Bulgarian Chemical Communications Volume 50, Special Issue C (pp.169 - 175) 2018

Glycine-rich peptides from *Cornu aspersum* snail with antibacterial activity

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Received October 15, 2017 ; Accepted November 22, 2017

Antimicrobial peptides are a unique and diverse group of molecules that have a great potential for use in new antimicrobial drugs, as many of them have a pronounced cytotoxicity to number of multi-drug resistant bacteria. We have been investigating different mucus extracts from the garden snail *Cornu aspersum* against the pathogen Gram-negative bacterial strain - *Escherichia coli* NBIMCC 878 and it has been found that the fraction below 10 kDa demonstrated strong antibacterial activity. Using tandem mass spectrometry we identified the primary structures of 9 novel antimicrobial peptides with molecular masses between 1000-3000 Da in this fraction. Most of them contain high level of glycine and leucine residues into the amino acid sequences.

Keywords: antimicrobial peptides, *Cornu aspersum*, HPLC, *Escherichia coli*, antibacterial activity, tandem mass spectrometry.

INTRODUCTION

In April 2014, the World Health Organization announced the beginning of a post-antibiotic era and declared antimicrobial resistance a public health priority demanding global action [1, 2]. New therapies to tackle multidrug resistant bacterial pathogens are urgently needed. This set the need of discovering novel molecules that could battle pathogen drug resistance. Antimicrobial peptides have proven to be a good natural alternative to chemical antibiotics [2, 3]. Antimicrobial peptides (AMPs) are important components of the nonspecific host defense or innate immune system in a variety of organisms. Besides direct antimicrobial activity, AMPs carry immunomodulatory properties [4, 5], which make them especially interesting compounds for the development of novel therapeutics. They have been found virtually in all organisms and they display a remarkable structural and functional diversity. So far more than 750 different antimicrobial peptides have been isolated and characterized from different sources – insects, plants and animals as well as humans [6 - 9]. Even bacteria themselves produce antimicrobial peptides – 50 AMPs have been isolated from different Gram (+) bacteria, especially those that produce lactic acid [10]. Most AMPs commonly consisting of 10–50 amino acids are amphipathic and hydrophobic α-helical peptides, with a positively charged domain. These properties are thought to be essential for their biological activity, with the amphipathic character causing disruption of the negatively charged bacterial membrane [11]. This property helps them enter the lipid bilayer and bring about the lysis of the attacked membranes [12]. Most AMPs have the ability to kill microbial pathogens directly, whereas others act indirectly by modulating the host defense systems. In general, peptide therapeutics are considered to have advantages from the safety perspective compared to small molecule drugs since their degradation products are natural amino acids and, because of their short half-life, few peptides accumulate in tissues. This reduces the safety risk and risk of complications caused by metabolites [2].

It has been proven that AMPs can be used to battle Gram (+), Gram (-) bacteria as well as viruses, fungi and even cancer cells [13]. Several peptides from the hemolymph of molluscs and arthropods exhibit a broad-spectrum of antimicrobial activity [14, 15]. Recently, a series of active peptides and glycopeptides with different physiological functions were extracted from marine molluscs [14]. Several novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *Rapana venosa snails* also were identified [16]. Some of them showed strong antimicrobial activities against *Staphylococcus aureus* (Gram+) and low activity against *Klebsiella pneumonia* (Gram-) [16]. Furthermore, it has been reported for several peptides from the hemolymph of the garden snail *H. lucorum* and *H. aspersa* that exhibit a broad spectrum of antimicrobial activity against *S.*

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aureus, S. epidermidis and *P. acnes* [17, 18]. The cysteine-rich peptide mytimacin-AF from the *Achatina fulica* snail, composed of 80 amino acid residues, and the mytilins A and B from the hemocytes and plasma of the bivalve mollusc *Mytilus galloprovincialis*, composed of 40 residues, exert the strongest antimicrobial activity against *S. aureus* [19].

We here report on the antibacterial properties of mucus extract below 10kDa containing AMPs, isolated from the mucus of *Cornu aspersum* snail against the Gram-negative bacteria *Escherichia coli*. We focused on *E.coli* because it has the ability to cause serious diseases and bacteremia is increasing worldwide. This is why the multidrug resistance in *E.coli* is becoming a serious concern in global healthcare. Using tandem mass spectrometry we have determined amino acid sequences of 9 new peptides in active fraction.

EXPERIMENTAL

Materials and Methods

Mucus collection and separation of different fractions. The mucus was collected and purified from *Cornu aspersum* snails, grown in Bulgarian farms using patented technology without suffering any snail [20]. The mucus extract was separated to different fractions using Milipore filters (10, 30 and 50 kDa). Three mainly fractions were obtained: Fraction 1 (masses between 0-10 kDa), Fraction 2 (masses between 30 and 50 kDa) and Fraction 3 (masses above 50 kDa). Fraction 1 (below 10 kDa) was then further separated using Milipore filters (3 and 5 kDa) into three fractions: Fraction A (masses below 3 kDa), Fraction B (masses between 3 and 5 kDa) and Fraction C (masses between 5 and 10 kDa).

HPLC isolation and purification of peptides from active fraction. Fraction 1 (below 10 kDa,) was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.1% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and solution B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min, over 75 min. Ultraviolet absorption was monitored at 216 nm. The eluted fractions were collected and dried by vacuum concentration Speed-110 vac. The fractions were reconstituted in Milli Q water containing 0.10% TFA (v/v).

Mass spectrometry analysis. The molecular masses of isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Some 50 pmol of the HPLC fractions were dissolved in 0.1% (v/v) TFA and applied to the target. Analysis was carried out using α-cyano-4-hydroxycinnamic acid as a matrix. A total of 3500 shots were acquired in the MS mode and a collision energy of 4200 was applied. A solution of protein standards was used to calibrate the mass scale. The mass values assigned to the amino acid residues are average masses. *De novo* sequencing of the peptides was performed by MS/MS in a 4700 proteomics analyzer with TOF-TOF optics (Applied Biosystems). The MS/MS spectra were carried out in reflector mode with external calibration, using the 4700 calibration mixture kit (Applied Biosystems). Peptide *de novo* sequencing was performed by precursor ion fragmentation.

Antibacterial assays of the peptides. The Gram-negative bacterial strain *Escherichia coli* NBIMCC 8785 was used in the antibacterial assays. *Escherichia coli* (Migula 1985) Castellani and Chalmers 1919 was representative of bacteria from family *Enterobacteriaceae* and was obtained from the National Bank of Industrial microorganisms and cell cultures.

The bacterial strain *E. coli* NBIMCC 8785 was initially grown in Nutrient Agar I to restore the lyophilized strain. After that the microbial culture was inoculated in nutrient broth and cultivated for 24 hours to obtain microbial suspension. The suspension was used to receive standardized suspension.

Five cm^3 standardized suspensions from the bacterial cultures (OD_{430}=0.600 abs) was inoculated in Nutrient agar, mixed, and poured in petri dishes layers with depth 2 mm. The petri dishes remained at room temperature (20°C) to solidify. The antibacterial tests were also performed in wells with no peptides added to serve as a negative control.

Fractions (A, B and C) were tested via the agar well diffusion method. Three wells (3 repetitions) were drilled using a punch, and then each hollow was filled with 50 µl of each fraction. Incubation for 24-72 hours at 37°C was then performed. The antibacterial effect was indicated in mm sterile zone around the wells.

The antibacterial tests were also performed on a medium with no peptides added to serve as a negative control. The fractions were also tested by a diffusion method of dripping a certain volume (10 µl) of each fraction on a certain spot on an inoculated Nutrient agar medium. Incubation for 24-72 hours at 37°C was then performed Results and discussion

RESULTS AND DISCUSSION

Antimicrobial activity

The rapidly increasing resistance towards conventional antibiotics suggests that, without urgent action, we are heading for a "post-antibiotic era," in which the previously effective therapeutic strategies are no longer relevant [2].

Mucus extract collected from the garden snails *Cornu aspersum* is a complex mixture of biochemically and pharmacologically active compounds. The mucus extract of *Cornu aspersum* was subdivided into four fractions, obtained after separation over Milipone filters with a cut-off of 10, 5 and 3 kDa, as described in section " Materials and methods".

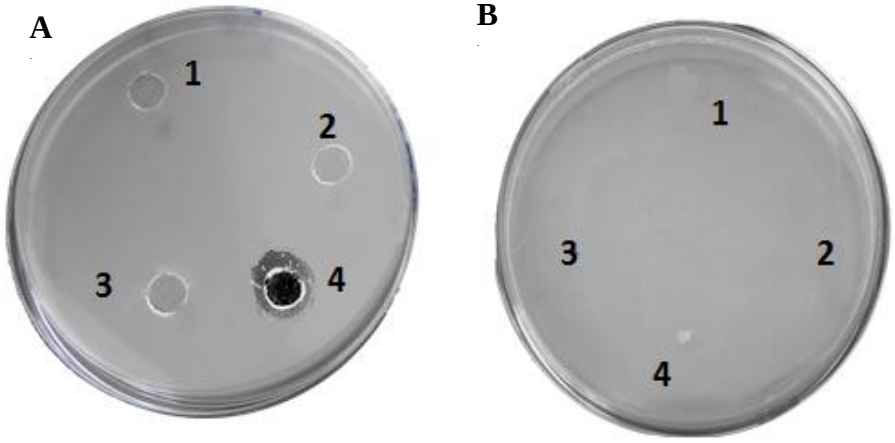

Fig. 1. A) Antimicrobial assays against Gram-negative *E.coli* of isolated fractions: A (spot 1), B (spots 2) and C (spots 3). Antimicrobial assays via agar well diffusion method against Gram-negative *E.coli* of isolated Fraction 1 (spot 4). Each fraction was applied on the agar medium in 50 µl of the peptide solution. **B)** No activity is shown in any of the fractions after dilutions (1/2, 1/4, 1/8 and 1/16) from the starting concentration.

The antimicrobial activity of fractions containing peptides, isolated from the mucus of garden snail *Cornu aspersum* were tested *in vivo*, against gram-negative bacterial strain *Escherichia coli* NBIMCC 8785. Upon testing their antimicrobial activity on agar medium after incubation for 24-72 hours at 37°C, in the agar well diffusion method only Fraction 1 appeared to generate a zone of 1,4 cm zone of inhibition of *E.coli* (Fig.1A spot 4).

The Gram-negative bacterial strain *Escherichia coli* NBIMCC 8785 was used in the antibacterial assays. It was chosen because it is a human pathogenic species and is commonly used in antimicrobial tests. To determine the minimal antimicrobial concentration of the active peptides, an aliquot of the *E.coli* strain was placed on Nutrient agar plates, mixed and allowed to solidify. Then the active fraction 1 (under 10 kDa) was tested by drilling a well in the agar using a punch and then each hollow was filled with 50µl of decreasing dilutions (1/2, 1/4, 1/8 and 1/8 from the starting concentration. Incubation for 24-48 hours at 37°C was then performed. It was found that the diluted peptide fraction 1 didn't show any activity meaning the initial concentration suppressed the growth of *E.coli* (Fig.1B).

These our results are preliminary testing the general effect of the isolated peptides. Further our efforts will be in direction to elucidate the mechanisms of the antibacterial effect in order to discover the best way for application of the peptides in pharmacy or cosmetics.

Characterization of peptides, containing in the fraction with antibacterial activity

Antibacterial active Fraction 1 (below10 kDa) was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.10% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min, over 75 min. Ultraviolet absorption was monitored at 216 nm. The eluted fractions were collected and dried by vacuum concentration. The fractions were reconstituted in Milli Q water. Additional purification of the isolated peptides was performed using the same equipment and conditions (Fig. 2). Mainly 23 sub-fractions were eluted and only a number of them were selected for further mass spectrometry analyses to determine their amino acid sequences in order to explain the antibacterial assessment results.

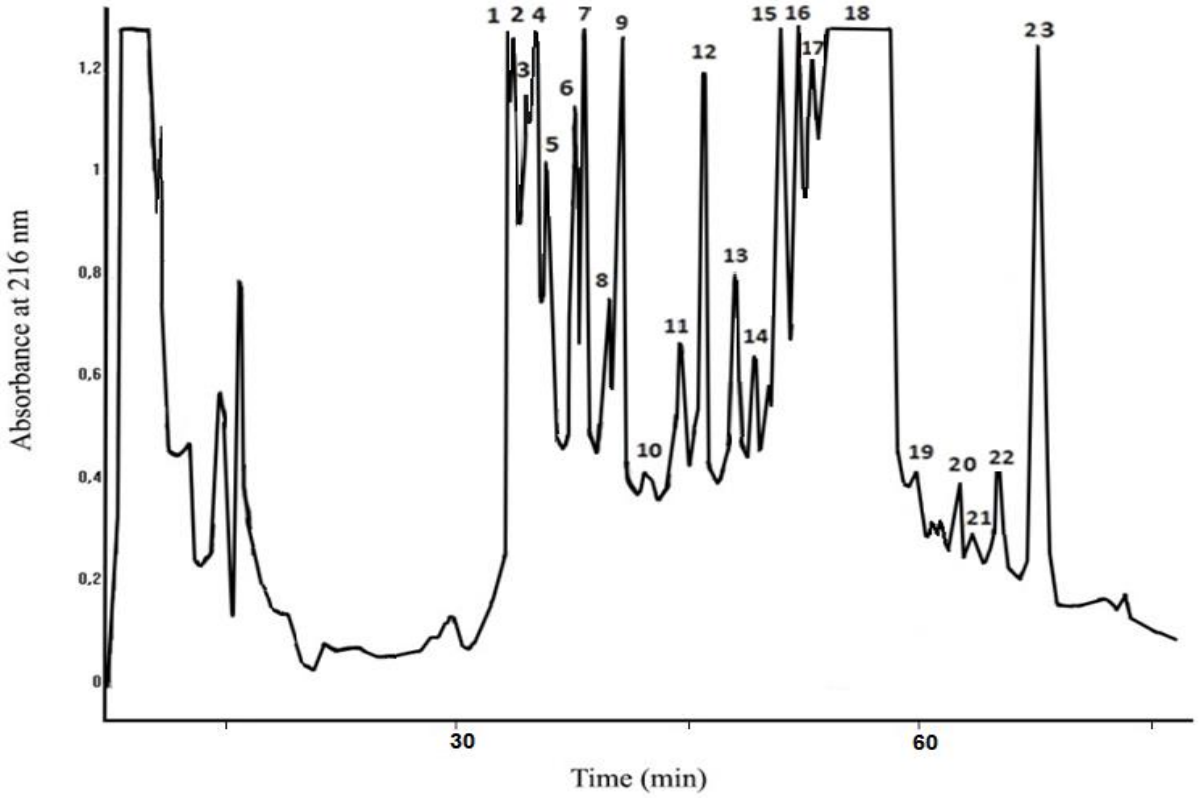

Fig. 2. RP-HPLC purification of the peptides with masses between 0-10 kDa residing in Fraction 1, to Nucleosil C18 RP-HPLC column (250 mm × 10 mm) with linear gradient of buffer A (0.1% TFA/water) and buffer B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min.

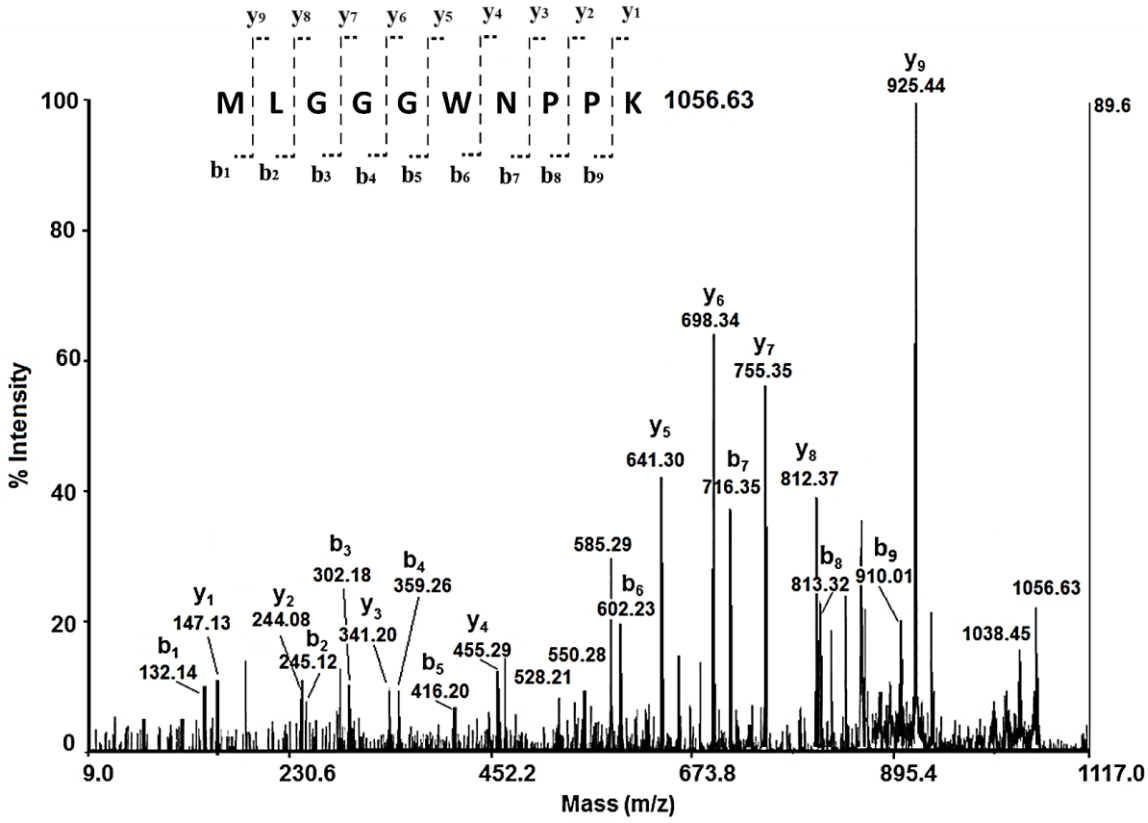

Fig. 3. MALDI-MS/MS spectrum of peptide at m/z 1056.63 Da (positive ion mode). Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics).

Table 1. Amino acid sequences of selected peptides in Fraction 1, which shows antibacterial activity against negative bacterial strain *Escherichia coli* NBIMCC 8785.

№	Amino acid sequence of peptides	MALDI-MS $[M+H]^+$ (Da)	pI (calculated)	Mass peptide (Da) (calculated)
1	M**LGGGW**N**PPK**	1056.63	8.50	1055.52
2	ML**GG**V**LGGGP**LK	1098.65	8.50	1097.63
3	M**GGLLGGGG**V**GGG**SLVG**P**GA**P**	1666.98	5.28	1665.85
4	LF**GGH**Q**GGGL**V**GG**L**W**RK	1738.99	11.00	1737.94
5	MGG**W**GG**LGGG**HN**GGW**M**PPK**	1853.06	8.52	1851.83
6	M**P**K**GGGL**V**GGLL**GD**W**MGHK	1910.08	8.37	1908.97
7	MVNLALV**GGLLGG**KCLA**P**AR	1953.08	9.50	1952.01
8	EN**LGGGL**V**GGLL**GG**W**FL**H**D**P**K	2136.24	5.32	2135.12
9	**H**AFDVAV**GGLLGGGG**A**GGGGL**V**GGGGLGGGG**A	2478.39	5.08	2477.24

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) was used to determine the molecular mass and sequence of the purified peptide. In the fraction with antibacterial activity, we determined 9 novel peptides with molecular masses between 1000-3000 Da (Table 1). Each of the peptides is detected in MS spectrum as the protonated molecular ions $[M+H]^+$ ion at m/z, respectively. These ions were selected for transmission through the first analyser, then fragmented in the collision cell and their fragments analysed by the second analyser to produce the following MS/MS spectrum. So, using tandem mass spectrometry, the primary structure of the peptides were identified by de novo MALDI-TOF-MS/MS sequencing experiments of the protonated molecule ions $[M+H]^+$ (Table 1) The amino acid sequence of peptide detected et m/z 1056,63 Da $[M+H]^+$, is shown in Fig. 2. Following the series of y- and b-ions from MALDI-TOF/MS/MS spectrum the sequence MLGGGWNPPK was deduced. The isoelectric points (pI) of the peptides were predicted by the ExPASy MW/pI tool program, (Table 1) [21]. In fraction1 were found six cationic peptides (№ 1, 2, 4, 5, 6 and 7) and three anionic peptides (№ 3, 8, 9). These peptides had a wide variety of structural motifs, but they belonged to two mainly groups AMPs. The first group is of α-helical antimicrobial peptides that usually contain an abundance of helix stabilizing residues such as alanine (A), leucine (L), and lysine (K). Often these peptides are not strictly α-helices and may contain an internal kink [22, 23]. In aqueous solutions these peptides are often unstructured but assume their amphipathic α-helical conformations when associated with a cell membrane or in a membrane mimetic environment. The second group AMPs is flexible rich in specific amino acids as such as proline (P), tryptophan (W), histidine (H), arginine (R), and glycine (G). Proline-rich AMPs, with a high content of Pro (P) and Arg (R) residues, are an important group of AMPs predominantly active against Gram-negative bacteria [24]. Our results for amino acid sequence of the peptides (Table 1) show that peptides No 1 and 5, contain composition –PPK to C -terminal end. Furthermore, peptides No 2, 3, 7 and 8 contain one Pro residue inserted into the sequences of α-helical to C-terminal region, only peptide No 6 - to N-terminal region. Probably, the biological activity is carried by the C-terminal region. Previous studies have shown that when Pro residues are inserted into the sequences of α-helical AMPs, the ability of these peptides to permeabilize the bacterial cytoplasmic membrane decreases substantially as a function of the number of Pro residues incorporated [25], and this could explain our results. On the other hand, the amino acid sequence sequences shown on Table 1 reveal a high content of glycine residues, especially for peptides № 2, 3, 4, 8 and 9. They belong to the class of AMPs, named the glycine-rich peptides. The percentage of glycine residues in these peptides varies considerably, from 10–30% in some species to more than 60% [26]. They are able to inhibit the growth of fungi and have been isolated and characterized from different taxonomic groups, including plants amphibians, and arthropods [26]. Recently, Lorenzini et al., reported for three isoforms of a novel glycine-rich AMP, named ctenidins from the hemocytes of unchallenged tarantula spider *Acanthoscurria gomesiana* [26]. They are also active against *E. coli* [26]. Another Gly-rich antimicrobial peptide is leptoglycin, isolated from skin secretion of

Leptodactylus pentadactylus. Leptoglycin was able to inhibit the growth of Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Citrobacter freundii* [27]. The amino acid sequence of leptoglycin with high level of glycine (GLLGGLLGPLLGGGGGGGGGLL) (59.1%) and leucine (36.4%) containing an unusual central proline suggests the existence of a new class of Gly/Leu-rich antimicrobial peptides. Similar to leptoglycin, the amino acid sequence of peptide No 9 shows high level of glycine and leucine into its amino acid sequence.

CONCLUSION

We determined by mass spectrometry the primary structures of 9 novel antimicrobial peptides with molecular masses between 1000 and 3000 Da which are contained in the fraction showed strong antibacterial activity against the Gram-negative bacterial strain - *E. coli* NBIMCC 878. Identified peptides contain high level of glycine and leucine as well as one or two proline residues inserted into the sequences of α-helical to C-terminal region, only one peptide – to N-terminal region. Probably this is important for their α-helical structures and antimicrobial activity. Our results may be considered as basic information for further investigations on bioactive peptides from *C. aspersum* and their potential applications in therapy.

In an era where we have run out of most 'off-the-shelf' antibiotics and are critically running out of last-resort options, it is imperative to continue to develop and accurately evaluate alternative antimicrobials [2]. We are persuaded that peptides have enormous potential as future therapeutics.

Acknowledgements: *This research was carried out with the support of a project under contract No. DN 01/14 of 19.12.16, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria and FWO-BAS project.*

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БОГАТИ НА ГЛИЦИН ПЕПТИДИ ОТ ОХЛЮВ *Cornu aspersum* С АНТИБАКТЕРИАЛНА АКТИВНОСТ

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Постъпила на 15 октомври, 2017 г.; приета на 22 ноември, 2017 г.

(Резюме)

Антимикробните пептиди са уникална и разнообразна група от молекули, които имат голям потенциал за използване в нови антимикробни лекарствени средства, тъй като много от тях имат силно изразена цитотоксичност към редица лекарствено-резистентни бактерии. Изследвахме различни екстракти от слузта градински охлюв *Cornu aspersum* срещу патогенния Грам-отрицателен бактериален щам - *Escherichia coli* NBIMCC 878 и установихме, че фракцията под 10 kDa проявява значителна антибактериална активност. Използвайки тандем масспектрометрия, ние идентифицирахме първичните структури на 9 нови антимикробни пептиди с молекулни маси между 1000-3000 Da в активната фракция. Повечето от тях имат високо съдържане на глицинови и левцинови остатъци в аминокиселинните си последователности.