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Study of Peptide Fractions from Hemolymph of *Galleria mellonella*

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Abstract—Changes in the peptide composition of hemolymph of *Galleria mellonella* larvae induced by their immunization have been studied, and some new peptides have been found. The composition of fractions exhibiting antibacterial activity was investigated. Known antibacterial peptides have been found in the hemolymph of control larvae and those immunized with bacteria.

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Defensive peptides are important elements of innate immunity to pathogenic microorganisms in vertebrates and invertebrates [1, 2]. In insects, antibacterial peptides play a key role in the defense against bacteria and fungi because of absence of the adaptive immune system [3]. Induction of antibacterial peptides by immunization of insects is currently an accepted way of finding new bacteriostatic components. However, in most of investigations little attention is paid to the presence of the inherent defensive barriers of insects [4-6]. Thus, investigation of the peptide composition of the hemolymph and its changes after immunization is of interest. The subject of the present investigation is the hemolymph of larvae of *Galleria mellonella* before and after immunization with bacteria *Escherichia coli* and *Bacillus cereus*.

MATERIALS AND METHODS

Chemicals. In the present work we used acetonitrile for HPLC from Aldrich (USA) (purity of 99.9% by gas chromatography), trifluoroacetic acid (TFA) of 99% purity (Alfa Aesar, Germany), and 2,5-dihydroxybenzoic acid (10 mg/ml) (Bruker Daltonics, Germany). Tridistilled water was purified using a Sub Boiling or Milli-P QG apparatus (Millipore, USA).

Equipment. An Agilent 1200 series liquid chromatograph and Agilent 1200 series multiple wavelength diode array detector were from Agilent Technologies (USA). The system was conditioned for 30 min. The detector was used at 210, 220, 224, and 280 nm. Spectra were taken in the range of 190-400 nm. A Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μ, 80 Å) was from Agilent Technologies and a HyperCarb column (100 × 2.1 mm, 5 μ, 250 Å) was from Fisher Scientific (USA).

Eluents for HPLC analysis: A) 0.04% (v/v) TFA; B) acetonitrile (flow rate, 0.5 ml/min, column temperature, 25°C, sample volume, 20 μl). A Bruker Ultraflex II MALDI TOF mass-spectrometer (Bruker Daltonics) equipped with a 20-Hz nitrogen laser (337 nm) was used for MALDI assay. Samples for MALDI were prepared using Anchor Chip targets with 600-μ wells in a thin layer of 2,5-dihydroxybenzoic acid used as the matrix: 20 μl of a tested sample was mixed with 0.3 μl of 2,5-dihydroxybenzoic acid (10 mg/ml in 20% aqueous solution of acetonitrile containing 0.5% TFA) and dried in air.

Sample preparation. Sixty larvae were immunized with the bacteria *E. coli* or *B. cereus* and incubated in a thermostat at 30°C for 24 h. Control larvae were incubated under the same conditions and were subsequently treated in the same way. To collect hemolymph, the larvae

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were dipped into 70% ethanol and then cooled on ice. The hemolymph was collected with a pipette and placed into a tube containing 0.5 ml of 0.01 μM phenylthiourea and 20 μg/ml of protease inhibitor. Cells and debris were removed by centrifugation at 100*g* for 5 min. The supernatant was centrifuged again at 2300*g* for 30 min.

To remove lipids, the supernatant was supplemented with an equal volume of hexane. The mixture was shaken for 10 min, left standing for 10 min, and then centrifuged at 100*g* for 15 min. The centrifugation resulted in phase separation. The hexane fraction (at the top) was removed. The remaining solution was supplemented with an equal volume of ethyl acetate, shaken for 5 min, left standing for 5 min, and then centrifuged at 100*g* for 15 min for phase separation. The ethyl acetate fraction was removed.

To precipitate proteins, 5 ml of methanol–glacial acetic acid–water (90 : 1 : 9 v/v) were added to the sample solution, and the mixture was centrifuged for 30 min at 20,000*g*. The supernatant was applied to a Sephadex G-10 column (0.7 × 7 cm) equilibrated with distilled water. The eluate (10 ml) was collected and lyophilized using an Inei-3.1 device. The lyophilizate was dissolved in 2 ml of 0.1% TFA and then diluted with 1 ml of water. The solution was centrifuged first at 4°C (2800*g*, 15 min) and then at 2°C (20,000*g*, 30 min). The supernatant was applied to a Sep-Pak C18 cartridge equilibrated with 0.05% TFA. Peptides were eluted with 5 ml of 10, 40, and 100% acetonitrile in 0.05% TFA. The resulting fractions were lyophilized, dissolved in 400 μl of water, and analyzed by HPLC. For each of three pairs of solutions, different programs were used for the separation and collection of fractions: for the fractions eluted with 10% acetonitrile, the gradient was set as 3-15% B in the range of 0-5 min and 15-50% B in the range of 5-6 min. Fractions were collected in the following time ranges: 4.2-7.5, 9.2-11.2, and 13-15 min. For the fractions eluted with 40% acetonitrile, the gradient was 10-50% B for 0-20 min and 50-80% for 20-22 min. Fractions of 1.5 ml were collected from the second minute (14 fractions). For the fractions eluted with 100% acetonitrile, the gradient was set to 35-80% B in the range of 0-20 min and 80-100% in the range of 20-22 min. Fractions of 3 ml were collected from the second minute (seven fractions).

Antibacterial test. Day culture of *E. coli* and *B. cereus* was used as the test microorganisms. Paper disks (0.6 cm in diameter, eight for each sample) were steeped in the analyzed fractions and placed on a fresh culture. Before plating, the disks were sterilized using UV radiation. The antibacterial activity was determined by the size of the zone of the limited growth of *E. coli* or *B. cereus* (singular bacterial clumps or their absence) around disks moistened with hemolymph fractions. In a sterile box, bacteria were plated into Petri dishes that were then incubated in a thermostat at 37°C for 24 h. The data were analyzed by statistical methods, calculating the mean value and the standard error. Each value is the mean of four independent experiments.

RESULTS AND DISCUSSION

Purified peptide extracts of the immunized and control larvae were separated by HPLC. All collected fractions were analyzed by mass-spectrometry and assayed for antibacterial properties. No peptides were found in the fractions eluted with 10% acetonitrile.

Results of microbiological tests of the fractions for antibacterial activity against *E. coli* and *B. cereus* are presented in the table.

The hemolymph fractions of the control (15 fractions of 21) and immunized (all fractions) larvae exhibited antibacterial activity. Some of the peptides found in the investigated fractions were known for the given organism [7, 8], and some of them have not been previously described. The control sample contained six of 10 investigated antibacterial peptides of *G. mellonella* with molecular masses of 4820, 4715, 4944, 4258, 6980, and 8400 Da [9-11]. The sample of the immunized larvae, besides the above-mentioned peptides, contained peptides of 4949 and 6528 Da [10, 12]. Some fractions exhibiting antibacterial activity did not contain known antibacterial peptides. Presumably, they contained antibacterial agents that have not been described in the literature. Some antibacterial components in the hemolymph of the larvae must be produced constitutively, while the immunization with bacteria results in the induction of synthesis of new antibacterial peptides. The mass-spectrometric analysis demonstrated that almost all fractions contained a mixture of peptides (table).

Different fractions were compared based on the data of the mass spectrometry and HPLC analyses. Fraction *1* (fractions eluted with 40% acetonitrile) yielded peaks at 3.16 min in the case of the sample obtained from the immunized larvae (Fig. 1a). Molecular weights of the compound that determined absorption of the control sample were not determined by MALDI. Some components were detected in fraction *1* of the immunized group (active against *E. coli*) that were absent in the corresponding fraction of the control group.

Chromatographic profiles of fractions *2* exhibiting antibacterial activity against *E. coli* are similar, but the number of peaks increases and the major peaks are shifted in the group of immunized larvae. The composition of the fractions is also different.

The peaks in the region of fractions *3* that are active against *E. coli* are weakly pronounced, but the sample of immunized larvae yielded an additional peak at 5.83 min (Fig. 1a). Fraction *3* is also active against *B. cereus*. Peptide compositions of the samples of the control and immunized groups are different.

The mass-spectrum of fraction *4* (Fig. 1a) of the control group in the range of 6.5-8 min exhibits four peaks corresponding to peptides of 2744, 3166, 3480, and 3664 Da. The mass spectrum of the active against *E. coli* fraction *4* of the immunized larvae exhibits the same peaks and also a peak corresponding to 2981 Da.

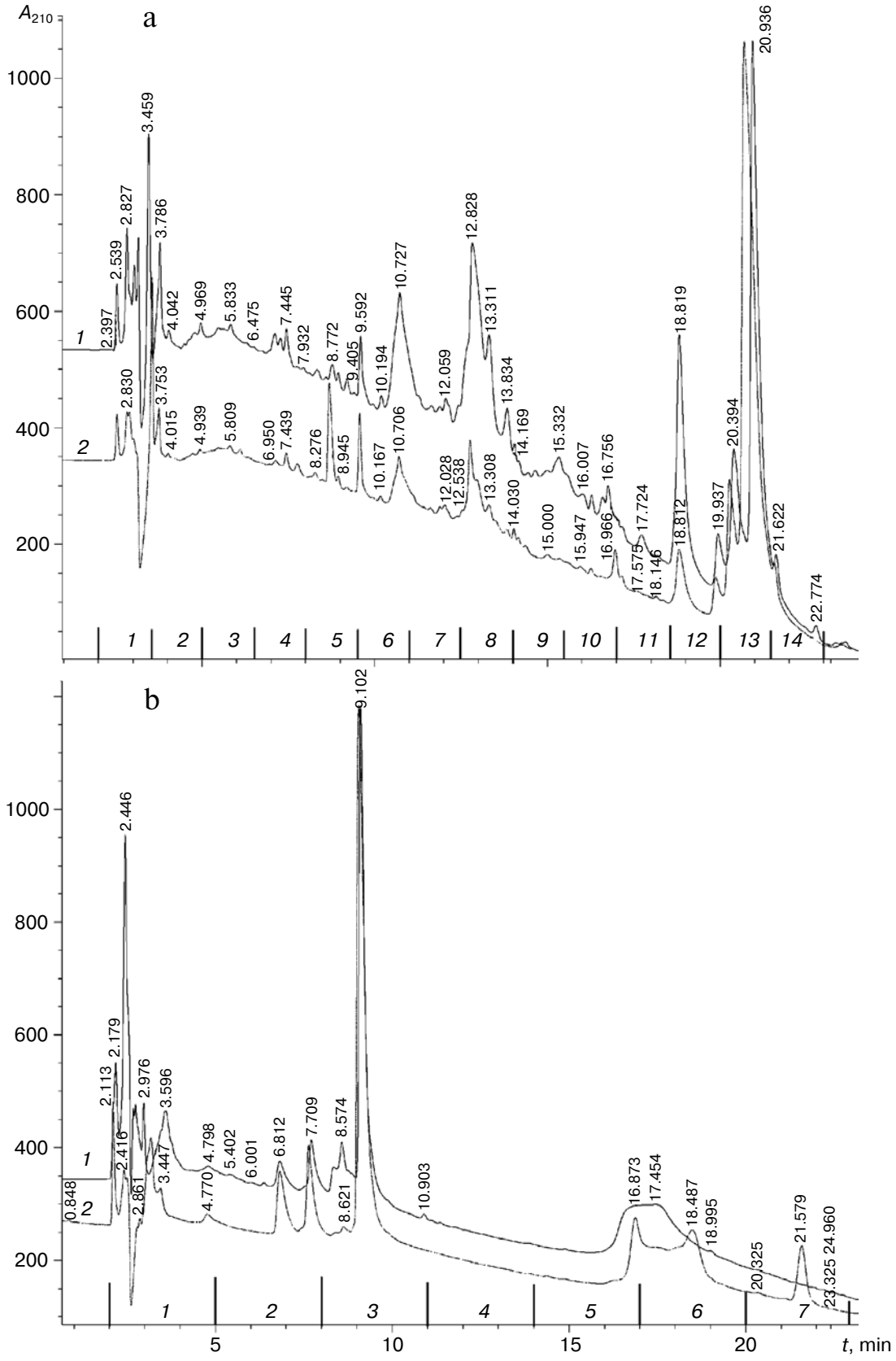

Fig. 1. Elution of peptides with 40% (a) and 100% (b) acetonitrile: *1*) sample from immunized larvae; *2*) control sample.

Antibacterial activity and peptide composition of hemolymph fractions with the same collection time range

Fraction number	Time, min	Zone of limited growth of *E. coli*, mm		Zone of limited growth of *B. cereus*, mm		Observed peptides, Da	
		control group	immunized group	control group	immunized group	control group	immunized group
1	2	3	4	5	6	7	8
				Fractions eluted with 40% acetonitrile			
1	2-3.5	6 ± 0	10 ± 0.9	6 ± 0	6 ± 0	875	875, 1013
2	3.5-5	7.3 ± 0.4	7.5 ± 0.6	6 ± 0	6 ± 0	875, 1062	1096, 1258, 6789
3	5-6.5	7.7 ± 0.8	7.8 ± 0.6	7.5 ± 0.9	6 ± 0	1480, 1979, 2183, **4820**	1064, 1226, 1682, 1845, **4820**
4	6.5-8	6.8 ± 0.4	8.8 ± 0.4	6 ± 0	6 ± 0	988, 1100, 1355, 2744, *3166*, *3481*, 3664	813, 878, 988, 1355, 1451, 2744, 2891, 2985, 3139, 3166, 3481, 3664
5	8-9.5	10.3 ± 2.2	9.8 ± 2.4	6.8 ± 0.4	8.3 ± 0.7	814, 1144, 1231, 1411, **1696**, 1798, 1856, 1983, 2397, 2435, 2933	814, 1058, 1144, 1231, 1495, **1696**, 1798, 1856, 1983, 2048, 2383, 2399, 2435, 2748, 2933, 4801, 6155
6	9.5-11	7 ± 0.5	10.3 ± 1.3	6 ± 0	6 ± 0	**1695**, 2211, 5595, 6318, 6459, 8340, **9161**	875, 1231, **1695**, 2211, 5595, 6318, 6459, 8340, **9161**
7	11-12.5	6 ± 0	8.5 ± 0.2	6 ± 0	6 ± 0	1731, 1822, 2075, 2323, 2566, *6318*, ***9161***, *6790*	875, 1731, 1822, 2075, 2323, 2566, *6318*, ***9161***, *6790*
8	12.5-14	6 ± 0	8.3 ± 0.4	6 ± 0	6 ± 0	1206, 1543, 1961, *2153*, *2211*, 2312, 2557, 2659, 2760, 2888, 3030, 3414, 3577, 3734, 4342, 6118, 7151, **8400**, 9311, 8023	1206, 1553, 1832, 1961, *2153*, *2211*, 2357, 2559, 2659, 2760, 3032, 3414, 3577, 3734, 4342, 4900, 7151, 6118, **8400**, 9311, 8023
9	14-15.5	7 ± 0.5	7.5 ± 0.8	6.3 ± 0.2	6 ± 0	*981*, 1243, **1386**, 1935, 2105, 2274, 2354, 2562, 2720, 2890, 3062, 4448, 4791, **4944**, 7154	981, 1243, **1386**, 1935, 2077, 2106, 2274, 2356, 2564, 2720, 2767, 2899, 3064, 3266, 3463, 4072, 4451, 4793, **4932**, **4949**, 5719, 7159, 9982
10	15.5-17	9.3 ± 0.75	9 ± 0.8	6 ± 0	7.3 ± 0.7	982, 2563, 2620, 2734, 2819, 2919, 3577, 4503, **4714**	982, 1129, 2494, 2563, 2620, 2733, 2819, 2919, 3577, 4585, **4714**, 5716, 6113, 12222
11	17-18.5	7.2 ± 0.7	8.8 ± 0.47	6 ± 0	6 ± 0	1680, 2463, 2564, 2734, 2820, 2929, 3034, 3425, 3614, 3844, 4183, 4463, 5039, 5105, 5710, **6980**, 9082, 12230	2463, 2701, *2818*, *2927*, 3032, 3423, 3612, 3842, 4180, 4461, 5035, 5576 5732, 6116, 6287, **6528**, 6683, 6999, 9075, 12217
12	18.5-20	6 ± 0	7.75 ± 0.75	6 ± 0	6 ± 0	3146, **4255**, ***4819***, **6979**	3146, **4255**, **4819**, **6979**

Table (Contd.)

1	2	3	4	5	6	7	8
13	20-21.5	9 ± 0.57	10.25 ± 0.63	6 ± 0	6 ± 0	2276, 3284, 4819, 4837, 5039, 9048, **18088**, 7036	1620, 2196, 2311 3614, **4255**, **4819**, 4837, 5039, 5959, 6687, **6980**, 7784, 9048, **18088**
14	21.5-23	6 ± 0	8.5 ± 0.86	6 ± 0	6 ± 0	*3614*, ***4255***, *4837*, *5039*, *9048*, ***18088***	3283, 3647, 3973, 4130, *4835*, 5237, **5384**, *9043*, ***18088***
Fractions eluted with 100% acetonitrile							
1	2-5	6.7 ± 0.4	11.3 ± 1.4	6 ± 0	6 ± 0	761, 1226, 2153, 2758, **4820**, 6320, 9174, 9314	761, 1063, 1226, 1768, 1843, 2275, 2491, 2590, 2620, 2848, 2991, 3288, 3658, 3771, 4003, 4131, 4588, 4888, **4820**, 5488, 6325, 9175, 9314
2	5-8	8 ± 0.8	7 ± 0	6 ± 0	6 ± 0	**4258**, 6863, **6980**, 7089, 12220	*761*, *875*, 3145, **4259**, 6290, **6532**, 6688, **6988**, 7785, 9062, 12221
3	8-11	6 ± 0	11 ± 1.91	6 ± 0	6 ± 0	*2276*, *3285*, *4835*, *9048*, ***18088***	875, *3614*, ***4257***, *5038*, *6289*, ***6980***, *7783*, *9048*, **18088**
4	11-14	8 ± 0.4	7.25 ± 1.25	6 ± 0	6 ± 0	761, *5240*, *5388*, *6032*, ***6980***, 9048	761, *2275*, *3283*, ***4820***, ***6980***
5	14-17	8 ± 1.3	8.2 ± 1.0	6 ± 0	8.5 ± 0.6	4375, **4820**, ***5384***, ***6980***	761, 1079, 1226, ***4258***, *4375*, ***4820***, *4932*, *5024*, ***5384***, *5797*, ***6980***
6	17-20	7.8 ± 0.2	9.2 ± 1.1	6 ± 0	6.5 ± 0.2	761, 1079, 1226	761, 1079, 1226
7	20-23	8.3 ± 0.85	11 ± 1.3	6.3 ± 0.2	6.75 ± 0.2	761, 1079, 1226, 1381, 1844, ***4257***	761, 1079, 1226, ***6980***

Note: Known peptides are shown bold [9-16], less intense signals are italicized, and the most intense signals are underlined.

However, the relative intensities of the peaks in this fraction 1.5-3-fold exceed those of the control fraction (Fig. 2a). According to the data of the microbiological test, the content of antibacterial components against *E. coli* significantly increased after the immunization. This chromatographic region exhibits small peaks, but in the case of the immunized larvae the absorbance markedly increased.

Chromatographic profiles of fractions *5* are different for the immunized and control groups and are characterized by an intense peak at 8.68 min (Fig. 1a) in the case of the control group that contains more peptides and exhibits higher antibacterial activity against *E. coli* and *B. cereus*. The fractions also contain common peptides, for example Q9TWE9 GALME, a 1695-Da fragment of prophenoloxidase [7].

Fractions *6* of both groups exhibit antibacterial activity against *E. coli*; however, no significant differences between them were found. By data of the mass-spectrometric analysis, the peptide composition of the two fractions is the same: they contain peptides of 5595, 6459, 8341, 6322, and 9161 Da (Sericin-1 SER1 GALME fragment [8]), and the peaks exhibit virtually the same high relative intensity. The peaks corresponding to the latter two peptides show the maximal intensity, and the peptides exist as dimers and doubly charged ions. There are three peaks in the chromatograms in this region, and two of them are intense.

In fractions *7* and *8*, no significant differences were found in the peptide composition or the relative intensity of the peaks between the control and immunized groups. The elution profiles of the fractions are similar, but in the case of the immunized larvae, two-fold increase in the absorbance is observed. The peak at 13.83 min (Fig. 1a) can be related to a substance of non-peptide nature that is contained in the hemolymph of the immunized larvae. This substance is not detected by mass spectrometry, but it

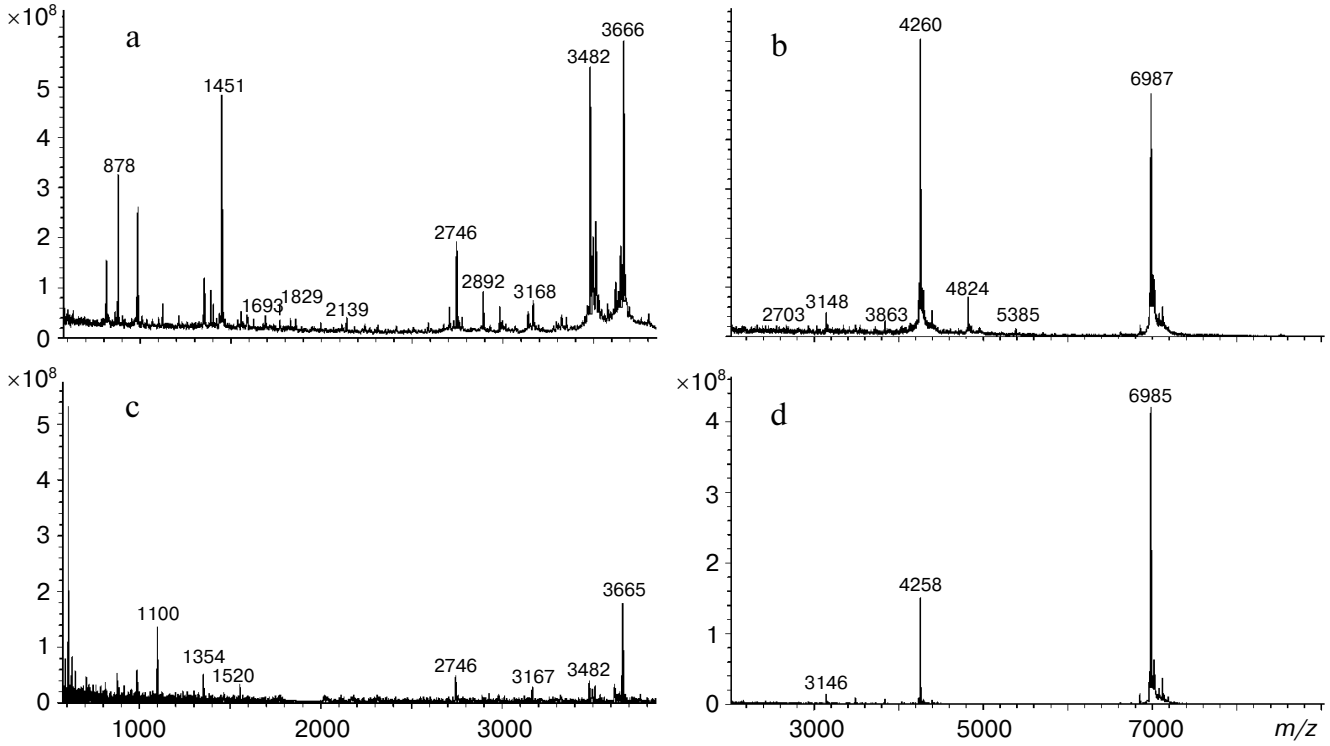

Fig. 2. Mass spectra of fractions *4* (6.5-8 min) (a, c) and *12* (18.5-20 min) (b, d) obtained after HPLC separation of fractions eluted with 40% acetonitrile. a, b) Immunized larvae; c, d) control larvae. Ordinate, relative intensity or share of particles with given *m/z*.

determines the significant increase in antibacterial activity of fractions *7* and *8* against *E. coli*. Fractions *8* of the control and immunized groups also contain the known antifungal peptide gallerimycin Q8MVY9_GALME of 8400 Da [9].

The chromatographic profiles corresponding to fractions *9* and *10* exhibiting activity against *E. coli* are different in the control and immunized groups. The peptide composition is also different. Fraction *9* of the hemolymph of the immunized larvae contains seven peptides (1386, 2077, 2720, 2989, 3266, 4932, and 4949 Da), the peptides of 2077, 3266, and 4949 Da being absent in the corresponding fraction of the control group. The peptide of 4932 Da is the proline-rich antimicrobial peptide 2 PROP2_GALME, the 4949-Da peptide is the defensin-like peptide DEF2_GALME [10], and the 1386-Da peptide is corazonin (20-30 amino acid fragment of CORZ_GALME) [15]. Fractions *10* have seven common peptides and differ in the presence of the 6113-Da peptide in the fraction of the immunized group and 4503-Da peptide in the fraction of the control group. Both fractions contain antibacterial peptide Galleria-defensin DEF1_GALME of 4714 Da [10, 11]. Fraction *10* of the control group and fraction *9* of the immunized group are active against *B. cereus*.

In the chromatographic profile of fraction *11* of the immunized larvae a slight increase in the absorbance is observed compared to that of the corresponding fraction of the control larvae. The mass spectra of the samples of the two groups exhibit identical peaks. The fractions possess antibacterial activity against *E. coli*, but no significant difference between the fractions in terms of the activity is observed. The fraction of the control larvae contains the moricin-like peptide C5 A5JSV1_GALME, whose presence was predicted previously [12].

The chromatographic profiles of fractions *12* contain two peaks. The corresponding mass spectra show the presence of three peptides in each case (Fig. 2, b and d), two of which correspond to antibacterial peptides anionic antimicrobial peptide 2 AP2_GALME (6979 Da) [10] and cecropin-D-like peptide CECD_GALME (4255 Da) [10, 13]. Fraction *12* of the immunized larvae contains one more antibacterial peptide, the lebocin-like anionic peptide 1 LEB1_GALME (4819 Da) [10]. The intensities of the peaks of the hemolymph fraction of the immunized larvae are two-fold increased in the mass spectrum and in the chromatogram. Presumably the presence of three antibacterial peptides determines the antibacterial activity of this fraction against *E. coli*.

The chromatographic profiles of fractions *13* that are active against *E. coli* are similar for the control and immunized groups. The peak with a high absorption is presumably due to the presence of the high-molecular-weight component, silk fibroin seroin SERO_GALME

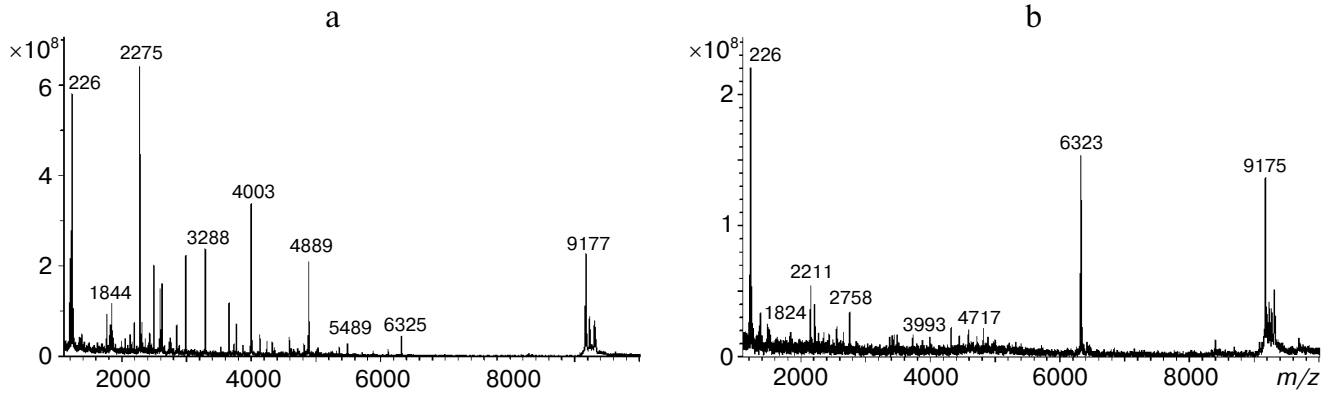

Fig. 3. Mass spectra of fraction *1* (2-5 min) obtained after HPLC separation of fractions eluted with 100% acetonitrile: a) sample of immunized group; b) control sample. Ordinate, relative intensity or share of particles with given *m/z*.

[14] of 18,088 Da (for doubly charged cation, 9045 Da). However, the peptide composition of the fractions is different, and the fraction of immunized larvae contains some new peptides. Among the known peptides, the fraction of the control group contains lebocin-like anionic peptide 1 LEB1_GALME (4819 Da), and the fraction of the immunized larvae contains anionic antimicrobial peptide 2 AP2_GALME (6979 Da), cecropin-D-like peptide CECD_GALME (4255 Da), and lebocin-like anionic peptide 1 LEB1_GALME (4819 Da).

The active fraction *14* of the immunized group contains six peptides that are also present in fraction *13* and a peptide of 5384 Da that is a fragment of the serine proteinase inhibitor ISP1_GALME [16]. The region of the active fraction corresponds to a single peak. Fraction *14* of the control group does not contain the 5384-Da peptide and exhibits no activity against *E. coli*.

The chromatography of the fractions eluted with 100% acetonitrile yielded different profiles in the region of fraction *1* (Fig. 1b) of the control and immunized groups. The immunization resulted in the emergence of a peak at 2.45 min. A number of peptide peaks sharply increased in the corresponding mass-spectrum (Fig. 3). The antibacterial activity also significantly increased.

An aliquot (100 μl) of fraction *1* was subjected to chromatography on a HyperCarb column. In the collected fractions, peptides of 4131 and 4820 Da were found (eluted with a 35-80% acetonitrile gradient at 8.2 min).

The chromatograms in the region of fraction *2* are characterized by two major peaks (Fig. 1b) whose intensity is higher in the case of the control larvae, like the antibacterial activity of this fraction against *E. coli*. The fraction of immunized larvae contains many new peptides that are absent in the corresponding fraction of the control group. These peptides presumably determine the diffuseness of the peaks.

Chromatographic profiles of fractions *3* are characterized by the presence of pronounced peaks at 9.10 min (Fig. 1b) in both cases. However, it is unclear what substance corresponds to these peaks since the intensities of the common peptides in these fractions are small. Fraction *3* of the immunized larvae also contains a component of 875 Da probably corresponding to the chromatographic peak at 8.57 min. Presumably the significantly increased activity against *E. coli* in this fraction is due to the compounds eluted at this retention time.

The chromatographic profiles of fraction *4* exhibit no pronounced peaks, and the mass spectra are characterized by weak peaks including those corresponding to known antibacterial peptides that presumably correspond to the activity against *E. coli*.

Chromatographic profiles of fractions *5* and *6* exhibit a number of diffuse peaks differing in shape. The mass spectra of fractions *5* contain weakly intensive peptide peaks, some of them corresponding to known antibacterial peptides. Besides, both fractions *5* contain a peptide of 5384 Da corresponding to a fragment of serine proteinase inhibitor ISP1_GALME [16]. Presumably it also makes a contribution to the antibacterial activity of these fractions. The mass spectra of fractions *5* and *6* of the immunized larvae and fraction *6* of the control group are characterized by pronounced peaks corresponding to peptides of 761, 1079, and 1226 Da. Presumably the activity of fractions *6* against *E. coli* is related to these components.

The chromatographic profile of fraction *7* of the control larvae contains a peak at 21.58 min (Fig. 1b) that is absent in the chromatogram of the immunized sample. The mass spectrum of this fraction exhibits peptide peaks corresponding to 1381 and 1844 Da that are absent in the fraction of the immunized larvae. Both fractions also contain small peaks of two known antibacterial peptides. The fractions are active against *E. coli* and *B. cereus*.

The fraction eluted with 100% acetonitrile was also separated on a HyperCarb column using a linear acetonitrile gradient. Fractions with maximal absorption were collected in the same time ranges. The known antibacter-

ial peptides of 4820 and 6980 Da (2-3.8 min) were found only in the sample of immunized larvae. The sample also contained a fragment of prophenoloxidase of 1692 Da (7.8-9.4 min).

The experiments revealed an increase in number of peptides after the immunization with bacteria, as well as the presence of some conserved peptide components. The comparative mass-spectrometric analysis of the corresponding fractions demonstrated that the larvae possess not only inducible, but also constitutive defense peptides. The spectra of the samples of the control and immunized groups exhibit similar peaks corresponding to the antibacterial peptides. However, in the case of the immunized larvae these peaks are more intensive. The peptide composition of the active fractions includes both known peptides and some new peptides that were not described previously.

The approach used here revealed changes in the peptide composition of hemolymph and the emergence of some new peptides, allowed study of the composition of fractions exhibiting antibacterial activity, and showed the presence of known antibacterial peptides in hemolymph of intact larvae and those immunized with bacteria.

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