PEPTIDES 28 (2007) 533–546

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Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph

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ARTICLE INFO

Article history:
Received 6 October 2006
Received in revised form
17 November 2006
Accepted 20 November 2006
Published on line 27 December 2006

Keywords:
Galleria mellonella
Insect immunity
Antibacterial/antimicrobial peptides
Hemolymph
Peptide purification

ABSTRACT

Defense peptides play a crucial role in insect innate immunity against invading pathogens. From the hemolymph of immune-challenged greater wax moth, *Galleria mellonella* (Gm) larvae, eight peptides were isolated and characterized. Purified Gm peptides differ considerably in amino acid sequences, isoelectric point values and antimicrobial activity spectrum. Five of them, Gm proline-rich peptide 2, Gm defensin-like peptide, Gm anionic peptides 1 and 2 and Gm apolipophoricin, were not described earlier in *G. mellonella*. Three others, Gm proline-rich peptide 1, Gm cecropin D-like peptide and *Galleria* defensin, were identical with known *G. mellonella* peptides. Gm proline-rich peptides 1 and 2 and Gm anionic peptide 2, had unique amino acid sequences and no homologs have been found for these peptides. Antimicrobial activity of purified peptides was tested against Gram-negative and Gram-positive bacteria, yeast and filamentous fungi. The most effective was Gm defensin-like peptide which inhibited fungal and sensitive bacteria growth in a concentration of 2.9 and 1.9 μM, respectively. This is the first report describing at least a part of defense peptide repertoire of *G. mellonella* immune hemolymph.

1. Introduction

Defense peptides are key factors in innate immunity against bacteria and fungi in vertebrates as well as invertebrates. Particularly, in insects which lack an adaptive immune system, antimicrobial peptides play a crucial role in fighting against invading pathogens. They are synthesized in response to microbial infection or septic body injury mainly in insect fat body (functional equivalent of mammalian liver) and in certain blood cells, and then rapidly released into hemolymph where they act synergistically against microorganisms [25,27,59]. From a large number of about 890 antimicrobial peptides of eukaryotic origin identified to date, more than 180 were described in insects [63].

Peptides exhibiting antimicrobial activity are mainly small (5 kDa), amphipathic, cationic molecules. On the basis of amino acid sequence and structural characteristics they are divided into three broad classes: (i) linear α-helical peptides without cysteine residues, e.g. cecropins; (ii) peptides whose structure is stabilized by disulfide bridges (cysteine-stabilized peptides), e.g. defensins; (iii) peptides with an overrepresentation of proline and/or glycine residues [5]. Most known antimicrobial peptides act toward microbial cell membrane causing permeability perturbations or even membrane disintegration due to pore-forming or carpet-like mechanisms of action [5,41,67]. However, the proline-rich peptides seem to have a protein target and are not membrane-active [6,47], while, on the other hand, the rare anionic antibacterial peptides kill bacterial cells, probably, by

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2006.11.010

causing cytoplasmic protein precipitation and intracellular content flocculation [3,4,31]. There are also known peptides affecting important intracellular processes, e.g. DNA and protein synthesis or proper folding of newly synthesized proteins [5,6,41,47,67]. Certain antimicrobial peptides demonstrate anticancer activity, e.g. insect cecropins [9,42] and magainins from frog skin [13,48]. Generally, antimicrobial peptides are assumed in the near future as an alternative for the nowadays classical antibiotics. The advantages of antimicrobial peptides are: selectivity, fast killing, broad antimicrobial spectra and no resistance development [1,41].

As it was stated earlier, many antimicrobial peptides have been discovered in insects. In *Drosophila melanogaster*, 20 antimicrobial peptide genes were identified and their peptide products were grouped into seven families: attacins, cecropins, defensins, diptericins, drosomycins, drosocin and metchnikowin [27]. Cecropins, defensins, drosomycin, drosocin and metchnikowin were isolated from immune-challenged flies and corresponding genes were cloned. Although *D. melanogaster* is nowadays the best characterized organism concerning insect innate immunity, the first insect inducible antibacterial peptides, cecropins, were isolated and characterized from bacteria-challenged pupae of the lepidopteran insect, giant moth *Hyalophora cecropia* [57]. Since then peptides with antimicrobial activity have been purified and described in many other insect species belonging to different orders: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Trichoptera and Odonata [25].

Recently, the lepidopteran insect, greater wax moth *Galleria mellonella* (Gm), has been developed as a model organism for studying innate immunity mechanisms and also for pathogenicity tests with different microorganisms, e.g. filamentous fungi *Aspergillus fumigatus* [53], *Aspergillus flavus* [37], yeast *Candida albicans* [2,12,16], *Cryptococcus neoformans* [45] and bacteria [29]. It is taken as a rule that a given insect species produces a unique repertoire of antimicrobial peptides with overlapping structural features but they are often targeted toward specific microorganisms. So far, five inducible *G. mellonella* peptides with antimicrobial activity have been characterized and genes of three of them have been cloned. Kim et al. [30] described a cecropin-like peptide homologous to *H. cecropia* cecropin A and Lee et al. [36] characterized a defensin-like peptide, named *Galleria* defensin. Cloning and expression of another *G. mellonella* antifungal peptide, gallerimycin, was also reported [56]. Antimicrobial peptide homologous to *Bombyx mori* cecropin D and a proline-rich peptide of unique amino acid sequence were purified by Mak et al. [40], however, the antimicrobial activity spectrum of both peptides was not determined.

In this paper we report on purification, characterization and antimicrobial activity spectrum of eight peptides present simultaneously in immune hemolymph of *G. mellonella* larvae.

2. Materials and methods

2.1. Materials and chemicals

If not otherwise stated, all materials and chemicals used were from Sigma-Aldrich-Fluka-Supelco Company, St. Louis, MO, USA.

2.2. Culture and immunization of insects

Larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) were reared on a natural diet—honeybee nest debris at 30 °C in the dark. Last instar larvae (250–300 mg in weight) were used throughout the study.

For immune challenge the larvae were pierced with a needle dipped into a pellet of viable *Escherichia coli* D31 cells. The larvae were kept at 30 °C in the dark and the hemolymph was collected 24 h after immune challenge, when (as was estimated in preliminary experiments) a very high level of low-molecular mass proteins and peptides expression was detected.

2.3. Collection and preparation of hemocyte-free hemolymph

Prior to hemolymph collection, the insects were chilled for 15 min at 4 °C and surface sterilized with 70% (v/v) ethanol solution. Hemolymph samples were obtained by puncturing larval abdomen with a sterile needle. Out-flowing hemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing a few crystals of phenylthiourea (PTU) to prevent melanization. The hemocyte-free hemolymph was obtained by centrifugation at $200 \times g$ for 5 min to pellet hemocytes and subsequently the supernatant was spun down at $20\,000 \times g$ for 15 min at 4 °C to pellet cell debris. The obtained hemocyte-free hemolymph was used immediately for extraction of peptides.

2.4. Preparation of hemolymph extracts

Acidic/methanolic extracts of hemocyte-free hemolymph were obtained by the method adapted from Schoofs et al. [55]. The hemolymph was diluted 10 times with the extraction solution consisting of methanol:glacial acetic acid:water (90:1:9, v/v/v) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at $20\,000 \times g$ for 30 min at 4 °C. The obtained supernatant was collected, freeze-dried and the pellet was dissolved in 0.1% trifluoroacetic acid (TFA). For lipid removal from the extract, the same volume of *n*-hexane was added, the sample was vortexed and centrifuged at $20\,000 \times g$ for 10 min at 4 °C. The upper fraction containing lipids was removed and an equal volume of ethylacetate was added to the water fraction. After vortexing and centrifugation the water fraction containing peptides was freeze-dried and stored at −20 °C until needed.

2.5. Purification of G. mellonella peptides

The immune hemolymph extract, deprived of lipids and freeze-dried, was redissolved in 0.1% TFA and subjected to the first step of purification using a Supelcosil LC-18-DB 4.6 mm × 250 mm column, two buffer sets—A: 0.1% TFA (v/v), B: 0.07% TFA, 80% acetonitrile (v/v), a linear gradient from 20 to 70% of buffer B over 30 min and 1 ml/min flow rate. This one and all next chromatographic steps were performed on a Dionex P680 HPLC system (Dionex, Sunnyvale, CA, USA). The resulting 12 fractions were subjected to freeze-drying, redissolved in water and visualized by staining with Coomassie

Brilliant Blue after gel electrophoresis. Antimicrobial activity of the obtained fractions was determined against Gram-negative bacteria *E. coli* D31 and Gram-positive bacteria *Micrococcus luteus* as described below (Section 2.8). Identified peptide-containing samples exhibiting antibacterial activity were then subjected to the second step of purification using gel filtration chromatography on a Superose 12 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden), 50 mM ammonium acetate buffer pH 7.5 supplemented with 30% (v/v) acetonitrile and 0.4 ml/min flow rate. The collected peptide-containing fractions were finally purified to homogeneity using the previously described Supelcosil LC-18-DB column and a TFA/water/acetonitrile buffer set. The gradient was individual for each peptide: from 30 to 50% of buffer B over 25 min in the case of Gm proline-rich peptide 1, from 35 to 55% B over 25 min in the case of Gm anionic peptide 1, from 40 to 70% B over 25 min in the case of Gm apolipophoricin, Gm proline-rich peptide 2 as well as *Galleria* defensin and Gm defensin-like peptide, and, finally, from 55 to 80% B over 25 min in the case of Gm anionic peptide 2 as well as Gm cecropin D-like peptide. The purified peptides were freeze-dried and stored at −20 °C until needed. Before use for antimicrobial activity tests, they were dissolved in apyrogenic water.

2.6. Protein chemistry techniques

Total protein concentration in hemolymph preparations was estimated using bicinchoninic acid (BCA) assay calibrated on bovine serum albumin. The concentration of the peptides was measured by the amino acid analysis. Briefly, peptide samples were hydrolyzed in gas phase using 6 M HCl at 115 °C for 24 h. The liberated amino acids were then converted into phenylthiocarbamyl (PTC) derivatives and analyzed by HPLC chromatography on a PicoTag 3.9 mm × 150 mm column (Waters, Milford, MA, USA).

Tris–tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Mini Protean II cell (BioRad, Sunnyvale, CA, USA) according to protocol of Schägger and von Jagov [54]. After separation, the gels were fixed by 30-min-long gentle shaking in 10% acetic acid, 50% methanol (v/v) and visualized by staining with Coomassie Brillant Blue R-250.

Fragmentation of Gm proline-rich peptide 2 at Asp-Pro bond was carried out by dissolving the peptide in 70% (v/v) formic acid and incubation of this mixture for 2 h at 55 °C followed by two additional hours at 75 °C. After incubation, the mixture was freeze-dried, redissolved in 0.1% TFA and the resulting peptides were separated using Supelcosil LC-18-DB column and a TFA/water/acetonitrile buffer set (see Section 2.5 for details) under a linear gradient from 20 to 70% of buffer B over 25 min.

Enzymatic fragmentation of Gm anionic peptide 2 after Lys and Arg residues by TPCK-treated trypsin was performed by incubation of peptide solution for 24 h at 37 °C in 50 mM ammonium bicarbonate at 1:50 weight ratio of trypsin to peptide. Identical conditions were applied in the case of digestion after Asp and Glu residues by the use of V8 protease (Biocentrum Ltd., Krakow, Poland). After digestion, the solutions were acidified by the addition of TFA and the obtained peptide fragments were separated using Supelcosil LC-18-DB column and a TFA/water/acetonitrile buffer set (see Section 2.5 for details) under a linear gradient from 0 to 100% of buffer B over 30 min.

Conversion of cysteine residues in *Galleria* defensin and Gm defensin-like peptide to *S*-pyridylethyl derivatives was performed by denaturation of peptides in 0.2 M Tris–HCl pH 8.3 supplemented with 6 M guanidine hydrochloride followed by reduction with β-mercaptoethanol and alkylation by the addition of excess of 4-vinylpyridine. The reaction mixture was then acidified by TFA addition and desalted, using Supelcosil LC-18-DB column and a TFA/water/acetonitrile buffer set (see Section 2.5 for details) under a linear gradient from 40 to 70% of buffer B over 25 min.

N-terminal amino acid sequences were determined using Procise 491 (Applied Biosystems, Foster City, CA, USA) automatic sequence analysis system and standard protocols of the manufacturer. Searching of sequence similarities was performed using BLAST server database release 2.2.13 available under http://www.ncbi.nlm.nih.gov/blast. Theoretical molecular masses and isoelectric point values were calculated using ExPASy proteomics server tools available under http://www.expasy.org.

2.7. Mass spectrometry

Esquire 3000 (Bruker-Daltonics, Bremen, Germany) mass spectrometer equipped with the electrospray ion-source was used for the measurements. Water solutions of peptides were mixed with an equal volume of methanol, water and formic acid mixture (30:69.9:0.1, v/v/v). The samples were injected into the ion-source using a syringe pump with the flow rate set to 3 μl/min. Scans were acquired in the scan range 150–1500 in the MS and MS/MS modes. Ions selected for the MS/MS experiments were isolated and fragmented with the isolation window of 4 Da and the fragmentation amplitude of the 1.2 unit. Peptides longer than 7 amino acid residues were fragmented using an enhanced method of peptide sequencing by N-terminal acetylation. A detailed procedure is described in our previous work [46]. Interpretation of the MS/MS spectra was performed manually with the help of Biotools v 2.0 software (Bruker Daltonics, Bremen, Germany).

2.8. Antimicrobial activity assays

2.8.1. Antimicrobial activity in hemolymph extracts—bioautography

Detection of antimicrobial activity in situ (bioautography) was performed after tricine SDS-PAGE of hemolymph extracts and the subsequent renaturation of polypeptides, as described earlier [14]. Briefly, for SDS removal the gels were washed in 2.5% Triton X-100 (Bio-Rad, Hercules, CA, USA) for 30 min. Then the gels were washed in 50 mM Tris–HCl pH 7.5 and subsequently in LB Broth (BioCorp, Poland). To localize the peptide bands with antimicrobial activity, the gels were overlaid with soft (0.7%) nutrient agar containing viable *E. coli* D31 cells and hen egg white lysozyme (EWL) in a concentration 2.5 mg/ml of the medium. After incubation at 37 °C for 12 h the zones of bacterial growth inhibition were observed.

2.8.2. *Antimicrobial activity in HPLC fractions*
Antimicrobial activity of fractions obtained after HPLC chromatography was estimated against *E. coli* D31 and *M. luteus* using a colony counting assay. Additionally, lysozyme activity was detected by the radial diffusion assay. For this purpose fractions were freeze-dried and redissolved in 50 μl of apyrogenic water.

2.8.2.1. *Colony counting assay*. One microliter of freeze-dried fractions redissolved in apyrogenic water was added to 10 μl of suspension containing 10^5 CFU of *E. coli* D31 or *M. luteus*, prepared in LB medium. The mixtures were incubated for 1 h at 37 °C (*E. coli*) or 30 °C (*M. luteus*), serial dilutions were prepared and plated on solid agar plates. After incubation at appropriate temperature for 24 h, bacterial colonies were counted. The antibacterial activity was expressed as percent of bacterial growth inhibition in comparison to control (bacterial suspension incubated without addition of fractions).

2.8.2.2. *Radial diffusion assay*. Lysozyme activity was detected using agarose plates containing freeze-dried *M. luteus* (Sigma) [28]. Wells (2.0 mm in diameter) were filled with 2 μl of each fraction and after 24 h of incubation at 28 °C the diameters of clear zones were measured. The relative activity was expressed in units (10 units = 1 mm) [68].

2.8.3. *Antimicrobial activity of purified peptides*
Antimicrobial activity tests of purified *G. mellonella* peptides were performed by measuring optical density A_{600} of microbial cultures incubated with peptides, mainly as described by Lee et al. [36] with small modifications. Antimicrobial activity of peptides was expressed as minimal inhibitory concentration at which microorganisms were unable to grow (MIC) or in some cases as the lowest concentration that caused 50% decrease in the optical density of the tested microorganism suspension (LC50) in comparison to the suspension incubated without the peptide addition. The obtained MIC values were presented as an interval (*A–B*) where *A* is the highest peptide concentration at which microbes were still growing and *B* is the lowest concentration which completely inhibited microorganism growth.

For antibacterial activity tests, bacteria were grown overnight in LB Broth at 30 °C (*M. luteus*, *Sarcina lutea*) or 37 °C (*E. coli* D31, *E. coli* ATCC 25922, *Salmonella typhimurium*, *Bacillus circulans*, *Listeria monocytogenes*, *Staphylococcus aureus*) to stationary phase. The cultures were diluted in a fresh LB medium, grown for an additional 3 h and then diluted in a fresh LB medium to A_{600} = 0.002.

For antifungal activity assays, yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*, *P. stipitis*, *Zygosaccharomyces marxianus*, *Pachysolon tannophilus*, *Schizosaccharomyces pombe*, *C. albicans*, *Candida fructus*, *C. wickerhamii*, *Cryptococcus albidus*) were grown overnight in YPD medium (0.1% yeast extract, 0.05% peptone, 0.2% dextrose) at 30 °C. Yeast suspensions were diluted with a fresh YPD medium, grown for an additional 6 h and diluted to A_{600} = 0.002. Aliquots (10 μl) of the culture were incubated with purified peptides (1 μl) for 24 or 48 h at the proper temperature, diluted 10 times and their optical density was measured.

Filamentous fungi (*Fusarium oxysporum*, *Aspergillus niger*, *Trichoderma harzianum*) were grown on solid PDA medium (5% potato extract, 0.5% dextrose, 1.7% agar) at 30 °C until spores were obtained. The fungal spores were suspended in potato dextrose broth to the final concentration of 200 spores/10 μl and aliquots of suspension (10 μl) were incubated with *G. mellonella* peptides for 24 or 48 h at 30 °C. Then, the suspensions were diluted seven times with sterile water and their optical density was measured. Additionally, the probes were tested microscopically.

Maximal final concentrations of purified peptides used in antimicrobial activity tests were as follows: Gm proline-rich peptide 1–110 μM; Gm proline-rich peptide 2–34.2 μM for fungi and 15.7 μM for bacteria; *Galleria* defensin −16.9 μM; Gm defensin-like peptide −2.9 μM; Gm anionic peptide 1–166.7 μM; Gm anionic peptide 2–86.6 μM; Gm cecropin D-like peptide −34.4 μM; Gm apolipophoricin −6.5 μM.

3. Results

3.1. *Comparison of polypeptide composition in G. mellonella non-immune and immune hemolymph extracts*

To obtain a hemolymph extract deprived of high molecular mass proteins we used an acidic/methanol extraction [55]. The resulted fraction contained several polypeptides of molecular mass below 30 kDa as revealed by Tris–tricine SDS-PAGE (Fig. 1A). In the extract of immune hemolymph at least two additional peptide bands with molecular mass 4–6 kDa were detected when compared to the extract prepared from non-immune hemolymph. This suggested that additional bands contained peptides appearing in the hemolymph in response to immune challenge (Fig. 1Ad). The antimicrobial activity of hemolymph extracts was tested by bioautography after resolution of polypeptides by Tris–tricine SDS-PAGE and subsequent renaturation (Fig. 1B). In the extract of immune hemolymph, but not of non-immune one, two *E. coli* growth inhibition zones, corresponding to molecular mass below 6.5 kDa were detected, confirming the presence of inducible antimicrobial peptides in the studied fraction (Fig. 1Be).

3.2. *Purification of immune hemolymph peptides*

The first step of purification – fractionation of immune hemolymph extract on a reversed phase C-18 column – allowed effective separation of 12 fractions containing mainly proteins and peptides of molecular masses below 20 kDa (Fig. 2, inset). The obtained fractions were tested for antimicrobial as well as lysozyme activity (Table 1). Relative high level of antibacterial activity against *E. coli* D31 and *M. luteus* was detected in fractions 1, 5, 9–12 and 5, 7, 9–12, respectively. Fractions 9–12 contained also lysozyme activity (Table 1). For further purification were chosen fractions 5, 7, 9, 10, 11 and 12, containing the most abundant low-molecular mass peptides (below 6.5 kDa) and exhibiting high antibacterial activity. The second step embraced gel filtration chromatography and allowed isolation of single peptide components from fractions 5, 7, 10, 11 and 12 (Fig. 3). Although fraction 5 resolved during gel filtration into three separate peaks, only one of them (named A) contained low-molecular mass peptide, whereas the two others contained higher molecular

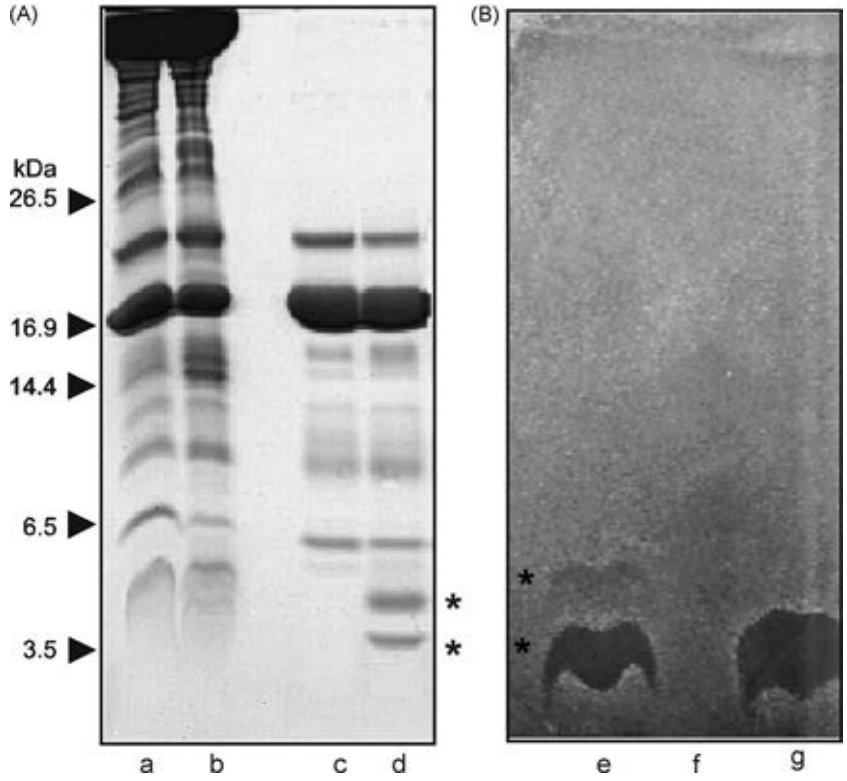

Fig. 1 – Tricine SDS-PAGE (A) and bioautography (B) of *G. mellonella* hemolymph extracts. (A) Hemolymph samples (100 μg of total protein) and acidic/methanolic extracts (20 μg of total protein) were resolved in polyacrylamide gel and visualized as described in Section 2: (a) non-immune hemolymph; (b) immune hemolymph; (c) non-immune hemolymph extract; (d) immune hemolymph extract. (B) Samples of immune (e) and non-immune (f) hemolymph extract (50 μg of total protein) and synthetic cecropin B (1 μg) (g) were resolved by SDS-PAGE and after renaturation their antibacterial activity was detected as described in Section 2. Asterisks indicate the position of additional peptide bands and zones of bacterial growth inhibition.

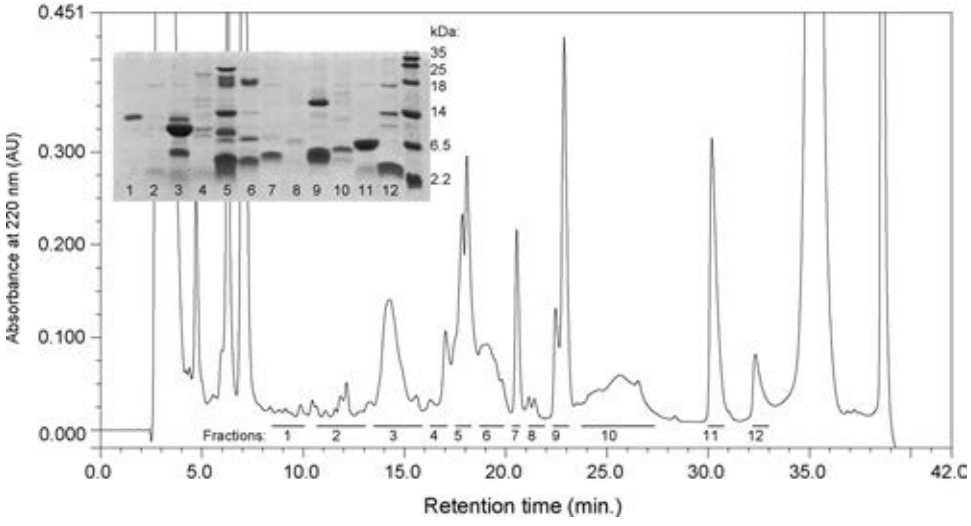

Fig. 2 – Reversed-phase HPLC fractionation of *G. mellonella* immune-hemolymph extract. Equivalent of 100 μl of hemolymph was separated on a C-18 column using water/TFA/acetonitrile buffers set. The denoted 12 fractions were collected, freeze-dried, dissolved in water and in quantities equivalent to 25 μl of hemolymph were visualized by SDS-PAGE (inset). The details of HPLC and SDS-PAGE techniques are described in Section 2.

Table 1 – Antibacterial activity of HPLC fractions obtained after chromatography of *G. mellonella* immune hemolymph extract

Fraction number according to Fig. 2	Anti-*E. coli* D31 activity (% of growth inhibition)[a]	Anti-*M. luteus* activity (% of growth inhibition)[a]	Lysozyme activity (U)[b]
1	66.8	24.8	–
2	0	31.5	–
3	0	25.3	–
4	0	23.1	–
5	78.2	99.3	–
6	24.5	32.5	–
7	0	91.5	–
8	22.8	49.3	–
9	97.9	98.5	113
10	98.9	98.6	87
11	95.6	70.6	60
12	92.4	85.8	50

–: no activity was detected.
[a] Inhibition of bacterial growth is expressed in percent in comparison to control incubated without fraction addition.
[b] Relative activity: diameters of clear zones are expressed as units (10 units = 1 mm).

mass polypeptides (not shown). Similarly, fraction 9 split up during this purification step into three peaks, but all of them contained separate small peptides (Fig. 3). All the eight peptides (A–H) were then desalted and purified to homogeneity by a reversed phase chromatography step on a C-18 column. The final peptide preparations gave single bands on SDS-PAGE gels (Fig. 3, inset), single peaks on a C-18 column, clear amino acid sequences and single ion peaks during mass spectrometry (not shown). The estimated amino acid sequences of purified *G. mellonella* peptides, theoretical and experimental molecular masses as well as calculated isoelectric points are summarized in Table 2. Alignment of the obtained sequences toward most similar microbicidal peptides is presented in Table 3.

Peptide A isolated from fraction 5 (the number according to Fig. 2) gave a clear sequence of a 37-mer peptide, identical to the so-called peptide 5.11.1, characterized in our previous work [40]. The peptide has a unique sequence and is relatively rich in proline residues (proline content about 13.5%), so it was finally called Gm (*G. mellonella*) proline-rich peptide 1. The estimated molecular mass of this peptide was 4322.0 Da and very well agreed with the theoretical molecular mass calculated from the sequence (4322.9 Da).

Fraction 7 (according to Fig. 2) contained a single peptide E giving a 42-mer sequence with 89% and 84% of identity to *B. mori* antimicrobial peptide lebocin 4 and 3 precursors, respectively [19]. The peptide E is relatively rich in anionic amino acids so we called it Gm anionic peptide 1. The estimated molecular mass was 4820.1 Da and very well agreed with the theoretical one, 4819.4 Da.

Fraction 9 (according to numeration from Fig. 2) split up during gel filtration into three peptide compounds B, C, and D. The first one, B, gave a clear sequence of a unique 42-mer peptide. The peptide B is relatively rich in positively charged amino acids and contains 11 proline residues (proline content 26.2%) so it was called Gm proline-rich peptide 2. The estimated molecular mass of this peptide was 4927.6 Da and well agreed with the theoretical molecular mass calculated from the sequence (4928.7 Da). The second and third compound from gel filtration column, peptides C and D, gave sequences of 43-mer and 44-mer peptides, respectively. Amino acid analysis demonstrated six cysteine residues in both peptides, so before sequencing, both compounds were derivatized with 4-vinylpyridine. Sequence analysis of both peptides showed that peptide C was identical, while peptide D exhibited 95% of identity, with *Galleria* defensin described by Lee et al. [36]. We called peptide D Gm defensin-like peptide. Additionally, Gm defensin-like peptide had a high degree (93%) of sequence identity to *Heliothis virescens* antifungal defensin [33] and to *Archaeoprepona demophon* defensin Ard1 [34]. Mass spectrometry measurements fully confirmed both obtained sequences and proved that all six cysteine residues in both peptides are involved in the formation of three intramolecular disulfide bonds: the 43-mer peptide (*Galleria* defensin) gave molecular mass of 4714.6 Da (theoretical mass regarding cysteines in a disulfide form is 4714.3 Da), while the 44-mer peptide (Gm defensin-like peptide) showed a molecular mass of 4943.9 Da (theoretical mass regarding cysteines in a disulfide form is 4943.5 Da).

Fraction 10 contained a single peptide compound, F. N-terminal sequencing up to residue 12 demonstrated 100% identity with the C-terminal part of *G. mellonella* protein named apolipophorin III (apoLpIII) [61]. Mass spectrometry analysis showed that this fragment has a molecular mass of 5712.7 Da, which is equivalent to theoretical 5711.5 Da molecular mass of a C-terminal fragment of apoLpIII, counting from residues 136 to 186 (according to numeration of apolipophorin precursor). The obtained C-terminal fragment of apoLpIII was named Gm apolipophoricin.

Fraction 11 contained peptide G, whose molecular mass was estimated by mass spectrometry to 6978.9 Da. Automatic N-terminal sequencing allowed determination of only the 40 first residues. The lacking C-terminal sequence was estimated in two stages. First, the peptide was digested by trypsin and the resulting peptides were separated on a reversed phase C-18 column. All peptide peaks were then subjected to molecular mass estimation on a mass spectrometer. An analysis of the obtained results revealed three peptide fragments that did not fit to the previously determined 40-mer N-terminus of peptide G. All these three new

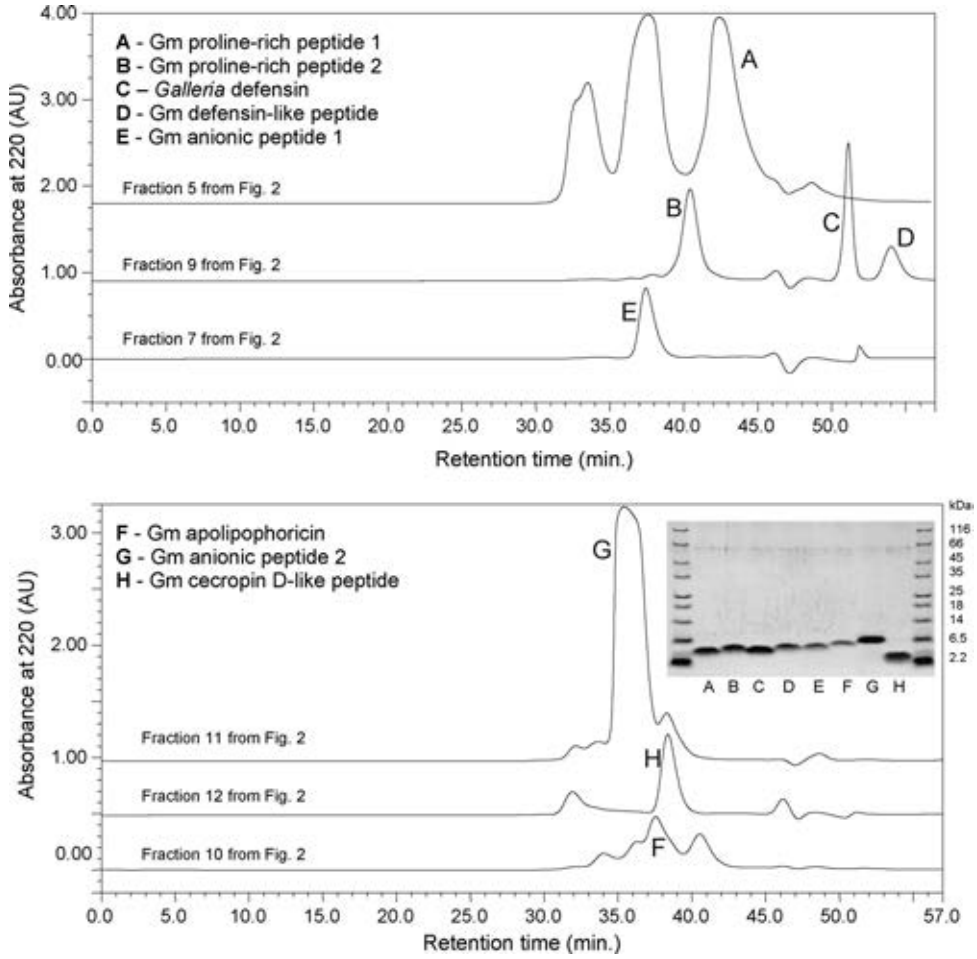

Fig. 3 – Gel filtration chromatography of fractions obtained after RP-HPLC. Fractions 5, 7, 9, 10, 11 and 12 from Fig. 2 were subjected to separation on a Superose 12 column using ammonium acetate/acetonitrile buffer. Fractions 5, 7, 10, 11 and 12 gave single peptide peaks denoted as A, E, F, G and H, while the fraction 9 split up into three peptide compounds, denoted as B, C and D. All 8 obtained peptides were then desalted on an additional RP-HPLC chromatography step (not shown), freeze-dried and visualized on a SDS-PAGE gel (inset). Each lane contains equivalent of about 5 μg of peptide. The details of chromatographic and SDS-PAGE techniques are described in Section 2.

peptides were subjected to automatic N-terminal sequence determination. The first peptide gave sequence EAPK, the second one gave ILNTEKK, while the third one was SEVNNFIESLGK. In the second stage of experiments, we determined the order of the above three peptide fragments in the C-terminal part of peptide G. Thus, the whole peptide G was again digested separately into short fragments by two proteases, trypsin and V8 endopeptidase, and the resulting peptide mixtures were analyzed by mass spectrometry in MS/MS mode. The resulting from MS/MS experiments overlapped sequences of short C-terminal peptides allowed estimation of the peptide sequence in the whole C-terminal part of the maternal peptide. The obtained complete amino acid sequence of *G. mellonella* peptide G shows a relatively anionic molecule with no similarity to known peptides and proteins and we designated it as Gm anionic peptide 2. Theoretical molecular mass of Gm anionic peptide 2 was 6979.7 Da and very well agreed with the experimental one (6978.9 Da).

The last analyzed peptide from *G. mellonella* immune hemolymph was a compound from fraction 12 designated as peptide H (according to Fig. 2). It is a 39-mer peptide of an amino acid sequence identical to the so-called peptide 8.4.1, characterized in our previous work [40]. The high level of sequence similarity of this peptide to cecropin D-like peptides, bactericidins, of *Manduca sexta* [15] was shown previously [40]. The peptide H exhibited also relatively high identity (82%) to cecropin D from Chinese oak silk moth *Antheraea pernyi* [51], domestic silkworm *B. mori* (73%) [66] and cecropin 6 of *M. sexta* (75%) [69]. We called our peptide Gm cecropin D-like peptide. The estimated molecular mass of this compound was 4255.0 Da and very well agreed with the theoretical molecular mass calculated from the sequence (4255.8 Da).

3.3. *Antimicrobial activity of purified G. mellonella peptides*

In the following experiments we examined antimicrobial activity of purified *G. mellonella* peptides against different Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. The obtained results are summarized in

Table 2 – Amino acid sequences, theoretical and estimated molecular masses, as well as calculated isoelectric points of peptides isolated from extract of *G. mellonella* immune hemolymph

Peptide name	Fraction according to Fig. 2	Fraction according to Fig. 3	Estimated amino acid sequence	Theoretical (calculated) molecular mass[a] (Da)	Obtained molecular mass (Da)	Calculated isoelectric point
Gm proline-rich peptide 1	5	A	1 11 21 31 DIQIPGIKKP THRDIIIPNW NPNVRTQPWQ RFGGNKS	4322.9	4322.0	11.0
Gm anionic peptide 1	7	E	1 11 21 31 41 EADEPLWLYK GDNIERAPTT ADHPILPSII DDVKLDPNRR YA	4819.4	4820.1	4.51
Gm proline-rich peptide 2	9	B	1 11 21 31 41 EIRLPEPFRF PSPTVPKPID IDPILPHPWS PRQTYPIIAR RS	4928.7	4927.6	9.97
Galleria defensin	9	C	1 11 21 31 41 DTLIGSCVWG ATNYTSDCNA ECKRRGYKGG HCGSFLNVKC WCE	4714.3[b]	4714.6	6.73
Gm defensin-like peptide	9	D	1 11 21 31 41 DKLIGSCVWG ATNYTSDCNA ECKRRGYKGG HCGSFWNVKC WCEE	4943.5[b]	4943.9	6.73
Gm apolipophoricin	10	F	1 11 21 31 41 51 VQETQKLAKT VGANLEETNK KLAPQIKSAY DDFVKQAQEV QKKLHEAASK Q	5711.5	5712.7	9.06
Gm anionic peptide 2	11	G	1 11 21 31 41 51 ETESTPDYLK NIQQQLEEYT KNFNTQVQNA FDSDKIKSEV NNFIESLGKI LNTEKKEAPK	6979.7	6978.9	4.79
Gm cecropin D-like peptide	12	H	1 11 21 31 ENFFKEIERA GQRIRDAIIS AAPAVETLAQ AQKIIKGGD	4255.8	4255.0	6.47

[a]Average isotopic mass. [b]Molecular mass calculated for cysteines in oxidized (disulfide) form.

Table 3 – Alignment of sequences of isolated *G. mellonella* hemolymph peptides toward most similar microbicidal peptides

Peptide		Sequence alignment [a]	
Gm anionic peptide 1	1	EADEPLWLYKGDNIERAPTTADHPILPSIIDDVKLDPNRRYA	42
Bombyx mori DNA for lebocin 4	44	AGQ------Q----P---S---------K------------V	85
Bombyx mori DNA for lebocin 3	44	AGQ------Q---VP---S---------K----Q-------V	85
Gm defensin-like peptide	1	DKLIGSCVWGATNYTSDCNAECKRRGYKGGHCGSFWNVNCWCEE	44
Galleria defensin [b]	1	-T----------L-------	43
Heliothis virescens antifungal defensin	1	----------V-------G---------------A-------T	44
Archaeoprepona demophon defensin Ard1	1	----------V----N-----------------A-------T	44
Gm cecropin D-like peptide	1	ENFFKEIERAGQRIRDAIISAAPAVETLAQAQKIIKGGD	39
Antheraea pernyi cecropin D	1	W-P---L------V-------G---A-V---TALA-	36
Manduca sexta cDNA for cecropin 6	25	WD-L--L-G----------Q-----I---TA-F--QS	63
Bombyx mori cDNA for cecropin D	25	G----DL-KM---V---V-------D---K-KALGQ-	61

[a]The table contains only peptides to whose statistically significant sequence similarities were found. The hyphens denote amino acids identical to respective residues in the compared peptide. Numbering of amino acid residues for sequences translated from nucleotide data concerns precursor forms of peptides. [b]Sequence of *Galleria* defensin according to Lee at al. [36].

Tables 4 and 5. Antimicrobial activity was calculated as MIC value but in some cases only LC50 values were determined.

Among the Gram-negative bacteria examined, only *E. coli* D31 was sensitive to Gm cecropin D-like peptide, whereas other purified peptides were not effective in inhibiting growth of Gram-negative bacteria used in this study (Table 4).

Gram-positive bacteria were more sensitive to purified *G. mellonella* peptides (Table 4). Five of the peptides were active against *M. luteus* and four of them to *L. monocytogenes*, but at a relatively high concentration range. The growth of *M. luteus* was most effectively inhibited by Gm anionic peptide 1. Interestingly, the growth of *S. lutea* was completely inhibited by Gm defensin-like peptide at a concentration of 1.9 μM.

Four of *G. mellonella* purified peptides inhibited yeast growth, namely Gm proline-rich peptide 1, *Galleria* defensin, Gm defensin-like peptide and Gm anionic peptide 2 (Table 5). The most effective antifungal peptide was Gm defensin-like peptide. This peptide completely inhibited growth of five examined yeast species and by 50% of two others at a concentration of 2.9 μM. Interestingly, Gm anionic peptide 2 seemed to selectively inhibit growth of *Pichia* species, although at high concentration.

The purified *G. mellonella* peptides were also effective in inhibition of filamentous fungi growth (Table 5). *Galleria* defensin and Gm defensin-like peptide inhibited growth of *A. niger* and *T. harzianum* at 2–4 μM concentration range, whereas *F. oxysporum* growth was inhibited by *Galleria* defensin at a concentration of 16.9 μM. Interestingly, Gm cecropin D-like peptide was effective in inhibition of *A. niger* growth at a concentration of 34.4 μM. Gm anionic peptide 1 exhibited also antifungal activity, however, at a relatively high concentration of 90.9 μM.

Table 4 – Antibacterial activity of purified *G. mellonella* hemolymph peptides

Microorganism	MIC[a] or LC50[b] doses of *G. mellonella* peptides (μM)							
	Gm proline-rich peptide 1	Gm proline-rich peptide 2	*Galleria* defensin	Gm defensin-like peptide	Gm anionic peptide 1	Gm anionic peptide 2	Gm cecropin D-like peptide	Gm apolipophoricin
Gram-positive bacteria								
M. luteus	31.4–55.0[a]	8.6[b]	–	–	11.4–22.7[a]	43.3–86.6[a]	34.4[b]	–
B. circulans	–	–	ND	ND	–	–	–	–
L. monocytogenes	–	–	–	–	45.5–90.9[a]	86.6[b]	34.4[b]	6.5[b]
S. aureus	ND	–	–	ND	–	–	ND	–
S. lutea	ND	–	–	1.4–1.9[a]	–	86.6[b]	34.4[b]	–
Gram-negative bacteria								
E. coli D31	–	–	–	–	–	–	6.9–8.6[a]	–
E. coli ATCC 25922	–	–	–	–	–	–	–	–
S. typhimurium	ND	–	–	–	–	–	–	–

ND: not determined; –: no activity was detected at the highest concentration tested.
[a] MIC values are expressed as an interval where the left value is the highest peptide concentration at which microbes are still growing and the right value is the lowest concentration that completely inhibits microorganism growth.
[b] LC50 values are expressed as the lowest concentration that causes 50% decrease in optical density of microorganism suspension.

Table 5 – Antifungal activity of purified *G. mellonella* hemolymph peptides

Microorganism	MIC[a] or LC50[b] doses of *G. mellonella* peptides (μM)							
	Gm proline-rich peptide 1	Gm proline-rich peptide 2	*Galleria* defensin	Gm defensin-like peptide	Gm anionic peptide 1	Gm anionic peptide 2	Gm cecropin D-like peptide	Gm apolipophoricin
Yeast and yeast-like fungi								
S. cerevisiae	ND	–	–	–	–	–	–	–
P. pastoris	8.3–16.5[a]	–	8.5–16.9[a]	1.4–2.9[a]	–	43.3–86.6[a]	–	–
P. stipitis	ND	ND	ND	2.9[b]	ND	43.3–86.6[a]	ND	ND
Z. marxianus	8.3–16.5[a]	–	4.2–8.5[a]	1.4–2.9[a]	–	–	–	–
P. tannophilus	ND	ND	4.2–8.5[a]	1.4–2.9[a]	ND	ND	–	ND
S. pombe	5.5–11[a]	ND	ND	–	ND	–	ND	ND
C. albicans	ND	–	4.2–8.5[a]	1.4–2.9[a]	–	–	–	–
C. fructus	ND	–	4.2–8.5[a]	1.4–2.9[a]	–	–	ND	ND
C. wickerhamii	8.3–16.5[a]	–	ND	2.9[b]	ND	–	–	–
C. albidus	–	ND	ND	–	ND	–	ND	ND
Filamentous fungi								
F. oxysporum	ND	–	8.5–16.9[a]	–	–	–	–	–
A. niger	–	–	1.1–2.1[a]	1.4–2.9[a]	46.4–90.9[a]	–	17.2–34.4[a]	–
T. harzianum	ND	–	2.1–4.2[a]	1.4–2.9[a]	46.4–90.9[a]	–	–	–

ND: not determined; –: no activity was detected at the highest concentration tested.
[a] MIC values are expressed as an interval where the left value is the highest peptide concentration at which microbes are still growing and the right value is the lowest concentration that completely inhibits microorganism growth.
[b] LC50 values are expressed as the lowest concentration that causes 50% decrease in optical density of microorganism suspension.

Among all the *G. mellonella* peptides tested, the peptides called Gm apolipophoricin and Gm proline-rich peptide 2, demonstrated the lowest antimicrobial activity. The peptides were able to partially inhibit growth of Gram-positive bacteria *L. monocytogenes* and *M. luteus*, respectively (Table 4).

4. Discussion

Defense peptides and proteins constitute key factors in insect humoral immune response against invading microorganisms. It is generally assumed that each insect species possesses an individual set of antimicrobial peptides synthesized in response to non-self recognition. In this study, we purified and characterized eight *G. mellonella* peptides which appeared in larval hemolymph after immune challenge. They probably comprise a part of the defense peptide repertoire of *G. mellonella*. Amino acid sequence analysis of purified peptides revealed that five of them, namely, Gm proline-rich peptide 2, Gm defensin-like peptide, Gm anionic peptides 1 and 2 and Gm apolipophoricin, were not described earlier in *G. mellonella*. Three others, Gm proline-rich peptide 1, Gm cecropin D-like peptide and *Galleria* defensin, are known *G. mellonella* peptides characterized by Mak et al. [40] and Lee et al. [36], respectively. Among purified by us new *G. mellonella* peptides, three, called Gm defensin-like peptide, Gm anionic peptide 1 and Gm apolipophoricin, exhibit homology to the previously described peptides and proteins involved in insect immune response. However, two others, Gm proline-rich peptide 2 and Gm anionic peptide 2, had a unique amino acid sequence and no homologs have been found for them.

Gm proline-rich peptide 1, described previously by Mak et al. [40], contains five proline residues (13.5%), whereas Gm proline-rich peptide 2 is richer in proline residues (26.2%). Both Gm proline-rich peptides lack the typical PRP motifs characteristic for short-chain proline-rich peptides but they do contain KP and PR motifs and could be classified to long-chain ones [5,6]. The proline-rich peptide, abaecin, lacking PRP motifs was purified and characterized from *Apis mellifera* (Hymenoptera) [5,6]. Members of long-chain proline-rich peptides are also lebocins isolated from *B. mori* [19,23,64]. Among *G. mellonella* hemolymph peptides, we purified a peptide named Gm anionic peptide 1 with unique characteristics. The peptide contains five proline residues (11.9%) and exhibits significant homology to the fragment of *B. mori* lebocin 4 and 3 precursors comprising amino acids from 44 to 85 of the propeptide sequence, while active processed lebocins 3 and 4 comprise amino acids from 121 to 152 of the precursor chain [19,23]. Isoelectric point values of the 44–85 amino acid fragment of lebocin 3 and 4 precursors were calculated for 4.82 and 5.51, respectively, and they resembled the pI 4.51 of Gm anionic peptide 1. Since lebocin-like peptide gene(s) of *G. mellonella* has not been cloned and the organization of this gene is unknown at present, it is difficult to determine if the peptide purified from the hemolymph of immune-challenged *G. mellonella* larvae represents an active processed lebocin peptide or rather a fragment of the propeptide sequence. Recently, antibacterial activity of proline-rich truncated form of *Drosophila* attacin C pro-domain, present in immune hemolymph, has been described [52].

G. mellonella proline-rich peptides were not active against Gram-negative bacteria but they exhibited anti-Gram-positive bacteria and antifungal activity. Similarly, *D. melanogaster* metchnikowins have no activity against Gram-negative bacteria but they inhibit growth of *M. luteus* and filamentous fungus *Neurospora crassa* [5]. Abaecins inhibit growth of Gram-negative and Gram-positive bacteria. It is known that proline-rich peptides like *Palomena prasina* metalnikowins and *B. mori*

lebocins, similarly to Gm proline-rich peptides, are active against sensitive microorganisms in relatively high concentrations. Metalnikowins inhibit Gram-negative bacteria growth at a concentration range from 50 to 200 μM depending on the isoform [10]. Similarly, the minimal inhibitory concentration of lebocin 3 tested against *E. coli* in nutrient broth was determined for 211.1 μM (800 μg/ml) [24]. It was suggested that lebocins can serve to reduce the minimum inhibitory concentration of other antimicrobial peptides acting synergistically [19,23,24,64].

Isoelectric points calculated on the basis of amino acid composition of purified *G. mellonella* peptides showed different values. Three of them, namely, Gm proline-rich peptide 1, Gm proline-rich peptide 2 and Gm apolipophoricin, exhibiting p*I* in basic pH range belong to cationic peptides. Three others, *Galleria* defensin, Gm defensin-like peptide and Gm cecropin D-like peptide, have p*I* values little below 7. The theoretical p*I* value for Gm defensin-like peptide is 6.73, exactly the same value can be calculated for *Galleria* defensin described by Lee et al. [36]. It was demonstrated that *Galleria* defensin exhibited antifungal activity and was not effective against bacteria *E. coli* and *Bacillus subtilis* [36]. In our studies, *Galleria* defensin was also active against fungi, whereas for Gm defensin-like peptide antifungal and antibacterial activity was detected. Although the p*I* value for both peptides is identical, they differ in amino acid sequence. Especially, the replacement of threonine residue in *Galleria* defensin by lysine residue in Gm defensin-like peptide noticed in position two of the polypeptide chain, could influence the activity. Such replacement could lead to local net charge increase, considering threonine and lysine p*I* values 5.19 and 8.75, respectively, and finally facilitate interaction of the peptide with the surface of microbial cells. Non-cationic defensin-like molecules were characterized from the tick *Amblyomma hebraeum*; p*I* values were calculated to be 6.71 and 4.44 for defensin 1 and defensin 2, respectively [32].

The predicted p*I* for Gm cecropin D-like peptide differs considerably from the p*I* values calculated for cecropin D-like molecules (p*I* 9.52–10.67) isolated from other insect species like *H. cecropia* [38], *B. mori* [22], *A. pernyi* [51], *M. sexta* [15]. The p*I* 6.47 of this molecule could, at least in part, explain its relatively low antimicrobial activity. However, it was demonstrated that *H. cecropia* cecropin D was less effective in growth inhibition of different bacteria in comparison to cecropin A and B [20]. Gm cecropin D-like peptide was active against Gram-negative and Gram-positive bacteria and also against the filamentous fungus *A. niger*. Antifungal activity of *D. melanogaster* cecropin A and B, *H. cecropia* cecropin A and cecropin-like peptide, andropin, was shown by Ekengren and Hultmark [17]. Gm cecropin D-like peptide in our studies was more active against *E. coli* (MIC 6.9–8.6 μM) than the identical peptide 8.4.1 (MIC 53 μM) described previously [40]. It is possible that anti-*E. coli* activity of peptide 8.4.1 was determined against another strain of *E. coli* than D31. It should be noted that Gm cecropin D-like peptide in our studies effectively inhibited growth of *E. coli* D31 but not of *E. coli* ATCC 25922. Similar reasons could explain different activity of Gm proline-rich peptide 1 determined in this report and identical peptide 5.11.1 described by Mak et al. [40] against *E. coli*.

Unexpectedly, two of the purified *G. mellonella* peptides were anionic, namely, Gm anionic peptide 1 (p*I* 4.51) and Gm anionic peptide 2 (p*I* 4.79). To date, only a few examples of anionic antimicrobial peptides were described. In ovine pulmonary surfactant, the presence of seven amino acid-long peptides containing five to seven aspartate residues and showing antimicrobial activity against *Pasteurella haemolytica* was demonstrated [4]. These peptides required zinc ions for maximum activity. Similar anionic peptides were also detected in cattle [3]. From the skin of the toad *Bombina maxima*, a 20 amino acid-long, anionic peptide, maximin H5, was described [31]. Maximin H5 had a limited antimicrobial activity and killed *S. aureus* with a MIC of 80 μM and was not dependent on zinc ions [31]. Another example of anionic antimicrobial peptide is the tick *A. hebraeum* defensin 2 exhibiting antibacterial activity against *E. coli* and *S. aureus* with MIC of 30 and 7.5 μM, respectively [32]. The purified *G. mellonella* anionic peptides were active against certain Gram-positive bacteria and also exhibited antifungal activity. Whether zinc ions might influence Gm peptides activity remains to be elucidated.

Some of the *G. mellonella* peptides which we purified exhibited antimicrobial activity at a micromolar concentration range, e.g. both defensins and Gm proline-rich peptide 1. However, inhibition of microbial growth by other purified peptides required higher concentrations in vitro. Yan and Hancock suggested that synergistic interactions are a very important determinant of antibacterial effectiveness of polypeptides [65]. It was demonstrated that an abundant hemolymph protein, apolipophorin III (apoLpIII), involved in lipid metabolism and immune response, acts synergistically with antibacterial peptides [21,49,60,62]. Purified *G. mellonella* apoLpIII enhanced the activity of synthetic cecropin A against *E. coli* [49] and the lytic activity of hen egg white lysozyme (EWL) against *M. luteus* [21]. Concerning this, the peptide called Gm apolipophoricin, representing a fragment of apoLpIII, seems to be an interesting molecule. However, without further detailed studies it is difficult to speculate if this peptide is synthesized de novo after immune challenge, released from storage places or if it is a fragment of partial proteolytic digestion of apoLpIII. Among different microorganisms tested, only *L. monocytogenes* growth was partially inhibited by Gm apolipophoricin. This could suggest that in *G. mellonella* hemolymph the peptide is probably not involved in direct killing of pathogens but rather plays some other role.

It is also well documented that lysozyme, exhibiting antibacterial and antifungal activity, is engaged in insect immune response, particularly in lepidopteran species like *G. mellonella*, *M. sexta*, *H. cecropia* [7,26]. The lepidopteran lysozyme genes are clearly induced by bacterial challenge and lysozyme activity in insect hemolymph, maintained constitutively at a low level, after bacterial infection increases considerably [11,35,39,43,44,50,58]. Observations obtained on *Aedes aegypti* and *H. cecropia* indicated synergy between lysozyme and antibacterial peptides against Gram-negative bacteria. Engström et al. [18] have shown that *E. coli* cells were susceptible to lysozyme in the presence of attacins. Chalk et al. [8] observed strong synergistic effect of EWL and *H. cecropia* cecropin B. They found that in the presence of lysozyme *E. coli* cells became susceptible to insect defensin [8].

One can speculate that in vivo high level of lysozyme and also apoLpIII in *G. mellonella* hemolymph allows lower

concentrations of antimicrobial peptides to act effectively against invading microorganisms. This could also be the answer to the question why after immune challenge with Gram-negative bacteria (*E. coli*) only a few from the purified peptides demonstrated anti Gram-negative bacteria activity, although in bioautography, in the presence of EWL, two distinct and clear zones of *E. coli* growth inhibition were observed. As was shown in Table 1, a strong anti-*E. coli* activity was present in several fractions obtained after HPLC chromatography. Importantly, most of the active fractions exhibited also lysozyme activity. The relative high lysozyme activity was detected in fraction 9, from which Gm proline-rich peptide 2 and both defensins were purified. This could suggest that indeed synergistic action of lysozyme and *G. mellonella* hemolymph peptides is important for anti-*E. coli* activity and could partially explain why most of the purified *G. mellonella* peptides did not inhibit growth of Gram-negative bacteria in vitro.

In summary, the presented paper demonstrates purification and characterization of eight peptides from hemolymph of immune-challenged *G. mellonella* larvae. The *G. mellonella* peptides differ considerably in amino acid sequences, isoelectric point values and antimicrobial activity spectrum. The appearance of peptides with such different properties in insect hemolymph in response to immune challenge indicates the complexity of the insect immune system. The simultaneous presence of described peptides in immune hemolymph suggests that they comprise a part of a defense peptide set involved in fighting against infection in *G. mellonella*.

Acknowledgements

The authors thank Prof. Teresa Urbanik-Sypniewska (Department of General Microbiology, Maria Curie-Skłodowska University, Lublin, Poland) and Prof. Janusz Szczodrak (Department of Industrial Microbiology, Maria Curie-Skłodowska University, Lublin, Poland) for providing some microbial species used in this study. The work was financially supported in part by the grant from the State Committee for Scientific Research (KBN, Poland) No. 2 P04C 054 26. P. Suder was supported by scholarship from the Foundation for Polish Science (FNP). P. Mak was supported by scholarship from Rector of Jagiellonian University.

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