

STATEMENT

from: Prof. Dr. Mariela Konstantinova Odjakova-Baytocheva, member of the Scientific Jury, appointed by order No. RD-09-15/30.01.2025 of the Director of the IOCCP, BAS, Prof. Dr. V. Kurteva

REGARDING: competition for the academic position of "professor" in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.2. Chemical Sciences, scientific specialty "Bioorganic Chemistry, Chemistry of Natural and Physiologically Active Substances", for the needs of the IOCCP, BAS - State Gazette, issue 104 of 10.12.2024

General presentation of the procedure and the candidate

In the competition for a professor in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional direction 4.2. Chemical Sciences, scientific specialty "Bioorganic Chemistry, Chemistry of Natural and Physiologically Active Substances", there is one candidate - Assoc. Prof. Dr. Lyudmila Georgieva Velkova, IOCCP, BAS. The submitted materials for participation in the competition are prepared in accordance with the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria (LDASRB), the Rules for the Implementation of LDASRB, and the Rules for the Conditions and Procedure for Acquiring Scientific Degrees and Holding Academic Positions at IOCCP, BAS. Assoc. Prof. Velkova graduated from Sofia University "St. Kliment Ohridski", Faculty of Chemistry and Pharmacy. In 2012, as a doctoral student of independent preparation, she obtained an educational and scientific degree "PhD".

Assoc. Prof. Velkova has worked at IOCCP, BAS successively as a chemist (2003-2009); assistant (2013); senior assistant (2013-2019). Since 2019, she has been an associate professor in the professional direction 4.2. Chemical Sciences, scientific specialty "Bioorganic Chemistry, Chemistry of Natural and Physiologically Active Substances."

She has had short-term specializations in prestigious foreign institutions: Institute of Biochemistry and Institute of Cell Biology, University of Tübingen - Germany; Institute of Biology, University of Padua - Italy; Institute of Virology in Kiev, Ukraine; University of Ghent, Belgium.

A list of 10 diplomas and awards received is presented, accompanied by supporting material.

Meeting the minimum state requirements for the academic position of Professor

Assoc. Prof. Velkova is a co-author in a total of 72 publications; 10 patents and utility models, two university textbooks and one collective monograph. The total number of publications outside competitions for the acquisition of the scientific and educational degree "PhD" and the academic position "associate professor" is 38, of which 28 are used in the current competition for the acquisition of the academic position "professor".

The presented scientific output and achieved scientometric data exceed the minimum requirements for awarding the academic position "professor," set out in the Rules for the Conditions and Procedure for Acquiring Scientific Degrees and Holding Academic Positions at IOCCP, BAS. Data is provided to meet the requirements according to indicators as follows: Indicators from Group A: dissertation work - 50 points; Indicators from Group B: habilitation work – 145 points when the required minimum is 100 points (5 publications with Q1; 1 - Q2); Indicators from Group C: publications in journals with IF/IR - 451 points when the required minimum is 250 points (5

publications with Q1; 6 - Q2; 8 – Q3; 3 - Q4 and 2 utility models). Indicators from Group D: cited works – 530 points when the required minimum is 200 points. Indicators from Group E: Data is provided for achieving 602 points when the required minimum is 150 points. Assoc. Prof. Velkova has participated and participates in 17 national and 15 international scientific projects and has led 2.5 national scientific projects. Assoc. Prof. Velkova actively participates in the educational process. She has been the supervisor of 4 students through student practices; she has led seminar sessions on "Good Laboratory and Production Practice in Bioindustries," Master's program "Biobusiness and Bioentrepreneurship." She has conducted exercises and lectures in the Master's program "Quality and Safety of Food" at BF, SU. Assoc. Prof. Velkova has also participated and continues to participate in the training of 14 diploma holders and specialists.

Meeting the minimum state requirements for the academic position of Professor

Assoc. Prof. Velkova is a co-author in a total of 72 publications; 10 patents and utility models, two university textbooks and one collective monograph. The total number of publications outside competitions for the acquisition of the scientific and educational degree "doctor" and the academic position "associate professor" is 38, of which 28 are used in the current competition for the acquisition of the academic position "professor".

The presented scientific production and the achieved scientometric data exceed the minimum requirements for the award of the academic position "professor", set out in the Regulations on the conditions and procedure for acquiring scientific degrees and occupying academic positions in the Institute of Physical Culture and Physical Culture, Bulgarian Academy of Sciences. Data are presented for meeting the requirements by indicators as follows:

Indicators from group A: dissertation work - 50 points;

Indicators from group B: habilitation work - 145 points. with a required minimum of 100 points (5 publications with Q1; 1 - Q2);

Group D indicators: publications in journals with IF/IR - 451 points with a required 250 points (5 publications with Q1; 6 - Q2; 8 - Q3; 3- Q4 and 2 useful models).

Group E indicators: cited works - 530 points with a required 200 points.

Group E indicators: Data on 602 points achieved with a required minimum of 150 points are provided. Assoc. Prof. Velkova has participated and is participating in 17 national and 15 international scientific projects and has led 2.5 national scientific projects.

Assoc. Prof. Velkova also actively participates in the educational process. She was the supervisor of 4 students in the line of student practices; She has led seminars on "Good Laboratory and Production Practice in Bioindustries", Master's Program "Biobusiness and Bioentrepreneurship". She has given exercises and lectures in the Master's Program "Food Quality and Safety" at the Faculty of Food Science, Sofia University. Assoc. Prof. Velikava has participated and is also participating in the training of 14 graduate and postgraduate students.

Evaluation of scientific and applied scientific activities

All scientific works submitted for participation in the competition are entirely in the field of the announced competition. The main emphasis in the presented research is the characterization of biologically active substances (peptides, proteins and glycoproteins) in the mucus and hemolymph of Gastropods with antimicrobial, antitumor, antioxidant and regenerative activity, clarification of their mechanism of action and the possibilities for their application. The scientific contributions

presented in the competition are the result of interdisciplinary research and can be summarized in several thematic areas:

I. Identification and characterization of biocomponents from Gastropods with antimicrobial activity.

For the first time, the antibacterial potential of 5 peptide fractions with different MT, obtained by ultrafiltration of purified extract from the mucus of the garden snail *C. aspersum*, against a number of Gram⁺ and Gram⁻ bacteria has been proven. Mass spectrometric analyses identified numerous peptides with MT below 3 kDa, as well as polypeptides with MT between 4 - 8 and 10 - 20 kDa. The primary structures of over 30 novel peptides with MT below 3 kDa were identified by de novo sequencing and interpretation of the results of MALDI-TOF-MS/MS spectra. Their characterization by physicochemical parameters such as molecular mass, isoelectric points (pIs), grand average hydropathicity (GRAVY) and net charge revealed a rich diversity of amphipathic structures, which is essential for their selectivity and antibacterial activity. Comparison of their amino acid sequences with an AMP database revealed high homology with other glycine-rich AMPs, such as acanthoscurin, ctenidin, procambarin, microcin B, with the Gly/Leu-rich antimicrobial peptide leptoglycin, as well as with defensin-like protein, various forms of gallinacin, scheferin, etc. Software analysis predicted antibacterial, antifungal and antiviral activity of the newly discovered peptides, making them potential candidates for new antimicrobial therapeutics. Based on these findings, a utility model for a composition with antibacterial activity against *Pseudomonas aureofaciens*, *Brevibacillus laterosporus* and *Escherichia coli* was protected. Based on the results of in silico molecular dynamics simulations, a hypothesis for the spontaneous formation of peptide nanostructures (clusters) in the mucus of *C. aspersum* was proposed. Through a developed methodology based on UV-Vis spectroscopic and fluorescence studies, and in vitro tests for antibacterial activity, it was found that the peptides p1 (KVKDNQWRP) and p3 (LFGGHQGGGLVGGGLWRK), as well as their mixture, self-associate and form aggregates at certain concentrations. Fluorescence studies show differences in the dynamics of aggregation between the peptides, with the mixture (p1+p3) and p3 having a greater tendency to aggregate compared to p1. Aggregation is the result of the balance of hydrophobic, electrostatic and π - π interactions between amino acid residues. Conformational changes at different pH values indicate different dynamics and stability of the mono- and bicomponent solutions. Antimicrobial tests against *E. coli* and *Bacillus subtilis* show that the bicomponent mixture (p1+p3) has higher inhibitory activity compared to the individual components, suggesting an additive or synergistic effect.

Studies of antimicrobial proteins in mucus and hemolymph of gastropods provide new data on the antibacterial potential of a protein fraction with a molecular weight above 20 kDa from the mucus of the snail *Cornu aspersum*. This fraction shows high activity against various aerobic and anaerobic bacterial pathogens. A new approach for protein characterization by electrophoretic analysis interpreted with ImageQuantTM TL v8.2.0 software is presented, which reveals a complex profile dominated by proteins with MT between 30 - 100 kDa. Proteomic analysis reveals the presence of important bioactive proteins and glycoproteins: NADH dehydrogenase, glutathione S-transferase, H-type lectins, agglutinin, antimicrobial protein, functional unit of hemocyanin, L-amino acid oxidase, FMRFamide protein, zinc finger protein, elastin-like protein, collagens and mucins. Many of these proteins are associated with antimicrobial and antioxidant

properties. The hypothesis of synergistic action between the different components of the fraction explains the high antibacterial activity against a wide range of pathogens. The antibacterial activity of the fraction is comparable to that of vancomycin, without cytotoxic effects on *Saccharomyces cerevisiae*. The methodology for identifying proteins in the mucus of *C. aspersum* can be used to develop multitargeted therapeutics from natural sources to overcome antibiotic resistance.

Antibacterial activity of a protein fraction with a molecular mass of 50-100 kDa from the hemolymph of the sea snail *Rapana venosa* has been demonstrated. This fraction shows high efficiency against *Escherichia coli* and its activity is due to the complex interaction of several types of proteins, including peroxidase-like protein, aplycyanin A and L-amino acid oxidase. Studies also show significant antifungal activity of fractions from the hemolymph of *R. venosa* and mucus of *C. aspersum* against fungal strains resistant to nystatin and amphotericin. This suggests the possibility of using these fractions as new therapeutic agents against fungal infections.

II. Antitumor potential of components from the hemolymph and mucus of Gastropods

Different isoforms and functional units of hemocyanins from several mollusc species, as well as fractions from the hemolymph and mucus of these organisms, were studied to evaluate their antitumor activity against various cancer cell lines. It was found that these components exhibit antiproliferative activity, which may be associated with the induction of apoptosis and autophagy in tumor cells. New methods for the isolation and purification of hemocyanins were applied, and the results showed that the specific oligosaccharide structures on the surface of these proteins play a key role in their antitumor activity. Particular attention is paid to the hemolymph of the sea snail *Rapana venosa*. A fraction with a molecular weight between 50-100 kDa (HRv 50–100 kDa) and two hemocyanin isoforms (RvH1 and RvH2) show promising antitumor potential against various breast cancer cell lines. This fraction is more effective than the hemocyanin isoforms and leads to cell death by apoptosis and autophagy. The glycosylation of proteins in this fraction also plays an important role in its antitumor activity.

Using modern methods such as proteomic analysis, de novo sequencing and bioinformatics, proteins in the active fraction of *R. venosa* hemolymph have been identified, including peroxidase-like proteins, Aplysianin-A and L-amino acid oxidase. These proteins may explain the antitumor effect and other biological activities of the fraction. The combination of HRv 50–100 kDa with cisplatin and/or tamoxifen showed synergistic and additive effects, which highlights the therapeutic potential of this fraction in the treatment of breast cancer. These studies reveal new possibilities for the use of natural bioactive compounds as antitumor agents, alone or in combination with conventional therapeutics.

The antitumor potential of hemocyanins from the sea snail *Rapana venosa* and the land snail *Helix lucorum* was investigated on the human bladder carcinoma cell line T-24. The hemocyanins from gastropods showed promising immunological and selective antitumor potential. The results showed that the structural subunit RvHII and the functional units β c-HIH-h and RvHIII-e demonstrated a highly selective cytotoxic effect on the proliferation of the T-24 cell line compared to the normal urothelial cell line HL 10/29. Treatment with the N-glycosylated functional unit β c-HIH-h of *H. lucorum* hemocyanin showed the highest antiproliferative effect, similar to that of doxorubicin, leading primarily to apoptotic changes in tumor cells. The hypothesis is that specific carbohydrate epitopes of the N-oligosaccharide structures on the surface of the functional unit β c-HIH-h are responsible for the observed antitumor effects.

III. Mechanism of action of snail biocomponents established by 2D-PAGE proteomic analysis.

The study focused on the mechanism of antiproliferative action of the functional unit β c-HIH-h of *H. lucorum* hemocyanin on the human bladder carcinoma cell line T-24 by proteomic analysis. Using a comprehensive approach including 2D-PAGE and bioinformatics, 40 proteins were identified whose expression was altered after treatment with β c-HIH-h. These proteins play important roles in the glycolytic pathway, lysosomal and proteasomal degradation pathways, as well as in the regulation of cytoskeletal and extracellular matrix components of cancer cells. Among them are proteins related to apoptosis induction, such as HSP27, G3P, ANXA1, TPI, GAPDH and PKM2. The downregulation of these proteins shows potential for new strategies in the therapy of bladder cancer. The results confirm the proapoptotic activity of keyhole limpet hemocyanins and their potential application in anticancer therapy. The mechanism of action of a standardized extract of the snail mucus *C. aspersum* in an animal model of Alzheimer's-type dementia induced by scopolamine was investigated. The extract showed moderate antioxidant properties and modulated the content of monoamines in brain structures related to memory. Proteomic analysis by 2D-PAGE and MALDI-TOF/MS analyses revealed changes in the expression of key proteins in the cortex of rats treated with the extract. Among the proteins identified were ubiquitin carboxyl hydrolase isoenzyme, calbindin, vacuolar ATP synthase, tropomyosin, 14-3-3 zeta/delta protein, kinesin and stathmin. These results highlight the potential of the extract of the mucus of *C. aspersum* as a therapeutic option for the treatment of Alzheimer's-type dementia.

IV. Characterization of other bioactive components from natural sources

17 novel peptides with molecular mass below 1 kDa were discovered in the hemolymph of *H. lucorum* by de novo sequencing. These peptides display cationic amphipathic structures with hydrophobic surfaces, different from those in the mucus of *C. aspersum*. The main amino acids in these peptides are Leu, Val, Phe, His, Pro, Lys and Tyr, which contribute to their high antioxidant potential, especially for scavenging hydroxyl radicals.

A methodology was developed for the isolation, purification and characterization of cyclolipopeptides from cell-free supernatants of *B. velezensis* R22 by LC-MS and LC-MS/MS analysis on a UHPLC-Q-TOF system. The main active compounds in the extract of *B. velezensis* R22 have been determined, including surfactins with molecular masses of 1035.698 Da and 1057.7 Da, and fengycins with molecular masses of 1462.8 Da, 1476.8 Da, 1490.8 Da and 1504.8 Da. Their primary structures have also been characterized. These studies provide valuable information on the potential applications of bioactive components from natural sources in medicine and biotechnology.

Conclusion

The documents and materials submitted by Assoc. Prof. Dr. Lyudmila Georgieva Velkova for participation in the competition for the academic position of "professor" meet all the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADSRB), the Regulations for its implementation and the Regulations on the terms and procedure for acquiring scientific degrees and occupying academic positions in the IOCCP, BAS, Bulgarian Academy of Sciences.

Assoc. Prof. Velkova has presented a sufficient number of significant scientific papers and convincing evidence of her scientific and scientific-applied work, illustrating the high quality of her overall academic activity. All this allows me to give a positive assessment and to recommend with conviction to the esteemed members of the Scientific Council of the IOCCP, BAS to vote positively for the election of Assoc. Prof. Dr. Lyudmila Georgieva Velkova to the academic position of "Professor".

17.04.2025 Prof. Dr. Mariela Odjakova