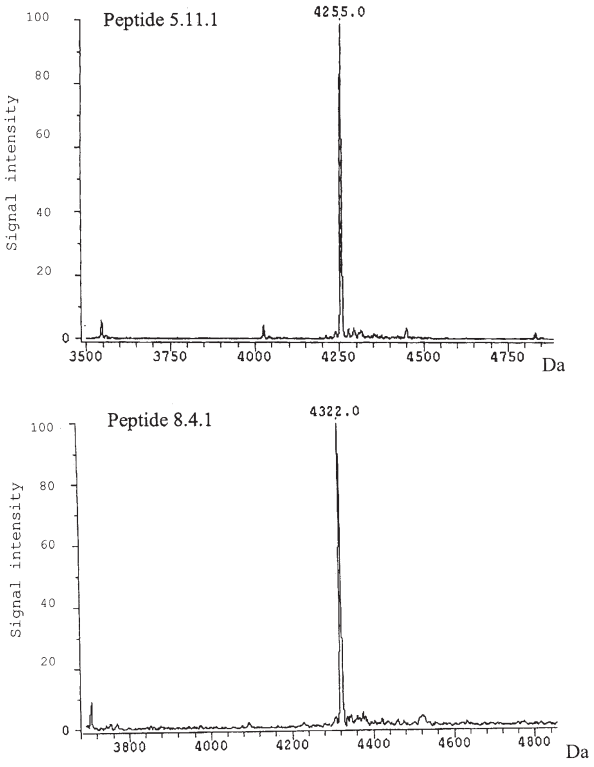

Figure 4. Mass spectra of the newly isolated *Galleria* antibacterial peptides.

biochemical investigations on immunized insects that resulted in isolation of novel classes of antimicrobial peptides called cecropins and attacins [7]. Presently, the insect hemolymph is one of the richest sources of novel antimicrobial agents; the number of novel inducible antibacterial peptides of insects exceeds now 150.

Galleria mellonella is a common subject of immunological investigations in insects [8–10], no detailed biochemical studies have so far been done on antibacterial peptides present in its hemolymph. The present work describes purification and biochemical characterization of two such compounds. One of them, bearing the working name 8.4.1, has 90% sequence identity with of cecropin D isolated from domestic silkworm (*Bombyx mori*). The peptide 8.4.1 is also similar to the bacteriocin family members from tobacco

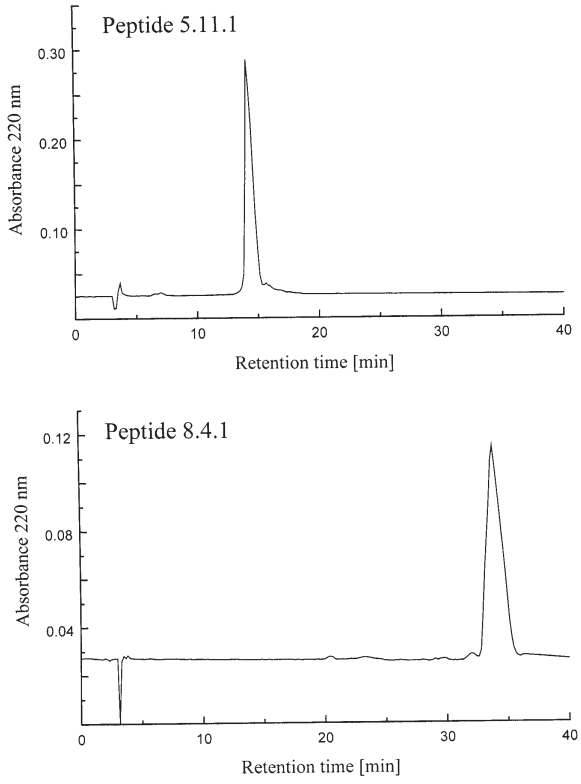

Figure 5. HPLC chromatography of the *Galleria* antibacterial peptides.

Fifty μg of a final preparation was loaded on a C-18 column and separated using a 40 min gradient and TFA containing buffers. See Materials and Methods for details.

hornworm (*Manducta sexta*) but this family is also referred as cecropin-like peptides. The known cecropins are 35–39 amino acid long, are highly basic and fold into two amphipatic α-helices [11]. Like the cecropins, to which the 8.4.1 is highly homologous, the peptide 8.4.1 has also one proline residue in position 23. This suggests the presence of a sharp bend in this region and also formation of a characteristic two-helical structure. A feature of the peptide 8.4.1 that is inconsistent with the common cecropin image is its charge. The peptide 8.4.1 in physiologic pH has six positive residues and six negative ones, the charge of the whole molecule is therefore neutral. Because the charge of a peptide is important in its interactions with negative charged bacterial membranes this may explain the poor value of MIC determined for 8.4.1. The known cecropins kill bacteria at a concentration range 0.2–20 μM [10], while the MIC for 8.4.1 toward *E. coli* is 53 μM.

The peptide 5.11.1 has a unique sequence and kills *E. coli* slightly more effective than 8.4.1. This may be related to its high net positive charge (+5). The peptide 5.11.1 is relatively rich in proline but the arrangement of these residues does not fit to the consensus sequences of insect proline-rich peptides, such as apidaecins, metchnikovin or abaecins [12]. The peptide 5.11.1 probably is a member of a novel family of antibacterial peptides. Further studies on both peptides isolated from *Galleria* should be directed to the determination of their activity toward different microorganisms, including specific insect pathogens.

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