

ДОКАЗАТЕЛСТВА

УЧАСТИЯ В НАУЧНИ КОНФЕРЕНЦИИ

на гл. ас. д-р Людмила Велкова

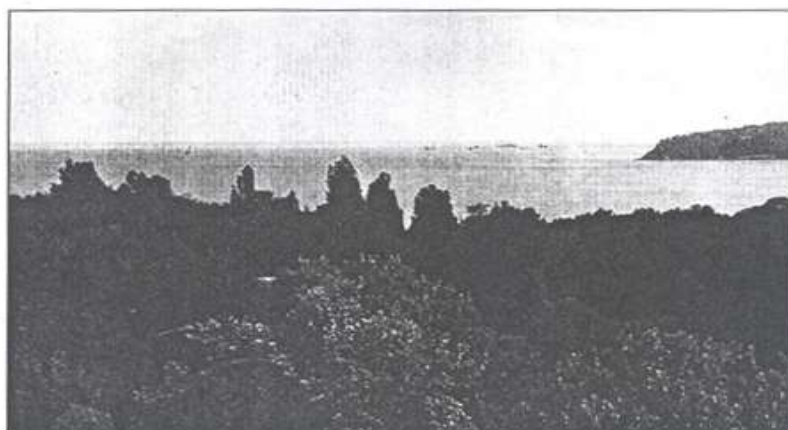
1. R. Toshkova, E. Ivanova, M.-D. Nastke, **L. Velkova**, S. Stevanovic, R. Hristova, A. Dolashki, M. Gardeva, I. Dimitrov, W. Voelter, P. Dolashka-Angelova. Hemocyanins as immunostimulators. П16, Eleventh Congress of the Bulgarian Microbiologists with International Participation International House of Scientists Frederic Joliot-Curie St. Constantine, 5-7 October 2006, Varna, Bulgaria.



ELEVENTH CONGRESS OF THE BULGARIAN MICROBIOLOGISTS

with International Participation

PROGRAM AND ABSTRACTS



St. Constantine, Varna, October 5-7, 2006

HEMOCYANINS AS IMMUNOSTIMULATORS

Reneta Toshkova¹, Emilia Ivanova², Maria-Dorothea Nastke³, Lyudmila Velkova⁴, Stefan Stevanovic³, Romyana Hristova⁴, Alexandar Dolashki⁴, Maria Gardeva⁴, Ivan Dimitrov⁴, Wolfgang Voelter⁵, **Pavlina Dolashka-Angelova⁴**

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Hemocyanin from the giant keyhole limpet *Megathura crenulata* has been a subject of biomedical interest because of its remarkable immunostimulatory properties in experimental animals and man. Molluscan *Helix vulgaris* (HvH) and *Rapana venosa* (RvH), and arthropodan *Carcinus aestuarii* (CaH) hemocyanins have been studied in order to evaluate their potential biochemical and medical applications.

It was established that the serum IL-2 production was better expressed in animals immunized by HvH and CaH than with the native molecule of KLH. Increased IL-2 production in supernatants of *in vitro* cultivated lymphocytes was observed in animals immunized with native CaH and KLH. Spleen cells from the mice immunized with other hemocyanins showed negligible stimulation. It was found that CaH causes increased

2. P. Dolashka-Angelova, **L.Velkova**, K. Sandra, A. Beck, A. Dolashki, B. Devreese, S. Stevanovic, J. Van Beeumen. Complete oligosaccharide structure of *Rapana venosa* hemocyanin. P21, Sofia School of Protein Science - 2007, For Students and Young Researchers, Institut Curie & Institute of Organic Chemistry with Center of Phytochemistry-BAS, 3-5 October 2007, Sofia, Bulgaria.



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Institute of Organic Chemistry with Centre of Phytochemistry

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SOFIA SCHOOL OF PROTEIN SCIENCE – 2007

FOR STUDENTS AND YOUNG RESEARCHERS

Sofia, 3-5 October 2007

Topics: Isolation, crystallization and crystallographic study of proteins, protein thermodynamics and kinetics, physico-chemical methods to study proteins and protein-ligand complexes, proteomics, protein bioinformatics and simulation

Invited speakers: J.Luis Arrondo (Spain), Jozef Van Beeumen (Belgium), Jacqueline Cherfils (France), Constantin Craescu (France), Plamen Demirev (USA), András Dér (Hungary), Christine Ebel (France), Ilian Jelesarov (Switzerland), Andrey Karshikoff (Sweden), Rudolf Ladenstein (Sweden), Wei Liu (Sweden), Maria Miteva (France), Liliane Mouawad (France), Stefan Stevanovic (Germany), Stefan Szedlacsek (Rumania), Nikolay Todorov (England)

Organizers: Petya Christova (Bulgaria), Andrey Karshikoff (Sweden), Constantin Craescu (France)

For further information, contact Dr. Petya Christova, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Akad. G.Bonchev Str. Bldg. 9, room 404, Sofia-1113, Bulgaria; tel. (359 2) 960 61 65, fax (359 2) 8700225, E-mail: petya02@yahoo.fr

Complete oligosaccharide structure of *Rapana venosa* hemocyanin

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Alexander Beck⁴, Alexandar Dolashki¹, Bart Devreese², Stefan Stevanovic³,
Wolfgang Voelter⁵, and Jozef van Beeumen²

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The complete oligosaccharide structures of *Rapana venosa* hemocyanin (RvH) were studied by sequencing of glycopeptides using LC/ESI-MS, Nanoflow-ESI mass spectrometry, CE or by MALDI-TOF-MS after enzymatically liberation of the N-glycans from both structural subunits RvH1 and RvH2. The structural information of these glycans was obtained by sequencing of them by Q-Trap mass spectrometer. Mass spectrometry revealed a highly heterogeneous mixture of different glycans from RvH2 comprising at least 37 different compositions of Hex_{0.9} HexNAc₂₋₄ Hex_{0.3} Pent_{0.3} Fuc_{0.3} and the deoxyhexose and pentose residues. Oligosaccharides with approximately the same structures were identified in structural subunit RvH1. Also novel types of N-glycans with a internal Fuc connecting one GalNAc(β1-2) and one hexuronic acid was confirmed in both structural subunits RvH1 and RvH2.

3. P. Dolashka-Angelova, L. De Smit, I. Dimitrov, T. Stefanova, E. Livaniou, **L. Velkova**, S. Stevanovic, B. Salvato, W. Voelter. Gene sequence and immunological properties of *Helix vulgaris* hemocyanin, isolated garden snails, in comparison with other molluscan hemocyanins. P26, Sofia School of Protein Science-2007, For Students and Young Researchers, Institut Curie & Institute of Organic Chemistry with Center of Phytochemistry-BAS, Sofia, 3-5 October 2007, Sofia, Bulgaria.



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Gene sequence and immunological potential of *Helix vulgaris* hemocyanin, isolated from garden snails, in comparison with other molluscan hemocyanins

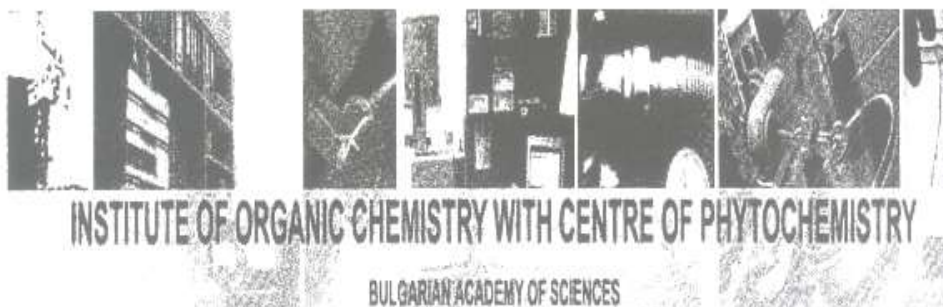
Pavlina Dolashka-Angelova, Lina De Smite, Ivan Dimitrov, Tsetanka Stefanova, Evangelia Livaniou, Persefoni Klimentzou, Ludmila Velkova, Stefan Stevanovic, B. Salvato, Hristo Neychev, Wolfgang Voelter
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A new hemocyanin was isolated from the hemolymph of garden snails *Helix vulgaris*, composed of two isoforms, HvH1 and HvH2 separated on an ion exchange column DEAE-Sepharose 6CL. Structural and immunological properties of *Helix vulgaris* hemocyanin were studied in comparison with molluscan Hcs *Rapana venosa* and *Megathura crenulata*. Quaternary structure of these Hcs is by didecameric isoforms, containing different structural subunits. However they show different reassociation behaviour by mixing them over a period of several days, using a stabilizing buffer (SB). Higher concentrations of Ca^{2+} and Mg^{2+} ions led to rapid reassociation of \square -HvH resulting in short and long multidecamers.

To obtain the complete coding sequence for *Helix vulgaris* hemocyanin (HvH), we performed several RT-PCRs using degenerated primers derived from the copper binding site A of *H. tuberculata* hemocyanin and sequences of HvH obtained by Edman degradation. All obtained fragments were cloned, sequenced and identified by "blasting" them, thus we finally were able to verify 80% of complete sequence of HvH1.

The possibility of using HvH and RvH as carriers of small molecules (haptens) in immunizing protocols was studied in comparison with KLH, which is a widely used, highly immunogenic carrier protein. By using HvH as a carrier of the well-known hapten TNBS (2,4,6-trinitrobenzene sulfonic acid), an increasing with time production of hapten-specific TFN- γ was detected in splenocyte cultures of mice, which lasted longer than in case of KLH and RvH carriers. Also, use of HvH or RvH as a carrier of the hapten ProT α [101-109] showed that antisera of higher titres than that of the control conjugate (ProT α [101-109]-KLH) were obtained immediately after the second bleeding. HvH and RvH may prove to be useful for the development of new antiviral, antibacterial and antitumor vaccines, since they seem to launch strong and specific immune response against the conjugated

4. V. Moshtanska, P. Dolashka-Angelova, **L.Velkova**, M. Angelova, W. Voelter, B. Atanasov. Electrostatic analysis of *Humicola lutea* 103 superoxide dismutase in comparison with bovine enzyme. P14. Technical Digest FEBS 2009, Sofia school of protein science from basic research to drug desing, FEBS Practical & Lecture Course, 21-26 September 2009, Sofia, Bulgaria.



TECHNICAL DIGEST

FEBS 2009

**SOFIA SCHOOL OF PROTEIN SCIENCE
FROM BASIC RESEARCH TO DRUG DESIGN**

FEBS Practical & Lecture Course

Sofia, Bulgaria

21-26 September 2009

Electrostatic analysis of *Humicola lutea* 103 superoxide dismutase in comparison with bovine enzyme

Vesela Moshtanska¹, Dolashka-Angelova P.¹, Velkova L.¹, Angelova M.²,
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There has been a considerable and continuing interest in the use of the antioxidant enzyme superoxide dismutase (SOD) in medicine over the last years, arising from its ability to reduce the deleterious effect of superoxide anion radicals (O₂^{•-}) in the cells. The fungal strain *Humicola lutea* 110 produces a manganese (Mn)- and a copper/zinc-containing (CuZn) superoxide dismutases (HLSODs). CuZnSOD enzyme is the first identified glycosylated fungal SOD with one mole of N-acetylglucosamine, connected to the polypeptide chain. The comparative investigations of the protective effect of HLSOD and commercially available bovine SOD show that intranasal inoculation of the virus produces a damaging infection of the lungs, which is highly lethal to the animals. HLSOD impressively protected mice from mortality; survival was increased by 66%, survival time was prolonged by 5.2 days. The pathological changes in the lungs were reduced, though viral infectious titers remained high. Thus, no correlation between the direct cause (viral replication) and effect (pathological changes) was observed. The bovine SOD at a high dose of 500 U/mice protected mice from mortality to a lesser extent. The protective effect of HLSOD was comparable to that of ribavirin. We assume that the clearance of HLSOD from the serum has been delayed due to the presence of polysaccharide chain in the molecule. This could explain the different protective effects of HLSOD which is naturally glycosylated and bovine SOD which is not glycosylated.

Because protein-virus interaction is primarily manifested by long-range electrostatics, we study in details Coulomb interactions of both enzymes. Their 3D structures are very homologous, but side chains distribution (by type and site) are quite different. First of all HLSOD has most alkaline stable ($\Delta G_{el} = -17$ kcal/mol at pH 11.3) if compare to bovine enzyme, which is more stable in wide pH region (more than $\Delta G_{el} = -35$ kcal/mol at pH 3-12). HLSOD has more hydrophobic residues on molecular surface than bovine enzyme, which facilitates and strengthens its dimerization and carbohydrate interaction. Bovine SOD has four abnormal Asp residues (D61, D122, D99 and D74, with calculated pK_a-s below 0), whereas no such anomalies in HL-enzyme. Most attractive is surface electrostatic potential (3D-SEP) distributed in different ways in both molecules: As seems from poster 3D-SEP figures HLSOD has wide neutral area (in green), but bovine enzyme has alternate negatively/positively fields. This difference should be functionally important, supporting carbohydrate moiety interaction with target structures (partially with capsid virus proteins).

5. I. Dimitrov, L. De smit., **L. Velkova**, S. Shishkov, K. Kostova, B. Devreese, J. van Beeumen, P. Dolashka - Angelova. *Helix vulgaris* hemocyanin: Gene sequence and antiviral properties. P9. Technical Digest FEBS 2009, Sofia school of protein science from basic research to drug desing, FEBS Practical & Lecture Course, 21-26 September 2009, Sofia, Bulgaria.

P9

Helix vulgaris hemocyanin: Gene sequence and antiviral properties

Dimitrov I.¹, De Smit L.², Velkova L.¹, Shishkov S.³, Kostova K.³, Devreese B.², Van Beeumen J.² and Dolashka-Angelova P.¹

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Rapana venosa hemocyanin is glycosylated proteine isolated from the hemolymph of marine snails from the Black Sea. Using LC/MS/MS, Q-Trap and MALDI techniques the most important class of glycans, one internal fucose branching to hexuronic acid and HexNAc, were analysed in both structural subunits RvH1 and RvH2. Though hexuronic acid moieties, which are common constituents of proteoglycans, occur rather rarely in glycoproteins.

Studies on antiviral activities of native molecules and functional units (FUs) from *Rapana venosa* (RvH), *Helix vulgaris* (HvH), keyhole limpet (KLH) and *Carcinus aestuarii* hemocyanin (CaSS2) revealed that only the glycosylated FU of RvH and CaSS2 presented antiviral properties against Herpes simplex virus type 1, strain Vic, (HSV-1). Thus, for the first time, our results revealed that the glycosylated functional unit of molluscan and arthropodan hemocyanin display antiviral activity. No inhibitory effect on this viruse could be observed for the native molecule RvH, HvH and structural subunit RvH1. The antiviral effect of glycosylated FU against the replication of HSV is established, but its antiviral mechanism is still unknown. Therefore, the further investigation of the inhibitory properties of HvH against HSV is under way.

6. **L. Velkova**, I. Iliev, I. Dimitrov, L. Yossifova, V. Moshtanska, S. Zacharieva, P. Dolashka-Angelova. (Lecture MB 17.) Isolation of molluscan hemocyanin *Helix vulgaris* with antitumor effect on Guerin ascites tumor-bearing animals. XI Anniversary Scientific Conference "Biology –traditions and challenges", 27–29 May 2009, Sofia, Bulgaria.

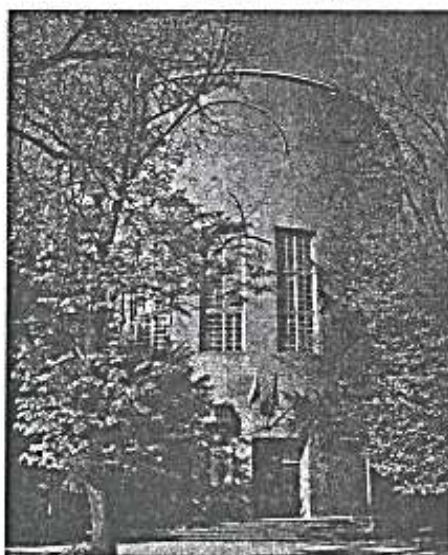


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ISOLATION OF MOLLUSCAN HEMOCYANIN *HELIX VULGARIS* WITH ANTITUMORE EFFECT ON GUERIN ASCITES TUMOR-BEARING ANIMALS

L. Velkova¹, I. Iliev², I. Dimitrov¹, L. Yossifova, V. Moshtanska¹, S. Zacharieva¹ and P. Dolashka-Angelova¹

¹ Institute of Organic Chemistry with Centrum of Phytochemistry, BAS, Sofia 1113, Bulgaria

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Keywords: hemocyanin, *Helix vulgaris*, Guerin tumore, glycoprotein

Three types of hemocyanin molecules were identified in the haemolymph of garden snails *Helix vulgaris*. This hemocyanin similar to *Helix pomatia* consists of β C-HvH, α D-HvH and α N-HvH. These components were separated by size exclusion chromatography and isoelectric fragmentation. They all show the cylindrical quaternary structure, typical for gastropodan hemocyanins, comprising 20 subunits with a molecular mass of approximately 500 kDa each.

The β C-HvH was crystallized by dialysis of total HvH against NaOAc-HOAc buffer, pH 5.3, I = 10mM. Seven functional units (FUs) with molecular masses about 50 kDa, determined by MALDI-TOF, were isolated after tryptic hydrolysis of structural subunit β C-Hc. N-terminal sequences of FUs showed high similarity with FUs from other molluscan hemocyanins.

HvH is a glycoprotein and its immunogenetical properties were studied in comparison with RvH and KLH. The mechanisms of immune response of spleen lymphocytes from experimental Guerin ascites tumor-bearing animals preliminarily immunized with vaccines supplemented with *Helix vulgaris*(HvH) *Rapana venosa* (RvH) and KLH were studied. Experimental data showed significant immune activation in animals treated with HvH, RvH, much higher than that in the control group immunized with keyhole limpet haemocyanin (KLH). Comparing the titres of specific antibodies against each subunit we found, that the second subunit of RvH2 is more immunogenic than the first one, although it is of lower molecular weight. This is probably due to the higher state of glycosylation and the greater heterogeneity in domain subunit organization.

7. **L. Velkova**, D. Todorov, I. Dimitrov, S. Shishkov, B. Atanasov, P. Dolashka-Angelova. (Lecture MB 13) *Rapana venosa* hemocyanin with antiviral activity. XI Anniversary Scientific Conference "Biology – traditions and challenges", 27 – 29 May 2009, Sofia, Bulgaria.

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MOLECULAR AND CELL BIOLOGY

MB 13

RAPANA VENOSA HEMOCYANIN WITH ANTIVIRAL ACTIVITY

L. Velkova¹, D. Todorov², I. Dimitrov¹, S. Shishkov², J. van Beeumen³ and P. Dolashka-Angelova¹

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Keyword: *Rapana venosa* hemocyanin, functional unit, glycosylatin, antiviral effect, mass spectrometry

Molluscan hemocyanins (Hcs) have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and highly specific monosaccharide composition. Our study revealed a highly heterogeneous mixture of different glycans isolation from structural subunit RvH2 of *Rapana venosa* hemocyanin at least 28 different compositions of Hex0-9 HexNAc2-4 Hex0-3 Pent0-3 Fuc0-3 and deoxyhexose and pentose residues. A novel type of N-glycan, with an internal Fuc connecting one GalNAc(B1-2) and one hexuronic acid, was detected in RvH2 as was previously found in subunit RvH1.

We compared investigation on antiviral effects of several molluscas hemocyanins (keyhole limpet hemocyanin, *Rapana venosa* hemocyanin and *Helix vulgaris* hemocyanin) and the arthropod *Carcinus aestuarii* hemocyanin. For the first time, we demonstrate here the inhibitory effect of one glycosylated functional unit of molluscan hemocyanin against viruses. The FU RvH-1 of *Rapana venosa* hemocyanin is the most effective inhibitor on the replication of Herpes simplex virus type 1, strain Vic, (HSV-1).



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8. **L. Velkova**, P. Dolashka-Angelova, W. Voelter, B. Atanasov. (Lecture MB 15). Thermodynamic and electrostatic analysis of functional unit RvH2-e of *Rapana Hemocyanin*. XI Anniversary Scientific Conference "biology –traditions and challenges", 27 – 29 May 2009, Sofia, Bulgaria.

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MOLECULAR AND CELL BIOLOGY

MB 15

THERMODYNAMIC ANALYSIS AND MOLECULAR MODELING OF RAPANA VENOSA HEMOCYANIN – FUNCTIONAL UNIT RVH2-E

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Keyword: *Rapana venosa Hemocyanin functional unit RvH2-e, Circular dichroism spectra, Reversible denaturation, Thermodynamic characteristics, 3D- structure*

pH-T diagram is typical "phase portrait" for stability of functional unit RvH2-e. Using different techniques the T-transition curves at different pH for RvH2-e were analyzed and the parameters of the thermodynamic functions were obtained. Increasing temperature and within the T range 25-55°C the reversibility increases and "opens a reversibility window" within the range of pH 5.5-9.0, for which were calculate at standard temperature the thermodynamic functions ΔH_o and ΔG_{oexp} .

Molecular modeling of correct 3D structure of functional unit RvH2-e was done which allows us to fix most probably position of missing 9 residues now presented in existed x-ray model at very poor resolution of 3.30Å.



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9. V. Moshtanska, P. Dolashka - Angelova, **L. Velkova**, M. Angelova, W. Voelter, B. Atanasov. Electrostatic analysis of Humicola Lutea 103 superoxide dismutase in comparison with bovine enzyme. XI ASC "Biology – traditions and challenges", 27 – 29 May 2009, Sofia, Bulgaria.

MB 18

ELECTROSTATIC ANALYSIS OF HUMICOLA LUTEA 103 SUPEROXIDE DISMUTASE IN COMPARISON WITH BOVINE ENZYME

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Keywords: *Humicola lutea* fungal strain, glycoprotein, stability

The fungal strain *Humicola lutea* 110 produces a manganese (Mn)- and a copper/zinc-containing (CuZn) superoxide dismutases (HLSODs). CuZnSOD enzyme is the first identified glycosylated fungal SOD with one mole of N-acetylglucosamine, connected to the polypeptide chain. The comparative investigations of the protective effect of HLSOD and commercially available bovine SOD show that intranasal inoculation of the virus produces a damaging infection of the lungs, which is highly lethal to the animals.

Because protein-virus interaction is primary manifested by long-range electrostatics, we study in details Coulomb interactions of both enzymes. Their 3D structures are very homologous, but side chains distribution (by type and site) are quite different. First of all HLSOD has most alkaline stable ($\Delta G_{el} = -17$ kcal/mol at pH 11.3) if compare to bovine enzyme, which is more stable in wide pH region (more than $\Delta G_{el} = -35$ kcal/mol at pH 3-12). HLSOD has more hydrophobic residues on molecular surface than bovine enzyme, which facilitates and strengthens its dimerization and carbohydrate interaction. Bovine SOD has four abnormal Asp residues (D61, D122, D99 and D74, with calculated pKa-s below 0), whereas no such anomalies in HL-enzyme. Most attractive is surface electrostatic potential (3D-SEP) distributed in different ways in both molecules: As seems from poster 3D-SEP figures HLSOD has wide neutral area (in green), but bovine enzyme has alternate negatively/ positively fields. This difference should be functionally important, supporting carbohydrate moiety interaction with target structures (partially with capsid virus proteins).



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10. L. Yossifova, L. Velkova, I. Iliev, I. Dimitrov, V. Moshtanska, S. Zacharieva, P. Dolashka-Angelova. *Helix vulgaris* hemocyanin: structure and anticancer activity. P2.9. Bulgarian-Japanese Symposium “Genomics and Proteomics in Personalized Medicine” 19–20 March 2009, Sofia, Bulgaria



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The logo symbolizes the graceful Danube, which flows through Austria, Hungary, Bulgaria and Romania, and the cherry blossoms which is widely known in these countries as a symbol of Japan. It represents a beautiful harmony created by these symbols.

Bulgarian-Japanese Symposium
“Genomics and Proteomics in
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March 19-20, 2009, Sofia, Bulgaria

P2.9. *Helix vulgaris* hemocyanin: structure and anticancer activity

Liliya Yossifova¹, Velkova L.², Iliev I.¹, Dimitrov I.², Moshtanska V.², Zacharieva S.¹, Dolashka-Angelova P.²

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Three types of hemocyanin molecules were identified in the haemolymph of garden snails *Helix vulgaris*. *Helix vulgaris* hemocyanin (HvH) similar to *Helix pomatia* consists of β c-HvH, α D-HvH and α N-HvH. They all show the cylindrical quaternary structure, typical of gastropodan hemocyanins, comprising 20 subunits with a molecular mass of approximately 500 kDa each. Seven functional units (FUs) with molecular masses about 50 kDa were isolated after tryptic

11. **L. Velkova**, D. Todorov, I. Dimitrov, S. Shishkov, B. Atanasov, P. Dolashka-Angelova. *Rapana venosa* hemocyanin with antiviral activity. P2.11. Bulgarian-Japanese Symposium “Genomics and Proteomics in Personalized Medicine” 19–20 March 2009, Sofia, Bulgaria.



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P2.11. *Rapana venosa* hemocyanin with antiviral activity

Lyudmila Velkova¹, Todorov D.², Dimitrov I.¹, Shishkov S.², Atanasov B.¹ and **Dolashka-Angelova P.**¹

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Rapana venosa hemocyanin is glycosylated protein isolated from the hemolymph of marine snails from the Black Sea. Using LC/MS/MS, Q-Trap and MALDI techniques the most important class of glycans, one internal fucose branching to hexuronic acid and HexNAc, were analysed in both structural subunits RvH1 and RvH2. Though hexuronic acid moieties, which are common constituents of proteoglycans, occur rather rarely in glycoproteins.

Studies on antiviral activities of native molecules and functional units (FUs) from *Rapana venosa* (RvH), *Helix vulgaris* (HvH), keyhole limpet (KLH) and *Carcinus aestuarii* hemocyanin (CaSS2) revealed that only the glycosylated FU of RvH and CaSS2 presented antiviral properties against Herpes simplex virus type 1, strain Vic, (HSV-1). Thus, for the first time, our results revealed that the glycosylated functional unit of molluscan and arthropodan hemocyanin display antiviral activity. No inhibitory effect on this virus could be observed for the native molecule RvH, HvH and structural subunit RvH1. The antiviral effect of glycosylated FU against the replication of HSV is established, but its antiviral mechanism is still unknown. Therefore, the further investigation of the inhibitory properties of HvH against HSV is under way.

12. V. Moshtanska, P. Dolashka-Angelova, **L. Velkova**, M. Angelova, W. Voelter, B. Atanasov. Antiviral and electrostatic analysis of *Humicola Lutea* 103 superoxide dismutase in comparison with bovine enzyme. P2.12. Bulgarian-Japanese Symposium "Genomics and Proteomics in Personalized Medicine" 19–20 March 2009, Sofia, Bulgaria.



P2.12. Antiviral and Electrostatic analysis of *Humicola Lutea* 103 superoxide dismutase in comparison with bovine enzyme

Vesela Moshtanska¹, Dolashka-Angelova P.¹, Velkova L.¹, Angelova M.², Voelter W.³, Atanasov B.¹

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There has been a considerable and continuing interest in the use of the antioxidant enzyme superoxide dismutase (SOD) in medicine over the last years, arising from its ability to reduce the deleterious effect of superoxide anion radicals ($O_2^{\bullet-}$) in the cells. The fungal strain *Humicola lutea* 110 produces a manganese (Mn)- and a copper/zinc-containing (CuZn) superoxide dismutases (HLSODs). CuZnSOD enzyme is the first identified glycosylated fungal SOD with one mole of N-acetylglucosamine, connected to the polypeptide chain. The comparative investigations of the protective effect of HLSOD and commercially available bovine SOD show that intranasal inoculation of the virus produces a damaging infection of the lungs, which is highly lethal to the animals. HLSOD impressively protected mice from mortality; survival was increased by 66%, survival time was prolonged by 5.2 days. The pathological changes in the lungs were reduced, though viral infectious titers remained high. Thus, no correlation between the direct cause (viral replication) and effect (pathological changes) was observed. The bovine SOD at a high dose of 500 U/mice protected mice from mortality to a lesser extent. The protective effect of HLSOD was comparable to that of ribavirin. We assume that the clearance of HLSOD from the serum has been delayed due to the presence of polysaccharide chain in the molecule. This could explain the different protective effects of HLSOD which is naturally glycosylated and bovine SOD which is not glycosylated.

Because protein-virus interaction is primarily manifested by long-range electrostatics, we study in details Coulomb interactions of both enzymes. Their 3D structures are very homologous, but side chains distribution (by type and site) are quite different. First of all HLSOD has most alkaline stable ($\Delta G_{el} = -17$ kcal/mol at pH 11.3) if compare to bovine enzyme, which is more stable in wide pH region (more than $\Delta G_{el} = -35$ kcal/mol at pH 3-12). HLSOD has more hydrophobic

13. И. Димитров, Л. Де Смет, **Л. Велкова**, Б. Девреезе, Ж. Ван Бимен, П. Долашка-Ангелова: Генна секвенция на структурна субединица β на хемоцианина от градински охлюв *Helix lucorum*. IX Национална конференция по химия за студенти и докторанти 18-19.05.2010, София, България.

IX Национална конференция по Химия за студенти и докторанти' 2010

**ГЕННА СЕКВЕНЦИЯ НА СТРУКТУРНА СУБЕДИНИЦА β НА
ХЕМОЦИАНИНА ОТ ГРАДИНСКИ ОХЛЮВ *HELIX LUCORUM***

**Иван Димитров, Лина Де Смет, Людмила Велкова, Барт Девреезе,
Жозеф Ван Бимен, Павлина Долашка-Ангелова**

*Българска Академия на Науките, Институт по органична химия с Център по
Фитохимия, ул. Акад. Г. Бончев, бл. 9, София 1113, e-mail: i.dimitrov7@gmail.com*

Изолирана и изследвана е структурната субединица β на хемоцианина от мекотелото, градинския охлюв *Helix lucorum*. Той, за разлика от други моллюсков хемоцианини, е представен в хемолимфата от три белтъчни изоформи - β -HvH, α_D -HvH и α_N -HvH. Определена е молекулната маса на β структурната субединица, с помощта на молекулно-ситова хроматография - 915 кДа (като димер). Същата е представена в хемолимфата, главно като хомогенни дидекамери, но има различно поведение при дисоциация и реасоциация в рН-стабилизиращ буфер, съдържащ 10 и 20 mM CaCl_2 . Клонирана и секвенирана е кДНК, кодираща целия полипептид на β структурната субединица. Тя се състои от 10 929 дв. бази, в които се включват 5'-нетранслируема област (5'UTR), съставена от 75 дв. бази, 3'-нетранслируема област (3'UTR), съставена от 555 дв. бази и отворена рамка на четене за сигнален пептид от 21 α -амино-киселини, както и полипептид от 3412 α -амино-киселини. Същият е съставен от осем отделни функционални единици, наименовани от „a” до „h” (FUs: a, b, c, d, e, f, g, h), които структурно съответстват на осемте различни функционални единици от хемоцианина от охлюва *H. pomatia* и притежаващи молекулна маса между 50 и 60 кДа, свързани помежду си, чрез кратки „линкерни” участъци. Същите са установени протеолитно, чрез анализ на N-крайната им аминокиселинна последователност, както и чрез N-крайни налагания спрямо съответстващи си функционални единици от хемоцианини от близки видове.



Вече втори ден се провежда IX Национална конференция по химия за студенти и докторанти.

ПРОГРАМА

19.05.2009 г.

Секция "БИОХИМИЯ И ФАРМАЦЕВТИЧНА ХИМИЯ"

8:30 - 8:50

Христо Н. Колев, Светла Петрова, Васил Атанасов, Мариана Митева

Софийски университет "Св. Кл. Охридски", Химически факултет

Флуориметричен метод за определяне на фосфолипазна активност

8:50 - 9:10

Младен Топалов, Ива Тодорова, Спиро Константинов, Стоян Парушев, Даниела Бътовска
Медицински университет София, Фармацевтичен факултет

Синтез и ин витро изследване цитотоксичната активност на серия халкони върху туморно-клетъчни популации IX Национална конференция по химия за студенти и докторанти 2010

НАУЧНА СЕСИЯ

9:10 - 9:30

Зорка Беджова, Георги Йорданов, Цецо Душкин

Софийски Университет "Св. Климент Охридски", Химически факултет

Вграждане на епирубин в наночастици от полиалкилцианоакрилати чрез нанопреципитация

9:30 - 9:50

Иван Димитров, Лина Де Смет, Людмила Велкова, Барт Девреезе, Жозеф Ван Бимен, Паулина Долашка-Ангелова

Българска Академия на Науките, Институт по органична химия, Център по Фитохимия

Генна секвенция на структурна субединица β на хемоцианина от градинскиохлюв *helix lucorum*

Секция "ОРГАНИЧНА ХИМИЯ"

9:50 - 10:10

Елеонора Илиева, Невена Петкова, Божидарка Иванова, Росица Николова

Софийски Университет "Св. Климент Охридски", Химически Факултет

Реакция на Нуклеофилно присъединяване на нитрометан към диетиловия естер на кумарин-3-фосфоновата киселина

10:10 - 10:30

Иван Свиляров, М. Богданов

Софийски Университет "Св. Климент Охридски", Химически факултет

Синтез на полизаместени цис-/транс-3,4-дихидроизокумарин-4-карбоксилни киселини



Снимка: <http://ase.tufts.edu>

14. O. S. Boyanova, P. Dolashka - Angelova, L. Yosifova, S. Zaharieva, **L. Velkova**, I. Dimitrov, D. Toncheva; P06.015 Testing the antitumor effect of hemocyanins on genomics and proteomics level European Human Genetics Conference 2010 Gothenburg, Sweden June 12 - 15, 2010.

Title: P06.015 - Testing the antitumor effect of hemocyanins on genomics and proteomics level

Keywords: bladder cancer; hemocyanins

Authors: **O. S. Boyanova**¹, **P. Dolashka-Angelova**², L. Yosifova³, S. Zaharieva³, L. Velkova², I. Dimitrov², D. Toncheva¹;

¹Department of Medical Genetics, Medical University- Sofia, Sofia, Bulgaria,

²Institute of Organic Chemistry with Centre of Phitochemistry, BAS, Sofia, Bulgaria, ³Institute of Experimental Pathology and Parasitology - Bulgarian Academy of Sciences, Sofia, Bulgaria.

Abstract Body: Urinary bladder cancer is the 7-th most common cancer worldwide and is a socially significant healthcare problem. Risk factors such as DNA mutation and environmental factors: tobacco smoking, infection by *Schistosoma haematobium*, diet and the aging of the world population may be responsible for its pathogenesis. One of the approaches for bladder cancer therapy is the use of Keyhole limpet hemocyanins (KLH). The hemocyanins are copper-containing respiratory proteins and serve as oxygen-carriers in the blood of some arthropods and mollusks. Our laboratory investigates hemocyanins and their derivatives from the species: *Helix lucorum* (HlH) and *Rapana venosa* (RvH) and their clinical use. The aim of our study is to examin their effect on genetic level against bladder cancer.

Three different hemocyanin isopolypeptides, named β -HlH, α D-HlH and α N-HlH, were isolated from the hemolymph of the *Helix lucorum* and were identified by their N-terminal sequences and molecular masses. The molecular masses were determined by PAGE and size exclusion chromatography. The mass of the β -HlH was found to be 1068 kDa and the masses of α D-HlH and α N-Hc - 1079 kDa. The anti-tumour effects were investigated on 647-V, T-24 and CAL-29 bladder tumor Gene expression profiling and Microarray CGH analyses of the tumor cells before and after haemocyanins treatment, and studies on the gene sequences of the haemocyanins isoformes will be disscused.

The European Society of Human Genetics 2010

15. L. Yossifova, I. Iliev, E. Gardeva, P. Dolashka-Angelova, V. Moshtanska, **L. Velkova**, S. Zacharieva. Research on the protective properties of haemocyanin from *Helix vulgaris* (HvH) and its derivatives against infection with *Trichinella spiralis*. P45. British Society for Parasitology Spring Meeting and Trypanosomiasis & Leishmaniasis Seminar Cardiff University, March 29th–April 1st 2010, Wales, UK.

P45 Research on the protective properties of haemocyanin from *Helix vulgaris* (HvH) and its derivatives against infection with *Trichinella spiralis*

Liliya Yossifova¹, Ivan Iliev¹, Elena Gardeva¹, **Pavlina Dolashka-Angelova²**, Vesela Moshtanska², Lyudmila Velkova² and Siya Zacharieva¹

¹Institute of Experimental Pathology and Parasitology – BAS, Sofia, Bulgaria

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We studied the effects of application of hemocyanins, derived from *Helix vulgaris* (garden snail) on experimental infection with *Trichinella spiralis* in laboratory animals. For this purpose we used white Wistar rats (age 30 days, weight 120g), which were immunized with hemocyanin alone or conjugated with total larval antigen according to immunization protocol. Rats immunized 20 days p.i. exhibited very low inhibition of muscle larval burden. Animals immunized once with the conjugate showed a significant reduction of larval burden up to 89%. After therapeutic application with the conjugate (single immunization, p.i.), pathomorphological alterations in *Trichinella* larvae were observed – i.e. a progressive destruction of capsule wall and resorption, which allowed the penetration of inflammatory cells. The matrix was reduced and altered and the capsule was infiltrated with cellular debris. This process ends in total destruction of larvae and their entire lysis. Our results showed low protective effects but strong therapeutic potential of HvH and its conjugates. Results obtained elucidate some function of hemocyanins as good carrier molecules and activators of the immune system and define their properties as feasible preparations in the therapy of parasitic zoonoses.



**British Society for Parasitology
Spring Meeting and
Trypanosomiasis & Leishmaniasis Seminar**



Cardiff University, Wales, UK March 29th – April 1st

BSP 2010



Evaluation of COST program
Dissemination of EuroKUP EV and other future plans

16. **L. Velkova**, A. Dolashki, S. Shishkov, P. Dolashka - Angelova. Complete oligosaccharide structure of *Rapana venosa* hemocyanin. P-77. VIII Chemistry Conference, 18-19 June 2010 r., Koprivshitsa, Bulgaria.

8th Chemistry Conference Koprivshitsa 18-19 June 2010

P-77

COMPLETE OLIGOSACCHARIDE STRUCTURE OF *RAPANA VENOSA* HEMOCYANIN

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Hemocyanins act as oxygen-transporting glycoproteins in many arthropod and mollusc species. There are large differences in their molecular masses, structure and monosaccharide composition.

Molluscan hemocyanins (Hcs) have recently received particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and highly specific monosaccharide composition. Now we analysed the oligosaccharides and the carbohydrate linkage sites of the *Rapana venosa* hemocyanin (RvH).

The oligosaccharide structures of the structural subunits RvH1 and RvH2 of *R. venosa* hemocyanin (RvH) were studied by sequence analysis of glycans using MALDI-TOF-MS and tandem mass spectrometry on a Q-Trap mass spectrometer after enzymatical liberation of the N-glycans from the polypeptides. Our study revealed a highly heterogeneous mixture of glycans of the compositions Hex₀₋₉ HexNAc₂₋₄ Hex₀₋₃ Pent₀₋₃ Fuc₀₋₃. A novel type of N-glycan, with an internal fucose residue connecting one GalNAc(β1-2) and one hexuronic acid, was detected, as also occurs in subunit RvH1. A glycan with the same structure but with two deoxyhexose residues was observed as a double charged ion. Several site-occupancies were subsequently elucidated by precursor ion scanning of the intact glycopeptides using the Q-trap-LC/MS and Nanoflow-ESI mass spectrometry.

Following reports on antiviral properties from arthropod hemocyanin, we conducted a study of antiviral effects of the native molecules of RvH and of *Helix lukorum* hemocyanin (HlH), of their structural subunits, and of the glycosylated functional unit (Fu) RvH2-e and the non-glycosylated unit RvH2-c on HSV virus type 1. Only glycosylated Fu RvH2-e exhibits this antiviral activity. The carbohydrate chains of the Fu are likely to interact with specific regions of glycoproteins of HSV, through van der Waals interactions in general or with certain amino acid residues in particular. Several clusters of these residues can be identified on the surface of RvH2-e.

Acknowledgement: This work was supported by a research grant by the Bulgarian National Science Fund TK01-496/2009 and UV-L-301, DAAD -17/2007 and DFG-01/2008 (Germany) and the Bulgaria Academy of Sciences (BAS).

17. P. Dolashka, **L. Velkova**, A. Dolashki, F. Zal, L. Francois, S. Stevanovic, W. Voelter L. Molin, P. Traldi, B. Salvato. Oligosaccharide structures of hemocyanins determined by mass spectrometry. Lecture, W2. 29th Informal Meeting on Mass Spectrometry, 15-19 May 2011, Fiera di Primiero, Italy.

29th IMMS

W2 OLIGOSACCHARIDE STRUCTURES OF HEMOCYANINS DETERMINED BY MASS SPECTROMETRY

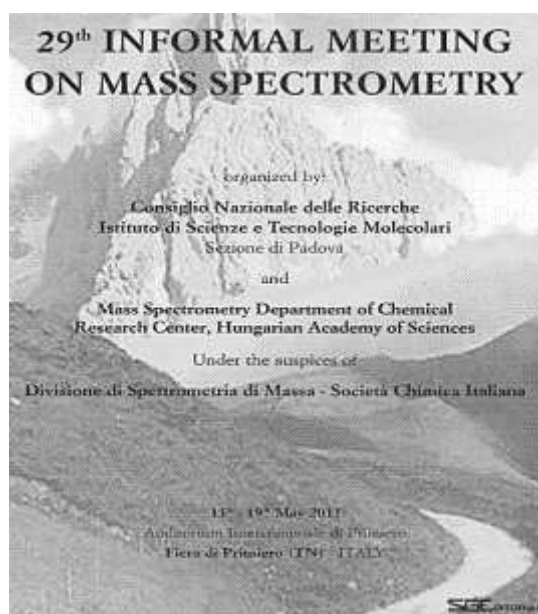
Pavlina Dolashka⁽¹⁾, Lyudmila Velkova⁽¹⁾, Aleksander Dolashki⁽¹⁾, Franck Zal⁽²⁾, Lallier François⁽²⁾, Stefan Stevanovic⁽²⁾, Wolfgang Voelter⁽³⁾, Laura Molin⁽⁴⁾, Pietro Traldi⁽¹⁾, Benedetto Salvato⁽⁵⁾

⁽¹⁾IOCCP-BAS, Sofia, Bulgaria, ⁽²⁾UPMC-CNRS UMR 7144, Roscoff, France, ⁽³⁾University of Tübingen, Tuebingen, Germany, ⁽⁴⁾CNR-ISTM, Padova, Italy, ⁽⁵⁾Department of Biology, University of Padova, Padova, Italy

Molluscan hemocyanins (Hcs) are glycosylated proteins with high molecular mass and carbohydrate content about 7-9%. Molecular masses of the native and dissociated molecules of molluscan *Octopus vulgaris* (OvH), *Sepia officinalis* (SoH), *Rapana venosa* (RvH) and *Helix lucorum* (HlH), as well as arthropodan *Carcinus aestuarii* (CaH) hemocyanins were determined to be in the region of 4000 to 9000 kDa by Electrospray ionisation mass spectrometry (ESI-MS), Matrix assistant laser light spectrometry (MALLS) and Matrix assistant laser desorption mass spectrometry (MALDI-MS) analyses. The differences of the masses measured by mass spectrometry and calculated by their gene sequences represent the carbohydrate content of Hcs.

Two approaches were applied and the oligosaccharide structures of several Hcs were proposed based on both, sequence analysis of the glycopeptides and the glycans by MALDI-TOF-MS, CE-MS μ LC/ESI-MS/MS and LC-MS-Q-Trap.

The oligosaccharide structures of the glycans were studied by preliminary sequence analysis by MALDI-TOF-MS before and after treatment with the specific exoglycosidases β 1-3,4,6-galactosidase and α 1-6(>2,3,4) fucosidase, followed by sequence analysis of Q-trap/MS/MS spectra. These analyses revealed specific differences in the oligosaccharide moieties of molluscan RvH (hexuronic acid and internal fucose), HlH (methyl-galactose), *Haliothis tuberculata* (methyl-hexosa and internal fucose), KLH (1,3 galactose linked to 1,4 GlcNAc) as well as high mannose, fucose and xylose residues. In fact, the presence of hexuronic acid residue, with a neighboring internal fucose to which also an N-acetylhexosamine is linked, stand for a novel N-glycan motif.



18. **L. Velkova**, L. Nikolaeva-Glomb, L. Mukova, A. Dolashki, P. Dolashka, A. Galabov, *Antivir. Res.*, 90, (2), A 47, Abstr. 94. Antiviral effect of molluscan haemocyanines. 24th International Conference on Antiviral Research (ICAR), May 8-11th, 2011, Sofia, Bulgaria

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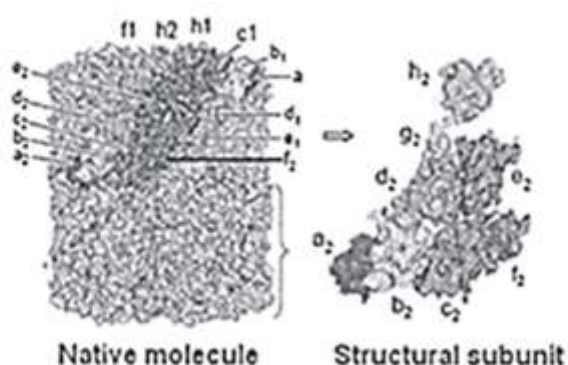
Antiviral Effect of Molluscan Haemocyanines

Lyudmila Velkova^{1,*}, Lubomira Nikolaeva-Glomb², Lucia Mukova², Aleksander Dolashki¹, Pavlina Dolashka¹, Angel S. Galabov²

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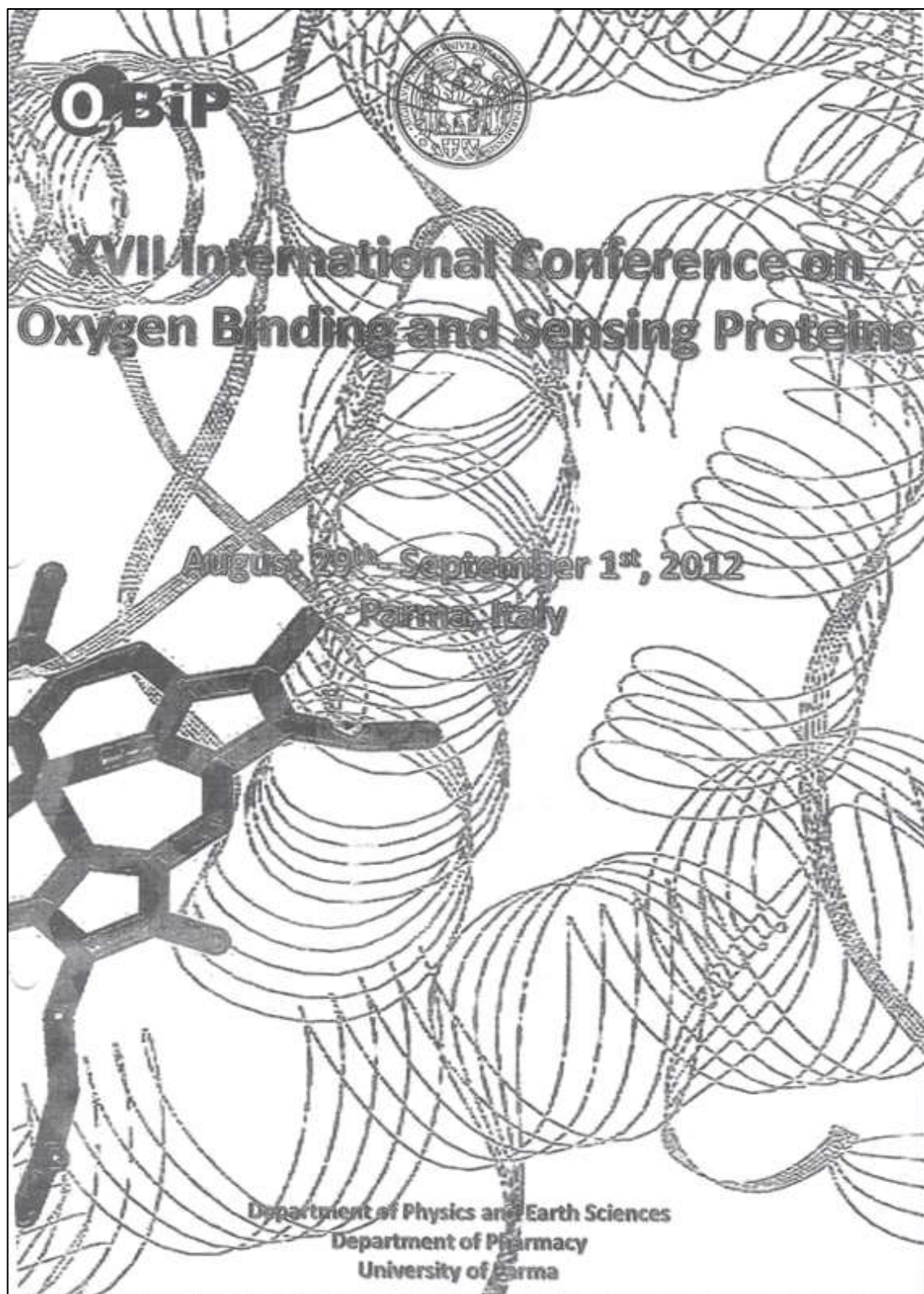
² The Stephan Angeloff Institute of Microbiology, Sofia, Bulgaria

Hemocyanins (Hcs) are oxygen-binding glycoproteins, freely dissolved in the hemolymph, of many arthropods and mollusks. The structure and oligosaccharide moieties of the molluscan Hcs *Rapana venosa* and *Helix lucorum* have been determined and recently received particular interest due to their immunostimulatory properties. Hemocyanins also have been found to show antiviral activity. In the present study the antiviral effect is tested against the *in vitro* replication of human respiratory syncytial virus (hRSV) and influenza virus A/Aichi/2/68/H3N2 by the CPE-inhibition assay. The complete molecules of Hcs do not show antiviral effect. But a marked antiviral activity of the structural subunits and the functional units is found against the replication of hRSV. Their effect against the replication of influenza A virus is weaker. The antiviral activity seems to be due to the glycosylation of the structural subunits RvH1 and RvH2 and of the functional units as well, where the carbohydrate chains are exposed on the surface of the molecule and some of the moieties can bind to viral proteins. It is assumed that the complete molecules of the hemocyanins do not possess any antiviral activity because of the fact that in this case the carbohydrate chains are buried in between the whole molecule and therefore, are unable to interact with viral proteins. The antiviral activity is present only in the case when the carbohydrate chains are exposed externally.



doi:10.1016/j.antiviral.2011.03.080

19. E. Kostadinova, P. Dolashka, S. Kaloyanova, **L. Velkova**, T. Deligeorgiev, W. Voelter, I. Petkov. Method for analysis of different oligosacchiride structures. XVII International meeting "Oxygen binding and sensing proteins", 29th August - 1st September 2012, Parma, Italy.





Method for analysis of different oligosacchiride structures

Elena Kostadinova^a, Pavlina Dolashka^{b*}, Stefka Kaloyanova^a, Ludmyla Velkova^b, Todor Deligeorgiev^a, Wolfgang Voelter^c, Ivan Petkov^a

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In this study, an improved, rapid, high yield synthesis of *N,N'*-4,4'-bis(benzyl-2-boronic acid)-bipyridinium dibromide (*o*-BBV) is described. The obtained *o*-BBV is applied in a two-component saccharide sensing system (complex) where it serves as a fluorescence quencher and a saccharide receptor. This system was applied to different natural oligosaccharides isolated from molluscan *Rapana venosa* (RvH1-a) and arthropodan *Carcinus aestuarii* (CaeH) hemocyanins (Hcs) and cyclodextrins (CDs). The carbohydrate contents of both Hcs were calculated in our previous work to be 1,6% and 7% for CaeH and RvH1-a, respectively. We propose that the difference in fluorescence increase of the native CaeH and RvH1-a when titrating them with the complex is due to the fact that the carbohydrate content of CaeH is lower and the carbohydrate chains are buried in between the structural subunits of the native molecule, while the glycans of the functional unit RvH1-a are exposed on the surface of the molecule leading to a 4-fold fluorescence's intensity change.

Acknowledgments

This work was supported by a research grant by the Bulgarian National Science Fund № BG051PO001-3.3-05/0001

20. O. Boyanova, P. Dolashka, **L. Velkova**, B. Rukova, I. Dimova, S. Hadjidekova, D.Toncheva. Expression profile of bladder cancer cell lines after treatment with potential new drug- Helix lucorum hemocyanin (HLH). European Human Genetics Conference, 23 – 26 June 2012, Nürnberg, Germany.



Expression profile of bladder cancer cell lines after treatment with potential new drug- Helix lucorum hemocyanin (HLH)

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Urinary bladder cancer is socially significant healthcare problem- negative environmental factor, infections and aging of the world population are responsible for its growing incidence. This requires elaboration of new drug development. Such a potential new drug is Helix lucorum haemocyanin (HLH) - a highly immunogenic glycoprotein with xenogenic nature and a lot of mannose residues.

The anti-tumor effect of HLH was investigated on CAL-29 and T-24 bladder tumor cell line and results suggest 35%-50% inhibition of cancer cell viability in a dose 500ug/ml of HLH after 72h of incubation.

Gene expression profiling of the tumor cells before and after HLH treatment using the panel for 84 genes for Human Inflammatory cytokines and receptors was performed.

Results presume more than 10 times upregulation of genes for inflammatory response activation, cell mediated immunity and proinflammatory cytokines: C4A, CCR4, CARD-18, IL36A, IL37, CXCR1, IL9, LTA, MIF and TNF in CAL-29 cell line treated with HLH (G1). In the same group more than 4 time overexpression of TOLLIP gene was achieved.

In T-24 cell line treated with HLH (G2): IFNA2, IL37, CXCR1, IL36B, IL5 and AIMP1 genes were significantly overexpressed. In the both group CXCR1 gene (for IL8 receptor) had the highest expression.

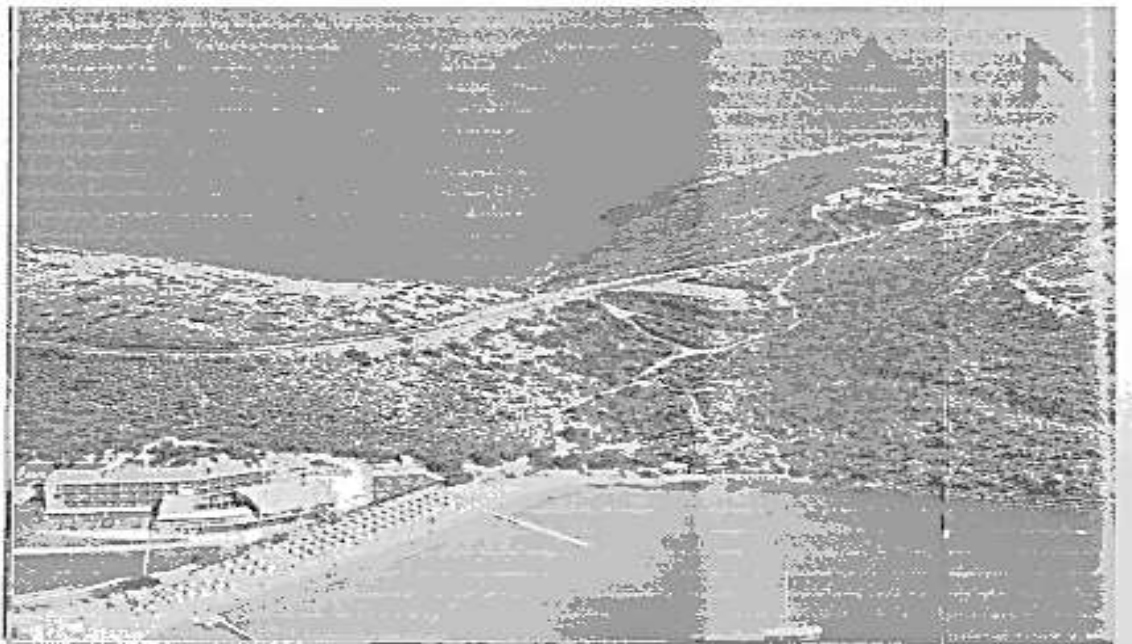
Gene for CXCL1 was notably downregulated compare to ABCF1, BCL6, IL1A, IL1B in both groups.

Our results suggest that HLH can perform both: growth inhibiting effect (on cell line level) and immune response activation (according gene expression profile) in bladder cancer.

Acknowledgements: Grant TK01-496/2009 Bulgarian National Science Fund, Erasmus, DAAD.

21. O. Boyanova, P. Dolashka, **L. Velkova**, B. Rukova, D. Toncheva: Presenting the results of experiments with *Helix lucorum* hemocyanin (HIH)-potential new agent in bladder cancer treatment. 8th Management Committee and Working Group meeting of the COST Action Kidney and Urine Proteomics EuroKUP, 29.03-1.04.2012, Sounion, Greece.

**8th Management Committee
and Working Group meeting
of the COST Action
Kidney and Urine Proteomics
EuroKUP (BM 0702)**



**Aegeon Hotel, Sounion, Greece
29 March – 1 April 2012**

Presenting the results of experiments with *Helix lucorum* hemocyanin (HIH)-potential new agent in bladder cancer treatment

Boyanova O.¹, Dolashka P.², Velkova L.², Rukova B.¹, Toncheva D.¹

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Searching of new therapeutic approaches to fight bladder cancer is essential because of relapses and growing incidence of the disease due to aging of the world population and unfavorable environmental factors.

Such a potential new drug is *Helix lucorum* haemocyanin (HIH) - glycoprotein with high molecular mass, xenogenic nature and a lot of mannose residues.

The antiproliferative effect of HIH have been investigated on CAL-29 and T-24 bladder tumor cell line and results suggest 35%-50% inhibition of cancer cell viability in a dose 500ug/ml of HIH after 72h of incubation.

Gene expression profiling of the tumor cells before and after HIH treatment using the panel for 84 genes for Human Inflammatory cytokines and receptors was performed.

Results presume more than 10 times upregulation of genes for inflammatory response activation, cell mediated immunity and proinflammatory cytokines: C4A, CCR4, CARD-18, IL36A, IL37, CXCR1, IL9, LTA, MIF and TNF in CAL-29 cell line treated with HIH (G1). In the same group more than 4 time overexpression of TOLLIP gene was achieved.

In T-24 cell line treated with HIH (G2): IFNA2, IL37, CXCR1, IL36B, IL5 and AIMP1 genes were significantly overexpressed. In the both group CXCR1 gene (for IL8 receptor) had the highest expression.

Gene for CXCL1 (overexpressed in metastatic bladder cancer) was notably downregulated compare to ABCF1, BCL6, IL1A, IL1B in both groups.

Proteins from treated and untreated with HIH cell lines were also isolated for the evaluation of changes in proteomic profile.

Our results suggest that HIH can perform both: growth inhibiting effect (on cell line level) and immune response activation (according gene expression profile) in bladder cancer.

Acknowledgements: Grant TK01-496/2009 Bulgarian National Science Fund, Erasmus and DAAD.

22. **L. Velkova**, P. Dolashka, A. Dolashki, S. Stevanovic, W. Voelter, J. Van Beeumen, B. Devreese. Analysis of glycoprotein from hemolymph of *Helix lucorum* garden snail. 33rd European Peptide Symposium, 31.08-05.09.2014, Sofia, Bulgaria.

Posters P162-170: Peptide Biochemistry & Ribosomal Peptide Synthesis

P162.

EXPRESSED PROTEIN LIGATION AS A MODIFICATION TOOL FOR A NOVEL NON-ANIMAL COLLAGEN-LIKE PROTEIN

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Collagen is the most abundant structural protein as substantial part of the extracellular matrix. The protein is built of three polypeptides which super-coil to form a triple-helix. These triple-helices form bundles and fibres to form larger tissue structures.[1]

Collagen is used in a variety of medical products, but concerns about possible transfer of diseases due to extraction from animal sources call for cost-effective production of recombinant collagen.[2]An alternative approach makes use of stable collagen-like triple-helical sequences, derived from various bacteria which are disease-free and their production by fermentation provides high yields.The bacterial collagen VCL (V: globular domain, CL: collagen-like domain) examined in this work has been fermented giving yields up to 19 g/L and being biologically inert which makes it a versatile template for further modifications.[3,4]

In the current project, expressed protein ligation (EPL) is used to modify VCL to introduce new and improved functions that will enhance the properties of this collagen-like protein. EPL allows the ligation via a native amide bond which is not limited to peptide sequences but also provides the opportunity to incorporate unnatural amino acids such as β - or D-amino acids, a single fluorescent label or a functional side chain which are not accessible by classic recombinant protein expression. Structural activity of collagen as material can be studied more extensively and information can be

collected about how the material properties change when modified.

Key words: expressed protein ligation, collagen, cell-biomaterial interaction, tissue engineering

- [1] B. Brodsky, J.A.M. Ramshaw, *Matrix Biology* 15,545(1997)
- [2] A. Werkmeister, J.A.M. Ramshaw, *Biomed Mater* 7, 012002(2012)
- [3] Y. Peng et al., *Microbial Cell Factories*, 11,14,(2012)
- [4] Y. Peng et al., *Appl Microbiol Biotechnol* , 98, 1807(2014)

P163.

ANALYSIS OF GLYCOPROTEIN FROM HEMOLYMPH OF *HELIX LUCORUM* GARDEN SNAIL

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Hemolymph of garden snail *Helix lucorum* is a complex mixture of biochemically and pharmacologically active components such as proteins, peptides and glycopeptides. Hemocyanin is a copper-containing, a respiratory glycoprotein. To analyse its oligosaccharide structures, peptides and glycopeptides are isolated by reverse-phase HPLC after treatment of the hemocyanin with trypsin. The isolated fractions are analyzed by the orcinol/H₂SO₄ test. Using the nano-LC/MS/MS-Q-Trap system allowed to determine the glycosylation sites and the carbohydrate structure. The glycopeptides,

23. A. Dolashki, P. Dolashka, S. Stevanovic, L. Molin, P. Traldi, **L. Velkova**, J. Van Beeumen, B. Devreese, W. Voelter. Antimicrobial activity of peptides isolated from the hemolymph of *Helix lucorum* snails 33rd European Peptide Symposium, 31.08-05.09.2014, Sofia.

Posters P232-274: Antimicrobial Peptides, Glycopeptides and Lipopeptides

- [1] R. González, F. Albericio, O. Cascone, NB Iannucci. *J Pept Sci.*, **16**, 424 (2010)
 [2] NB. Iannucci, LM. Curto, F. Albericio, O. Cascone, JM Delfino. *Pept. Sci.*, **102**, 49 (2013)

P253.

SYNTHETIC PEPTIDES DERIVED FROM BOVINE LACTOFERRICIN: EFFECT OF L-, D-AMINO ACIDS EXCHANGE

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Bovine Lactoferricin (LfcinB) is a peptide with demonstrated antimicrobial activity. This activity is directly related with its structure. Structure-activity studies have established two main requirements for its antimicrobial activity: a cationic charge and an amphipathic conformation [1,2].

In this study we have evaluated the effect of the replacement of L by D-amino acids, on the secondary structure peptide and its antimicrobial activity. As model, a sequence derived from LfcinB was selected (⁴RRWQWRMKKLG¹⁴). Designed peptides were synthesized, purified, and characterized using RP-HPLC, MALDI-TOF MS and Circular Dichroism (DC). For each peptide, the antibacterial activity against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 was evaluated. RP-HPLC analysis showed that D-amino acids in sequence peptide, induce a variation in retention times with respect to L-amino acids analogue, indicating differences in the physicochemical behavior of the peptides. Furthermore, it was possible to identify, by DC, changes in secondary structural elements, which depends on type and position of D-amino acid. Finally, inclusion of D-amino acid residues in the peptide sequence had minor influence on the

antimicrobial activity against both studied strains (MIC~130 µM).

The results underlined the importance of secondary structural elements and suggest that an appropriate position of D-amino acid residues might be affecting the antimicrobial activity.

Keywords: Peptides, Bovine Lactoferricin, D-Amino Acids, Secondary Structure.

- [1] D Chan. *Biochimica et Biophysica Acta*, **1758**, 1184 (2006)
 [2] D. Chapple. *Infection and Immunity*, **66**(6), 2434 (1998)

P254.

ANTIMICROBIAL ACTIVITY OF PEPTIDES ISOLATED FROM THE HEMOLYMPH OF *HELIX LUCORUM* SNAILS

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The recent appearance of a growing number of bacteria resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph of *Helix lucorum* garden snail is a complex mixture of biochemically and pharmacologically active

24. P. Dolashka, A. Dolashki, **L. Velkova**, S. Stevanovic, L. Molin, P. Traldi R. Velikova, W. Voelter. Bioactive compounds isolated from garden snails. Втора Национална младежка конференция „Биологически науки за по-добро бъдеще” 30-31.10.2015, Пловдив.

„БИОЛОГИЧЕСКИ НАУКИ ЗА ПО-ДОБРО БЪДЕЩЕ”, 2015 г.



Секция: Биотехнологии, Бионанотехнологии и Приложна биология



Bioactive Compounds Isolated from Garden Snails

Dolashka P.^{1*}, Dolashki A.¹, Velkova L.¹, Stevanovic S.², Molin L.³, Traldi P.³, Beeumen J.⁴, Devreese B.⁴, Voelter W.⁵

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Abstract. The recent appearance of a growing number of resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph and mucus of *Helix lucorum* and *Helix aspersa* garden snails are a complex mixture of biochemically and pharmacologically active components.

Glycoprotein 'hemocyanin' and antimicrobial peptides from the hemolymph and mucus are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens.

The in vitro antitumor activity of *Helix hemocyanin* and its isoforms with different oligosaccharide structures was established on the bladder carcinoma permanent cell lines Cal-29, T-24. This is probably due to the specific oligosaccharide structures of hemocyanins which are exposed on the surface of the molecule.

25. P. Dolashka, A. Dolashki, **L. Velkova**, S. Stevanovic, L. Molin, P. Traldi R. Velikova, W. Voelter. Poster P80. Bioactive compounds isolated from garden snails. Втора Национална младежка конференция „Биологически науки за по-добро бъдеще“ 30-31.10.2015, Пловдив.



„БИОЛОГИЧЕСКИ НАУКИ ЗА ПО-ДОБРО БЪДЕЩЕ“, 2015 г.

P80

Bioactive Compounds Isolated from Garden Snails

Dolashka P.^{1*}, Dolashki A.¹, Velkova L.¹, Stevanovic S.², Molin L.³, Traldi P.³, Beeumen J.⁴, Devreese B.⁴, Voelter W.⁵

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Abstract. The recent appearance of a growing number of resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph and mucus of *Helix lucorum* and *Helix aspersa* garden snails are a complex mixture of biochemically and pharmacologically active components.

Glycoprotein 'hemocyanin' and antimicrobial peptides from the hemolymph and mucus are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens.

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26. **L. Velkova**, P. Dolashka-Angelova, A. Dolashki, B. Devreese, J. Van Beeumen. Carbohydrate structures of molluscan hemocyanins from snails *Helix lucorum* and *Rapana venosa*, determined by mass spectrometry. (Lecture) Втора Национална младежка конференция „Биологически науки за по-добро бъдеще” 30-31.10.2015, Пловдив.



„БИОЛОГИЧЕСКИ НАУКИ ЗА ПО-ДОБРО БЪДЕЩЕ“, 2015 г.

*Carbohydrate Structures of Molluscan Hemocyanins from Snails *Helix lucorum* and *Rapana venosa*, Determined by Mass Spectrometry*

Velkova L.¹, Dolashka-Angelova P.¹, Devreese B.²,
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Abstract. Hemocyanins (Hcs) are copper-containing respiratory glycoproteins with quaternary structure, freely dissolved in the hemolymph of several arthropods and molluscs. The carbohydrate structure of molluscan Hcs has recently received particular interest due to their immunostimulatory properties, antitumor and antiviral activity. This is mainly related to their high carbohydrate content and specific monosaccharide composition. We have determined and analysed oligosaccharide structures of molluscan hemocyanins, isolated from the hemolymph of Bulgarian garden snails *Helix lucorum* and of marine snail *Rapana venosa*. The oligosaccharide structures of the structural subunits of *Rapana venosa* hemocyanin (RvH) and the isoform β c-*Helix lucorum* hemocyanin (β c-HIH) were studied by sequence analysis of N-glycans using MALDI-TOF-MS and tandem mass spectrometry on a Q-Trap mass system, after enzymatical liberation of the N-glycans from the polypeptides. Our study revealed a highly heterogeneous mixture of N-glycans with compositions Hex3-7HexNAc2-5MeHex0-4Pent0-1Fuc0-1 and Hex0-9HexNAc2-4MeHex0-1Pent0-2Fuc0-3, in the isoform β -HIH and subunits of RvH, respectively. Identified glycans of β -HIH have predominantly monoantennary and diantennary structure with complex type and hybrid type. As in most molluscan hemocyanins, the glycans of β -HIH contain mainly a terminal MeHex residue, in some cases even two, three and four. Several carbohydrate chains in the HIH we analyzed are core-fucosylated, and also possess a high degree of methylation. Identified glycans in RvH have mainly

27. **L. Velkova**, P. Dolashka-Angelova, A. Dolashki, B. Devreese, J. Van Beeumen. Carbohydrate structures of the β c-HIH structural subunit of *Helix lucorum* hemocyanin. P100. Втора Национална младежка конференция „Биологически науки за по-добро бъдеще“ 30-31.10.2015, Пловдив.



„БИОЛОГИЧЕСКИ НАУКИ ЗА ПО-ДОБРО БЪДЕЩЕ“, 2015 г.

P100

Carbohydrate structure of the β c-HIH structural subunit of *Helix lucorum* hemocyanin

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Devreese B.², Van Beeumen J.²*

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Abstract. Hemocyanins (Hcs) are copper-containing respiratory glycoproteins with quaternary structure localized in the hemolymph of several arthropods and molluscs. Molluscan Hcs usually have are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition. We analyzed the oligosaccharides and the carbohydrate linkage sites of the structural subunit β c-Helix lucorum hemocyanin (β c-HIH) isolated form hemolymph of garden snail *Helix lucorum* using tandem mass spectrometry.

Monosaccharide sequencing and determination of the configurations of N-glycans released from β c-HIH after PNGase F treatment was performed by Q-Trap mass spectrometry. The carbohydrate structures of β c-HIH contained various larger N-glycans with a number of methylated sugars, which could be identified as 3-O-methyl-mannose and 3-O-methylgalactose, in addition Fuc and/or Xyl residues were found in polysaccharides. In total 26 glycans, were identified as a highly heterogeneous mixture with compositions Hex3-7HexNAc2-5 MeHex0-4 Pent0-1Fuc 0-1. The primary structures of N-glycans from high mannose, complex- and hybrid type have been determined.

Acknowledgements. This work was supported by Bulgarian Ministry of Education, projects DHRC/01/6 and Youth and Science, DMU 03/26, Bulgaria and co-financed by FWO – VS.025.12N, Belgium and DFG-STE 1819/5-1/2012 – Germany

28. **L. Velkova**, P. Dolashka. Identification of glycosylated sites in β c-structural subunit of *Helix lucorum* hemocyanin (β c-HIH) using analysis of glycopeptides by mass spectrometry.(Poster, P 55). 7-th Bulgarian Peptide Symposium – 2016 with international participation. 10-12.06.2016, Blagoevgrad, Bulgaria.

7th Bulgarian Peptide Symposium
JUNE 10 - 12, 2016, BLAGOEVGRAD, BULGARIA

7th Bulgarian Peptide Symposium – 2016 with international participation is organized by the Bulgarian Peptide Society and South-West University "Neofit Rilski", Blagoevgrad, Bulgaria

The Symposium is organized under the auspices of European Peptide Society and is dedicated of the

40th Anniversary of South-West University "Neofit Rilski"

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P 55

Identification of glycosylated sites in β c-structural subunit of *Helix lucorum* hemocyanin (β c-HIH) using analysis of glycopeptides by mass spectrometry

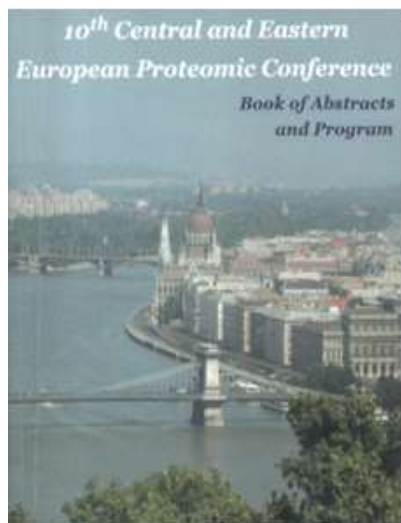
L. Velkova, P. Dolashka

Hemocyanins (Hcs) are large, oligomeric blue coppercontaining glycoproteins that play a role as dioxygen carriers in the hemolymph of most molluscs and several arthropods. Molluscan Hcs usually have powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition. Although that β c-structural subunit of *Helix lucorum* hemocyanin (β c-HIH) manifested immunological potential and antitumor activity, its oligosaccharide structure is still incomplete.

Glycopeptides were isolated from the structural subunit β c-HIH after overnight digestion with trypsin and the resulting peptides were subjected to reversed phase by high-performance liquid chromatography (RP-HPLC). Fractions testing positive in the orcinol/H₂SO₄ glycosylation test were lyophilized and further studied by tandem mass spectrometry, using a Q-Trap LC/MS/MS system.

Glycopeptides, selectively detected in a proteolytic fraction by the appearance of collisionally induced marker oxonium ions such as m/z 163 [Hex+H]⁺, 204 [HexNAc+H]⁺, or 366 [HexHexNAc+H]⁺, were sequenced. Additional carbohydrate structures were analyzed in the same way and authentic binding sites could be revealed. We analyzed a number of glycopeptides by Q-Trap-LC/MS/MS, identified the sugar chains and peptide sequences and confirmed six N-glycosylation sites from gene sequence of structural subunit β c-HIH.

29. P. Dolashka, A. Dolashki, O. Antonowa, **L. Velkova**, R. Velikova, J. Van Beeumen, S. Stevanovic, W. Voelter, H.-G. Rammensee, B. Devreese. Proteomic analysis of antitumor activity of molluscan hemocyanin. 10th Central and Eastern European Proteomic Conference (11-15.10.2016) Budapest, Hungary.



FrOr02

10th CEEPC 2016

PROTEOMIC ANALYSES OF ANTITUMOR ACTIVITY OF MOLLUSCAN HEMOCYANINS

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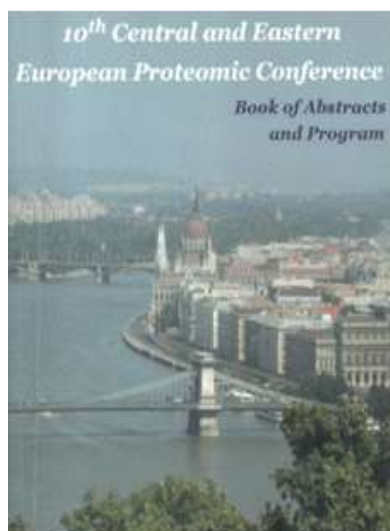
Bladder cancers are wide-spread in the world with a high morbidity rate and considerable social and economic implications. Therefore, the development of novel agents with mechanisms of action is a persisting issue. Hemocyanins, the huge oxygen-transporting glycoproteins of some mollusks, are used as immunomodulatory proteins with proven anti-cancer properties.

In our previous studies 'hemocyanins' glycoproteins, isolated from the hemolymph of molluscan brown garden snail *Helix lucorum* (HHH) and marine snail *Rapana venosa* (RvH) were analysed in comparison to the hemocyanin from keyhole limpet hemocyanin *Megatura crenulata* (KLH). The antitumoral effect of the mentioned hemocyanins and its isoforms were studied *in vitro* on the bladder cancer permanent cell HT-1197, CAL-29, T-24, and the normal urothelial cell line HL 10/29 (1). We demonstrated that the total molecule of HHH has a superior growth inhibiting effect than KLH and RvH on CAL-29 and T-24 bladder cancer cells and the mechanism of antitumor activity of the tested hemocyanins includes the induction apoptosis. Gene expression data showed upregulation of genes involved in the apoptosis as well as of the immune system activation and downregulation of genes associated with response to angiogenesis (2).

Inhibition of Cal-29 tumor cell growth was observed after incubation with native hemocyanin of *H. lucorum* hemocyanin. The effect and mechanism of action of active hemocyanins were studied at the level of their molecular organization, amino acid sequence, and carbohydrate structure using several new methods and techniques. In this study, the antitumor activities of HHH were investigated before and after treatment with hemocyanin by changes in protein expression of T-24 and CAL-29 bladder cancer cell lines. Since KLH is used increasingly in biomedicine as a carrier for vaccines and immunotherapeutic agent for bladder transitional cell carcinoma was used as a control.

Proteomic profiling of bladder carcinoma permanent cell lines T-24 and CAL-29, treated with HHH, show different protein expression by two-dimensional (2D) gel electrophoresis. 2D profiles of protein expression before treatment were compared to those after treatment with HHH and the different spots were analysed by MALDI-TOF-TOF. Mass spectrometry analysis

30. **L. Velkova**, P. Dolashka, B.Lieb, J. Van Beeumen, B.Devreese Glycan structures of the structural subunit hth2 of *haliotis tuberculata* hemoc. 10th Central and Eastern European Proteomic Conference. 11-15.10.2016, Budapest, Hungary.



10th CEEPC 2016

ThPo03

GLYCAN STRUCTURES OF THE STRUCTURAL SUBUNIT HTH2 OF HALIOTIS TUBERCULATA HEMOCYANIN, DETECTED BY MASS SPECTROMETRY

L. Velkova¹⁾, P. Dolashka¹⁾, B. Lieb²⁾, J. Van Beeumen³⁾ and Bart Devreese³⁾

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Hemocyanins (Hcs) are copper-containing respiratory glycoproteins with extremely high molecular weight and complex quaternary structure, dissolved in hemolymph of different species of molluscs and arthropods. Molluscan hemocyanins have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and specific monosaccharide composition.

To identify the carbohydrate structure of Hth2, the glycans were removed after digestion with the specific glycosidase PNGase F. Two approaches were applied to analyse the isolated glycans. The first approach based on MALDI-TOF-MS before and after treatment with the specific exoglycosidases β 1-3,4,6-galactosidase and α 1-6(\rightarrow 2,3,4) fucosidase and analysis of the fragments via MS giving only preliminary results about the structures of the glycans. Therefore, the second approach, tandem mass spectrometry was applied, and the glycan structure being derived from their MS/MS spectra, obtained on a hybrid quadrupole - linear ion trap mass spectrometer.

In total, 24 glycans were identified as a highly heterogeneous group of structures with compositions MeHex0-2HexNAc2-4Hex2-5Pent0-1Fuc0-2. The novel structural motif for Hcs: MeHex[Fuc(α 1-3)-]GlcNAc, including thus MeHex and (α 1-3)-Fuc residues being linked to an internal GlcNAc residue, was detected, as also occurs in subunit Hth1. As a particular feature, we finally detected 6 xylose-containing structures in Hth2, but none of these types of glycans has been found in Hth1, so far. The oligosaccharide chains are mostly modified at the inner core by β 1,2-linked xylose to the β -mannose, by α -fucosylation (mainly α 1,6-) of the innermost GlcNAc residue and via methylation.

The oligosaccharide moieties found in Hth2 reveal a complex N-glycan pattern combining typical structural features of different higher organisms. Our results may be considered as basic information for further investigations on *Haliotis tuberculata* hemocyanin, as a potential source of novel N-glycans that are important for the stimulation of the immune response and/or for the production of antibodies used in diagnosis and therapy.

31. P. Dolashka, A. Dolashki, **L. Velkova**, S. Stevanovic, L. Molin, P. Traldi R. Velikova, W. Voelter. (Lecture BO3). Bioactive compounds isolated from garden snails. XIth работна среща с международно електронно участие “Биологична активност на метали, синтетични съединения и природни продукти” 14-16.12.2016 г.

На **14-16 декември 2016** г. в Института по експериментална морфология, патология и антропология с музей при Българска академия на науките ще се проведе:

ЕДИНАДЕСЕТА РАБОТНА СРЕЩА
с международно електронно участие

**“БИОЛОГИЧНА АКТИВНОСТ НА МЕТАЛИ,
СИНТЕТИЧНИ СЪЕДИНЕНИЯ И
ПРИРОДНИ ПРОДУКТИ”**



Главен организатор: Институт по експериментална морфология, патология и антропология с музей

Bioactive compounds isolated from garden snails

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ABSTRACT

The recent appearance of a growing number of resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph and mucus of *Helix lucorum* and *Helix aspersa* garden snails and *Rapana venosa* marine snail are a complex mixture of biochemically and pharmacologically active components.

Glycoprotein 'hemocyanin' and antimicrobial peptides from the hemolymph and mucus are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens.

The *in vitro* antitumor activity of *Helix* and *Rapana* hemocyanins and their isoforms with different oligosaccharide structures was established on the bladder carcinoma permanent cell lines T-24. This is probably due to the specific oligosaccharide structures of hemocyanins which are exposed on the surface of the molecule.

Key words: Antibacterial activity, Antitumore activity, Hemocyanins, *Helix lucorum*, *Helix aspersa*, peptides.

32. **L. Velkova**, A. Dolashki, P. Dolashka. (Lecture BO4). N- Linked carbohydrate structures of molluscan hemocyanins from snails. XIth работна среща с международно участие “Биологична активност на метали, синтетични съединения и природни продукти” 14-16.12.2016 г.

На **14-16 декември 2016** г. в Института по експериментална морфология, патология и антропология с музей при Българска академия на науките ще се проведе:

ЕДИНАДЕСЕТА РАБОТНА СРЕЩА
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**“БИОЛОГИЧНА АКТИВНОСТ НА МЕТАЛИ,
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ПРИРОДНИ ПРОДУКТИ”**



Главен организатор: Институт по експериментална морфология, патология и антропология с музей

N- Linked carbohydrate structures of molluscan hemocyanins from snails

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Abstract

Molluscan hemocyanins (Hcs) have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and specific monosaccharide composition. We present comparative studies in oligosaccharide structures of structural subunits from *Rapana venosa* (RvH), *Haliotis tuberculata* hemocyanin and *Helix lucorum* hemocyanin by mass spectrometry.

Two approaches were applied to analyse the isolated glycans. The first approach included sequencing of the glycans by specific glycosidases and analysis of the fragments via MALDI-TOF-MS before and after treatment with the enzymes giving only preliminary results about the structures of the glycans. Therefore, the second approach, tandem mass spectrometry was applied, and the glycan structure being derived from their MS/MS spectra, obtained by tandem mass spectrometry, on a hybrid quadrupole-linear ion trap mass spectrometer - ESI-Q-Trap system.

The characterization of the N-linked glycans found in HlH, RvH and HtH, in this study, is revealed in part novel structural motifs which might contribute to the pronounced immunogenicity of this gastropod glycoprotein. It is obvious that gastropods have a wide capacity to modify the basic biantennary N-glycan structure with many species-specific peculiar structures. The oligosaccharide moieties found in HlH, RvH and HtH are a potential source of novel N-glycans that are important for the stimulation of the immune response and/or for the production of antibodies used in diagnosis and therapy.

Keywords: molluscan hemocyanins (Hcs), *Rapana venosa* (RvH), *Haliotis tuberculata* hemocyanin (HtH), *Helix lucorum* hemocyanin (HlH), structural subunit; mass spectrometry; ESI-Q-Trap; N-glycans.

33. A. Dolashki, P. Dolashka, A. Stenzl, S. Stevanovic, W.K. Aicher, **L. Velkova**, R. Velikova, W. Voelter. Poster Proteomics analysis of antitumor activity of Helix and Rapana hemocyanins. First Balkan Conference on Personalized Medicine 26-27.10.2017, Sofia, Bulgaria.



Proteomics analysis of antitumoractivity of Helix and Rapana hemocyanins

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Hemocyanins (Hcs) are copper-containing glycoproteins that act as oxygen transporting proteins in many arthropods and mollusk species. Hemocyanins from the molluscs *Helix aspersa* (HaH), *Helix lucorum* (HIH) and *Rapana venosa* (RvH) exhibiting different oligosaccharide structures have been investigated for potential use in therapy of bladder cancer permanent cells. In vitro studies on the antitumor activities of these proteins were performed in T-24 cells and compared to doxorubicin and mitomycin-C. Control experiments were performed using normal urothelial HL 10/29 cells. The obtained results show that the human tumor T24 cell lines are sensitive to the action of the tested hemocyanins and their isoforms. The inhibition of the tumor cell growth was dose and time dependent and was observed after incubation with native HaH and HIH and FUs β c-HIH-h and RvH-c. Cells treated with both FUs, β c-HIH-h and RvH-e, showed apoptotic and necrotic cells and this inhibition was stronger than the effect measured for doxorubicin treated cells. No growth inhibition of the normal urothelial cell line HL 10/29 was observed after treatment with HIH, HaH, RvH and their isoforms. The impact of hemocyanins on tumor cells was investigated by 2D-gel PAGE and several proteins showed indeed altered abundancies. The most effective inhibition of tumor cells is probably caused by a specific novel and unusual N-glycan oligosaccharide structure on HIH with methylated hexoses, an internal fucose residue connecting one GalNAc(β 1-2) and one hexuronic acid.

34. R. Velikova, **L. Velkova**, A. Dolashki, P. Dolashka. Structure and conformational stability of hemocyanin from garden snail *Cornu aspersum*. (Lecture) XV Национална конференция с международно участие „Природни науки 2017“ (NCNS2017) 29.09.2017 - 01.10.2017, Варна, България.



XV НАЦИОНАЛНА КОНФЕРЕНЦИЯ С МЕЖДУНАРОДНО УЧАСТИЕ

“ПРИРОДНИ НАУКИ '2017”

СЕКЦИЯ

БИОРАЗНООБРАЗИЯ, БИОМЕДИЦИНА, БИОРЕСУРСИ И ЕКОЛОГИЯ

Section

Biodiversity, Biomedicine, Bioresources and Ecology

Structure and conformational stability of hemocyanin from garden snail *Cornu aspersum*

R. Velikova, L. Velkova, A. Dolashki, P. Dolashka

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Abstract

The hemolymph of molluscan snails is a complex mixture of biochemically and pharmacologically-active components such as peptides and proteins. The various aspects of biomedical applications of molluscan hemocyanins, associated with their immunogenic properties and antitumor activity, give major importance to structural studies of these glycoproteins. Hemocyanins (Hcs) are copper-containing respiratory glycoproteins with quaternary structure localized in the hemolymph of several arthropods and molluscs. Molluscan hemocyanins are large glycoproteins, usually have a higher carbohydrate content with different structures and quantities of the oligosaccharide moieties.

The native molecule hemocyanin CaH isolated from hemolymph of garden snail *C. aspersum* is constructed from three different subunits - β c-CaH, α N-CaH and α D-CaH (MW~ 400 kDa), each of them containing eight functional units (MW~ 50kDa).

The structural and conformational stability of the CaH, β c-CaH subunit and the β c-CaH-g functional unit were analyzed by CD in wide pH-T range ($T^{\circ}\text{C}$ 20÷85°C and pH 2.5÷12). The obtained results lead to the conclusion that:

- The mechanism of thermal denaturation of CaH is of a complicated character and the process of thermal unfolding is irreversible. The relatively small changes of initial especially $[\theta]_{222}$ at high temperatures indicate that many secondary structure elements are preserved, especially at neutral pH and even at extreme high temperatures.
- The native CaH molecule is more stable compared to the β c-CaH subunit and β c-CaH-g functional unit, it was explained by the formation of quaternary structures, which introduces additional factors, namely non-ionic forces (intra-subunit, hydrophobic and hydrogen-bonded networks of carbohydrate moiety interactions). The difference in the stability of the native CaH and β c-CaH SU indicates the influence of the quaternary structure and the difference in the stability of β c-CaH and β c-CaH-g Fu shows the influence of the tertiary structure.
- The carbohydrate structure influences the stability of the native molecule CaH and β c-CaH subunit, but does not affect the stability of one isolated functional unit.

Keywords: hemocyanin, β c-CaH subunit (SU), β c-CaH-g functional unit, circular dichroism (CD), stability, temperature and pH-dependent denaturation.

Acknowledgments: This work was supported by a research grant for young scientists №72-00-40-111/09.05.2017, by the Bulgarian Academy of Sciences.

35. **L. Velkova**, A. Dolashki, A. Nissimova, Y. Voinikov, R. Velikova, P. Dolashka. „Analysis of peptides and glycopeptides from garden snail *Cornu aspeprsum* by mass spectrometry“(Poster). XV Национална конференция с международно участие „Природни науки 2017“(NCNS2017) Варна, България, 29.09.2017 - 01.10.2017



XV НАЦИОНАЛНА КОНФЕРЕНЦИЯ С МЕЖДУНАРОДНО УЧАСТИЕ

“ПРИРОДНИ НАУКИ '2017”

СЕКЦИЯ

БИОРАЗНООБРАЗИЯ, БИОМЕДИЦИНА, БИОРЕСУРСИ И ЕКОЛОГИЯ

Section

Biodiversity, Biomedicine, Bioresources and Ecology

Analysis of peptides and glycopeptides from garden snail *Cornu aspeprsum* by mass spectrometry

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Abstract

The rapid increase in drug-resistant infections has presented a serious challenge to antimicrobial therapies, because it emphasizes the urgent need to develop novel antimicrobials drugs agents. One area of interest is peptides and proteins produced by invertebrates as part of their defenses system. Extract collected from hemolymph of the garden snails *C. aspersum* is a complex mixture of biochemically and pharmacologically active compounds as peptides and proteins.

After applying ultrafiltration and reverse-phase high-performance liquid chromatography (RP-HPLC) were isolated 10 different fractions, with molecular weights below 3000 Da. The precise molecular weight of the peptides was determinate by MALDI-TOF-MS. All fractions testing positive in the orcinol/H₂SO₄ glycosylation test were lyophilized and further studied by tandem mass spectrometry, using a Q-Trap LC/MS/MS system. Glycopeptides, selectively detected in a fraction by the appearance of collisionally induced marker oxonium ions such as m/z 163 [Hex+H]⁺, 204 [HexNAc+H]⁺, or 366 [Hex-HexNAc+H]⁺, were sequenced. We analyzed a 5 of glycopeptides by Q-Trap-LC/MS/MS, identified the sugar chains and peptide sequences.

Our *in vitro* study has shown that several fractions, containing peptides and glycopeptides showed antimicrobial activities against tested microorganisms including Gram-positive and Gram-negative bacteria. We suggest that these compounds therefore have the potential to become a substitute for the commonly used antibiotics against which bacterial resistance has gradually been developed.

Keywords: snail *C. aspersum*, glycopeptides, MALDI-TOF-MS, Q-Trap LC/MS/MS system.

Acknowledgments: This work was supported by a research grant № ДН 01/14 from 19.12.2016, to the National Science Fund of Republic Bulgaria.

36. A. Dolashki, **L. Velkova**, P. Dolashka. Mass spectrometric analyses of bio-antioxidants: peptides and glycoproteins. (Lecture) "First International Conference on Bio-antioxidants" 25-29.07.2017, Sofia, Bulgaria.

YSSBA 2017 Keynote Lectures

Mass spectrometric analyses of bio-antioxidants: peptides and glycoproteins

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Several peptides and glycoproteins with antioxidante activity have excellent potential as pharmaceutical products. Inhibitory effect of the fraction, containing, on the reduction of NBT by photochemically generated superoxide anion radicals.

Tandem mass spectrometry, combines MALDI-MS/MS and Q-Trap MS/MS, was applied to analyse the structure of ten biologically active peptides isolated from *Cornu aspersum* with antioxidant activity. The molecular masses of peptides were measured by their MS spectra and the primary structures were determined by their MS/MS spectra.

Tandem mass spectrometry as a very useful method was also apply to analyse the primary structure of glycoproteins, superoxide dismutase from *K. marxianus* NBIMCC 1984 yeast (Cu/Zn-KmSOD) and from fungal strain *Humicola lutea* 103 (Cu/Zn-HISOD). The obtained fractions after treatment of the enzyme with trypsin were separated by HPLC and their amino acid sequences were determined by MALDI-TOF-TOF.

Both SODs are glycoproteins and their carbohydrate structures were also identified by MALDI-MS/MS and Q-Trap MS/MS. After treatment of the Cu/Zn-KmSOD with PNGase F a single peak at 1257.3 [M+Na]⁺ was detected which suggests a uniform oligosaccharide chain. The structure of this carbohydrate chain was determined by Q-Trap MS/MS.

One putative linkage site was observed in the sequence of both glycoproteins. This linkage site (-Asn-Leu/Ile-Thr-) at position 33-35 is the glycosylated and is conserved in several SODs.

Keywords: antimicrobial peptides, glycopeptides, glycoproteins, *Cornu aspersum*, mass spectrometry

Acknowledgement: 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.



37. A. Nissimova, A. Dolashki, **L. Velkova**, P. Dolashka. Structure and antibacterial activity of glycopeptide, isolated from snail *Cornu aspersum*. "First International Conference on Bio-antioxidants" 25-29.06.2017, Sofia, Bulgaria.

YSSBA 2017 Oral Presentations

Structure and antibacterial activity of glycopeptide, isolated from snail *Cornu aspersum*

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The world provides a rich source of peptides with antimicrobial, antiviral and antitumor activity. Glycopeptides are an expanding group of structurally complex anti Gram positive antibacterial agents, representatives of which have been 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.

We now report on the primary structure and mass of a peptide isolated from the mucus of the molluscan garden snail *Cornu aspersum*, and compare its antimicrobial activities with those of other similar peptides isolated from the hemolymph of the marine snail *R. venosa* and garden snail *H. lucorum*.

The structure of a glycopeptide isolated by high performance reverse phase chromatography (HPLC) from the mucus of the garden snail *Cornu aspersum* was determined by mass spectrometric analyses. Its molecular mass of 4113 Da was measured by MALDI-TOF and the amino acid sequence of the glycopeptide KARYCGAEVTAN was determined by its MS/MS analysis.

In the test against Gram+ (*C. perfringens*, *P. acnes* и *S. aureus*) and Gram- (*E. coli*) bacteria the peptide shows an inhibition effects against *S. aureus* and *E. coli*.

Keywords: antimicrobial peptides, glycopeptides, *S. aureus*, *E. coli*, *Helix lucorum*, *Rapana venosa*, *Cornu aspersum*, mass spectrometry

Acknowledgement: 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.



29. R. Velikova, **L. Velkova**, A. Dolashki, P. Dolashka. Structure and stability of peptides and hemocyanin from garden snail *Cornu aspersum* (Poster) „First International Conference on Bio-antioxidants (ICBA2017) 25-29.07.2017, Sofia, Bulgaria.

YSSBA 2017

Poster Presentations

Structure and stability of peptides and hemocyanin from garden snail *Cornu aspersum*

Radostina Velikova¹, Lyudmila Velkova¹, Aleksandar Dolashki¹ and Pavlina Dolashka¹

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The hemolymph of molluscan snails is a complex mixture of biochemically and pharmacologically-active components such as peptides and proteins with antioxidante activity.

The peptides with antioxidant activity were isolated from the extract of garden snail *C. aspersa* and the temperature and pH-dependent denaturations of one isolated peptide were determined by circular dichroism (CD). Loss of α -helical structure occurred in the range of 50-85°C depending on pH. The peptide was not stable towards thermally-induced unfolding.

A copper-containing respiratory glycoprotein (hemocyanin) was also isolated from the hemolymph of garden snail *C. aspersa* (CaH). The structural and conformational stability of CaH, β c-CaH subunit and functional unit β c-CaH-g were analyzed by CD in wide pH-T range (T°C 20-85°C and pH 2-12).

The obtained results show that the native CaH is more stable compared to the β c-CaH subunit and β c-CaH-g functional unit, which was explained by the formation of quaternary structures, and non-ionic forces (intra-subunit, hydrophobic and hydrogen-bonded networks of carbohydrate moiety interactions). The difference in the stability of the native CaH and β c-CaH indicates the influence of the quaternary structure and the difference in the stability of β c-CaH and β c-CaH-g shows the influence of the tertiary structure.

The carbohydrate structure influences the stability of the native molecule CaH and β c-CaH subunit, but does not affect the stability of one isolated functional unit.

Keywords: β c-CaH subunit (SU), β c-CaH-g functional unit (Fu), circular dichroism (CD), stability, temperature and pH-dependent denaturation.

Acknowledgments: This work was supported by a research grant № ДН 01/14 from 19.12.2016, to the National Science Fund of Republic Bulgaria.



39. Y. Voynikov, R. Abrashev, N. Kostadinova, E. Krumova, A. Dolashki, **L. Velkova**, P. Dolashka. Antioxidant Screening of Peptides Isolated from the Hemolymph of Garden Snail *Cornu aspersum*. “Biomedicine and Quality of Life” Conference 26-26.07.2017.



Antioxidant Screening of Peptides Isolated from the Hemolymph of Garden Snail *Cornu aspersum*

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Abstract: Antioxidative peptides have been utilized by various species to combat pathogenic microorganisms and stress. In the present study, the antioxidant potential of protein fractions obtained from the hemolymph of the garden snail *Cornu aspersum* was evaluated. Proteins were separated from the isolated moluscan hemolymph into three fractions with masses of < 10 kDa, 10-30 kDa and > 30 kDa. Two complementary test methods were employed for the preliminary antioxidative screening, involving measurement of the radical scavenging activity on 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH), and the inhibition of *nitro blue tetrazolium* (NBT) reduction by photochemically generated O₂⁻. Caffeic acid was used as a positive control. On the DPPH assay all fractions were able to reduce the stable free radical DPPH to the yellow-colored 1,1-Diphenyl-2-picrylhydrazyl, with the peptide fraction below 10 kDa exhibiting better free radical scavenging activity than those with higher molecular weight. All tested fraction revealed comparable inhibition on the reduction of NBT by photochemically generated superoxide anion radicals, but the effect was less pronounced than that of caffeic acid.

Keywords: *Cornu aspersum*, Peptide, Protein, DPPH, NBT.

Acknowledgement

This work was supported by a research grant № DN 01/14 from 19.12.2016 of the National Science Fund of Bulgaria.

40. A. Nissimova, A. Dolashki, **L. Velkova**, P. Dolashka. Antibacterial Activity of Peptides from Snail *Cornu aspersum*. "Biomedicine and Quality of Life" Conference 26-26.07.2017.

Antibacterial Activity of Peptides from Snail *Cornu aspersum*

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Abstract: The emergence of multi-resistant bacteria requires the development of antibiotics with new mechanisms of action. 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 a number of multi-drug resistant bacteria. It is known that *Cornu aspersum* snail mucus contains a number of biologically active components. Ten biologically active peptides from *Cornu aspersum* snails contained in a fraction below 10 kDa were isolated by high pressure reverse phase chromatography (HPLC) on a Nucleosil column C18.

The molecular masses of these peptides between 1 and 5 kDa were determined by mass spectrometry analysis (MALDI-TOF). The amino acid sequences of the peptides were determined by their MS/MS spectra which indicate that they are tryptophan, tyrosyl and proline rich peptides. We also analyzed a glycopeptide with a mass of 4113 Da, whose amino acid sequence was determined by MS/MS analysis – KARYCGAEVTAN.

Some of these peptides show antibacterial activity against bacterial strains *E. coli*, *C. perfringens*, *P. acnes* and *S. aureus*.

Keywords: Antimicrobial peptides, *Cornu aspersum*, Mass spectrometry, *E. coli*, *C. perfringens*, *P. acnes*, *S. aureus*.

Acknowledgements

This work was supported by a research grant № DN 01/14 from 19.12.2016 of the National Science Fund of Bulgaria.



41. R. Velikova, **L. Velkova**, A. Dolashki, P. Dolashka.. Structure and Stability of Hemocyanin and Peptides from Garden Snail *Cornu aspersum*. "Biomedicine and Quality of Life" Conference 26-26.07.2017

Structure and Stability of Hemocyanin and Peptides from Garden Snail *Cornu aspersum*

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Abstract: The hemolymph of molluscan snails is a complex mixture of biochemically and pharmacologically-active components such as peptides and proteins. The structures stability, temperature and pH-dependent denaturation of one of isolated peptide was determined by CD. Loss of α -helical structure occurred in the range 50-85 °C depending on pH. The peptide was not stable towards thermally-induced unfolding.

The native molecule of CaH, isolated from hemolymph of garden snail *C. aspersa*, is constructed from three different subunits – β c-CaH, α s-CaH and α D-CaH (Mw ~ 400 kDa), and each of them containing eight functional units (Mw ~ 50kDa).

The structural and conformational stability of the CaH, β c-CaH subunit and the β c-CaH-g functional unit (Fu) were analyzed by CD in wide pH-T range (T °C 20÷85 °C and pH 2÷12). To provide details on the stability of the protein, Fu β c-CaH-g was compared with the native molecule of CaH and the subunit β c-CaH via pH–T diagrams, typical phase portraits for stability and denaturation reversibility. Increasing the temperature from 25 °C to 60 °C, the reversible denaturation of the molecule of protein also increases, opening a reversibility window within the range of pH 5.5-8.0 for subunit β c-CaH and the range of pH 5.0-9.0 for functional unit β c-CaH-g and as a result of which the real thermodynamic parameters (ΔC_p , ΔH°_{exp} , and ΔG°_{exp}) were determined.

The obtained results lead to the conclusion that the carbohydrate structure influences the stability of the native molecule CaH and β c-CaH subunit, but does not affect the stability of one isolated functional unit.

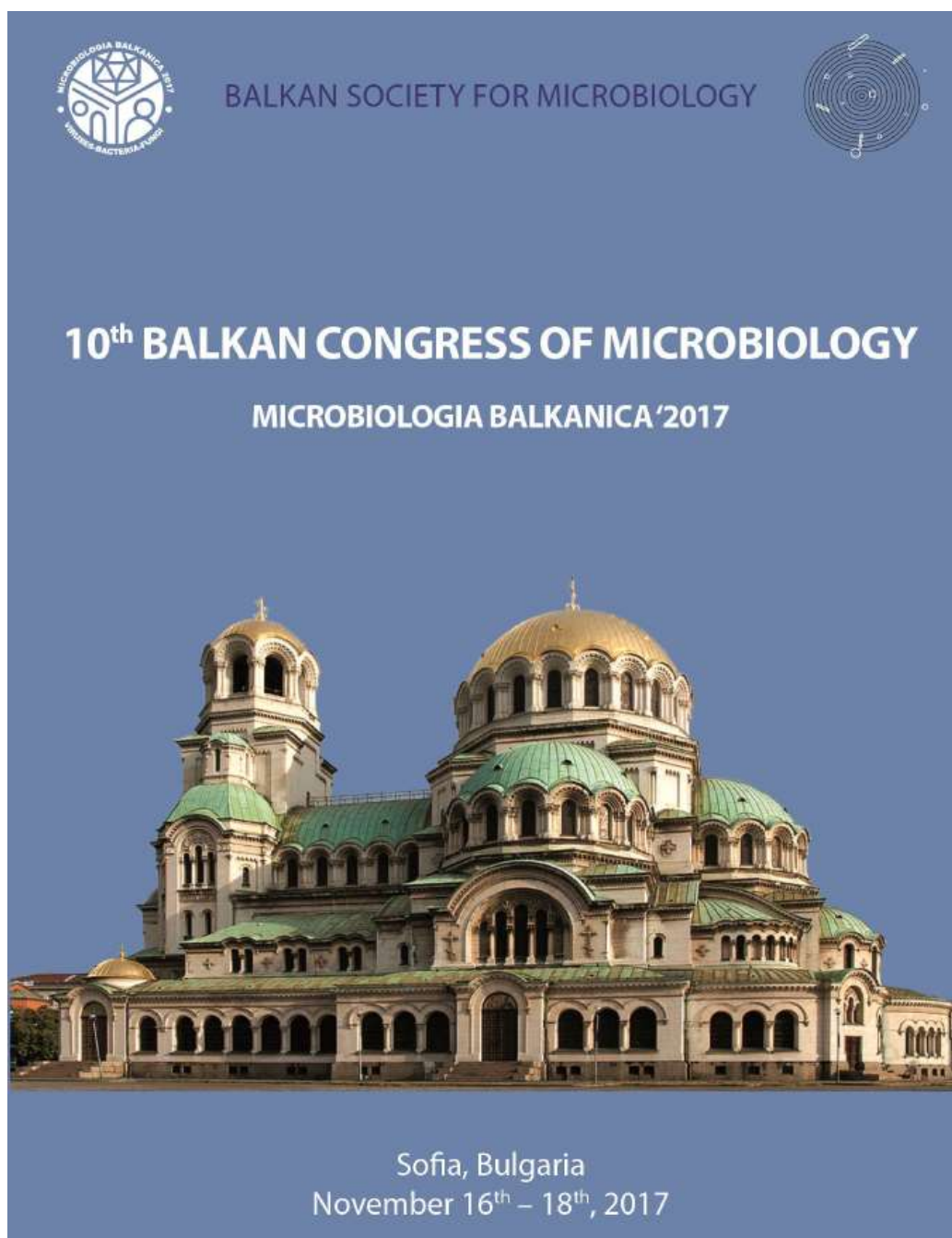
Keywords: Photosystem β c-CaH subunit (SU), β c-CaH-g functional unit (Fu), Circular dichroism (CD), Stability, Temperature and pH-dependent denaturation.

Acknowledgements

This work was supported by a research grant № DN 01/14 from 19.12.2016 of the National Science Fund of Bulgaria.



42. **L. Velkova**, A. Dolashki, L. Molin, P. Traldi, Y. Voinikov, R. Velikova, W. Voelter, P. Dolashka. Antimicrobial activity of proteins and peptides isolated from extract of the garden snail *Cornu aspersum*. (Lecture), 10th Balkan Congress of Microbiology Microbiologia Balkanica '2017 Sofia Park Hotel Moskva, November 16th – 18th, 2017.



ANTIMICROBIAL ACTIVITY OF PROTEINS AND PEPTIDES ISOLATED FROM EXTRACT OF THE GARDEN SNAIL *CORNU ASPERSUM*

L. Velkova¹, A. Dolashki¹, L. Molin², P. Traldi², Y. Voinikov¹, R. Velikova¹, W. Voelter³, P. Dolashka¹

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The rapid increase in drug-resistant infections has presented a serious challenge to antimicrobial therapies, because it emphasizes the urgent need to develop novel antimicrobials drugs agents. One area of interest is peptides and proteins produced by invertebrates as part of their defence system.

Extract collected from the garden snails *C. aspersum* is a complex mixture of biochemically and pharmacologically active compounds as peptides and proteins.

The structural subunits (β -CaH, α -CaH and α_0 -CaH) with molecular mass of about 420 kDa of oxygen transporter glycoprotein *C. aspersum* hemocyanin (CaH) were isolated. Surprisingly, the structural subunit β -CaH not only shows strong antimicrobial activities against *S. aureus* and the likewise Gram-positive *Streptococcus epidermidis*, but also against the Gram-negative bacterium *Escherichia coli*. Furthermore, the subunit β -CaH, carries immunomodulatory properties, which makes it especially interesting compound for the development of novel therapeutics.

The isolated 14 fractions from the extract (mucus and hemolymph) of *C. aspersum* after applying ultrafiltration and reverse-phase high-performance liquid chromatography (RP-HPLC), containing different peptides, have molecular weights between 600-7000 Da, determined by MALDI-TOF-MS.

Our *in vitro* study has shown that the three fractions from the extract of *C. aspersum*, have a demonstrable antimicrobial activity against two different strains of *Propionibacterium acnes* (PA266 and KPA) and *E. coli* (3548 and 3397). Two of the fractions show strong antimicrobial activities against *S. epidermidis*, as a β -CaH subunit.

We suggest that these compounds therefore have the potential to become a substitute for the commonly used antibiotics against which bacterial resistance has gradually been developed.

Acknowledgments. This work was supported by a research grant № ДН 01/14 from 19.12.2016, to the National Science Fund of Republic Bulgaria.

Keywords. antimicrobial activity, extract, snail *C. aspersum*, β -CaH, peptides

43. R. Velikova, **L. Velkova**, A. Dolashki, P. Dolashka. AntM-39 Antimicrobial properties of molluscan hemocyanins. (Poster), 10th Balkan Congress of Microbiology Microbiologia Balkanica '2017 Sofia Park Hotel Moskva November 16th – 18th, 2017, Sofia, Bulgaria.

AntM-39

ANTIMICROBIAL PROPERTIES OF MOLLUSCAN HEMOCYANINS

R. Velikova, L. Velkova, A. Dolashki, P. Dolashka

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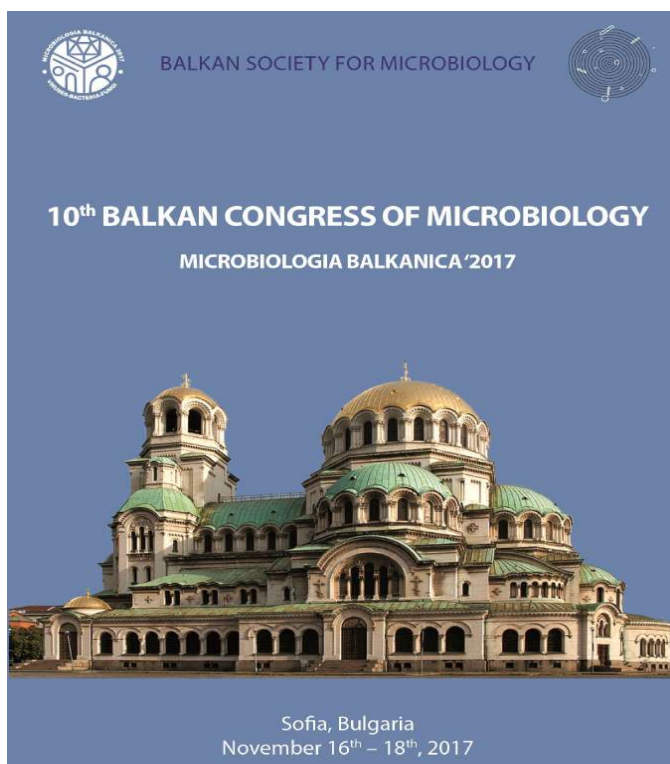
Hemocyanins (Hcs) are oxygen-binding glycoproteins, freely dissolved in the hemolymph, of many arthropods and mollusks. They have been shown to exhibit antitumor, antiviral and antimicrobial activity and are therefore considered to be "natural antibiotics". Hemocyanins are a promising alternative to conventional preparations.

The native molecule and three structural subunits were isolated from the hemolymph of the garden snail *Cornu aspersum* from the farms. Physicochemical analyzes and conformational stability of hemocyanin by fluorescence spectroscopy and circular dichroism confirmed that the protein is very stable. The antibacterial activity of hemocyanin from snail *Cornu aspersum* and its isoforms were also determined *in vitro* against bacterial strains of *E. coli* and *S. aureus*, identified as pathogenic with a high risk of resistance.

Hemocyanin from *C. aspersum* and its isoforms are new biologically active natural compounds, alternatives to chemical antibiotics, potential for application and treatment of therapeutic problems. Information will be gathered to create new concepts to overcome multi-drug resistance and create new types of antimicrobials.

Acknowledgments. This work was supported by a research grant of Bulgarian Academy of Science № 72-00-40-111/ 09.05.2017.

Keywords. antibacterial activity, circular dichroism (CD,) snail *C. aspersum*



44. P. Dolashka, Y. Voynov, **L. Velkova**, A. Dolashki. Proteomics analysis of Alzheimer's and antitumor activity of glycoproteins against bladder carcinoma permanent cell lines. (Lecture) 9th International Conference and Expo on Molecular Medicine Proteomics & Bioinformatics November 13 - 15, 2017 Paris, France.

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Pavlina Dolashka et al., J Proteomics Bioinform 2017, 10:11(Suppl)
DOI: 10.4172/0974-276X-C1-106

JOINT EVENT

9th International Conference and Expo on

Proteomics and Molecular Medicine

9th International Conference on

Bioinformatics

&

November 13-15, 2017 Paris, France

Proteomics analysis of Alzheimer's and antitumor activity of glycoproteins against bladder carcinoma permanent cell lines

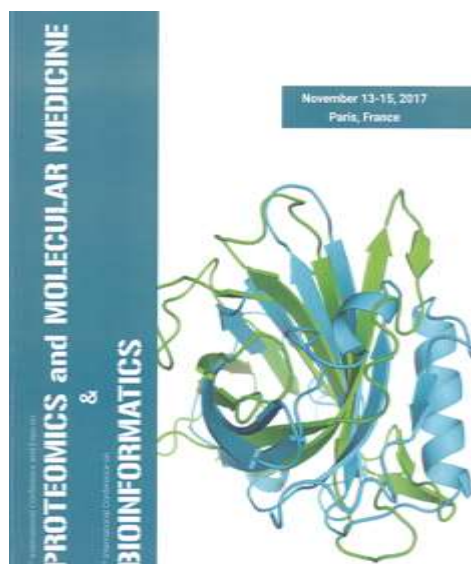
Pavlina Dolashka, Voynikov Y, Velkova L and Dolashki A
Bulgarian Academy of Sciences, Bulgaria

Alzheimer's disease (AD) is the most common form of dementia. It is the sixth leading cause of death and affects nearly 30 million people worldwide. Scopolamine and streptozotocin are widely utilized in chemically-induced dementia animal models to mimic specific pathophysiological pathways thought to underlie AD. To the best of our knowledge, there is no report describing proteome analysis on scopolamine or streptozotocin AD animal models. Therefore, we conducted a comparative proteome analysis on CSF isolated from rats with chemically-induced dementia with the purpose of identifying protein biomarkers. Rodents were divided into three groups: rats with scopolamine-induced dementia, rats with streptozotocin-induced dementia and healthy controls. Proteins and peptides were separated from the isolated CSF into four fractions. Two low molecular peptide fractions, with mass below 3 kDa and another with mass ranging from 3 to 10 kDa were analyzed by mass spectrometry, while two other protein fractions, with mass between 10 and 50 kDa and with mass higher than 50 kDa, were characterized by 2D-PAGE and the results were compared. The impact of hemocyanin on tumor cells was investigated by 2D-gel PAGE and several proteins showed indeed altered abundancies. The most effective inhibition of tumor cells is probably caused by a specific novel and unusual N-glycan oligosaccharide structure on HH with methylated hexoses, an internal fucose residue connecting one GalNAc(β 1-2) and one hexuronic acid.

Biography

Pavlina Dolashka and her group has wide experience in the isolation, purification and characterization of biologically active compounds. She has more than 130 publications on these topics, 3 book chapters and 6 patents. She is Editor-in-board of 3 journals and representative IUPAC. She is coordinating several international research projects, sponsored by NATO (Brussels), the European Commission, Germany (DFG and BMBF), CNR (Italy), FWO (Belgium), China and Ukraine.

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45. R. Velikova, A. Dolashki, A. Stenzl, S. Stevanovic, W.K. Aicher, J. Van Beeumen, L. Velkova, W. Voelter, P. Dolashka. Proteomics analysis of antitumor activity of Helix and Rapana hemocyanins. (Poster) 9th International Conference and Expo on Molecular Medicine Proteomics & Bioinformatics November 13 - 15, 2017 Paris, France.

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Radostina Velikova et al., J Proteomics Bioinform 2017, 10:11(Suppl)
DOI: 10.4172/0974-276X-C1-107

JOINT EVENT

9th International Conference and Expo on

Proteomics and Molecular Medicine

9th International Conference on &

Bioinformatics

November 13-15, 2017 Paris, France

Proteomics analysis of antitumor activity of Helix and Rapana hemocyanins

Radostina Velikova¹, Dolashki A¹, Stenzl A², Stevanovic S³, Aicher WK², Jozef Van Beeumen², Velkova L¹, Voelter W², Bart Devreese² and Dolashka P^{1*}

¹Bulgarian Academy of Sciences, Bulgaria

²University of Tübingen, Germany

³ Ghent University, Belgium

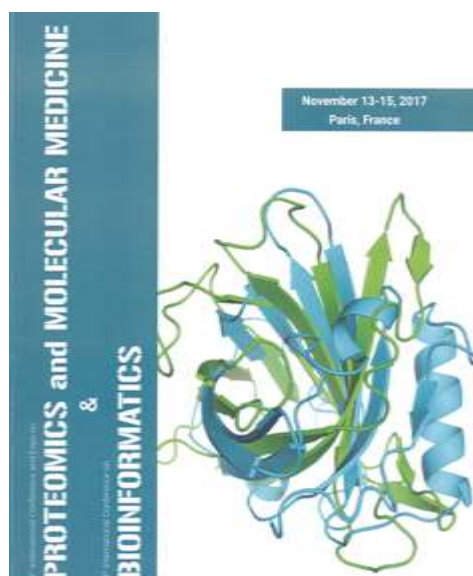
Hemocyanins (Hcs) are copper-containing glycoproteins that act as oxygen transporting proteins in many arthropods and mollusk species. Hemocyanins from the molluscs *Helix aspersa* (HaH), *Helix lucorum* (HIH) and *Rapana venosa* (RvH) exhibiting different oligosaccharide structures have been investigated for potential use in therapy of bladder cancer permanent cells. *In vitro* studies on the antitumor activities of these proteins were performed in T-24 cells and compared to doxorubicin and mitomycin-C. Control experiments were performed using normal urothelial HL 10/29 cells.

The obtained results show that the human tumor T24 cell lines are sensitive to the action of the tested hemocyanins and their isoforms. The inhibition of the tumor cell growth was dose and time dependent and was observed after incubation with native HaH and HIH and FUs β c-HIH-h and RvH-c. Cells treated with both FUs, β c-HIH-h and RvH-e, showed apoptotic and necrotic cells and this inhibition was stronger than the effect measured for doxorubicin treated cells. No growth inhibition of the normal urothelial cell line HL 10/29 was observed after treatment with HIH, HaH, RvH and their isoforms. The impact of hemocyanins on tumor cells was investigated by 2D-gel PAGE and several proteins showed indeed altered abundancies. The most effective inhibition of tumor cells is probably caused by a specific novel and unusual N-glycan oligosaccharide structure on HIH with methylated hexoses, an internal fucose residue connecting one GalNAc(β 1-2) and one hexuronic acid.

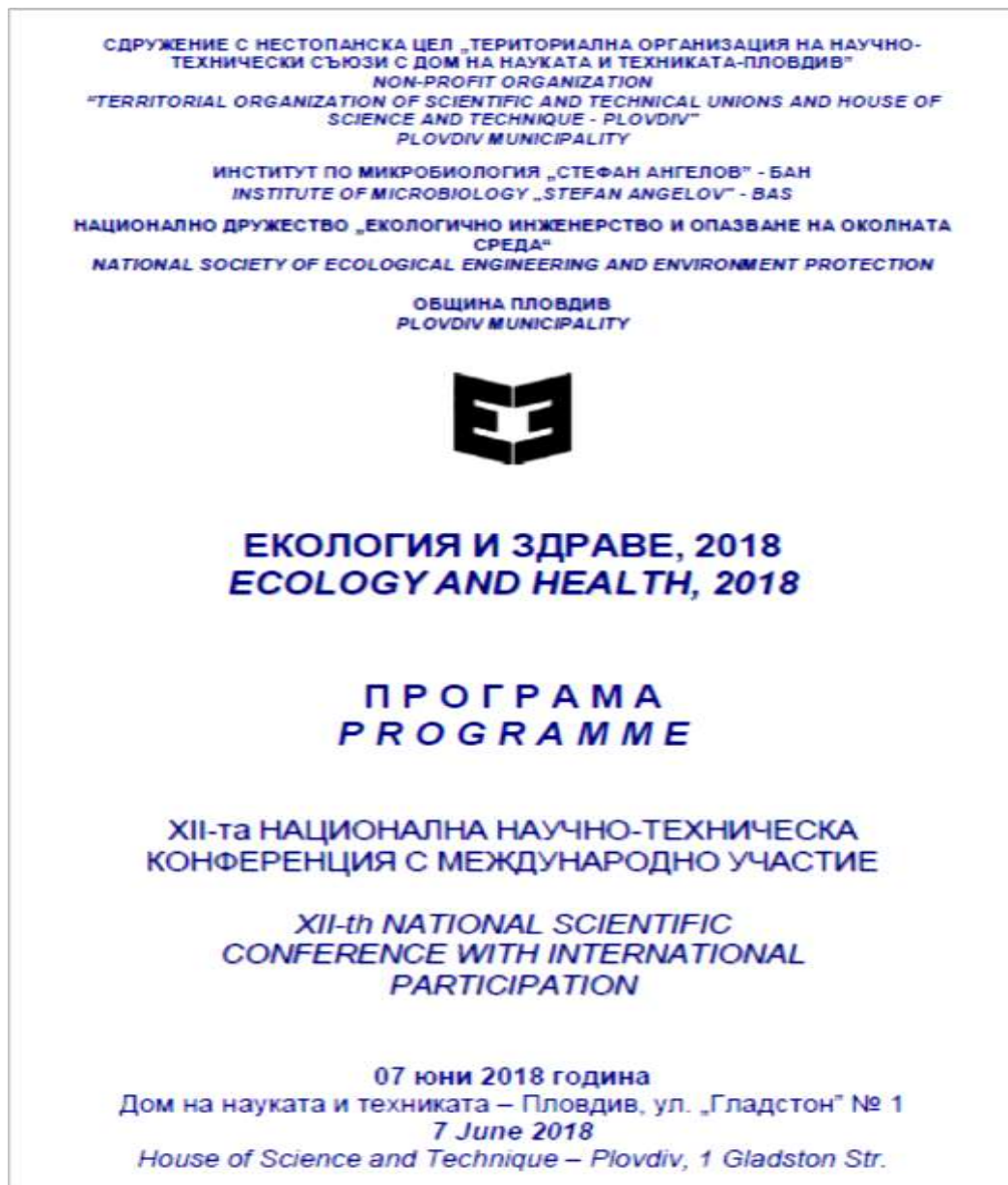
Biography

Radostina Velikova is currently working in the Institute for Organic Chemistry with Center for Phytochemistry, Bulgarian Academy of Sciences.

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46. **L. Velkova**, A. Dolashki, Y. Voinikov, R. Velikova, E. Daskalova, Y. Topalova, P. Hristova, E. Krumova, J. Miteva-Staleva, M. Angelova, P. Dolashka. Antibacterial and antifungal activity of mucus extracts from garden snail *Cornu aspersum*. (Lecture) "Ecology and health" XVII-the National Scientific conference with international participation, 7 June 2018, Plovdiv, Bulgaria.



**Antibacterial and antifungal activity of mucus extracts from
garden snail *Cornu aspersum*
(Lecture)**

Lyudmila Velkova¹, Aleksandar Dolashki¹, Yulian Voynikov¹, Radostina Velikova¹, E. Daskalova², Yana Topalova², Petya Hristova², Ekaterina Krumova³, Jeni Miteva-Staleva³, Maria Angelova³, Pavlina Dolashka¹

¹*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences*

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Abstract

Natural compounds have a great potential for use in new antimicrobial drugs, as some of them have a pronounced cytotoxicity to a number of multi-drug resistant bacteria. Snails produce biological fluid (mucus) which protects them against microbial invasion. The mucus of garden snail *C. aspersum* is a complex mixture of bioactive compounds with potential pharmacological application.

We have performed in vitro studies on the antimicrobial activities of different extracts, obtained from mucus of *C. aspersum*, against Gram⁺ and Gram⁻ bacteria and a fungal strain *Aspergillus niger*. Our preliminary results have shown that the mucus fraction below 10kDa possess strong antibacterial activity against the pathogen *E. coli*. The fraction having below 20kDa displayed the highest antibacterial activity against *Propionibacterium acnes* 266 AI, followed from fraction above 100kDa. The fraction between 10-50 kDa inhibits the strongest mycelial growth of *A. niger* than fractions below 10 kDa, between 50-100 kDa and over 100 kDa.

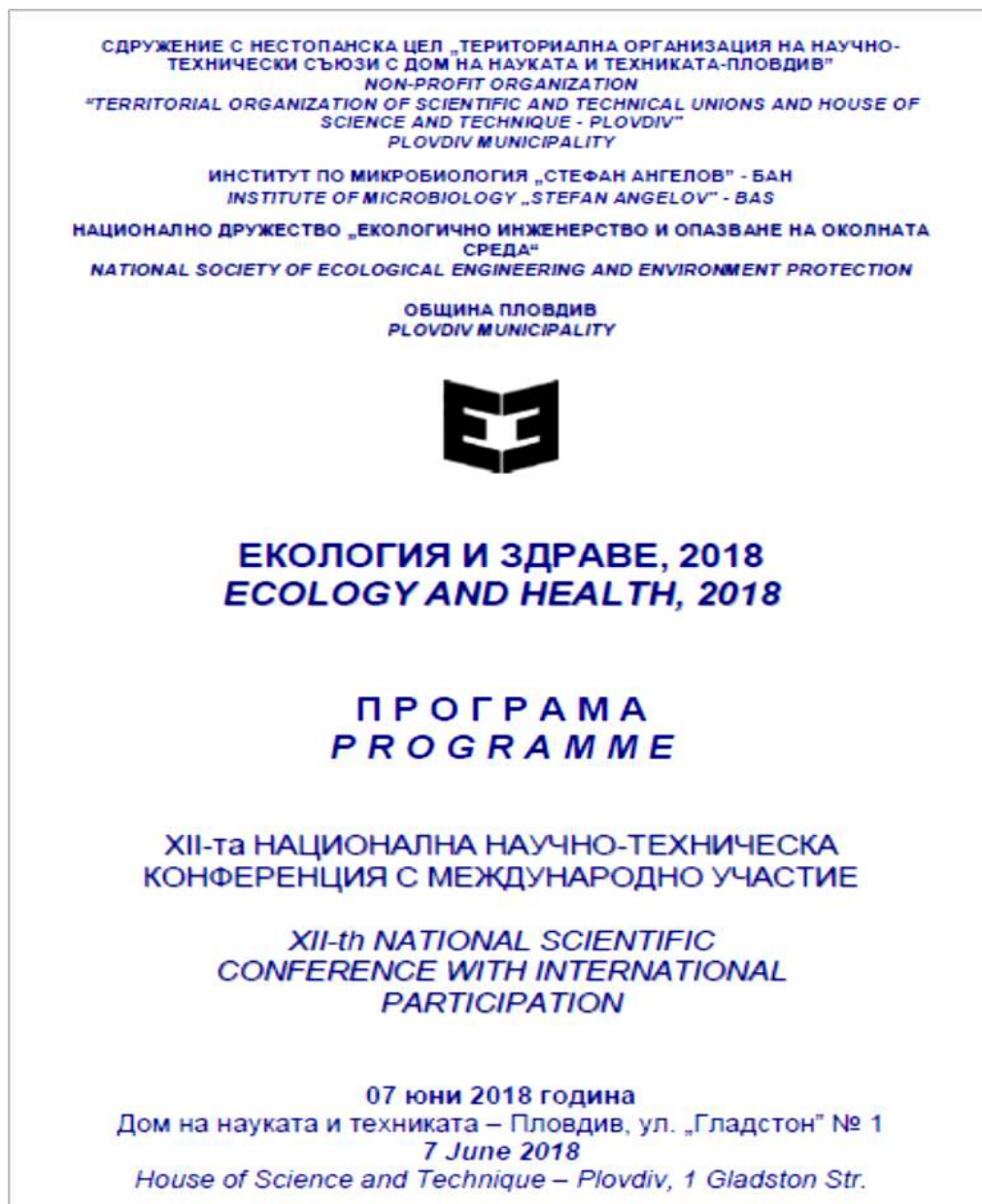
Using tandem mass spectrometry we identified the primary structures of many novel antimicrobial peptides in active fraction below 10kDa. Most of them contain high level of glycine and leucine residues into the amino acid sequences and belong of a class of Gly/Leu-rich AMPs, but others, contain proline, tryptophan and valine residues which are typical for peptides with antimicrobial activity. We compared of protein expression profiles of bacterial cells of strain *P. acnes* 266 AI before and after treatment with the fraction below 20kDa and found a significant change in the expression of seven proteins in bacterial cells. After analyzed by MALDI-MS, Mascot and searches across the different databases, these peptides were identified.

Our results may be considered as basic information for further investigations on bioactive compounds from *C. aspersum* and their potential biomedical applications.

Keywords: mucus extracts, garden snail *Cornu aspersum*, antibacterial and antifungal activity, antimicrobial peptides (AMPs), mass spectrometry.

Acknowledgement: 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.

47. P. Dolashka, Y. Voynikov, **L. Velkova**, A. Dolashki, L. Tancheva, L. Alova, W. Voelter. Exothermic processes in rat brain homogenate investigated by proteomic analyses. (Lecture) "Ecology and health" XVII-the National Scientific conference with international participation, 7 June 2018, Plovdiv, Bulgaria.



Exothermic processes in rat brain homogenate investigated by proteomic analyses

(Lecture)

P. Dolashka¹, Y. Voynikov¹, L. Velkova¹, A. Dolashki¹, L. Tancheva², L. Alova², W. Voelter³

¹*Institute of Organic Chemistry with Centrum of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, 1113 Sofia, Bulgaria*

²*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

³*Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany*

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Abstract

Alzheimer's disease (AD) is now considered the most prevalent progressive neurodegenerative disorder, responsible for 75% of all dementia cases. It affects approximately 35.6 million people worldwide, mainly the elderly ones. At present time only symptomatic treatments are available. From recent data it is suggested that for the development of AD, mainly β -amyloid peptides and neurofibrillary assemblies consisting of phosphorylated tau proteins are responsible for plaque formation. The structure, dynamic behavior and spatial organization of microtubules in neurons in the brain are regulated by their associated tau proteins. Although tau's structure function has been intensively studied for many years, little is known about the molecular mechanisms of interaction between tau proteins and microtubules and promotion of the assembly of microtubules.

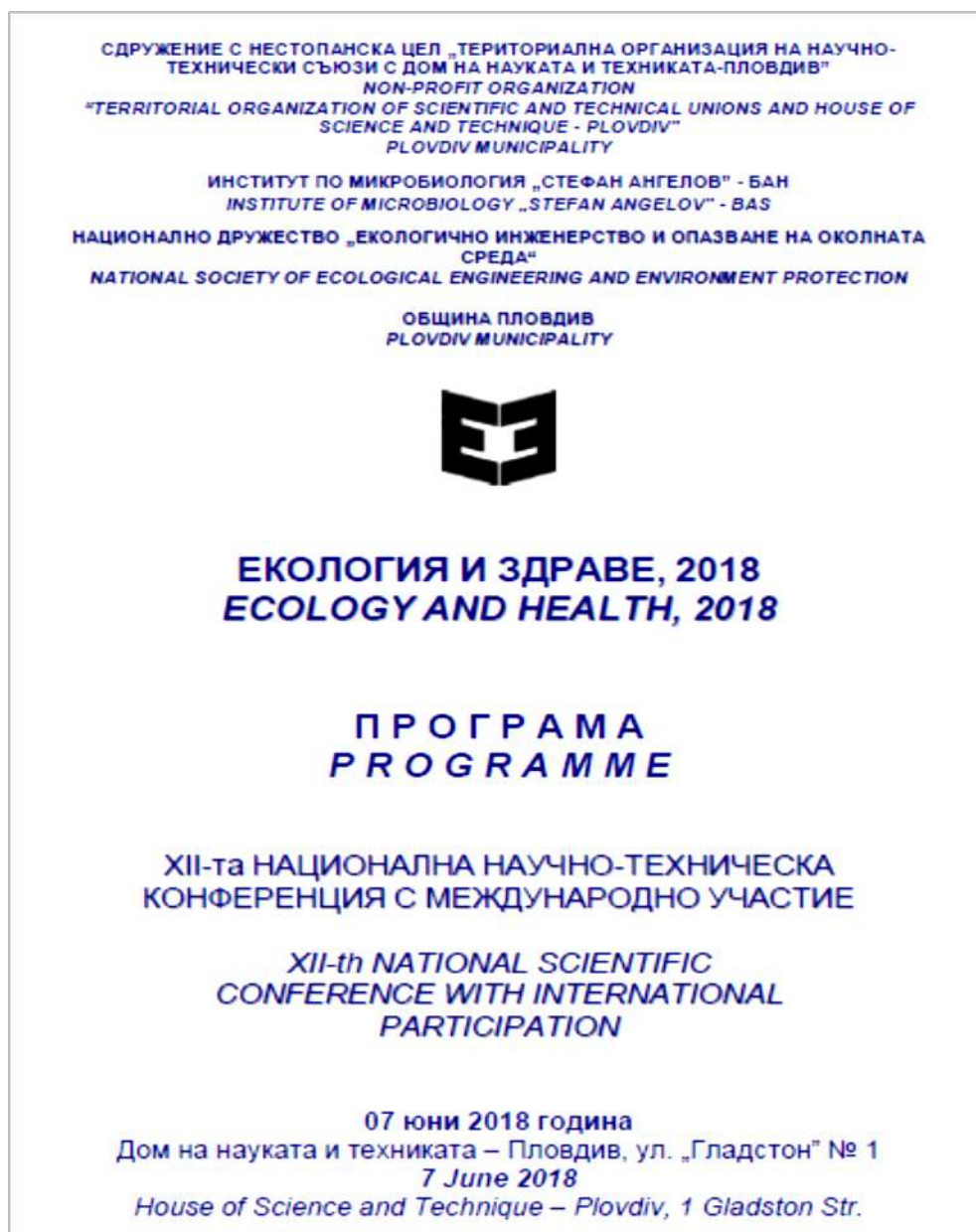
Herein, we present results of changes occurred at 45-50°C in the brain proteome of an experimental animal model (rat) with scopolamine-induced neurodegenerative disorder, type AD, using two-dimensional gel electrophoresis (2D-PAGE) coupled with MALDI-TOF-TOF. After homogenization of brain tissue from hippocampus, striatum and cortex, a comparative 1D- SDS-PAGE analysis was performed on samples with and without heating to 45°C.

The main observation from our investigations is the different behavior of the hippocampal proteome from the healthy rats before and after heating to 45°C. Due to the demonstrated change in protein level of tau and tubulin in the rat hippocampus after heating to 45°C, it was suggested that the observed exothermic process at 35-45°C in mice may be due to the partial unfolding of tau protein which leads to the release of tubulin. Both proteins together are involved in protein fibrillation and aggregation. The reported results from this study can help clarify the molecular mechanisms of scopolamine-induced dementia and neurodegenerative processes in general.

Keywords: Scopolamine-induced neurodegenerative disorder; Brain homogenates; Hippocampus; Tau protein; Proteomic analyses; Mass spectrometry.

Acknowledgement: *This research was carried out with the support of a project under contract No. 03/13/2016, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.*

48. P. Dolashka, **L. Velkova**, A. Dolashki, Y. Voynikov, R. Velikova, E. Daskalova, Y. Topalova, P. Hristova. Proteomic assay of *Propionibacterium acnes* treatment with mucus extract from garden snail *Cornu aspersum*. (Poster) "Ecology and health" XVII-th National Scientific conference with international participation, 7 June 2018, Plovdiv, Bulgaria.



**Proteomic assay of *Propionibacterium acnes* treatment with mucus extract from
garden snail *Cornu aspersum*
(Poster)**

Pavlina Dolashka¹, Lyudmila Velkova¹, Aleksandar Dolashki¹, Yulian Voynikov¹, Radostina Velikova¹, E. Daskalova², Yana Topalova², Petya Hristova².

¹*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences*

²*Sofia University "St.Kliment Ohridski", Faculty of Biology, Bulgaria*

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Abstract

Acne vulgaris is one of the most common an inflammatory skin disease, which is observed in about 80% of young adults. The pathophysiological factors that cause the development of acne are excessive sebum production, abnormal keratinization and desquamation of pilosebaceous follicular epithelium (comedogenesis), follicular proliferation of *Propionibacterium acnes* and inflammation. *P. acnes* appears to play a central role in the induction of inflammation. Recent microbiological and dermatological studies have reproducibly pointed to the strong associations between *P. acnes* and acne vulgaris, *S. aureus* and atopic dermatitis, and Malassezia species with dandruff. Resistance development in *P. acnes* is another serious problem in using conventional antibiotics such as erythromycin. In these contexts, we investigated several mucus extracts from garden snail *Cornu aspersum* for antibacterial activity.

The isolated mucus extract was separated into several fractions by ultrafiltration on Millipore membrane filters from 10kDa, 20kDa, 30kDa and 100 kDa. The obtained fractions were tested for antimicrobial activity by the well diffusion method against *Propionibacterium acnes* 266 (AI).

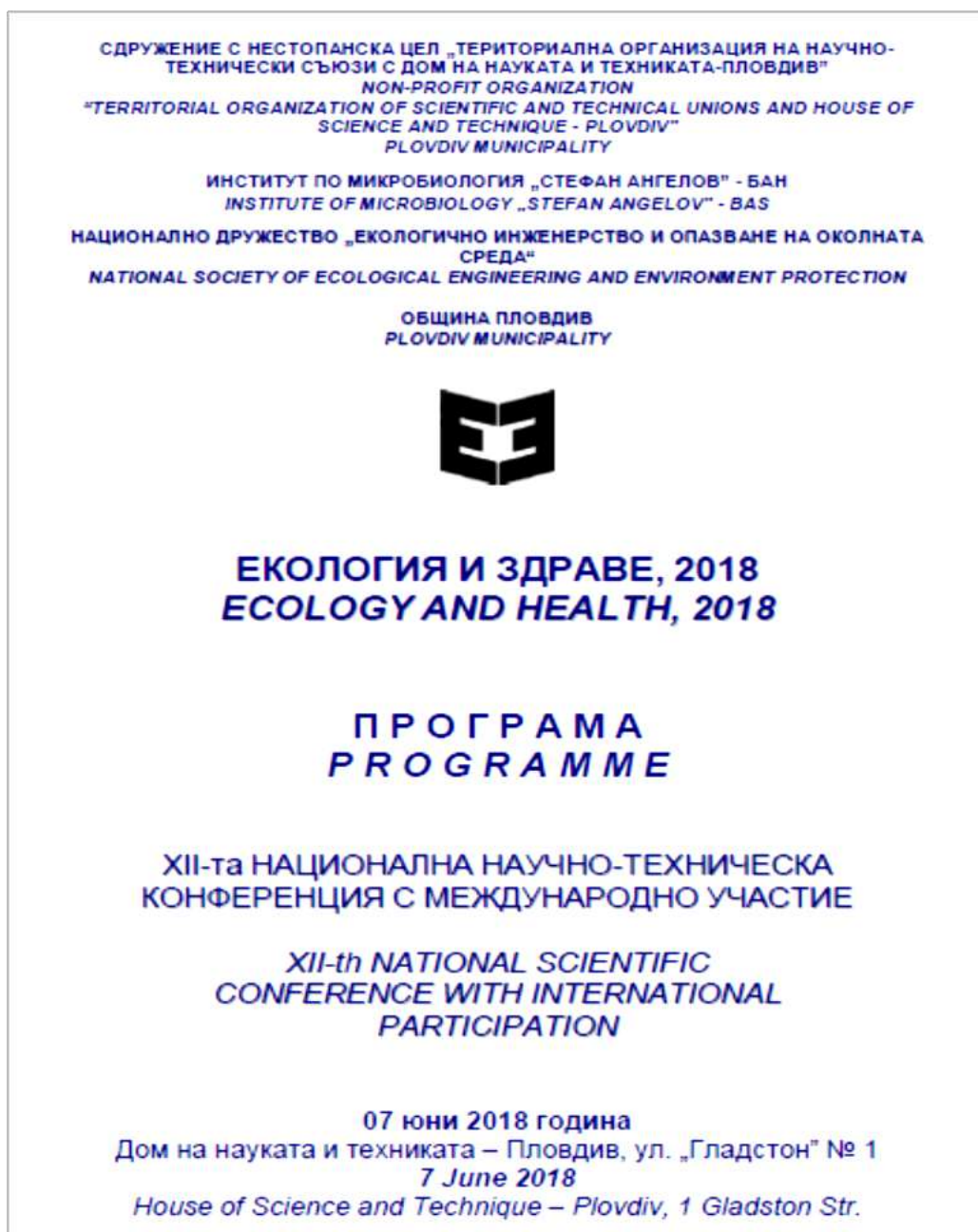
The fraction having below 20kDa displayed the highest antibacterial activity against *P. acnes* 266 (AI), followed from fraction above 100kDa. Using tandem mass spectrometry we identified the primary structures of many novel antimicrobial peptides in active fraction below 20kDa. Most of them contain high level of glycine, proline, tryptophan and valine residues which are typical for peptides with antimicrobial activity.

We have applied a combination of two-dimensional electrophoresis (2-DE) and mass spectrometry (MALDI-MS) to identify changes in expression of proteins secreted by *P. acnes* 266 (AI), before and after treatment with active fraction below 20kDa. These proteins are: glycerol-ester hydrolase A PPA2105 (GehA; MW 35972Da, pI 6.59); PROAC0001_1849 (MW 31987Da, pI 5.00) putative lysozyme M1 [*P. acnes* SK137]; PPA0687 (MW 28612 Da, pI 9.61) CAMP factor 2; hypothetical protein, specific to *P. acnes* PPA1715 (MW 49335Da); hypothetical protein specific to *P. acnes*; PPA1939 (MW 16862Da, pI 6.17); PPA2106 (MW 54063Da, pI 6.34) putative endoglycoceramidase; PPA0721 (MW 40716Da, pI 9.44) putative invasion-associated protein, NlpC/P60 family.

Keywords: mucus extracts, garden snail *Cornu aspersum*, proteomic assay, two-dimensional electrophoresis (2-DE), mass spectrometry MALDI-MS.

Acknowledgement: 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.

49. **L. Velkova**, Y. Voynikov, A. Dolashki, L. Tancheva, L. Alova, W. Voelter, P. Dolashka. Proteomic analyses of brain homogenate and cerebrospinal fluid of an experimental animal model (rat). (Poster) "Ecology and health" XVII-the National Scientific conference with international participation, 7 June 2018, Plovdiv, Bulgaria.



**Proteomic analyses of brain homogenate and cerebrospinal fluid of an experimental animal model (rat)
(Poster)**

L. Velkova¹, Y. Voynikov¹, A. Dolashki¹, L. Tancheva², L. Alova², W. Voelter³, P. Dolashka¹

¹*Institute of Organic Chemistry with Centrum of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, 1113 Sofia, Bulgaria*

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Abstract

The most common neurodegenerative disorder and the most important cause of dementia in elderly people appear to be Alzheimer's disease (AD). The AD-associated problems decrease life expectancy, reduce quality of life, cause physical disability, and eventually lead to serious problems in daily life activities such as social and occupational functions. To reduce the burden of the disease on patients and their families and the social and economic costs, many scientific teams make the remarkable efforts to find diagnostic markers which predict the disease earlier. Recently, it is suggested that for the development of AD, mainly β -amyloid peptides and neurofibrillary assemblies consisting of phosphorylated tau proteins are responsible for plaque formation.

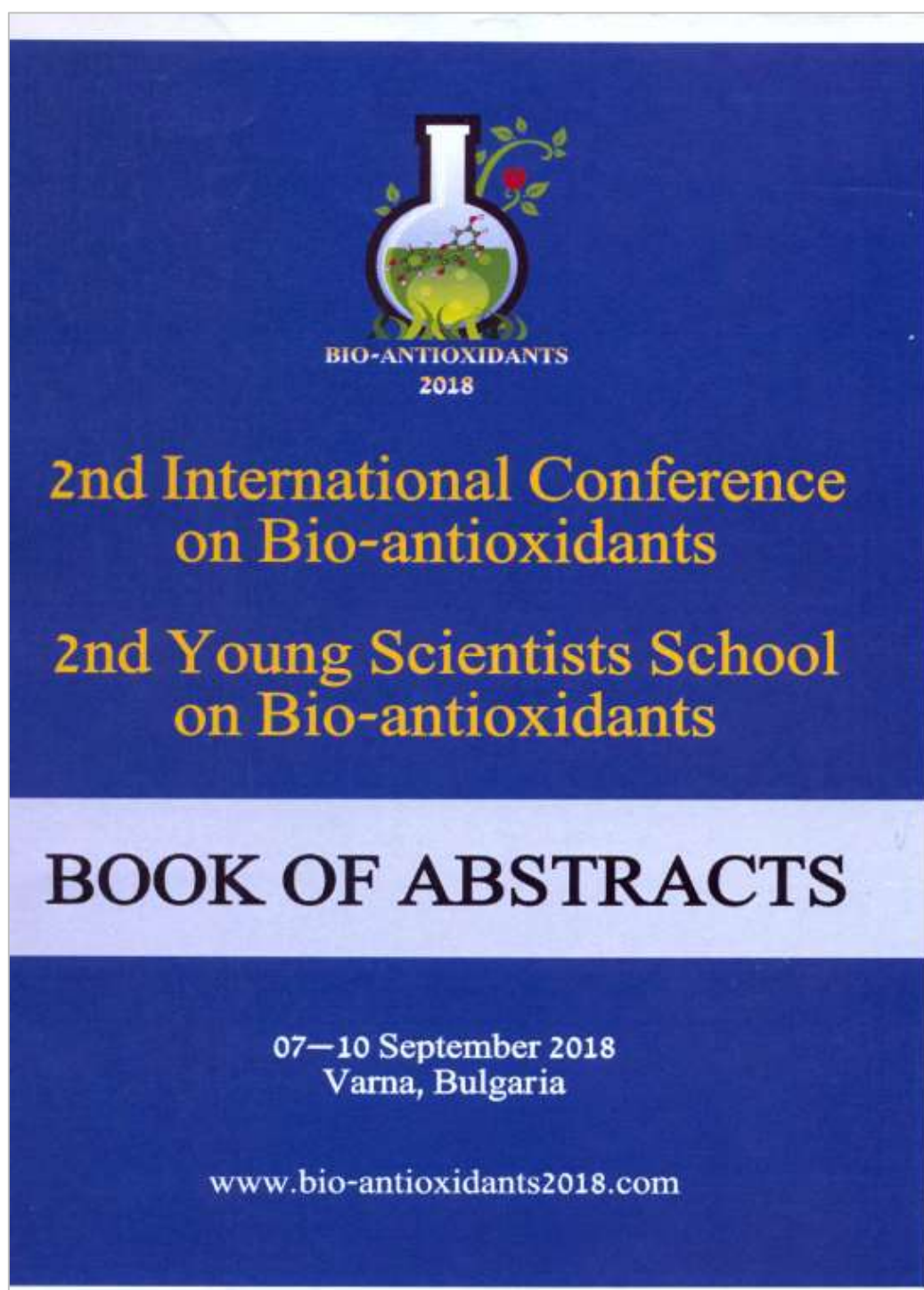
In this study, brain homogenate (BH) and cerebrospinal fluid (CSF) of an experimental animal model (rat) in normal and scopolamine-induced neurodegenerative disorder (type AD), were analyzed by using proteomic techniques, mass spectrometry (MALDI-TOF-TOF) and bioinformatics' analysis.

Comparisons between controls and scopolamine-induced neurodegenerative disorder (type AD) of BH and CSF have showed several common proteins (as microtubule associated proteins, tau proteins, tubulins, and beta amyloid precursor) which are expressed significantly differences. Recent studies have shown, the amyloid-beta ($A\beta$) and amyloid β 1-42 play a key role in the pathogenesis of AD. The intact $A\beta$ from soluble aggregates comperes are especially relevant biochemical marker because they are believed to be the most toxic form of $A\beta$. Therefore, we investigated different fractions <10kDa from BH and CSF, using mass spectrometry (MALDI-TOF-TOF). We found a diversity of $A\beta$ peptides between 3.0-6.5kDa, and identified some of them. We observed high heterogeneity of proteoforms, which associates with post-translational modifications. This heterogeneity of $A\beta$ proteoforms deepens our understanding of AD and offers many new avenues for investigation into pathological mechanisms of the disease, with implications for therapeutic development.

Keywords: Scopolamine-induced neurodegenerative disorder (type AD); Brain homogenate (BH); Cerebrospinal fluid (CSF); Amyloid-beta ($A\beta$) peptides ; Proteomic analyses; Mass spectrometry.

Acknowledgement: *This research was carried out with the support of a project under contract No. 03/13/2016, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.*

50. P. Dolashka, A. Dolashki, **L. Velkova**. From science to business. Opening Lecture, 2nd International Conference on Bio-antioxidants, 07 – 10 September 2018, Varna, Bulgaria.





Final Scientific Program

Friday, 7 th September 2018		
Registration and information <i>Company for International Meetings</i>	<i>Romance Splendid Hotel, St. Constantine and Helena Resort</i>	12.00-18.00
Welcome and Opening Ceremony		17.00-18.30
Prof. Vessela Kancheva Chair of organizing committee, IOCCP – BAS, Bulgaria	Welcome address	17.00-17.10
Dr. Stefan Dochev Coordinator Young Scientists School, Austria	Welcome address	17.10-17.20
Prof. Pavleta Shestakova IOCCP-BAS, Bulgaria	Welcome address	17.20-17.30
Prof. Pavlina Dolashka, IOCCP-BAS, Bulgaria	Opening Lecture <i>From science to business</i>	17.30-18.00
Mrs. Anelia Vasileva, Orange Holiday, Varna, Bulgaria	Welcome to Varna	18.00-18.10
Students from Varna, Bulgaria	Welcome to Bulgaria	18.10-18.30
Poster session I	Topics A, B and C	18.30-20.00
Welcome Reception		20.00-21.30

51. P. Dolashka, A. Dolashki, **L. Velkova**, B., Devreese Y. Topalova, P. Hristova, J. Van Beeumen, W. Voelter. Food supplements with novel bioactive extracts from garden snails. (Lecture), 2-nd International Conference on Bio-antioxidants, 07 – 10 September 2018, Varna, Bulgaria



E-KL7. FOOD SUPPLEMENTS WITH NIVEL BIOACTIVE EXTRACTS FROM GARDEN SNAILS

Pavlina DOLASHKA,^a Aleksander DOLASHKI,^a Lyudmila VELKOVA,^a Bart DEVREESE,^b Yana TOPALOVA,^c Petya HRISTOVA,^c Jozef VAN BEEUMEN,^b Wolfgang VOELTER^d

^a Institute of Organic Chemistry, Bulgarian Academy of Sciences, G. Bonchev 9, Sofia 1113, BULGARIA

^b Laboratory of Protein Biochemistry and Biomolecular Engineering, Ghent University, KL Ledeganckstraat 35, 9000 Ghent, BELGIUM

^c Sofia University "St. Kliment Ohridski", Faculty of Biology, BULGARIA

^d Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, GERMANY

Hemolymph and mucus of *Cornu aspersum* garden snails provide a rich source of peptides and proteins with antioxidant, antimicrobial, antiviral and antitumor activity. Antimicrobial peptides and glycoproteins from the mucus and hemolymph are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens. Therefore quite a series of proline-rich peptides isolated from the mucus of *C. aspersum* garden snails were analysed and were considered to be promising candidates for the treatment of microbial infections and suppression of microbial resistance.

Using tandem mass spectrometry the primary and oligosaccharide structures of many novel peptides with molecular masses between 1 and 20 kDa displayed the highest antibacterial and antifungal activities were identified. Most of them contain high level of glycine, proline, tryptophan and valine residues which are typical for peptides with antimicrobial activity.

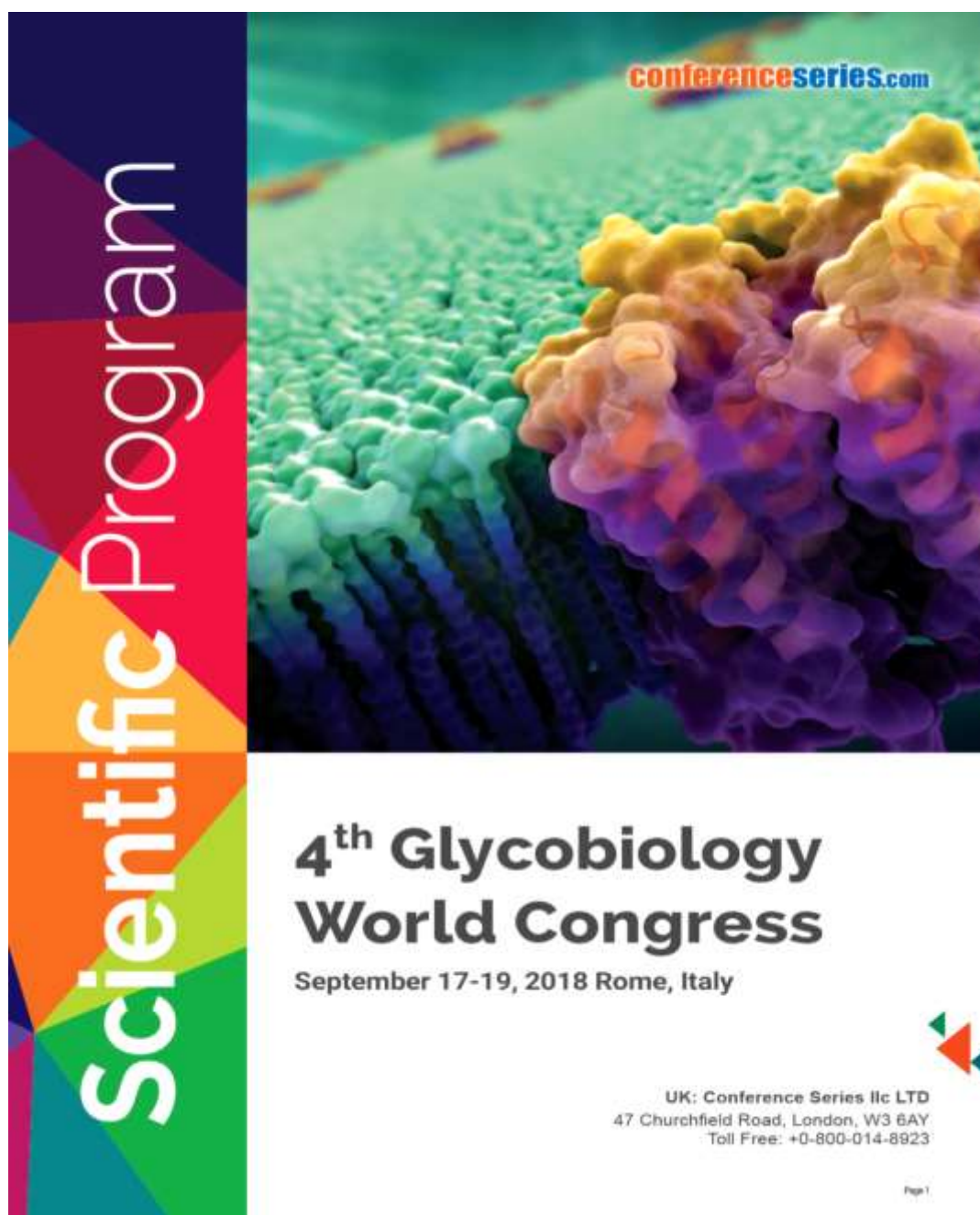
Applying a combination of two-dimensional electrophoresis (2-DE) and mass spectrometry (MALDI-MS) the proteins secreted in the mucus also were identified. The carbohydrate structures of both glycoproteins, superoxide dismutases (SODs) and hemocyanins, having also antioxidant activity were determined by MALDI-MS/MS and Q-Trap MS/MS.

Based on obtained results the active compounds were applied to develop several novel food supplements to overcome the recent appearance of a growing number of resistant to conventional antibiotics, which has become a serious medical problem.

Keywords: antimicrobial peptides, **bio-antioxidants**, glycopeptides, glycoproteins, *Cornu aspersum*, mass spectrometry

Acknowledgement: 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.

52. P. Dolashka, A. Dolashki, **L. Velkova**, V. Petrova, A. Kujumdzieva. Specific glycosidases in quiescent *S. cerevisiae* culture and their functions. (Lecture), 4-th Glycobiology World Congress, 17-19 September 2018, Rome, Italy.



4th Glycobiology World Congress

September 17-19, 2018 | Rome, Italy

Specific glycosidases in quiescent *S. cerevisiae* culture and their functions

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Several glycosidases such as alpha-glucosidase I and alpha-mannosidase are located in the endoplasmic reticulum of *Saccharomyces cerevisiae* and they cleave the terminal alpha1, 2-linked glucose alpha1, 2-mannose. Therefore, the effect of *S. cerevisiae* in quiescent state G0 and active cells on the glycosylated hemocyanin *Rapana venosa* (RvH) and *Haliotis tuberculata* (HtH) is analyzed in comparison to the enzyme zymolysis. The carbohydrate structure of both the hemocyanins is known to have mainly mannose-type structures. The structural units of RvH1 and HtH1 are composed of various functional units with 45–60 kDa molecular masses measured by MALDI-TOF/TOF-MS ligand-bound by protease-sensitive peptides. However, we propose the model that the individual polypeptides of the various FUs are linked together in a network generated by oligosaccharides. Our hypothesis is confirmed by the generated fragments, with molecular masses of about 50 kDa, after incubation of HCS in *S. cerevisiae* growth medium. It is based on the assumption that yeasts produce glycosidases that break the oligosaccharides connecting the different functional units. After four days incubation of RvH and HtH in medium with *S. cerevisiae* in quiescent state G0 and active cells, several different fractions were isolated which showed a negative orcinol/H₂SO₄ test. The results show a higher effect after treatment of both hemocyanins in *S. cerevisiae* active cells medium than in quiescent state G₀. However, the observed effect is lower than that after incubation of both HCS with zymolyase.

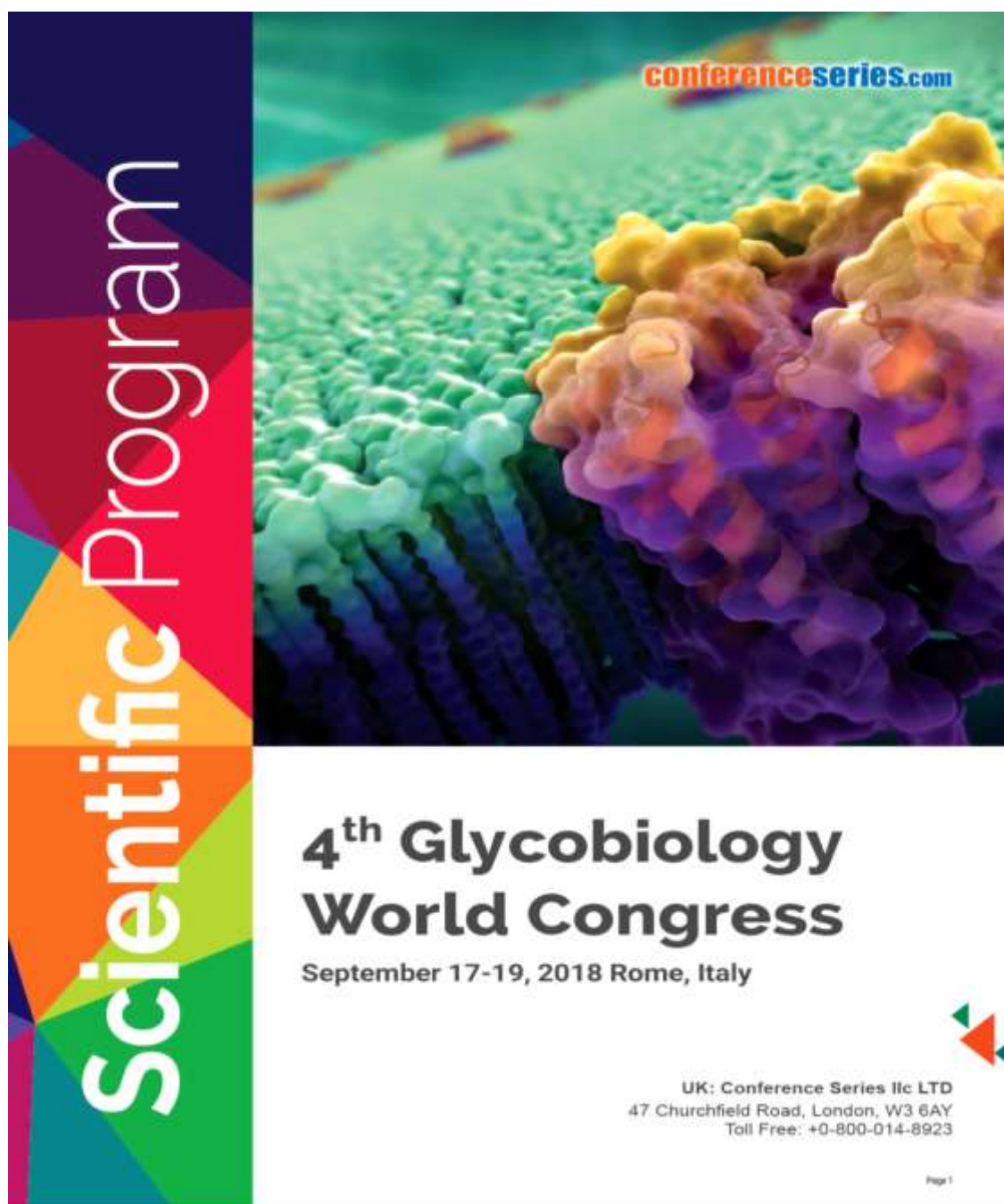
Biography

Dolashka P has wide experience in the isolation, purification, and characterization of biologically active compounds. She has more than 130 publications on these topics, three book chapters and six patents. She is the Editor-in-board of three journals and Representative of IUPAC. She is coordinating several international research projects, sponsored by NATO (Brussels), the European Commission, Germany (DFG and BMBF), CNR (Italy), FWO (Belgium), China, and Ukraine.

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Notes:

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Identification of peptides and glycopeptides with antimicrobial activity from garden snail *C. aspersum* using mass spectrometry

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The recent appearance of a growing number of bacteria resistant to conventional antibiotics has become a serious medical problem. Therefore the development of antibiotics, with novel mechanisms of action is a current issue. The mucus from the garden snail *C. aspersum* is a complex, multi-component mixture including different biochemical active substances. The mucus extract was separated into several fractions using Millipore filters with different size (10, 20 and 100 kDa). We have performed *in vitro* studies on the antimicrobial activities of different extracts, obtained from mucus of garden snail *C. aspersum*, against Gram+, Gram- bacteria and fungal strains *A. niger* and *A. fumigatus*. Our results have shown that fractions < 10 kDa, <20 kDa and between 1-10 kDa possess strong antimicrobial activity against the tested pathogens. To explain the observed effects against various microbial organisms, the peptides and glycopeptides in active fractions were purified by reversed phase high-performance liquid chromatography (RP-HPLC). All fractions were tested of the orcinol/H₂SO₄ test. The positive fractions (glycopeptides) were lyophilized and further studied by Q-trap-LC/MS/MS. We identified the carbohydrate chains and amino acid sequences of several glycopeptides. Using tandem mass spectrometry MALDI-TOF-MS/MS, we identified the primary structures of 15 novel antimicrobial peptides in active fraction below 10kDa. Most of them contain high levels of glycine and leucine residues, but others contain proline, tryptophan and valine residues, typically for peptides with antimicrobial activity. Our results may be considered as basic information for further investigations on bioactive compounds from mucus of garden snail *C. aspersum*.

Keywords: mucus extracts, garden snail *Cornu aspersum*, antimicrobial peptides (AMPs), glycopeptides, mass spectrometry

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Biography

Lyudmila Velkova works at laboratory "Chemistry and Biophysics of proteins and enzymes" at the Bulgarian Academy of Sciences (BAS). Her research interests include isolation, characterization and application of bioactive substances from natural sources, primarily proteins, glycoproteins, antimicrobial peptides, investigation of carbohydrate structures of glycoproteins by mass spectrometry, as and proteomics analysis. She defended PhD on the topic "Structure and function of carbohydrate chains of hemocyanin, isolated from the marine snail *Rapana venosa*" in Institute of Organic Chemistry with Centre of Phytochemistry - BAS, 2013. She has published more than 25 papers in reputed international journals.

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Notes:

54. R. Abrashev, E. Krumova, A. Dolashki, N. Kostadinova, J. Miteva-Staleva, **L. Velkova**, B. Spasova, M. Angelova, P. Dolashka. Cornu aspersum - derived peptides with biological activity. (Poster), 4-th Glycobiology World Congress, 17-19 September 2018, Rome, Italy.



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Cornu aspersum-derived peptides with biological activity

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Recently, bioactive peptides have received close scientific attention for their broad scope of bioactivities, mainly including antioxidant and antimicrobial properties. Such peptides are especially prominent for their notable contributions to human health improvement through the scavenging of dangerous free radicals. Moreover, they have shown to be good candidates for the development of alternative antimicrobial agents. The aim of the present study was to derive different fractions from the mucus of the garden snail *Cornu aspersum* and to evaluate their antioxidation and antifungal activity. The snails, *Cornu aspersum* were collected in Bulgaria and the mucus was purified. After that, the fractions with different molecular mass were obtained by ultrafiltration on Millipore filters. Mass spectrometry analyses on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectron spray ion source was performed for the peptide characterization. The total antioxidant potential of the tested fractions was assessed by the DPPH and ABTS radical scavenging activity methods and the nitroblue tetrazolium (NBT) reduction assay; superoxide dismutase (SOD) activity was evaluated as well. The results showed that the natural peptides derived from *C. aspersum*, specifically the low molecular mass fractions possess potential antioxidant activity confirmed by hydroxyl and superoxide radical scavenging activity and radical cation decolorization assay. These peptides exhibited fungicidal and fungistatic activity against *Candida membranifaciens*, *Aspergillus fumigatus* and *Aspergillus niger*.

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Biography

Radoslav Abrashev is an Associate Professor in the Department of Mycology at The Stephan Angeloff Institute of Microbiology (IMiB), Bulgarian Academy of Sciences. He has completed his PhD from IMiB and Post-doctorate from the University of Strathclyde, Glasgow, UK. He has published more than 30 papers in reputed journals. His research interest is focused on production of biological active compounds from natural sources, their purification, chemical and biological characterization in terms of antioxidant and antifungal properties.

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Notes:

55. N. Kostadinova, P. Dolashka, E. Krumova, **L. Velkova**, A. Dolashki, R. Abrashev, J. Miteva-Staleva, B. Spasova, M. Angelova. Mucus from Cornu aspersum – a perspective resource of antioxidant substances. 14th Congress of Microbiologists in Bulgaria with International Participation, October 10th – 13th, 2018, Hisarya, Bulgaria.



**14th Congress of Microbiologists
in Bulgaria
with International Participation**

PROGRAM AND ABSTRACTS



Hisarya, October 10th – 13th, 2018

MUCUS FROM *CORNU ASPERSUM* – A PERSPECTIVE RESOURCE OF ANTIOXIDANT SUBSTANCES

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Introduction. Snail mucus is a rich source of bioactive natural compounds with a variety of functions, including facilitating of the treatment of wounds and skin conditions. More importantly, the mucus is well known for its anti-aging properties and frequently investigated for antibacterial potency. Generally, both of these properties facilitate the ability of the organism to cope with the generation of free radicals.

Aim. The aim of the present study was to evaluate the extent of antioxidant potential of fractions from mucus of garden snail *Cornu aspersum*.

Methods. The model snail species were collected in Bulgaria and the mucus was purified and subjected to ultrafiltration. Three different samples were investigated – lyophilized fractions (molecular mass < 10 kDa and > 20 kDa) and liquid fraction (> 20 kDa). Inhibitory effect of the fractions was determined by calculating of the inhibition of NBT reduction by photochemically generated superoxide. Superoxide dismutase (SOD) activity and dose-dependence of the superoxide anion scavenging effect were studied as well.

Results. The fraction with molecular mass <10 kDa showed the highest SOD activity. It is possible that the detected SOD value represents the so called “SOD-like” activity that could be displayed by some peptides with low molecular mass capable of superoxide scavenging.

Conclusions. The results from the antioxidant screenings of *C. aspersum* mucus and its fractions show that this naturally derived product (specifically the low molecular weight fractions), has a good potential to counteract the formation of reactive oxygen radicals.

Keywords: *Cornu aspersum*, superoxide radicals, SOD, antioxidant activity

Acknowledgment: 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.