INFORMATION ON SCIENTIFIC CONTRIBUTIONS

of Senior assistant Dr. Lyudmila Velkova

Extended Habilitation Report

This Extended Habilitation Report is prepared and intended for participation in the contest for associate professor in professional division 4.2 Chemical Sciences: scientific speciality "Organic Chemistry, Chemistry of Natural and Physiologically Active Substances" for the needs of the laboratory "Chemistry and Biophysics of Proteins and Enzymes".

A habilitation report is presented for the competition, which reflects my scientific contributions, published on 8 scientific publications (Section B 4), and additional 16 publications (Section Г 7) and 4 National patents (Section Г 9,) all of which are outside of the 5 publications used for my PhD thesis. Overall, my research papers are citated 167 times in scientific information databases, of which 152 citates were not used to defend my PhD thesis, h-factor 8.

All scientific papers submitted for participation in the competition are in the field of bio-organic chemistry. My scientific interest is directed at the isolation and characterization of biologically active substances from natural sources, primarily hemocyanins and antimicrobial peptides from mollusks and their possibile application.

The increased interest in hemocyanins (Hcs) in recent years is due both to their important biological function and complex structure, as well as to the possibilities for their potential application in medicine and pharmacy. Hcs are oxygen transporter copper-containing proteins with extremely high molecular weight and complex quaternary structure freely dissolved in the hemolymph of many species of arthropods and molluscs. Most of them are glycoproteins containing *O*- and *N*-linked carbohydrate structures. Hcs don't form a homogeneous class of proteins. Depending on their structure they are divided into two main classes: Arthropods and Molluscs. Although the Hcs of two biological species have the same physiological function, there are large differences in their molecular masses, structural organization, amino acid sequences and carbohydrate content.

The structural and biophysical characteristics of hemocyanins have been extensively studied during the recent decades. Mollusc hemocyanins are oligomer proteins with molecular masses ranging from 3.3 to 13.5 MDa. Their molecules are represented in the hemolymph as cylindrical decamers, didecamers or multidecamers aggregates, composed from one, two or three isoforms (structural subunits). The basic quaternary structure is a cylindrical decamer 35nm in diameter, containing 10 subunits (350-400 kDa) with identical amino acid sequence (AAS). Despite the difference in the quaternary structure of molluscan hemocyanins, the structural subunits are composed of seven or eight different functional units (FUs) with molecular mass 45-65 kDa, each with an oxygen-binding site.

An important feature of hemocyanins is their carbohydrate structure which plays a fundamental role in their structural organization, conformational stability, immunological properties and their biomedical application. Studies on the *N*-glycan structures of molluscan hemocyanin revealed that they contain an enormous potential for generating a large set of structural elements commonly found in eukaryotic *N*-glycosylation. Molluscan Hcs usually have a higher carbohydrate content (2 – 9%,w/w) than arthropodan Hcs, complex carbohydrate structure and specific monosaccharide content. Of key

importance to the antitumor activity is the *N*-linked carbohydrate structures which possess diverse epitopes and can cross-react, binding to equivalent epitopes on the surface of tumour cells.

The investigation of the conformational stability of Hcs is of fundamental interest in understanding the structural and functional linkages in these glycoproteins. Various aspects of biomedical applications of molluscan hemocyanins, associated with their immunogenic properties and antitumor activity, prompted for studies of the structural and conformational stability on these glycoproteins.

Another interesting subject of my research is antimicrobial peptides (AMP) - diverse and a unique group of endogenous compounds widely distributed in nature. They are essential for the innate immune response of organisms of all branches, presenting activity against a wide range of pathogens, like Gram-positive and Gram-negative bacteria, fungi, viruses and even cancer cells. Most AMPs commonly consisting of 10–50 amino acids are amphipathic and hydrophobic α-helical peptides, with a positively charged domain and show a remarkable structural and functional diversity. So far more than 750 different AMPs have been identified in various organisms ranging from plants to insects and animals, including human. Due to their broad spectrum of antibiotic activity, as well as anti-inflammatory and immunomodulatory properties, AMPs became a model for the discovery of novel antimicrobial drugs that could answer the problem of the increasing antibiotic resistance of pathogenic microorganisms.

The results presented in the habilitation report are related to the development of methods for isolating new hemocyanins and antimicrobial peptides and their in-depth characterization. Another important aspect of my work is to clarify the relationship between structure, function and biological activity of these molecules. The study of the carbohydrate structure of molluscan hemocyanins is essential to my scientific research and is a continuation of my dissertation work.

The main scientific contributions of my research can be summarized as follows:

I. Isolation, purification and characterization of molluscan hemocyanins.

The biodiversity of hemocyanins has promoted interest in the discovery of new structures with increased biomedical properties. Despite the study of a large number of molluscan hemocyanins, there is limited evidence of hemocyanins of the family Helicidae, genus Helix. So far, three subunits of hemocyanins have been identified only in *H. pomatia* and *H. aspersa* but have only been studied incompletely. Therefore, my research has been related to isolation, characterization and study of the structure of new hemocyanins, and to closer investigation of hemocyanins with three subunits found in *Helix lucorum* (*Helix vulgaris*) and *Cornu aspersum* (*H. aspersa*).

1. A new molluscan hemocyanin and its three different structural subunits are isolated and characterized from the hemolymph of the garden snail *H. lucorum*. **(№ 1, №2)** The determined molecular weight of the isoforms 1068 kDa (β-HlH) and 1079 kDa (αD-HlH, and αNHlH) correlates very well with the masses of the structural subunits of *H. pomatia* Hc, which is from the same family Helicidae. **(№1)**

2. For the first time the quaternary structure of native hemocyanin from *H. lucorum* and its isoforms was determined using transmission electron microscopy (TEM). Native HlH exhibits a predominantly didecameric structure (typical for most gastropods) and additionally a few tridecamers

resulting from the association of a further decamer with one didecamer. The three isoforms are mainly represented as homogeneous didecameric structures. The protein characterization, is further confirmed by different methods: UV absorption spectroscopy, fluorescence and CD spectra of the native molecule and its units, confirmed the structure of multimer protein complexes. **(№1)**

3. A new approach to the isolation of three different structural subunits from native HlH was applied. The approach is based on the difference between these isoforms to precipitate or crystallize during dialysis against sodium acetate buffer at a low ionic strength, which allows isolation of βc-HlH in the form of crystalline precipitate and purification of ion-exchange chromatography on a DEAE Sepharose CL-6B column. The α-components (α_D-Hc and α_N-Hc) dissolved into the supernatant were isolated and purified by anion exchange chromatography on a Flow Sepharose Q column of FPLC. **(№1, №2)**

4. There is a significant difference in the structure of the native HlH organization compared to other molluscs Hc which are represented by one or two structural subunits. Undisputed proof of the structural composition of HlH is the identification of N-terminal sequences of three different structural subunits β-HlH, α_D- and α_D-HlH by Edman's degradation. The comparative analysis of N-terminal AAS of the three isoforms with N-terminal sequences of other molluscan Hcs *(R. venosa, H. tuberculata, O. dofleini, N. pompilius, A. californica* and *H. pomatia)* shows similarity of about 50-67% and presence of -LVRKNVDXLT- conserved fragment. Other evidence is based on different isoelectric points (pI) of the three isoforms determined by 2D-gel electrophoresis, different from those of *H. pomatia* and *H. aspersa* Hcs. It was demonstrated that α_D-HlH and βc-HlH isoforms are acidic proteins with pI= 4.3 and 5.2, whereas α_N-HlH is a neutral protein with pI=7.0. **(№1)**

5. The tertiary structure of β-HlH was analyzed in detail, after limited proteolysis with low trypsin concentrations. The tryptic cleavage products were isolated on a FPLC system and purified on RP-HPLC system. Eight different functional units were identified by their N-terminal sequences and molecular masses in the interval (region) of 47 to 55 kDa, determined by SDS/PAGE and MALDI-MS. The multiple sequence alignment of N-terminal sequences of the separated FUs shows that HlH shares a higher identity with *A. californica* Hc than with *Haliotis* Hc. In all of them a typical molluscan hemocyanin fragment (-Val-Arg-Lys-As-) was identified. **(№1)**

For the purposes of the investigation, native *Rapana venosa* hemocyanin (RvH) and its two structural subunits (RvH1 and RvH2), the native *Cornu aspersum* hemocyanin (CaH), structural subunit HtH1, and glycosylated functional unit RvH2-e were isolated, using different methods and chromatographic techniques. **(№2**, **№3, №4, №5, №6, №7**)

II. Determination of carbohydrate structures of molluscan hemocyanins

Glycosylation is one of the most important post-translational modifications found in nature and holds key role in the stability and function of proteins. The relevance of glycosylation of hemocyanins as a factor for their immunostimulatory properties has been revealed from studies on the hemocyanin isoforms KLH1 and KLH2 of *Megathura crenulata* which are widely used in experimental immunology and clinical practice. The clinical success of intravesical administration of KLH to patients with bladder carcinoma is assumed to be based on the presence of the disaccharide Gal(*β*1-

3)GalNAc determinants which are cross-reactive with an equivalent epitope on the bladder tumor cell surface.

Although it is generally accepted that the oligosaccharide constituents of Hcs are of prime significance for its antigenicity and biomedical properties, knowledge of the carbohydrate structures of these glycoprotein is still incomplete. Therefore, the study of the carbohydrate structure of hemocyanins with potential medical applications is an important part of my research.

The purified isoforms βc-HlH (*H. lucorum* Hc), HtH1 (*H. tuberculata* Hc) and RvH2 (*R. venosa* Hc) were subjected to deglycosylation with the specific endoglycosidase PNGase F. The resulting reaction mixtures of *N*-glycans were purified by solid phase extraction on a Carbograph column and applied to mass spectrometry analysis. **(№ 3, № 4, № 5)**

1. A large structural diversity of *N*-glycans in the 3 structural subunits of different molluscan hemocyanins (βc-HlH, HtH1 and RvH2), was proved after interpretation of the MS/MS spectra from the Q-trap system and MALDI-TOF-MS analysis. **(№ 3, № 4, № 5)**

2. The identified oligosaccharide structures of βc-HlH (32 glycans), HtH1 (15 glycans) and of RvH2 (28 glycans) reveal a complex *N*-glycan pattern combining typical structural features of different higher organisms (mammals, plants, insects, nematodes, trematodes). These results have a fundamental significance and broaden the knowledge about the diversity of *N*-glycan structures of molluscan hemocyanin. **(№ 3, № 4, № 5)**

3. The natural carbohydrate structure database was enriched, with novel structural motifs discovered in βc-HlH and HtH1. A new important class of *N*-glycans for the hemocyanins, including an internal Fuc residue connecting one GalNAc(β1-2) and one hexuronic acid confirmed in RvH2. A similar structure was not identified in the *N*-glycans of HtH1 and βc-HlH. Obviously, this important class of acidic glycans is characteristic only of RvH. **(№ 3, № 4, № 5)**

4. The structural 3D-models demonstrate that the putative glycosylated sites and the oligosaccharide chains are exposed on the surface of the functional units. This data suggests their important role in the composition of the quaternary structure, because it may prevent the formation of larger multidecamers. **(№3, №4)**

<u>*N*-Glycan structures of structural subunit βc-HlH of *H. lucorum* hemocyanin isolated from garden snail</u>

- In total, 32 structures of *N*-glycans with composition $Hex_{3-7}HexNAc_{2-5}MeHex_{0-4}Pent_{0-1}Fuc_{0-1}$ were determined. Their oligosaccharide chains contain three main modifications at their inner core: β1-2-linked xylose to β-mannose, α1-6-fucosylation of the innermost GlcNAc residue (the Asn-bound GlcNAc), and methylated Man. Primarily mono- and bi-antennary *N*-glycans from high mannose and complex type were found as well as two hybrid type structures with or without core-fucosylation. The hybrid type structures are not typical of hemocyanins and similar structures have until now been identified only in KLH. **(№3)**
- Methylation of sugar residues is an unusual modification. As far as we know, mammals do not carry this glycan modification, but many other organisms such as nematodes and molluscs do. A high degree of methylation in the oligosaccharide structures of βc-HlH was found. Most of the identified glycans contain mainly terminal MeHex residue, but in some structures an inner residue is found, determined so far only for *H. pomatia* hemocyanin. Novel structural motifs with partly or fully

methylated terminal hexoses are identified, such as MeHex(*β*1-4)GlcNAc(*β*1-2)Man(*α*1-3), Hex(*β*1-3)GalNAc(*β*1-4)GlcNAc(*β*1-2), MeHex(*β*1-3)-[Hex(*β*1-6)-]-MeHex(*β*1-3)GalNAc and Hex(*β*1-3)[Hex(*β*1-6)]GalNAc(*β*1-4)GlcNAc(*β*1-2), which were previously not found in other molluscan Hcs. **(№3)**

- *N*-glycans which contain a modification of the pentasaccharide core with α1-6-linked fucose on the reducing GlcNAc and possess a high degree of methylation were identified. Most of these structures have been found for the first time in βc-HlH. (**№3**)
- Several glycopeptides were isolated and purified by RP-HPLC, after trypsinolysis of βc-HlH. Their structures were analyzed by μLC/ESI-MS, and μLC/ESI-CID-MS. Two glycopeptide with mass 2745.12 Da and 2907.10 Da from oligomannose type, with a glycan structure $Hex_5Man_3HexNAc_2$ and $Hex_6Man_3HexNAc_2$ were identified. **(№ 2)**
- The 3D-model demonstrates that the putative glycosylated sites and the oligosaccharide chains are exposed on the surface in both domains of the functional unit βc-HlH-g. **(№ 3)**.

<u>*N*- Glycan structures identified in subunit HtH1 of *Haliotis tuberculata* hemocyanin</u>

- For the first time, the oligosaccharide structure of the subunit HtH1 of *Haliotis tuberculata* hemocyanin (HtH) were studied by mass spectral sequence analysis of the glycans. 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 MS before and after treatment with specific exoglycosidases 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, was obtained by tandem mass spectrometry of ESI-Q-Trap. The proposed 15 structures from high mannose and complex type are based on MALDI-TOF-MS data 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 via electrospray ionization MS/MS-spectra **(№ 4)**
- A novel structural motif MeHex[Fuc(*α*1-3)-]GlcNAc, including MeHex and (α1-3)-Fuc residues that are linked to an inner (internal) GlcNAc residue in the oligosaccharide chain was identified. The *N*-glycans of HtH1 contain mainly one or two terminal MeHex-residues, as it is characteristic for most molluscan hemocyanins. **(№ 4)**
- Structures with a high degree of fucosylation containing (*α*1-3)-Fuc-linked to an internal GlcNAc residue in the oligosaccharide chain and (*α*1-6)-Fuc-linked to the terminal GlcNAc-residue of the pentasaccharide core were identified in HtH1. The presence of (*β*1-2)-Xyl and (*α*1-3) Fuc-residue is relatively common in plant and insect glycoproteins, and very rarely found in animal glycoproteins. Core fucosylated *N*-glycans are widely distributed in a variety of glycoproteins but little information is available about α1-6-fucosylation in land snails. **(№4)**
- Based on 3D-models of the FUs of HtH1, the positions of the glycosylation sites were shown to likely occur on the surface of the subunits, at least for seven of these FUs. Such a site is missing in FU-c, but there are two putative sites in FU-h. The finding that HtH1 is not able to form multidecameric structures in vivo could be explained by the presence of the exposed glycans on the surface of FU-h, thus preventing the formation of stable multidecamers and longer tubule polymers. **(№4)**

<u>*N*-Glycan structures identified in RvH2 of *R.venosa* hemocyanin</u>

- In total, 28 *N*-glycans from high mannose and complex type with the composition: Hex_{0-9} $HexNAc_{2-4}$ Hex_{0-3} $Pent_{0-3}$ Fuc_{0-3} were characterized by Q-Trap analysis. **(№ 5)**
- The presence of unusual novel type *N*-glycan structure was confirmed, with an internal fucose residue connecting one GalNAc (β1-2) and one hexuronic acid in RvH2 as was previously found in subunit RvH1. This is a confirmation for a new important class of *N*-glycans of hemocyanins. **(№5)**
- An innovative approach was applied to differentiate between the HexA and MeHex residues, which differ by 0.036 Da. The method involves the modification of a carboxyl group of HexA residue by converting the acid to the amide. Analysis of the glycan mixture from RvH2 via MS before and after amidation revealed that molecular ions of two glycans were reduced by ~1 Da. In this way, 2 glycans containing terminal HexA residue and 3 glycans with MeHex were identified. **(№5)**
- The activity of both glycosylated and non-glycosylated forms of different hemocynins HlH and RvH and arthropodan hemocyanin *C. aestuarii* was investigated against *Herpes simplex* virus type 1, strain "Vic" (HSV type 1). For the first time, it was demonstrated the inhibitory effect of one glycosylated functional unit RvH1-Fu of molluscan hemocyanin against the replication of HSV type 1, which is the confirmation of the link between the carbohydrate structures and the antiviral properties of hemocyanins. **(№5)**

<u>III. Study of structural and conformational stability of molluscic hemocyanin</u>

1. The dissociation/reassociation behavior of hemocyanins isolated from garden snails *H. lucorum* and *C. aspersum* and structural subunits (βc-HlH, α_D-HlH and α_N-HlH) is studied by transmission electron microscopy. It has been found, that the reassociation of the investigated hemocyanins occurs differently but depends on the pH of the solution and the concentration of Ca^{2+} and Mg^{2+} ions. **(№1, №6).**

- Higher concentrations of Ca^{2+} and Mg^{2+} ions, lead to a more rapid reassociation and to formation of multidecamers with different lengths for both hemocyanins, but neither hemocyanin forms tubules. **(№ 1, № 6)**
- The three structural subunit (βc-HlH, α_D-HlH and α_N-HlH) demonstrate different behavior than HlH and CaH. They are reassociated predominantly into didecamers and tubules with varying length. In contrast to α_D-HlH isoform, longer tubules were observed in βc-HlH. A similar capacity for the formation of tubules has been observed in other molluscan hemocyanins, as those from *R. venosa* and *H. pomatia*. **(№ 1, № 6)**

2. So far, the analyses provide no explanation why some hemocyanins form very long multidecamers, tubules, others form very short ones, and still others are restricted to didecamers, or just decamers, as in the gastropod chitons. A hypothesis is suggested of a relation between glycosylation and the formation of decamers and multidecamers, namely that the carbohydrate chains linked to the putative glycosylation centers located on the surface of the decamer make it difficult to form long multidecamers and tubules. **(№ 1**)

3. The conformational changes of native CaH and one functional unit RvH2-e were investigated in a wide pH-temperature range by CD spectroscopy. **(№ 6, № 7**)

- The relatively small changes of initial ellipticity $[\theta]_{222}$ indicated that many secondary structural elements are preserved in the native CaH and FU RvH2-e, even at high temperatures (above 80°C), especially at neutral pH. **(№ 6, № 7**)
- From the analysis of conformational changes in a wide pH-temperature range, it was found that the oligomeric CaH has a greater thermal stability than FU RvH2-e, which can be explained by the formation of the quaternary structure and the action of additional stabilizing factors, such as non-ionic interactions. **(№ 6, № 7**)
- It was found that the thermal unfolding of the oligomeric protein CaH is an irreversible process and the mechanism of thermal unfolding has a complicated character. **(№ 6**)

4. The pH-induced conformational changes of one functional unit RvH2-e and the native molecule CaH have compared via pH–T diagram. From a phase diagram are determined the areas of reversibly T- and pH-dependent unfolding of both hemocyanins. **(№ 6, № 7)**

- A small interval of partial reversibility close to pH 5.5, et 25^{o}C was determined for the oligomeric hemocyanin CaH **(№ 6)**. In contrast to the native molecule CaH, the reversibility for one FU RvH2-e is possible at 25°C, in a relatively wide pH range 5.5-7.0. **(№ 7)** 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 the thermodynamic functions ΔH^{o} and ΔG^{o}_{exp} were calculated at standard temperature. The large value of specific heat capacity ΔCp (ΔCp=0.550 kcal/mol.grad) is in agreement with the opening of hydrophobic protein interior at denaturation. The curve of Gibbs free energy of reversiby denaturation of RvH2-e [ΔG^{o}_{exp}(pH)] is pH-dependent with extreme minimum at pH 7.6 (ΔG^{o}_{exp}=33.4 kcal/mol), but the ΔH^{o} is pH-independent, which is characteristic for processes of hydrophobic rearrangment of the quaternary structure. **(№ 7)**

5. For the first time, the unfolding of native CaH in water solutions in the presence of increasing concentrations of four different denaturants (Gdn.HCl, urea, (urea + LiCl) and LiCl) was investigated by CD spectroscopy. It was found that unfolding of native CaH with (urea + LiCl) is similar to the unfolding in the presence of Gdn.HCl. The quantitative comparison of Gdn.HCl by bi-component solution of urea + LiCl unfolding could serve as a new tool to explore refolding pathways by varying the concentrations of urea or LiCl, independently. **(№ 6)**

6. The conformational stability of native CaH as a function of concentration of used denaturants was determined by $\Delta G_D^{H_2O}$. The $\Delta G_D^{H_2O}$ was calculated in the range of 15.48–16.95 kJ.mol^{-1} and in good agreement with the values of other investigated Hcs. The conformational stability of the native CaH toward various denaturants indicates that hydrophilic and polar forces stabilize the quaternary structure, similar to KLH and RvH. The presented results will facilitate the further investigation of the properties and potential applications of CaH. **(№ 6)**

IV. Isolation and characterization of antimicrobial peptides

Antimicrobial peptides (AMPs) are a unique and diverse group of amphipathic molecules with a great potential for use in new antimicrobial drugs, because many of them have a pronounced cytotoxicity against various multi-drug resistant bacteria.

1. The antimicrobial activity of different peptide fractions isolated from the mucus of garden snail *C. aspersum* were tested against gram-negative bacterial strain *Escherichia coli* NBIMCC 8785. Only a fraction below 10 kDa exhibits significant antibacterial activity. To explain the observed effect we purified this fraction by RP-HPLC and eluted 23 peptide fractions and analyzed them by tandem mass spectrometry. **(№ 8)**

2. Using *de novo* MALDI-TOF-MS/MS sequencing we identified the primary structures of 9 novel antimicrobial peptides with molecular masses between 1000-3000 Da in this fraction. Most of them contain a high level of glycine and leucine and belong to a new class of Gly/Leu-rich AMPs. Several peptides contain one or two proline residues inserted into the α-helical to C-terminal sequences, only one peptide to the N-terminal region. This might be important for their structural stability and antimicrobial activity. **(№ 8)**

PERSPECTIVES FOR SCIENTIFIC RESEARCH IN THE NEXT 3 YEARS

My research will continue in the above-mentioned directions and will be accomplished with new objectives and developing new approaches for isolating and characterizing glycoproteins and AMPs.

My future scientific research is closely related to my participation in the following research projects:

1. BNSF № ДН 01/14 from 19.12.16 „Proteome analysis of new natural peptides with antibacterial and antifungal activity isolated from snail *Cornu aspersum*“.
2. BNSF № ДН 01/14 (2016-2020) „Proteome analysis of new natural peptides with antibacterial and antifungal activity isolated from snail *Cornu aspersum*.“
3. BNSF № ДН 03/13 from 18.12.2016 „Changes in the composition and the thermodynamic properties of the brain proteome et neurodegenerative disorders - connection of the exothermic processes in the proteome with the mechanism of plate formation“.
4. BNSF № ДН 11/10. (2017-2020) “*Saccharomyces cerevisiae* quiescence – smart model for toxicological and stress response research“.
5. BNSF № КП-06-ОПР-03/10 from 20.12.2018 г. (2018-2021)“Development and validation of an in silico method to identify biotherapeutics in peptide mixtures of natural origin“.
6. BNSF № КП-06-21/13 from 18.12.2018 г. (2018-2021),"New enzymes from the group of sialidases in filamentous fungi".
7. FWO (2018-2021) Belgium, „Proteomics investigation of the antibacterial effect of molluscan bio-active peptides“.

National Science Program

1. ДО1-2017/30.11.2018 (2018-2022) National Scientific Program "Innovative Low-Toxic Biologically Active Precision Medicine (BioActiveMed)”.

Center of Competence

1. BG05M2OP001-1.002-0012-C01. "Sustainable Recovery of Bio-Resources and Medicinal and Aromatic Waste for Innovative Bioactive Products" .
2. BG05M2OP001-1.002-0019. "Clean Technologies for Sustainable Environment - Water, Waste, Energy for Circular Economy".

My work will continue in these two main directions:

Topic 1:

- Isolation, characterization and identification of the primary structure of new natural peptides and glycopeptides with antibacterial and antifungal activity of molluscs and arthropods, and the generation of a database for their potential pharmacological effect.
- Preparation and characterization of new proteins and glycoproteins isolated from the hemolymph of arthropods and mollusks, using new approaches and chromatographic methods.
- The study of the carbohydrate structures of novel glycoproteins isolated from molluscs.
- Identification of proteins from intracellular extracts of proliferating cells responsible for oxidative and toxic stress.

Topic 2:

- Analyzing and ranking the impact of pollution sources on the environmental status of water;
- Development of innovative technologies for the treatment of waters containing toxic pollutants by creating a selective adaptive algorithm;
- Development of clean technologies for processing of waste products from different biological sources.

List of presented publications:

1. **Velkova, L.;** Dimitrov, I.; Schwarz, H.; Stevanovic, S.; Voelter, W.; Salvato, B.; Dolashka-Angelova, P. *„Structure of hemocyanin from garden snail Helix lucorum“* Comparative Biochemistry and Physiology - B Biochem.and Mol. Biology **2010**, 157(1), 16-25.
2. Dolashka, P.; **Velkova, L.;** Iliev, I.; Beck, A.; Dolashki, A.; Yossifova, L.; Toshkova, R.; Voelter, W.; Zacharieva, S. *„Antitumor activity of glycosylated molluscan hemocyanins via Guerin ascites tumor“* Immunological Investigations **2011**, 40(2), 130-149.
3. **Velkova, L.*;** Dolashka, P.; Van Beeumen, J.; Devreese, B. *„N-glycan structures of b-HlH subunit of Helix lucorum hemocyanin“* Carbohydrate Research **2017**, 449, 1-10.
4. **Velkova, L**.; Dolashka, P.; Lieb, B.; Voelter, W.; Dolashki, A.; Van Beeumen, J.; Devreese, B. *„Glycan structures of the structural subunit (HtH1) of Haliotis tuberculata hemocyanin“* Glycoconjugate Journal **2011**, 28, 385-395.
5. **Velkova, L**.; Todorov, D.; Dimitrov, I.; Shishkov, S.; Van Beeumen, J.; Dolashka- Angelova, P. *„Rapana venosa hemocyanin with antiviral activity”* Biotech. Biotech. Equip. **2009**, 23(2), 606-610.
6. Dolashki, A.; **Velkova, L.*;** Voelter, W.; Dolashka, P. *„Structural and conformational stability of hemocyanin from the garden snail Cornu aspersum“* Zeitschrift für Naturforschung - Section C Journal of Biosciences **2019,** 74(5-6), 113-123.
7. **Velkova, L.;** Dolashka-Angelova, P.; Dolashki, A.; Voelter, W.; Atanasov B. *„Thermodynamic analysis and molecular modeling of Rapana venosa hemocyanin–functional unit RvH2-e”* Biotech. Biotech. Equip. **2009**, 23(2), 601-605.
8. **Velkova, L.*;** Nissimova, A.; Dolashki, A.; Daskalova, E.; Dolashka, P.; Topalova, Y. *„Glycine-rich peptides from Cornu aspersum snail with antibacterial activity“* Bulgarian Chemical Communications **2018**, 50C, 169 – 175.