REFEREE REPORT

By Prof. Ivan Georgiev Ivanov, D.Sc.

Evaluation of candidates for the position of "Associate 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"* announced at the Institute of Organic Chemistry with Center for Phytochemistry (IOCCP), Bulgarian Academy of Sciences (BAS)

1. General comments

The "Associate Professor" position 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" is announced in State Gazette issue. 43/31. 05. 2019. The only candidate is Dipl. Eng. Alexander Konstantinov Dolashki, Ph.D. from the same institute. The overview of the presented documents shows that the procedure for announcing of the academic position has been complied with the requirements of the Law for Development of the Academic Staff of the Republic of Bulgaria (LDASRB) and the Rules for its implementation as well as with the internal rules and regulations of BAS.

2. Biography

Alexander K. Dolashki, Ph.D., was born on December 29, 1977. He graduated from the University of Chemical Technology and Metallurgy, Sofia in 2000 with a master degree in "Chemical Technology Processes and Systems". He continued his education at the University of Tubingen, Germany, where in 2005 he obtained a Ph.D. degree in Biochemistry. Since then Dr. Dolashki is Assistant Professor at the IOCCF, BAS. After graduation he has visited a number of outstanding universities such as the University of Tubingen (Germany), Mainz University (Germany), University of Padova (Italy), University of Ghent (Belgium), University of Kiev (Ukraine), Qingdao University (China), etc. Dr. Dolashki is the carrier of the prestigious Pythagoras Science Award for 2017.

3. Research activity and scientific achievements

3.1. Overview of the candidate's scientific publications

Dr. Dolashki is the author of 50 scientific papers, 45 of which in peer reviewed journals with IF (total IF 80.458). The papers have been cited so far 262 times and his h-index is 10. He is co-author of 4 applications for national patents.

Dr. Dolashki has appended to his application for Assoc. Prof. 11 new papers published in the period 2008-2019 - all in IF journals (total IF 19.176). They are classified into quadrilles as follows: Q1 - 3, Q2 - 5, Q3 - 2 and Q4 - 1. In 9 of them he is first author. All papers are in the field of bio-organic chemistry. They are dedicated to studying the structure and properties of copper-containing proteins. In my review I will refer to the papers showing their numbers as in the original list of publications.

Dr. Dolashki has reported his results at 53 national and international scientific forums. The abstracts of these presentations will be taken into consideration in my final evaluation but will not be explicitly reviewed.

3.2. Evaluation of the candidate's scientific achievements

All the eleven papers to be reviewed are dedicated to the isolation and characterization of copper-containing proteins, such as superoxide dismutases (SODases), hemocyanins and tyrosinases and can be classified into four thematic groups: *a) Copper-containing proteins*

Type I (No 1 and 2); (b) *Copper-containing proteins Type II* (No 3, 4 and 5); (c) *Copper-containing proteins Type III* (No 6, 7, 8 and 9); (d) *Other papers* (No 10 and 11).

(a) Type I copper-containing proteins (No 1 and 2).

Type I copper-containing proteins includes Cu / Zn-superoxide dismutases (Cu / Zn-CODases) bearing one copper ion in their active center. These enzymes play a key role in neutralizing the superoxide anion radical ($\bullet O^{2-}$), thus limiting formation of H_2O_2 in the cell.

Cu / Zn-CODases are obtained from the filamentous fungus *Humicola lutea* 103 and *Aspergilus niger* 26. From *H. lutea* the enzyme is isolated from three cellular fractions - cytosol, mitochondrial matrix and mitochondrial membranes. Since the three proteins show equal molecular mass, N-terminal sequence and both cyanide and H_2O_2 sensitivities, the author concludes that they are identical and launches the hypothesis that in *H. lutea* the enzyme is located in the mitochondrial matrix and intermembrane lumen (No. 1). It must be noted that the *H. lutea* Cu / Zn-SOD is the first naturally-occurring glycosylated enzyme found in fungi.

Dolashki has isolated two species of SODases from *A. niger*, one of which is Cu / Zn- and the other is Mn-containing (No. 2). Their molecular masses determined by MALDI-MS and ESI-MS are 15821 Da and 15912 Da, respectively. The two enzymes consist of 153 amino acid residues and share high homology with other fungal Cu / Zn-SODs. They do not carry glycosylation sites and show high temperature and pH stability (No 1 and 2).

(b) Type II copper-containing proteins (No 3, 4 and 5).

Type II glycoproteins include oxygen carrying hemocyanins from molluscs and arthropods containing two copper ions. Despite their identical biological function, the proteins of the two kinds of organisms have different structure and properties.

Dr. Dolashki has isolated a new hemocyanin from the Black Sea crustacean *Eriphia verrucosa*. He proved its hexameric organization (6x75 kDa) and purified four of its subunits (EvH1, EvH2, EvH3 and EvH4). He has also obtained a cDNA sequence of the subunit 5 (No. 3). Using ESI, MALDI and MALS, Dolashki has characterized the hemocyanins from three other molluscs such as *Octopus vulgaris*, *Sepia officinalis* and *Rapana venosa*. He proved that the hemocyanins of *O. vulgaris* and *S. officinalis* consist of one type of subunits (359.3 kDa and 443.8 kDa, respectively), whereas the *R. venosa* hemocyanin is composed of two different subunits (422.8 kDa and 400.0 kDa) aggregating into didecamers. Each of the two subunits is composed of eight functional monomers of 50 kDa each (No 4). The re-association of the *R. venosa* hemocyanin, as observed by electron microscopy, shows that its subunits aggregate into helical tubules and multidecamers of different length. The aggregation itself is affected by both pH and Ca^{2+} and Mg^{2+} concentrations, but their effect is different for the different hemocyanins (No 5). The conformational stability of *R. venosa* hemocyanin has also been investigated by CD. Many of its secondary structures have been found to remain stable over a wide pH and temperature range. At neutral pH, their structure was preserved even at 80-90 °C, which is explained by the stabilizing effect of the quaternary structure on the corresponding secondary structures (Nos. 5 and 6). On the other side, the quaternary structure itself is stabilized by the glyosidic residues attached to the hemocyanin molecule. In the *E. verrucosa* hemocyanin Dolashki has identified three potential O-glycosylation sites at positions 444-446, 478-480 and 547-549 (No. 3).

(c) Type III copper-containing proteins (No 6, 7, 8 and 9).

The copper-containing proteins with three copper atoms in their active center are tyrosinases and catechol oxidases. Dolashki has focused his attention to the tyrosinases from

Streptomyces albus and *Laceyella sacchari* - not studied before. Using ammonium sulfate precipitation and ultrafiltration he has isolated from the two bacteria tyrosinases with molecular masses of 30096 Da and 30910 Da respectively. They share homology with other *Streptomyces* tyrosinases but unlike the eukaryotic enzymes, they are not glycosylated (No. 8). The *S. albus* tyrosinase has been investigated for both monophenolase and diphenolase activities using L-tyrosine and L-DOPA / dopamine as substrates. The pH optimum of this enzyme was set to pH 6.8 (No 6 and 8).

Amongst the original studies of Dr. Dolashki it is worth mentioning the discovery of a latent o-diphenoloxidase activity of the RvH1 subunit of *R. venosa* hemocyanin. This activity is manifested after SDS, trypsin and urea treatment, and in the presence of L-dopa and/or dopamine. Since the native RvH1 is devoid of such activity, it is assumed that the reagents "open" the active center, which is screened in the native protein (No. 9).

(d) Other studies (No 10 and 11).

The last two publications of Dr. Dolashki are devoted to studying the biological effects of molluscan hemocyanins. Hemocyanins of *Helix lucorum, Rapana venosa* and *Megatura crenulata* as well as their functional units have been tested for antitumor activity against human tumor cell lines derived from bladder cancer cells (CAL-29 and T24). It has been shown that all tested proteins cause apoptosis and necrosis, with an effect greater than that of the reference doxorubicin. The hemocyanins influence also the tumor cells gene expression, causing a visible change in their protein pattern. A decreased expression was registered for eight major proteins and an increased level for two others (No 10).

Hemocyanins of *R. venosa* and *H. aspersa* and their subunits have also been tested for antibacterial activity against *Staphylococcus aureus* pathogenic strains. The strongest effect is observed with the βc-HaH subunit of *H. aspersa* hemocyanin, which exhibits also antibacterial activity against *Escherichia coli* (No. 11).

4. Research projects

Dr. A. Dolashki is a participant in 27 research projects with national and foreign funding. He is principle investigator of two projects funded by the Ministry of Education and Science and leader of the Bulgarian teams in three joint projects with Germany, China and Belgium. Currently he is involved in the development of the National Science Program "BioActMed", funded by the Bulgarian Ministry of Education and Science. Dr. Dolashki maintains stable partnership with researchers from many national institutes and universities, as well as with scientists from the universities of Tübingen, Germany; Mainz, Germany; Padova, Italy; Ghent, Belgium; Kiev, Ukraine and Qingdao, China.

5. Personal impressions

I have reviewed several projects and scientific papers of Dr. A. Dolashki's and I have been highly impressed of their quality. I am also impressed of his ability to seek and find appropriate implementation and business realization of his fundamental results. He is able to work in a team and he is also capable of creating and managing research teams, which is a prerequisite for his further progress and success in science.

Conclusion: Dr. Alexander Dolashki is an established scientists in the field of bio-organic chemistry, in particular in copper-containing glycoproteins. In this area, he has published 50 scientific papers in the best international journals of biochemistry, bio-organic chemistry and molecular biology. His papers have been cited so far 262 times. He is also a co-author of four national patent applications. The name of Dr. Dolashki is well known and respected in

Bulgaria and abroad. He satisfies (even exceeds) the formal national minimum criteria, the requirements of LDASRB and the internal rules and regulations of IOCCPh for the academic position "Associate 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"*. Taking into consideration all this, I am highly recommending the distinguished Scientific Jury and the Scientific Council of IOCCPh to award Dr. Alexander Dolashki the academic position **Associate Professor**.

Sofia
12.09.2019

Signature:

/Ivan Ivanov/