REVIEW

by Prof. Dr. Mariela Konstantinova Odjakova-Baytocheva,
Sofia University "St. Kliment Ohridski"
of the materials submitted for the competition
to occupy the academic position of 'Associate Professor'
at the Institute of Organic Chemistry with the Center for Phytochemistry (IOCCP), BAS
in the field of higher education 4.2 Chemical sciences: scientific specialty "Bioorganic chemistry, chemistry of natural and physiologically active substances" for the needs of the laboratory "Chemistry and biophysics of proteins and enzymes".

In the competition for 'Associate Professor', announced in the State Gazette, issue 43 of 31.05.2019 and on the web site of IOCCP, BAS, as a candidate participates Ch. Assistant Professor Alexander Dolashki, Ph.D. IOCCP, BAS.

1. General presentation of the received materials

For the participation in the announced competition documents have been submitted by a single candidate Assistant Professor Alexander Dolashki, Ph.D. IOCCP, BAS. The set of paper materials presented by Dr. Alexander Dolashki is in accordance with the Regulations for the Development of the Academic Staff of IOCCP and meets the criteria of the IOCCP - BAS for the occupation of the academic position "Associate Professor". According to the scoring system, Dr. Dolashki has 1840 points and exceeds the NACID requirements. Dr. Alexander Dolashki has published a total of 50 scientific papers. I accept 24 scientific papers for review. Of these, 11 are presented as habilitation work and 13 are in the competition area. Of the remaining 26 - 7 are related to the PhD dissertation and 19 are out of the field of competition. The total distribution of the 24 scientific papers by the relevant Quartiles (Q) is as follows: Q1 - 7; Q2 - 10; Q3 - 5 and Q4 - 2. Dr. Dolashki has co-authored one textbook and one help textbook. The relevant documents for participation in national and international scientific forums after 2006 are presented: 53 national and 51 international.
Attendance documents and project guides are also attached, as follows: participations in national scientific and educational projects - 16, participations in international scientific and educational projects - 13, national project management - 2 and leadership of the Bulgarian team in international projects – 3. The funds raised for the Institute are BGN 300000. A list of four patents is presented.

2. Short biography of the applicant

Alexander Dolashki graduated from the University of Chemical Technology and Metallurgy, Sofia in 2000 with a qualification as a chemical engineer. From 2001 to 2005 he holds a PhD from the University of Tübingen, Germany. In 2005 he defended his dissertation on the structure, functions and properties of copper-containing proteins: hemocyanins and superoxide dismutase. By protocol 26 of 22.11.2006, the HAC approves the Doctor's degree obtained in Germany in the specialty 01.05.10 "Organic Chemistry, Chemistry of Natural and Physiologically Active Substances". From 2006 until now he has been working as a chief assistant at IOCCP, BAS.

Dr. Dolashki has numerous collaborations in scientific projects and specializations at the following institutes: Institute of Biochemistry, University of Tubingen, Germany (2000-2015); Institute of Biology, University of Padova, Italy (2008, 2011); Max Planck Institute

and University of Tübingen, Germany (2002 and 2011); Ghent University, Belgium (2005 - 2011); Institute of Cell Biology, Tubingen University, Germani (2001-2011: Institute of Zoology, Mainz University, Germany (2011-2013); Kiev University, Ukraine (2011); Qingdao University, China (2012).

As a result, he has acquired a high level of computer literacy and considerable experience in the operation of specific equipment and machines needed for research, such as a mass spectrometer, circular dichroism, sequencer, etc.

3. Evaluation of the applicant's scientific and applied scientific activity

An extended habilitation report is presented to reflect the scientific contributions published in 11 scientific papers. In addition to the competition, another 13 scientific publications have been attached, which have been referenced and indexed in world-renowned databases of scientific information with IF ~ 43. The total IF of the articles is **80.458**. 53 published abstracts from participation in international and national scientific forums were also presented. Dr. Dolashki is a co-author of one textbook (Nature and Biological Application of Mass Spectrometry). All the scientific papers submitted for the competition are in the field of bio-organic chemistry and in particular the structure and properties of proteins and glycoproteins.

The main scientific contributions of the conducted research can be summarized thematically in the following directions:

- Isolation and characterization of the structure and properties of proteins with one copper ion in the active center - superoxide dismutases (SOD) - Articles No. 1 and No. 2 (according to the numbering of the articles given in the contribution reference).

Studies have been conducted to provide information on the localization of new Cu/Zn-superoxide dismutase in lower eukaryotes - the fungal strain *Humicola lutea* (Cu/Zn-HlSOD). Results based on electrophoretic motility, KCN and H_2O_2 sensitivity and immunoblot analysis support the existence of Cu/Zn-SOD in the mitochondrial intermembrane space (IMS) and in the cytosol of cells. Enzymatic activity is almost the same in the two compartments, thus suggesting that the membrane space may be one of the major sites of exposure to superoxide anion radicals. The mitochondrial Cu/ Zn-SOD was purified and compared with the cytosolic enzyme. They have identical molecular weight, cyanide and H_2O_2 sensitivity, N-terminal amino acid sequence, glycosylation sites, and carbohydrate composition. The results show that the same Cu/Zn-SOD functions in both the IMS and the cytosol.

Superoxide dismutase (Cu/Zn-AnSOD) from the fungal strain *Aspergillus niger* were isolated and characterized. The primary structure of Cu/Zn-HlSOD and Cu/Zn-AnSOD was determined by Edman decomposition. The molecular weights (15821 Da and 15912 Da) were determined by MALDIMS and ESI-MS, and confirmed by the calculations for the amino acid sequences.

The mitochondrial Cu/Zn-SOD of *H. lutea* is the first naturally occurring glycosylated enzyme from a fungal strain. The isolated enzyme from *A. niger* is not a glycoprotein since no N-binding center (-Asn-Ile-Thr-) is identified in the carbohydrate chain.

Fluorescence spectroscopy and circular dichroism data on temperature and pH stability indicate high enzyme stability, which can be explained by the stabilizing effect of the disulfide bridge.

- Isolation and characterization of the structure and properties of proteins with two copper ions in the active center - hemocyanins (Articles 3, 4 and 5)

New crustacean hemocyanins *Eriphia verrucosa* (EvH) and Black snail *Rapana venosa* (RvH) were isolated and analyzed by mass spectrometry and circular dichroism. Additional information on the structure and properties of hemocyanins from Molluscs and Artropods is also provided.

Arthropod hemocyanin from *E. Verrucosa* (EvH) was isolated for the first time. The hexameric quaternary structure is based on the binding of six 75 kDa subunits. Four structural subunits (EvH1, EvH2, EvH3, and EvH4) were purified by ion-exchange chromatography and characterized. Subunit 3 (EvH3) showed a high percentage of compliance (75.0, 87.5, 91.7 and 75.0%, respectively) compared to the N-terminal sequence of *Cancer pagurus* subunit 1 (Cp1), *Cancer magister* subunits 3 and 6 (Cm3 and Cm6) and *Carcinus aestuarii* subunit 2 (CaSS2), respectively. The partial cDNA sequence (1309 bp) of *E. verrucosa* hemocyanin encoding 435 amino acids was also isolated, showing a high degree of homology (81-84%) with *Cancer magister* subunits 3, 4, 5 and 6. (№3).

The structure and behavior of the association / dissociation of native macromolecular complexes of hemocyanins from molluscs *Octopus vulgaris*, *Sepia officinalis* and *Rapana venosa* and their subunits are characterized by mass spectrometric techniques (electrospray ionization).) and polygonal laser light scattering (MALS). The differences in the quaternary and tertiary structures of the hemocyanins have been demonstrated, with only one subunit type building the intact and dissociated molecule of the cephalopod hemocyanin *O. vulgaris* (respectively with Mm 3545 kDa and 359.3 kDa) and *S. officinalis* (respectively Mm 4134 kDa).), while the presence of two subunits of different masses (with Mm 422.8 kDa and 400.0 kDa, respectively) were established for the gastropod hemocyanin *R. venosa*, which aggregates into didecamers.

Differences in structural subunits were determined after limited proteolysis with tripsin. The two subunits of RvH and one isoform of *S. officinalis* are composed of eight functional units (FUs) with molecular masses of ~ 50 kDa, while seven FUs are established in hemocyanin by *O. Vulgaris*. Mass spectrometric studies show glycosylation, which is also confirmed by the difference in molecular weights measured by ESI-MS and calculated from the amino acid sequence. (№4).

The stability and behavior of the reassociation of the native hemocyanin molecules of *Rapana venosa* and its two subunits (RvH1 and RvH2) were studied. In the presence of different concentrations of Ca^{2+} and Mg^{2+} ions and pH values, the subunits differ not only in their dissociation but also in the formation of helical tubules and multidecamers. Higher concentrations lead to a faster reassociation of the native RvH molecule and its subunits, resulting in stable multidecams of different lengths. RvH1 shows greater stability at higher pH values than RvH2. It is generally found that the stability of bound RvH and its structural subunits is pH dependent. (№5).

The conformational stability of RvH, studied by circular dichroism over a wide pH range, indicates the preservation of many secondary structural elements, even at high temperatures above 80 ° C and 90 ° C, especially at neutral pH. Most of the hemocyanins are glycosylated and three putative O-binding centers are identified in the partial amino acid sequence of EvH at positions 444-446, 478-480 and 547-549, respectively. The higher stability of native hemocyanin *Eriphia verrucosa* (EvH) and *R. venosa*, compared to their

subunits determined by circular dichroism, may be explained by the formation of a quaternary stabilizing structure. The increase in stability, both of the whole hemocyanin molecule and of the subunit constituents shown by pH-induced CD transitions (acidic and alkaline denaturation), can also be explained by the oligosaccharide structure. (№5 и №6).

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The results presented will facilitate further study of the properties and potential applications of hemocyanins.

- Isolation and characterization of the structure and properties of glycoproteins with three copper ions in the active center - tyrosinases (Articles 6, 7, 8 and 9)

For the first time, bacterial strains of *Streptomyces albus* and *Laceyella sacchari* were tested for tyrosinase activity.

After centrifugation, ammonium sulfate precipitation and ultrafiltration, two tyrosinases were purified from the supernatants of S. albus and L. sacchari, purified on an Servacell DEAE 52 anion exchange column and SEC Sephacryl S-100 column (No. 6.7). The molecular weights of the enzymes obtained (30096 Da and 30910 Da, respectively), determined by SDS-PAGE, MALDI mass spectrometry and analysis of the N-terminal amino acid sequences, confirmed their homology with other tyrosinases. The amino acid sequences determined by MALDI-MS / MS of the SDRQVTTGPFAYRHG, WVGGQMATGVSPN and DTDSGERTGHR amino acids of several isolated peptides show very similarities to the sequences of the database for other tyrosinases from *Streptomyces* species (No. 8). The monophenolase and diphenolase activity of *S. albus* tyrosinase has been demonstrated and the enzyme activity is induced in the presence of L-methionine and CuSO4. Using diphenol L-DOPA and monophenol L-tyrosine as substrates, the kinetic parameters Km and kcat were determined as well as the optimum pH values for the activity of the purified enzymes. Bacterial tyrosinases, unlike eukaryotic organisms, are not glycosylated, as confirmed by the orcinol / H_2SO_4 test. (№ 6.8).

Analyzes show that *Streptomyces albus* and *Laceyella sacchari* may be future sources of increased tyrosinase production. The preparation, purification and characterization of these tyrosinases are also the basis of three funded projects led by Dr. Dolashki (Fund for Scientific Research–Flanders (FWO-Vlaanderen) VS.016.09N/2008 (20102012) “Structural characterization of bacterial tyrosinases by *Streptomyces albus* and *Laceyella sacchari* of biotechnological importance"; Project EAP.RIG No 982552 NATO (2007-2009) Isolation and structural study of bacterial tyrosinases for biotechnological applications; POSTDOC-06 Scholarship - MES (2007 - 2009) Isolation and structural study of bacterial tyrosinases for biotechnological use).

The o-diphenoloxidase activity (o-diPO) of a chemically modified functional unit RvH1-a of *Rapana venosa* hemocyanin using L-Dopa and dopamine as substrates was studied. Native RvH1-a has been shown to exhibit no diphenoloxidase activity, but after

treatment with SDS, trypsin or urea, and at different pH values, it converts to an enzymatically active form. The highest induced o-diphenoloxidase activity was determined after incubation of the functional unit with 3.0 mM SDS, with RvH1-a showing activity on both substrates, dopamine and L-Dopa due to detection of the enzyme's active center and better access to substrates. The Km value for SDS-activated RvH1-α and dopamine substrate is higher than published values for hemocyanins from *Helix vulgaris, Helix pomatia*, and tyrosinase from *Ipomoea batatas*, but much lower than tyrosinase from Illex argentinus (ST94) hemocyanin from Carcinus aestuarii. The Km value of SDS-activated RvH1-a relative to L-Dopa is higher than that of hemocyanins from *Helix vulgaris* and *Cancer magister*, but lower than that of tyrosinase from *Streptomyces albus.* The results demonstrate that, despite the fact that hemocyanins and tyrosinases are from the type-3 copper protein family, which have a similar structure to their active center, differences in substrate accessibility to the active centers also determine their function. (№9).

- Proteomic analyzes of the antitumor activity of hemocyanins (Articles 10 and 11)

The effect of hemocyanins of *Helix luc*orum (HlH), *Rapana venosa* (RvH), *Megatura crenulata* (KLH) molluscs and their functional units (FEs) on the growth of human BAL tumor cells and normal cell bladder CAL-29 was investigated line and T10 / 29 compared with doxorubicin. The results obtained indicate that the human tumor cell line CAL-29 is sensitive to the action of the tested hemocyanins and their isoforms. The dose and time dependence of inhibition of tumor growth after incubation with HlH and its functional units (bc-HlH-a and FU bc-HlH-h) is shown. bc-HlH-h shows a surprisingly stronger effect than doxorubicin treatment, observing apoptotic and necrotic cells. For the first time, a proteomic map for the cytostatic action of H. lucorum hemocyanin on the human CAL-29 cell line has been provided. Reduced expression of eight different proteins was identified, as well as increased expression of two proteins that may be associated with the observed apoptosis. No inhibition of normal ueroepithelial cells HL10 / 29 after treatment with HlH and its isoforms has been established. The specific role of oligosaccharide structures of proteins for their biological action against bladder cancer has been suggested. (No. 10)

For the first time, the antimicrobial activities of the hemocyanins of the molluscs *R. venosa* and *H. aspersa* were examined. One subunit structural unit (βc-HaH) and 8 functional units (FUs, βc-HaH-a to βc-HaH-h) were isolated and their N-terminal sequences and molecular masses were determined. Antimicrobial tests show that only two FEs of *R. venosa* (RvH1-b and RvH1-e) have a low inhibitory effect on the growth of the bacterial strain *Staphylococcus aureus.* Of interest is the structural subunit βc-HaH of *H. aspersa*, which exhibits strong antimicrobial activity against the growth of bacterial strains of *S. aureus* and *Streptococcus epidermidis,* but also against *Escherichia coli*. (No. 11). βc-HaH has the potential to be incorporated into pharmaceuticals and used as a substitute for commonly used antibiotics that develop bacterial resistance.

Contributions from the other 13 publications are in the same scientific fields as habilitation work.

Combining enzymatic and non-enzymatic methods, hemocyanins and their functional units from different sources are isolated and purified. Their structures, properties and function were investigated. (1, 2, 3, 4, 5, and 12). Studies have shown the glycosylated character of hemocyanins from various organisms from Arthropoda and Mollusca. The results show that carbohydrate moieties play a major role in the organization of structural units. The molluscan

hemocyanins produced bind complex carbohydrate structures to predominantly N-linked glycans. Using the various mass spectroscopic techniques, the heterogeneous nature of the glycans from the functional units of Rapana hemocyanin ($Hex_{0–9}$ $HexNAc_{2–4}$ Hex0–3 $Pent_{0–3}$ $Fuc_{0–3}$) has been demonstrated. A new type of N-glycan has been found in which an internal fucose residue is bound to GalNAc and one hexuronic acid (6). 15 different glycan structures have been identified in the hemocyanin structural unit HtH1 of Haliotis tuberculata. Like most molluscan hemocyanins, HtH1 glycans contain terminal MeHex. A novel MeHex [Fuc (α1-3) -] GlcNAc structural motif associated with an internal GlcNAc residue (9) has been identified. The specific glycosylation positions identified add to the information on the structure of hemocyanins, which enables a deeper insight into the glycosylation process and clarifies its importance for these huge molecules.

The structural and conformational stability of hemocyanin from garden snail *Cornu aspersum* was investigated. For the first time, its behavior in aqueous solutions in the presence of various denaturing agents is demonstrated (12). Three structural units of the garden snail *Helix lucorum* were cloned and sequenced. Comparing their nucleotide sequences with the hemocyanin database of published sequences for other molluscs, a phylogenetic tree was constructed to illustrate the molecular evolution of the molluscan hemocyanins (4).

The superoxide dismutase from *Humicola lutea* (7) and the L-phenylalanine aminopeptidase from *Cicer arietinum* L. cotyledons (10) have been isolated, purified and characterized, and the O-diphenol oxidase activity of molluscan hemocyanins (11) has been demonstrated.

The immuno-adjuvant properties of hemocyanins, their derivatives and conjugates related to cell-mediated immunity in experimental animals with Guerin ascites tumor have been investigated. They activate the immune system of experimental animals and could be included in non-specific anti-tumor vaccines to enhance their effects (8). The cytotoxic activity of the hemocyanin by *Rapana venosa* and its structural subunits was tested *in vitro* on bladder cancer cell lines CAL-29, T-24 and normal urothelial cell line HL 10/29. A significant cytotoxic effect was observed for the RvH1-c functional unit in contrast to native RvH (13).

Dr. Dolaski is a co-author of four national patents: "Bioactive product containing hemocyanin". Protected No 66374 B1/31.10.2013; "Gastric Disease Prevention and Treatment Composition" Protected No 2194 B1/31.03.2016; "Biologically active peptides from the hemolymph of Rapana venosa". Protected No 66614 B1/31.10.2017, and "Composition of biologically active slime mucus mixtures of Helix aspersa for use in food additives and the cosmetic industry". Protected No. 66832 B1 / 04/02/2019.

Dr. Dolashki's scientific contributions can be grouped as contributions containing new and original information for science; contributions of a confirmatory nature; methodological contributions and applied contributions.

The importance of scientific works can be judged by the rating of the journals in which they were published (total IF - ~ 80.458 - 43 for the competition) and their high citation. A total of over 450 Scopus citations (h-index - 14) have been found, and the publications with which Alexander Dolashki has participated in the competition have been

cited 262 times by independent authors in prestigious international publications. Dr. Dolashki has received recognition among the scientific community at home and abroad. This is evidenced by the 18 awards and nominations received.

All publications by Dr. Dolashki are co-authored. In the published 11 scientific papers, presented as habilitation work, Al. Dolashki is the first author in 8 articles and the second in two of them, which is an indicator of his personal involvement in their drafting.

According to Al. Dolaski in the future, his scientific activity will be related to his participation in two projects: National scientific program K1-2017 / 30.11.2018, "Innovative low-toxic biologically active precision medicine (BioActMed)" and Competence Center BG05M2OP001-1.002-0019/03.2018-12.2023 “Clean technologies for a sustainable environment - water, waste, energy for a circular economy”. Scientific studies are related to the isolation of native peptides and glycopeptides from mollusc extracts and their characterization by MALDI-TOF/TOF-MS, Q-Trap MS and MS / MS, and ESI-MS measurements, for which methods Dr. Dolaski is extremely competent. Particular attention should be paid to establishing the therapeutic effect of biologically active substances and clarifying their mechanism of action.

CONCLUSION

The documents and materials presented by Dr. Dolashki meet all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the Implementation of the LDASRB, the Rules for the Implementation of the LDASRB of BAS and the Regulations of IOCCP-BAS.

The candidate submitted a significant number of scientific papers published after the materials used in the defense of the Doctor 's NSA. The applicant's works have original scientific and applied contributions that have received international recognition as a representative part of them have been published in international journals with a high rating. Its theoretical developments have practical applicability. The scientific and methodological qualifications of Alexander Dolashki are beyond doubt.

The results achieved by Alexander Dolashki in the research activity are fully in compliance with the specific requirements of the IOCCP-BAS Regulations for the application of LDASRB.

After getting acquainted with the materials and scientific works presented in the competition, an analysis of their importance and the scientific, applied and applied contributions contained therein, I find it reasonable to give my positive opinion and to recommend to the Scientific Jury to prepare a report proposal to IOCCp-BAS Scientific Council for the selection of Alexander Dolashki at the academic position of Associate professor at IOCCP-BAS in the professional field 4.2 Chemical Sciences: scientific specialty "Bioorganic chemistry, chemistry of natural and physiologically active substances

09/15/2019

(Prof. Dr. Mariela Odjakova)