

Structure and antibacterial activity of isolated peptides from the mucus of garden snail *Cornu aspersum*

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The world provides a rich source of peptides with antimicrobial, antiviral and antitumor activity. Peptides and glycopeptides are an expanding group of structurally complex anti Gram positive antibacterial agents, which are used in human and veterinary medicine. Quite a series of proline-rich peptides, isolated from arthropods and molluscs, were considered to be promising candidates for the treatment of microbial infections and suppression of microbial resistance. In the present study, we report the primary structure and antimicrobial activity of peptides produced by the mucus of garden snail *Cornu aspersum* in comparison to similar peptides isolated from molluscs. Several peptides with molecular masses between 1 and 4 kDa measured by mass spectrometric analysis were identified in the mucus. Their amino acid sequences were determined by MS/MS analysis as is shown for peptide at m/z 1438.87 [M+H]⁺ (ML/INVAVNQ/KGEVKH). The fraction with peptides with molecular masses below 3 kDa exhibited antibacterial activity against Gram-negative *Pseudomonas aeruginosa* AP9 and Gram-positive *Brevibacillus laterosporus* BT271 bacteria and the inhibition effects of the peptides can be explained with the amino acid residues. The *de novo* sequence of six peptides revealed that most of them contain glycine, proline, tryptophan and valine which are typical for peptides with antimicrobial activity.

Keywords: Antimicrobial peptides, *Pseudomonas aeruginosa*, *Brevibacillus laterosporus*, *Helix lucorum*, *Rapana venosa*, *Cornu aspersum*, Mass spectrometry

INTRODUCTION

Most living organisms are constantly exposed to potentially harmful pathogens *via* direct contact, inhaling and intake [1]. Their survival in an environment with an enhanced presence of microorganisms depends on a multicomponent mechanism system for protection. On one side there are the T and B cells against different antigens [2, 3]. In contrast to this primary defense mechanism are endogenous peptides which are constitutively expressed or induced in some cases and provide a rapid and effective protection against pathogens. Nowadays the number of pathogens that are resistant to the antibiotics used in treating them is rapidly increasing. This set the need of discovering novel molecules that could overcome pathogen drug resistance. Antimicrobial peptides have proven to be a good natural alternative to chemical antibiotics [4]. So far more than 750 different antimicrobial peptides have been isolated and characterized from different sources – insects,

plants and animals, as well as humans [5-8]. Many of these active peptides have demonstrated antibacterial effects against a wide range of Gram-positive and Gram-negative bacteria [9, 10].

The antimicrobial peptides composed of less than 10 amino acid residues, comprising two groups of amino acid sequence as: group 1) arginine, lysine, valine, or isoleucine and group 2) tryptophan, phenylalanine or proline, were identified as new antibiotic peptides, useful in treating microbial or viral infections or in inactivating Gram-positive and -negative bacteria, protozoa, fungi and viruses [11].

Our group identified several peptides from the hemolymph of the garden snail *Helix lucorum* and marine snail *Rapana venosa* that exhibit a broad spectrum of antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Helicobacter pylori*, *Propionibacterium acnes* and others [12-14].

We here report on the antibacterial properties of new antimicrobial peptides isolated from the mucus of the snail *Cornu aspersum* against the Gram (-) bacteria *Pseudomonas aeruginosa* AP 9 and the Gram (+) *Brevibacillus laterosporus* BT-271.

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Pseudomonas bacteria are a serious problem in hospital environments since the infections species are the second-most common infectors in hospitalized patients. This might be because of the wide range of secreted proteins and their ability to cause serious diseases and bacteremia is increasing worldwide [14].

EXPERIMENTS

Purification of the fractions from the mucus of garden snail Cornu aspersum

The snails *Cornu aspersum* were collected in Bulgaria and the mucus was purified. The crude extract was separated using Millipore filters (10, 30 and 50 kDa) into three fractions: Fraction 1 (compounds with Mw between 0-10 kDa), Fraction 2 (Mw 30 - 50 kDa) and Fraction 3 (Mw above 50 kDa). Fraction 1 was then further separated using Millipore filters of 3 kDa into two fractions: Fraction A (compounds with Mw <3 kDa) and Fraction B (compounds with Mw > 3 kDa).

Molecular mass analysis and de novo sequencing of low molecular weight peptides

Isolated fraction A was lyophilized and analyzed by MALDI-TOF-TOF mass spectrometry on an AutoflexTM 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. Samples were prepared by mixing 2.0 µl of the sample with 2.0 µl of matrix solution (7 mg/ml of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA) and 1 µl of the mixture was spotted on a stainless steel 192-well target plate. They were dried at room temperature and subjected to mass analysis. A total of 3500 shots were acquired in the MS mode and collision energy of 4200 was applied. A solution of protein standards was used to calibrate the mass scale. The mass spectrometer was externally calibrated with a mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1-17), and ACTH (18-39). For MS/MS experiments, the instrument was externally calibrated with fragments of Glu-fibrino-peptide B. The mass values assigned to the amino acid residues are average masses.

Antibacterial assays of the peptides

The Gram-negative bacterial strain *Pseudomonas aeruginosa* AP9 and the Gram-positive *Brevibacillus laterosporus* BT271 were used in the antibacterial assays. They were chosen because of their antibiotic resistance. Beside that they were chosen as model pathogenic bacteria

from different essential Gram-negative and Gram-positive groups, with specific relations towards antibiotics and xenobiotics, as well as specific permeability of their cell wall.

The strain *Pseudomonas aeruginosa* AP9 was isolated by Topalova (1989) and was characterized as resistant towards aryl-containing xenobiotics and aryl-containing antibiotics, possessing the ability to degrade these compounds.

The strain *Brevibacillus laterosporus* BT-271 was isolated and characterized by Topalova, 1982, as resistant against aryl-containing xenobiotics and antibiotics. The two bacteria were used as model Gram-negative and Gram-positive bacteria, with a potential to resist and to degrade aryl-containing antibiotics. Five cm³ of standardized suspensions of bacterial cultures (OD₄₃₀ = 0.600 abs) were inoculated in Nutrient agar, mixed, and poured in petri dishes layers with depth of 2 mm. Petri dishes were left at room temperature (20°C) to solidify. Fraction 1, Fraction A and Fraction B in concentrations 13.1, 6.9 and 5.3 mg/ml, respectively, determined by Lowry method, 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 and incubated for 24-72 hours at 37°C. The antibacterial effect was indicated in mm sterile zone around the wells. The antibacterial tests were also performed in a medium with no peptides added, to serve as a negative control.

RESULTS AND DISCUSSION

Several bioactive compounds with antimicrobial and antiviral activities were isolated from molluscs. It was found that the isolated fractions, containing compounds with different molecular masses from the mucus of garden snail *Cornu aspersum*, had a strong antibacterial effect against several strains of *Pseudomonas aeruginosa* and a weak effect against *Staphylococcus aureus*. It was published that the inhibition effect of separated fractions from *Helix aspersa* on the growth of bacteria was not caused by bacteriophages; the experiments indicated that the substance with Mw between 30 and 100 kDa in *H. aspersa* has antimicrobial activity [16].

We have analyzed the biochemically active peptides in the mucus of *Cornu aspersum* with molecular weights ranging from 1 to 10 kDa (Fraction 1) in a concentration of 13.1 mg/ml. This fraction was additionally subdivided into two fractions, Fraction A (Mw<3 kDa and concentration of 6.9 mg/ml) and Fraction B (Mw>3 kDa and concentration of 5.3 mg/ml), obtained after separation over Millipore filters with a cut-off of 3

kDa. Upon testing their antimicrobial activity in agar medium after incubation for 24-72 hours at 37°C, no inhibition effect was observed of isolated Fraction A (Fig. 1A, spot 1) and Fraction B (Fig. 1B, spot 3) against *Pseudomonas aeruginosa* AP9 and *Brevibacillus laterosporus* BT-271. However,

applying agar well diffusion method, only Fraction 1 in concentration of 13.1 mg/ml appeared to generate a zone of 1.5 and 1.2 cm, respectively, inhibition of both *Pseudomonas aeruginosa* AP9 (Fig. 1A, spot 4) and *Brevibacillus laterosporus* BT271 (Fig. 1B, spot 4).

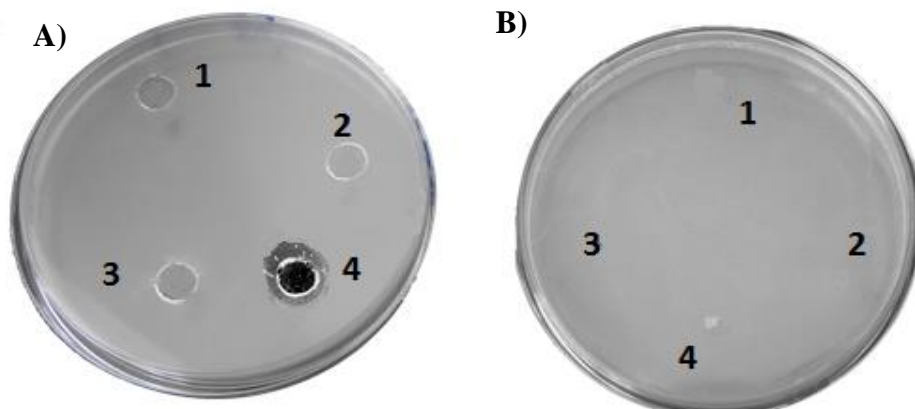


Fig.1. A) Antimicrobial assays against Gram-negative *E.coli* of isolated fractions: A (spot 1), B (spots 2) and C (spots 3). Antimicrobial assays via agar well diffusion method against Gram-negative *E.coli* of isolated Fraction 1 (spot 4). Each fraction was applied on the agar medium in 50 μ l of the 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.

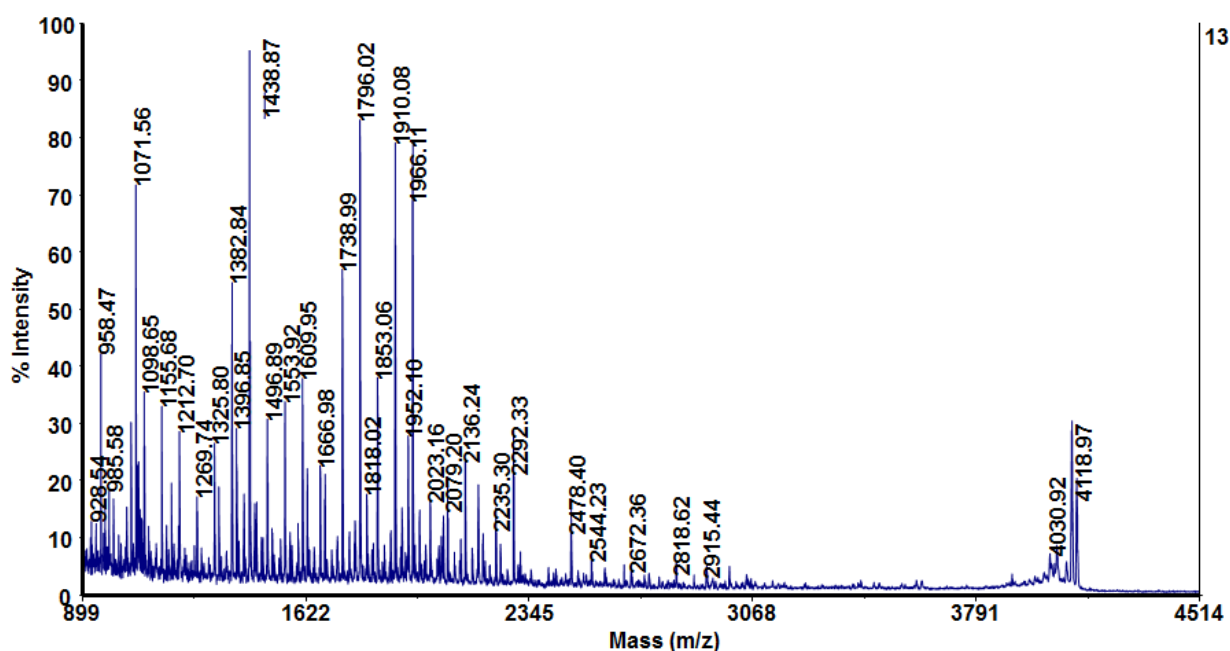


Fig. 2. MALDI-MS spectrum of Fraction A containing peptides with molecular masses between 1-5 kDa. The sample was measured by MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany).

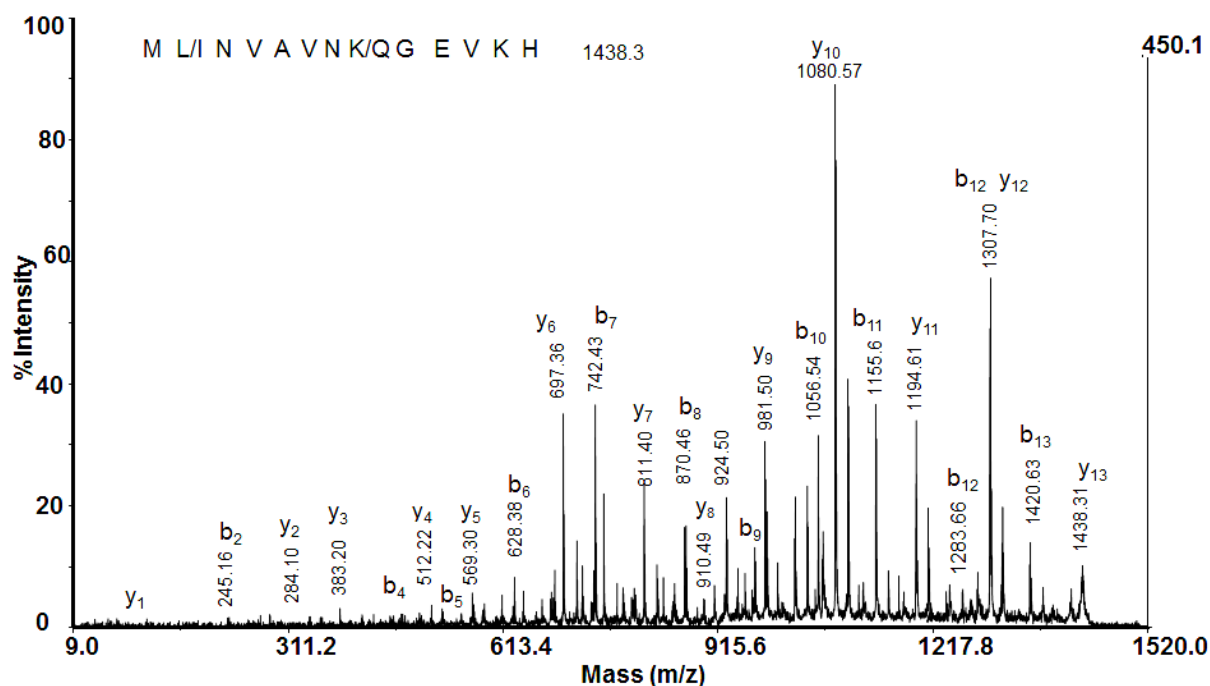


Fig. 3. *De novo* sequence by MALDI-MS spectrum (positive ion mode) of the peptide at m/z 1438.87. Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics). All labeled fragment ions are y-ions (y13) and other fragment ions are indicated (b-ions).

To determine the minimal antimicrobial concentration of the active peptides the above described method was applied and the effect of Fraction 1 with 50 μ l of decreasing dilutions (1/2, 1/4 and 1/8 from the starting concentration) was tested. The obtained results didn't show any activity for diluted peptide fractions against both bacteria. Only the initial concentration of Fraction 1 (13.9 mg/ml) suppressed the growth of the bacteria. To explain the mechanism of antimicrobial activity of mucus against *P. aeruginosa* AP9 and *B. laterosporus* BT271 Fraction 1 was analyzed by mass spectrometry. Several peptides were identified by their MS spectra (Fig. 2) with molecular masses determined by MALDI/MS (Table 1). The primary structure of six peptides with m/z at 1155.68, 1325.8, 1438.87, 1496.89, 1796.02 and 2292.33 Da were analyzed by their MALDI-MS/MS spectra. *De novo* sequence was derived for the antimicrobial peptide generated by *de-novo* explorer with the highest score value (b ion values and y ion values). Following the series of y- and b-ions the sequence ML/INVAVNQ/KGEVKH was deduced for Peptide 3 (Fig.3). The primary structures of six peptides from Fraction 1, shown in Table 1, were analyzed by their MALDI-MS/MS spectra.

The amino acid sequence of the peptides determined by MS/MS analysis showed that they are rich in glycine (Gly), proline (Pro), tryptophan (Trp) and isoleucine/leucine (Leu). A number of antibacterial and antifungal peptides rich in Cys, Pro, Ser or Gly residues have been isolated and characterized in the past from other mollusks like *Helix lucorum* [18]. Amino acid sequence comparisons of peptides with molecular masses between 1 and 3 kDa of the mucus of garden snail *C. aspersum* with peptides from the extract of *H. lucorum* determined by MS/MS analyses and peptides from the hemolymph of the marine snail *R. venosa* determined by Edman degradation show homology with this category of peptides. They are rich in Pro, Val, Trp and Gly residues.

Eleven proline-rich peptides exhibiting high antimicrobial activity against *S. aureus* and low activity against *K. pneumoniae* with molecular weights ranging from 3000 and 9500 Da were isolated from the hemolymph of *R. venosa* snails [18]. Antimicrobial activity exhibit also cysteine-rich peptides identified in the *Achatina fulica* snail and bivalve mollusc *Mytilus galloprovincialis* [17].

Table. 1 Amino acid sequence comparisons of peptides from Fraction 1 of the mucus of garden snail *Cornu aspersum* and extract of *H. lucorum* determined by MS/MS analyses with peptides from the hemolymph of the marine snail *Rapana venosa* determined by Edman degradation. Proline, valine, tryptophane and glycine residues are in boldface.

	Amino acid sequence of peptides	MALDI_MS [M+H] ⁺ (Da)	pI	Mass (Da) calculated
CaP1	M G V G A V W N G H K	1155.68	8.52	1154.59
CaP2	M L G G G V N S L R P P K	1325.80	11.00	1324.73
CaP 3	M L N V A V N K G E V K H	1438.87	8.37	1437.78
CaP 4	N L V G G L S G G G R G G A P G G G G	1496.89	9.75	1495.75
CaP 5	L L L D G F G G G L L V E H D P G S	1796.02	4.02	1794.92
CaP6	M P K R A L G G G L V G G L L G G G G E G L L V N	2292.33	8.50	2291.24
HIP 1	V P K A R V M T S G K K K			
HIP2	R T V P F G G A E E E L L D L G V G			
HIP 3	G S G G A D D G C L P V Y R R F P A N M L			
Pep 2	L G G K S P P N Q P S I M T F D Y A K T N K			
Pep 4	S L P P T L E E E F N M K K M G			
Pept5	S P P P G E S K V D M S F N Y A L S N P A Q			
Pep 6	S P P S E Q L G K S F N F			
Pep7	A P P P G L S A G V			
Pep 8	A P P P G Y A M E S D S F S			
Pep 9	F P P P G E S A V D M S F F Y A L S N P			

CONCLUSIONS

This study showed that the mucus from the common brown garden snail *C. aspersum* contains bioactive compounds exhibiting a demonstrable antimicrobial activity against bacterial strains of *P. aeruginosa* AP9 and *B. laterosporus* BT271. The obtained results show higher inhibiting effect of the peptides with molecular weight between 1000 and 10000 Da on the growth of the bacterial strain *P. aeruginosa* AP9 (1.5 cm zone) than of *B. laterosporus* BT271 (1.2 cm zone). The effect may be explained with the amino acid sequence comparisons of peptides from Fraction A which are rich in proline, valine, tryptophane and glycine residues.

These results are preliminary testing of 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.

Understanding the function and mechanism of action of antibacterial peptides can contribute to the development of new anti-bacterial therapeutics.

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СТРУКТУРА И АНТИБАКТЕРИАЛНА АКТИВНОСТ НА ПЕПТИДИ, ИЗОЛИРАНИ ОТ СЛУЗТА НА ГРАДИНСКИ ОХЛЮВ *Cornu aspersum*

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(Резюме)

Светът осигурява богат източник на пептиди с антимикробна, антивирусна и антитуморна активност. Пептидите и гликопептидите са разширяваща се група от структурно сложни антибактериални пептиди с ефект срещу Грам-положителни бактерии, които се използват в медицината, както за лечение на хора, така и на животни. Голям брой богати на пролин пептиди, изолирани от артроподи и мекотели, се считат за обещаващи кандидати за лечение на микробни инфекции и потискане на микробната резистентност. В представените изследвания ние докладваме за първичната структура и антимикробната активност на пептиди, изолирани от слузта на градинския охлюв *Cornu aspersum* в сравнение със сходни пептиди, изолирани от мекотели. Няколко пептида, с молекулни маси между 1 и 4 кДа, измерени чрез маспектрометричен анализ, бяха идентифицирани в слузта. Аминокиселинните им последователности са определени чрез MS/MS анализи, както представения пептид при m/z 1438.87 [M+H]⁺ (ML/INVAVNQ/KGEVKH). Фракцията, която съдържа пептиди с молекулни маси под 3 кДа, проявява антибактериална активност срещу Грам-отрицателен *Pseudomonas aeruginosa* AP9 и Грам-положителните *Brevibacillus laterosporus* BT271 бактериални щамове, като инхибиращият ефект на тези пептиди може да бъде обяснен с аминокиселинните остатъци. Де ново фрагментирането на шест пептида разкри, че повечето от тях съдържат глицин, пролин, триптофан и валин, които са типични за пептиди с антимикробна активност.