INFORMATION ON SCIENTIFIC CONTRIBUTIONS

of Assoc. Prof. Dr. Aleksandar Konstantinov Dolashki

for participation in the contest for associate professor on 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"

An extensive habilitation report is presented for the competition, which reflects my scientific contributions published in 11 scientific papers. There are also 13 scientific papers, other than those used in the dissertation thesis, to acquire the educational and scientific degree "doctor". Overall, my research papers are 49 published in journal with Impact Factor and 250 times citated. h-index 10.

All scientific papers submitted to the competition are in the field of bio-organic chemistry, in the field of protein chemistry and in particular the structure and properties of proteins and glycoproteins.

One of the most widespread chemical element in nature is copper. It participates in many biological processes involving electron transfer reactions, oxygen activation, and oxygen transport.

Copper proteins are classified as type I, type II, or type III centers, depending on the environment of the metal ion and spectroscopic characteristics. Therefore, my work is a continuation of the research done during my PhD work which included research on copper containing glycoproteins superoxide dismutases, hemocyanins and tyrosinases.

The Cu/Zn-superoxide dismutase (Cu/Zn-SOD) enzyme (type I), which includes one copper ion in the active site, plays a key role in the elimination of superoxide anion radical ($\bullet O_2^-$) and restriction of the formation of hydrogen peroxide (H_2O_2). The enzymes Cu/Zn-SODs play a key role in the body's antioxidant protection.

A different function is found for the glycoproteins 'hemocyanins' (type II) with two copper ions in the active site that bind a oxygen molecule and carry it to the cells in the respiratory organisms. Detailed information about hemocyanins is available and refers to two major species Molluscs and Arthropods. Comparative analyzes show that these hemocyanins perform the same function of transporting the oxygen to the cells but have significant differences in structure and properties.

Another oxygen binding glycoproteins tyrosinases (type III) and the related catechol oxidases comprise a family of enzymes with three copper ions in the molecule found in many species of animals, plants, fungi, and bacteria. They use phenol-like starting materials to produce a variety of biologically important compounds, such as melanin and other polyphenolic compounds. The copper ions present in their active site bind one molecule of atmospheric oxygen to catalyze two different kinds of enzymatic reactions: (I) ortho-hydroxylation of monophenols (cresolase activity) and (II) oxidation of o-diphenols to o-diquinones. The complicated hydroxylation mechanism at the active centre is still not completely understood.

The research conducted shows some specific characteristics for hemocyanins and tyrosinases, such as enzyme with phenoloxidase activity.

Recently, copper glycoproteins have been increasingly used to treat a number of diseases. The Cu/Zn-SOD enzyme is used to improve the antioxidant protection of patients and accelerate the recovery process after brain injury. Although there is much evidence of the therapeutic effect of SODs, their use is still limited due to very few isolated natural glycosylated SODs and inadequate production of suitable preparations.

Other oxygen-binding copper glycoproteins 'hemocyanins' are also widely used in the pharmaceutical and medical fields. They are well known as immunostimulants and hapten carriers. The application areas of hemocyanins from Molluscs are considered to be potential new adjuvants for immunization and possible immunotherapy of certain types of tumors, as investigated antitumor effect of KLH in the treatment of bladder cancer. Hemocyanins from Arthropods are suitable for application against certain viral and bacterial infections.

Recently, studies on the properties and function of tyrosinase received enormous interest due to their usefulness in numerous biotechnological applications. Tyrosinases are essential for pigmentation, important factors in wound healing and primary immune response. They have been used as phenol biosensors, to cross-link proteins, or for the removal of phenols from waste waters.

In spite of the presented information on the importance and the different application areas of tyrosinases, hemocyanins and Cu/Zn-SODs, the mechanisms of these effects are not yet fully explained, and only assumptions have been made. This requires detection and purification of new tyrosinases, Cu/Zn-SODs and hemocyanins which will help expand research and gain additional insight into the complex structure of glycoproteins that are so important to organisms.

The main scientific contributions of the research can be summarized thematically follows:

I. ISOLATION AND CHARACTERIZATION OF THE STRUCTURE AND PROPERTIES OF PROTEINS WITH ONE COPPER ION IN THE ACTIVE SITE (SUPEROXIDE DISMUTASE) (paper №1)

1. Novel studies were carried and information about the unusual location of new Cu/Zn-superoxide dismutase in lower eukaryotes such as filamentous fungi *Humicola lutea* (Cu/Zn-HlSOD) was provided. The purified mitochondrial and cytosolic Cu/Zn-SOD have identical molecular mass, cyanide- and H_2O_2 -sensitivity, N-terminal amino acid sequence, and these findings suggest that the same Cu/Zn-SOD exists in both the mitochondrial intermembrane space and cytosol **(№1**).

2. Two species of superoxide dismutases, Cu/Zn-SOD and Mn-SOD, were also isolated from the fungal strain *Aspergillus niger* (Cu/Zn-AnSOD). The molecular masses of 15821 Da and 15912 Da, respectively, were determined by MALDI-MS and ESI-MS for Cu/Zn-SOD from both fungal strain, and calculated by their amino acid sequences **(№2**).

3. The primary structure of Cu/Zn-HlSOD and Cu/Zn-AnSOD, determined by Edman degradation and mass spectroscopy, consisting of 153 amino acid residues, reveals a very high degree of structural homology with the amino acid sequences of other Cu/Zn-SODs **(№1 and №2).**

4. It was found that *H. lutea* mitochondrial Cu/Zn-SOD is the first identified naturally glycosylated enzyme from fungal strain. However, isolated enzyme from *A. niger* is not a glycoprotein, because a carbohydrate chain was not identified on *N*-linkage site -Asn-Ile-Thr-.

5. Temperature and pH stability, analysed by fluorescence and CD spectroscopic, confirmed that the enzyme is very stable, which can be explained by the stabilising effect of the disulfide bridge **(№1and №2).**

II. ISOLATION AND CHARACTERIZATION OF THE STRUCTURE AND PROPERTIES OF PROTEINS WITH TWO COPPER IONS IN THE ACTIVE SITE (HEMOCYANINS)

(**papers 3,4,5**)

Oxygen transporting glycoproteins hemocyanins dissolved in the hemolymph of many arthropodan and molluscan organisms bind two copper ions in the active site and have very complicated structure. Various aspects of biomedical applications of hemocyanins prompted for structural studies on these glycoproteins.

We have been isolated new hemocyanins from crab *Eriphia verrucosa* (EvH) and

marine snail *Rapana venosa* (RvH), living in the Black Sea, and analysed them by mass spectrometry and circular dichroism. Additional information about the structure and properties of hemocyanins from Molluscs and Artropods have been represented.

1. For the first time, an arthropodan hemocyanin was isolated from crab *E. verrucosa*. Its multi-hexameric molecular assembly (6×75 kDa) was identified using ion-exchange chromatography and four structural subunits EvH1, EvH2, EvH3, and EvH4 were purified and characterized. Moreover, a partial cDNA sequence (1309 bp) of *E. verrucosa* hemocyanin encoding a protein of 435 amino acids with a high degree of similarity to subunit 5 was determined **(№3**).

2. The understanding of the structure and association/dissociation behavior of native macromolecular complexes and of their subunits of molluscan *Octopus vulgaris*, *Sepia officinalis* and *Rapana venosa* hemocyanins have been characterized using mass spectrometric techniques (electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)) and multi-angle laser light scattering (MALS) (**№4**).

3. The differences in the quaternary and tertiary structures of Hcs have been proven, as only one type subunit organizes the native and dissociated molecule of cephalopodan Hcs *O. vulgaris* (with Mw 3545 kDa and 359.3 kDa, respectively) and *S. officinalis* (with Mw 4134 kDa and 443.8 kDa, respectively), while the presence of two isoforms with different masses (422.8 kDa and 400.0 kDa) has been determined for gastropodan *R. venosa* Hc, aggregated into didecamers.

Both subunits of RvH and isoform of *S. officinalis* are arranged by eight functional units (FUs) with masses of ~ 50 kDa, while seven FUs were purified from *O. vulgaris* hemocyanin (**№4**).

4. The reassociation behavior of hemocyanins from marine snail *Rapana venosa* (**№5**) in comparison with hemocyanis from Molluscs have been analysed by electron microscopy. Higher concentrations of Ca^{2+} and Mg^{2+} ions led to a more rapid reassociation of the native molecule of RvH, and its isoforms (structural subunits), resulting in stable multidecamers with different lengths. Reassociation behaviour of structural subunits of both hemocyanin in the presence of different concentrations of Ca^{2+} and Mg^{2+} ions and pH values differ not only in their reassociation behaviour, but also in formation of helical tubules and multidecamers. RvH1 revealed a greater stability at higher pH values compared to the other subunits (**№5)**.

5. The conformational stability of the native RvH, investigated by circular dichroism within a wide pH-temperature range, indicated that many secondary structural elements are preserved, even at high temperatures above 80°C and 90°C, respectively, especially at neutral pH.

The mechanism of thermal unfolding of *Cornu aspersum* hemocyanin (CaH) has a complicated character, and the process is irreversible. The increasing stability of both native Hcs and their subunits, shown by pH-induced CD transitions (acid and alkaline denaturation), can be explained with the formation of quaternary structure (**№5 and** №**6)** .

6. Most of the hemocyanins are glycosylated, and three putative O-linkage sites were identified in the partial amino acid sequence of Eriphia verrucosa hemocyanin (EvH) at positions 444-446, 478-480, and 547-549, respectively. The higher stability of native EvH and R. venosa hemocyanin in comparison to their subunits determined by circular dichroism (CD) could be explained with the formation of a stabilizing quaternary structure. The increasing stability of both native Hcs and their subunits, shown by pH-induced CD transitions (acid and alkaline denaturation), can also be explained with oligosaccharide structure **(№3 and №5).**

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

III. ISOLATION AND CHARACTERIZATION OF THE STRUCTURE AND PROPERTIES OF GLYCOPROTEINS WITH TREE COPPER IONS IN THE ACTIVE SITE (TYROSINASES).

(**papers № 6,7,8,9**)

The bacterium *Streptomyces albus*, and the soil bacterium *Laceyella sacchari* have not been thoroughly investigated for tyrosinase activity until now. The studies show that **t**his bacterium is maybe a future source for larger production of tyrosinase. In the cours of three projects, I led, two bacteria tyrosinases were purified from bacteria *S. albus* and *L. sacchari* and analysed by different methods.

1. After centrifugation, ammonium sulfate precipitation and ultrafiltration, the supernatants from *S. albus* and, and *L. sacchari* bacteria were isolated on an anion-exchange Servacell DEAE 52 column and additionaly purified by SEC Sephacryl S-100 column **(№ 6 and №7)**. The molecular masses of both purified tyrosinases were determined by MALDI mass spectrometry to be 30 096 Da and 30 910 Da, respectively, and their *N*-terminal sequence analysis confirmed that the isolated enzymes are homologous to other tyrosinases.

Additionaly, several isolated peptides characterized by MALDI-MS/MS, deduced the sequences SDRQVTTGPFAYRHG, WVGGQMATGVSPN and DTDSGERTGHR, respectively, with very high similarity to database sequences for other tyrosinases from Streptomyces species (№**8)**.

2. Bacterial tyrosinases in contrast to eukaryotic organisms are not glycosylated and it was confirmed by an orcinol/H_2SO_4 test.

3. The enzyme isolated from *S. albus* shows both, monophenolase and diphenolase activities. The enzymatic activity of isolated tyrosinases was induced in the presence of L-methionine and $CuSO_4$. The kinetic parameters for the diphenol substrates L-DOPA and dopamine and for the monophenol substrate L-tyrosine were determined at pH 6.8. These characteristics make *Streptomyces albus* tyrosinase an interesting candidate for future analytical and biotechnological applications **(№ 6 and №8).**

4. Hemocyanins and tyrosinases represent the 3-type proteins, whereas the chemically modified functional unit RvH1-a of molluscan hemocyanin *R. venosa* exhibited o-Diphenol oxidase activities using L-Dopa and dopamine as substrates. The native FU RvH1-a did not show anyo-diPO activity, but after treatment with SDS, trypsin, urea and different values of pH it was converted to enzymatic active form. The highest artificial induction ofo-diPO activity was observed after incubation of FU with 3.0 mM SDS, and RvH1-a shows both, dopamine andL-Dopa activity due to a more open active site of the enzyme and better access of the substrates. The K_m value of SDS-activated RvH1-a against L-Dopa is higher than those of hemocyanins from *H. vulgaris* and *Cancer magister*, but lower than that of the tyrosinase from *Streptomyces albus* **(№ 6and №8**).

V. –PROTEOMIC ANALISES OF ANTITUMOR ACTIVITY OF HEMOCYANINS (**papers 10**)

Haemocyanins are well studied and their antitumor activity are well known. Therefore, the growth of human bladder tumor cell lines, CAL-29 and T24, is detected in the presence of *Helix lucorum* (HIH), *Rapana venosa* (RvH), *Megatura crenulata* (KLH) hemocyanins and their functional units.

1. Cells treated with β_c-HlH-h showed a stronger effect than that the doxorubicin-treated cells and both, apoptotic and necrotic cells were observed. The most effective inhibition of CAL-29

tumour cells after treatment with β_c-HlH-h, was probably caused by a specific oligosaccharide structure of HlH with methylated hexoses.

2. Two-dimensional polyacrylamide gel electrophoresis was used to analyse the changes in protein expression in cells after treatment with hemocyanins. Eight different down-regulated and two up-regulated proteins were identified, which may be associated with the apoptosis pathway. No inhibition of the normal urothelial cell line HL 10/29 was observed after treatment with HlH and its isoforms. These results suggest that hemocyanin glycosylation plays an important role in its anticancer activity (№ 9,10).

3. For the first time the antimicrobial activities of hemocyanins from the molluscs *R. venosa* and *H. aspersa* have been tested. The antimicrobial test of the hemocyanins against different bacteria showed that only two FUs from Rapana, RvH1-b and RvH1-e, exhibit a low inhibition effect against *Staphylococcus aureus*. In contrast, the structural subunit βc-HaH shows strong antimicrobial activities against Gram-positive *S. aureusand* and *Streptococcus epidermidis*, but also against the Gram-negative bacterium *Escherichia coli*. We suggest that this subunit therefore has the potential to become a substitutefor the commonly used antibioticsagainst which bacterial resistance has been developed (№ 11)

GUIDELINES FOR FUTURE RESEARCH

The work will continue in two main directions:

1. Center of competence BG05M2OP001-1.002-0019/03.2018-12.2023 "Clean Technologies for Sustainable Environment - Water, Waste, Energy for Circular Economy".

2. National Scientific Program DO1-2017/30.11.2018, "Innovative Low-Toxic Biologically Active Precision Medicine (BioActiveMed)".

Research includes:

TOPIC 1:

1. Isolation of natural peptides and glycopeptides from natural products/

2. Characterization of the resulting natural peptides and glycopeptides by their molecular masses, amino acid sequences using mass spectrometry by MALDI-TOF/TOF-MS, Q-Trap and ESI-MS measurements;

3. Establishing the therapeutic effect of biologically active substances and clarifying the mechanism of action;

4. Analysis of proteomic profiles of bacterial, fungal and tumor cells to predict the effect of bioactive compounds (peptides, glycopeptides, proteins and glycoproteins) after treatment.

• Proteomic profiling of model cells treated with isolated peptides with antimicrobial activity and modeled cells treated with the peptide and nanodiamond complexes;

Comparison of protein expression profiles of bacterial, fungal and tumor cells before and after treatment with various bioactive compounds;

TOPIC 2:

1. Determination of the ecological status of the water intake (water and sediments);

2. Analyzing and ranking the impact of pollution sources on the environmental status of water;

3. Development of innovative technologies for the treatment of waters containing toxic pollutants by creating a selective adaptive algorithm;

4. Development of clean technologies for processing of waste products.

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