

Insect antimicrobial peptides: isolation, separation and antimicrobial activity

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Abstract. In modern conditions of intensification of agriculture the industrial animal breeding involves the use of antibacterial drugs aimed at the prevention of infectious diseases. However, uncontrolled use of antibiotics leads to the selection of antibiotic-resistant strains, it should be noted that the treatment of diseases caused by these strains is difficult. Insect peptides have a barrier function and are able to protect the macroorganism from various pathogenic agents. In this study the original method of isolating protein fractions from the biomass of insect *G. mellonella* and *M. domestica* larvae was developed. The 19 protein fractions (PF) were obtained and their antimicrobial activity was studied as related to *S. aureus* ATCC 6538 (209-P), *S. typhimurium* 1626, *C. albicans* PKIIIY-401/NCTC-885-653, *B. cereus* ATCC 10702. It was found that protein fractions No. 2, 2.3, 2.6, 3.4, 3.6 had antimicrobial activity as related to *S. aureus* ATCC 6538 (209-P); protein fractions No. 2, 2.6, 3.2, 3.6 inhibited the growth of *S. typhimurium* 1626; protein fractions No. 2, 2.3, 2.6, 3.3, 3.6 had an inhibiting effect on *C. albicans* PKIIIY-401/NCTC-885-653, PF 2, 2.6, 3.2, 3.6, 3.7 had antimicrobial activity as related to *B. cereus* ATCC 10702.

1 Introduction

Nowadays there are research investigations related to the discovery of antimicrobial peptides (AMPs), which are effective in the treatment of infections caused by microorganisms also related to substances produced by insects. The reason for this is the evolutionary success that allows insects to occupy different habitats. Due to the lack of acquired immunity in invertebrates upon contact with pathogens, the protective effect can only be due to innate immunity. In addition, there are more than 30 million species of insects, that is, a huge source of resources for finding new substances for further use in medicine and veterinary [1-4].

Antimicrobial peptides *in vivo* have a detrimental effect on microorganisms not only due to direct action, but also due to the indirect killing through immunomodulatory effects. The increased production of AMP in the focus of inflammation causes the concentrations in such foci, which leads to the death of the microbial cell. In addition, *in vivo* AMPs of different classes and various antimicrobial substances have a synergistic antimicrobial effect [5]. According to some studies, the development of resistance in microorganisms to antimicrobial peptides *in vitro* is unlikely [6].

Furthermore, antimicrobial peptides do not adversely affect eukaryotic cells and can be used to treat animals as well as in animal feeding. The search for new alternative drugs to antibiotics in animal husbandry is primarily associated with the growing problem of antibiotic resistance [7].

Currently, the use of "classic" antibiotics to fight infectious diseases is due to their specific effect, the speed of exposure to the body of animals and the elimination of complications in the treatment of viral infections. However, such therapy does not always pass without consequences for the macroorganism and can lead to the selection of multi-resistant strains of pathogenic bacteria, a decrease in immunity and disruption of some organs [8]. Unlike classical antibiotics, antimicrobial peptides have a low probability of developing antibiotic resistance; however, they have antimicrobial activity against a wide range of bacteria, fungi and viruses, causing their rapid killing and have anti-inflammatory properties.

High antibacterial activity against different groups of microorganisms, including antibiotic-resistant strains, low probability of selection of strains resistant to AMP, makes them a promising drug agents for the design of new effective antimicrobial drugs in veterinary medicine [9].

2 Materials and methods

The method of isolation of water-soluble peptides from the biomass of *G. mellonella* and *M. domestica* larvae, included of homogenization of the larval biomass, extraction, repeated centrifugation, salting with ammonium sulfate and repeated dissolution was developed. The pellet was collected in a separate tube after each centrifugation and the supercharger was poured out. All the samples obtained after centrifugation were frozen for subsequent manipulations [10].

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The resulting fractions were analyzed and separated by high-performance liquid chromatography (HPLC). Separation and determination were performed by HPLC with a preliminary search for the optimal set of solvents and conditions for chromatography.

A small amount of phosphate buffer salt solution (FSB) was added to the samples to dissolve the precipitation before chromatographic separation and then the solution was injected into the chromatograph.

The following model was selected for high-performance liquid chromatography: BioSep S2000 300x2120 mm. The separation was conducted with 0.1 M FSB solution as the eluent, the flow is 1 ml/min, with a loop volume of 1575 µl, at a wavelength of 280 nm. The analysis time for a single sample takes 60 min. The protein content in the test samples was determined by the Lowry method [11].

The bovine albumin is used to construct the calibration curve. A new calibration graph was constructed for each newly prepared Folin reagent. The protein content of the analyzed fractions was determined by the Lowry colorometric method on the spectrophotometric equipment of the company "ShimadzuUV-1280" at a wavelength of 450 nm. The molecular weight of the isolated peptides was determined by polyacrylamide gel electrophoresis (PAAG) in the presence of sodium dodecyl sulfate (SDS-Na) using a Helicon camera and a BIO-RAD power supply [12].

The antibacterial activity was determined according to the Methodological guidelines 4.2.1890-04 Determination of the sensitivity of microorganisms to antibacterial drugs. Methodological guidelines (MG 4.2.1890-04, 2004) [13].

The study of antibacterial activity was carried out by the macrotube method. The following cultures of microorganisms were used in the studies: *S. aureus* ATCC 6538 (209-P); *S. typhimurium* 1626, which were obtained from the Public Collection of Pathogenic Bacteria FGHI Russian Research Antiplague Institute «Microb», the Russian Agency for Health and Consumer Rights; *B. cereus* ATCC 10702, obtained from the Federal State Unitary Enterprise State-run Think Tank Genetics; *C. albicans* PKPIFY -401/NCTC-885-653, *E. coli* 1027 obtained from the Laboratory «Russian Collection of Pathogenic Fungi» SFEI HPE North-Western State Medical University named after I. I. Mechnikov of the Ministry of Health of the Russian Federation.

3 Results and discussion

Thus, we obtained 19 protein fractions from the biomass of *G. mellonella* and *M. domestica* larvae by HPLC.

The column exit of protein fraction No. 2.1 was 5.24 % with a retention time of 2.15 minutes; No. 2.2 – 7.38 % with a retention time of 14.27 minutes; No. 2.3 – 16.12 % with a retention time of 22.35 minutes; No. 2.4 – 68 % with a retention time of 25.52 minutes; No.2.5 – 0.65 % with a retention time of 34.87 minutes; No. 2.6 – 2.30 % with a retention time of 43.46 minutes; No. 2.7 –

0.31 % with a retention time of 56.65 minutes (Figure 1, Table 1).

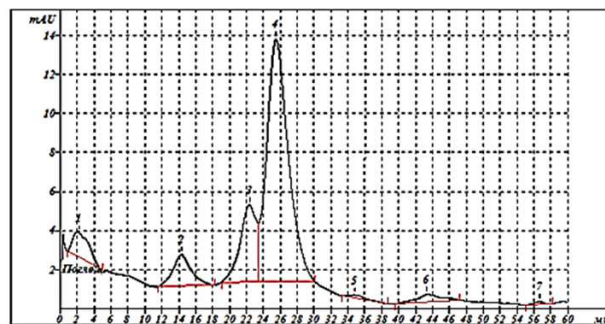


Fig. 1. Chromatogram protein fraction 2, isolated from biomass of *G. mellonella* larva

Table 1. Column exit of protein fraction, isolated from biomass of *G. mellonella* larva (Figure 1), %

No.	tR – retention time, min	Area response, mAU · sec	Column exit, %
2.1	2.15	170.32	5.24
2.2	14.27	240.26	7.38
2.3	22.35	524.57	16.12
2.4	25.52	2212.25	68.00
2.5	34.87	21.03	0.65
2.6	43.46	74.91	2.30
2.7	56.65	10.11	0.31
Sum		3253.44	100.00

Table 2. Protein concentration of the obtained protein fractions (Figure 1), mg/ml

No.	tR – retention time, min	Sample volume, ml	Protein concentration, mg/ml
2.1	2.15	3	6.98±0.19
2.2	14.27	4	7.39±0.21
2.3	22.35	4	16.12±0.11
2.4	25.52	6	45.33±0.14
2.5	34.87	1	2.59±0.09
2.6	43.46	2	4.61±0.08
2.7	56.65	1	1.24±0.05

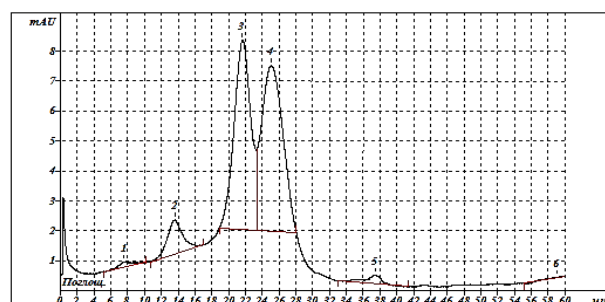


Fig. 2. Chromatogram protein fraction 3, isolated from biomass of *G. mellonella* larva

The column exit of protein fraction 3.1 was 0.88 % with a retention time of 7.63 minutes; No. 3.2-6.81 %, with a retention time of 13.58 minutes; No. 3.3-42.95 % with a retention time of 21.67 minutes; No. 3.4 – 47.43% with a retention time of 25.06 minutes; No. 3.5 – 1.91% with a retention time of 37.43 minutes; No. 3.6 – 0.01% with a retention time of 59 minutes (Figure 2, Table 3).

Table 3. Column exit of protein fraction, isolated from biomass of *G. mellonella* larva (Figure 2), %

No.	tR – retention time, min	Area response, mAU • sec	Column exit, %
3.1	7.63	18.10	0.88
3.2	13.58	139.46	6.81
3.3	21.67	879.51	42.95
3.4	25.06	971.21	47.43
3.5	37.43	39.11	1.91
3.6	59.00	0.28	0.01
Sum		2047.67	100.00

Table 4. Protein concentration of the obtained protein fractions (Figure 2), mg/ml

No.	tR – retention time, min	Sample volume, ml	Protein concentration, mg/ml
3.1	7.63	2	2.65±0.09
3.2	13.58	4	10.22±0.11
3.3	21.67	5	51.54±0.12
3.4	25.06	3	94.86±0.21
3.5	37.43	2	5.73±0.08
3.6	59	2	0.04±0.03

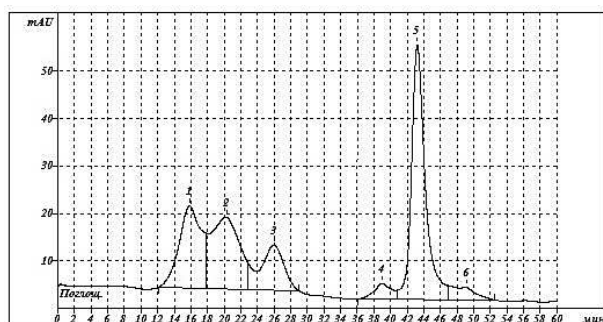


Fig. 3. Chromatogram protein fractions 1-6, isolated from biomass of *M. domestica* larva

The column exit of protein fraction No. 1 was 20.33 % with a retention time of 15.85 minutes; No. 2 – 22.43 % with a retention time of 20.20 minutes; No. 3 – 11.87% with a retention time of 25.98 minutes; No. 4 – 2.99% with a retention time of 38.96 minutes; No. 5 – 38.84% with a retention time of 43.23 minutes; No. 6 – 3.54% with a retention time of 49.02 minutes (Figure 3, Table 5).

Table 5. Column exit of protein fraction, isolated from biomass of *M. domestica* larva (Figure 3), %

No.	tR – retention time, min	Area response, mAU • sec	Column exit, %
1	15.85	3239.77	20.33
2	20.20	3574.26	22.43
3	25.98	1892.03	11.87
4	38.96	476.09	2.99
5	43.23	6188.91	38.84
6	49.02	563.49	3.54
Sum		15934.55	100

Table 6. Protein concentration of the obtained protein fractions (Figure 3), mg/ml

No.	tR – retention time, min	Sample volume, ml	Protein concentration, mg/ml
1	15.85	6	20.33±0.45
2	20.20	4	33.65±0.65
3	25.98	4	17.81±0.78
4	38.96	5	3.59±0.91
5	43.23	6	38.84±0.31
6	49.02	5	4.24±0.15

Then protein fractions were dialyzed after separation for 24 hours against 0.9% sodium chloride solution. When studying the molecular weight of the isolated peptides, it was found that it was in the range of 3.4-6 kDa.

The antimicrobial activity of protein fractions No. 2.1-2.7; 3.1-3.7 isolated from the biomass of *G. mellonella* larvae and PF No. 1-6 isolated from the biomass of *M. domestica* as related to *S. typhimurium* 1626, *S. aureus* ATCC 6538 (209-P), *C. albicans* PKHIFY -401/NCTC-885-653, *B. cereus* ATCC 10702 were determined by the macrotube method. The experiment was performed in three replications for each culture of microorganisms. The test tubes were placed in a thermostat at 37 °C for 24 hours. After the time had elapsed, the results were recorded.

Protein fractions 2.3 and 2.6 inhibit the growth of the *S. aureus* ATCC 6538 (209-P), and PF 2.6 inhibit the growth of *S. typhimurium* 1626 was found. Protein fractions 2.3 and 2.6 have activity as related to *C. albicans* PKHIFY -401/NCTC-885-653, 2.6 – *B. cereus* ATCC 10702. Antimicrobial activity of PF 2.1; 2.2; 2.4, 2.5 and 2.7 was not detected (Table 7).

Then, the antimicrobial activity of PF 3.1-3.7 was studied by the same method. It was found that PF 3.4 and 3.6 have antimicrobial activity as related to *S. aureus* ATCC 6538 (209-P). Protein fractions 3.2 and 3.6 inhibit the growth of the *S. typhimurium* 1626, PF 3.3 and 3.6 – *C. albicans* PKHIFY-401/NCTC-885-653. Protein fractions 3.2, 3.6, and 3.7 exhibit antimicrobial

activity as related to the *B. cereus* ATCC 10702 (Table 8).

Table 7. Antimicrobial activity of protein fraction No. 2.1 - 2.7 (*G. mellonella*)

Protein fractions	Strains of microorganisms			
	<i>S. aureus</i> ATCC 6538 (209-P)	<i>S. typhimurium</i> 1626	<i>C. albicans</i> PKIIFY-401/NCTC-885-653	<i>B. cereus</i> ATCC 10702
2.1	-	-	-	-
2.2	-	-	-	-
2.3	+	-	+	-
2.4	-	-	-	-
2.5	-	-	-	-
2.6	+	+	+	+
2.7	-	-	-	-

Notes – ‘+’ – transparent broth (no microbial growth);
 ‘-’ – cloudy broth or there is a precipitate (microbial growth).

Table 8. Antimicrobial activity of protein fractions No. 3.1 - 3.7 (*G. mellonella*)

Protein fractions	Strains of microorganisms			
	<i>S. aureus</i> ATCC 6538 (209-P)	<i>S. typhimurium</i> 1626	<i>C. albicans</i> PKIIFY-401/NCTC-885-653	<i>B. cereus</i> ATCC 10702
3.1	-	-	-	-
3.2	-	+	-	+
3.3	-	-	+	-
3.4	+	-	-	-
3.5	-	-	-	-
3.6	+	+	+	+
3.7	-	-	-	+

Notes – ‘+’ – transparent broth (no microbial growth);
 ‘-’ – cloudy broth or there is a precipitate (microbial growth).

Table 9. Antimicrobial activity of protein fraction 1-6, isolated from biomass of *M. domestica* larva

Protein fractions	Strains of microorganisms			
	<i>S. aureus</i> ATCC 6538 (209-P)	<i>S. typhimurium</i> 1626	<i>C. albicans</i> PKIIFY-401/NCTC-885-653	<i>B. cereus</i> ATCC 10702
1	-	-	-	-
2	+	+	+	+
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-

Notes – ‘+’ – transparent broth (no microbial growth); ‘-’ – cloudy broth or there is a precipitate (microbial growth).

The antimicrobial activity of protein fractions 1-6 isolated from the biomass of *M. domestica* larvae was determined by a macrotube method (Table 9). It was found that PF 2 had antimicrobial activity as related to *S. aureus* ATCC 6538 (209-P), *S. typhimurium* 1626, *C. albicans* PKIIFY-401/NCTC-885-653, *B. cereus* ATCC 10702.

4 Conclusions

Our studies allowed us to develop an original method for isolating protein fractions from the biomass of insect larvae including homogenization; extraction; centrifugation; salting out with ammonium sulfate; dissolution; salting out with ammonium sulfate, and to work out an experimental technique for the separation of protein fractions in the course of high performance liquid chromatography. The chromatographic conditions were selected: BioSep S2000 300x2120 mm. The separation was conducted with 0.1 M FSB solution as the eluent; the flow is 1 ml/min, with a loop volume of 1575 µl, at a wavelength of 280 nm. The analysis time for a single sample takes 60 min.

As a result of studying the antimicrobial activity by the macrotube method, it was found that protein fractions No. 2.3 and 2.6 isolated from the biomass of *G. mellonella* larvae inhibit the growth of *S. aureus* strain ATCC 6538 (209-P), PF No. 2.6 - *S. typhimurium* 1626. Protein fractions No. 2.3 and 2.6 inhibit the growth of the *C. albicans* PKIIFY -401 / NCTC-885-653, No. 2.6 - *B. cereus* ATCC 10702. Antimicrobial activity of PF No. 2.1; 2.2; 2.4; 2.5 and 2.7 were not identified.

When studying the antimicrobial activity of protein fractions 3.1-3.7 isolated from the biomass of *G. mellonella* larvae, it was revealed that PF 3.4 and 3.6 have antimicrobial activity against the *S. aureus* ATCC 6538 (209-P). Protein fractions 3.2 and 3.6 inhibit the growth of *S. typhimurium* 1626, PF 3.3 and 3.6 - *C. albicans* PKIIFY-401 / NCTC-885-653. Protein fractions 3.2, 3.6, and 3.7 exhibit antimicrobial activity in relation to the *B. cereus* strain ATCC 10702. The study of the antimicrobial activity of the protein fraction 2 isolated from the biomass of *M. domestica* larvae showed an antimicrobial activity in relation to *S. aureus* ATCC 6538 (209-P), *S. typhimurium* 1626, *C. albicans* PKIIFY -401 / NCTC-885-653, *B. cereus* ATCC 10702.

Research work on the isolation of the most promising AMPs can become a prerequisite for the development of highly effective antimicrobial drugs. In addition, it will help to solve the problem of disruption of microbiocenoses of living organisms, will contribute to the treatment of diseases caused by antibiotic-resistant strains and can be used to prevent diseases of various etiologies [14].

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