REVIEW

by *prof. Dr. Svetla Dimitrova Petrova, Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski" Member of the Scientific Jury appointed by order* ***№ 111-ОБ/28.06.2024*** *of the Director of the Institute of Molecular biology "Acad. Roumen Tsanev"- BAS*

on a **Doctoral Thesis** entitled:

"DNA deglycating activity of the glycolytic enzyme phosphoglucose isomerase"

for awarding the Educational and Scientific Degree "PhD" in the Area of Higher education: 4. Natural sciences, mathematics and informatics, Professional field: 4.3. Biological Sciences
PhD program: ***Molecular Biology***

Author of the PhD Thesis: Elitsa Hristova Boteva
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1. General characteristics of the dissertation work and the presented materials

Elitsa Boteva's dissertation is written following a standard principle - Introduction, Literature review (41 pp.), Aims and objectives (1 p.), Materials and Methods (26 pp.), Results and Discussion (90 pp.), Summary (1 p.) and Contributions (1 p.), Supplements - list of publications and participations in scientific forums (4 publications with impact factor, 1 without IF and 1 book chapter; 10 participations in scientific forums - 2 p.) and a Bibliography (cited 287 scientific sources reflecting the actuality of the problem). The dissertation contains 177 pages, 55 color figures and 4 tables, excellently designed. The set of documents and materials presented meets all the requirements of the Act on Development of Academic Staff in the Republic of Bulgaria (ADASRB) and the Regulations for its implementation.

2. Brief biographical data and personal impressions about the candidate

PhD student Elitsa Boteva graduated Sofia Mathematical High School "Paisiy Hilendarski" in 2005 and immediately after that she was admitted to the Faculty of Biology (Molecular Biology major) at "St. Kliment Ohridski" University, where she graduated "Bachelor" degree in Molecular biology with honors, defending a Diploma thesis with excellence. Elitsa Boteva obtained a Master's degree in "Genetics" at SU "St. Kliment Ohridski", so choosing PhD program in "Molecular Biology" at IMB-BAS was the absolutely reasonable and natural development of Elitsa Boteva as a future researcher. She presents an enviable experimental experience acquired successively at: Medical Center "Prosel" Sofia (2 years), where she worked on the isolation and manipulation of hematopoietic stem cells and cryopreservation of stem cells from umbilical cord; National HIV/AIDS Reference Laboratory at the National Center, working on genotyping and conservation; in IMB-BAS, she began (as biologist and PhD student) her scientific work on glycobiology and DNA glycation. Her specializations as a molecular biologist are impressive: in Japan (Tokyo, 1 year), at the Hasumi International Research Foundation's Electrochemical and Cancer Institute, she specialized in the development of peptide vaccines for cancer immunotherapy; in Utrecht (Netherlands, 6 months) at the Department of Biochemistry and Cell Biology, University of Utrecht, she studied the host-pathogen interaction; and in Stockholm (Sweden), she trained in Flow Cytometry at the Department of Cell and Molecular Biology of the Karolinska Institute, essential method

for any molecular biologist these days. I am confident that Elitsa Boteva possesses not only the skills, but also the mindset of a researcher and should devote herself to scientific research in the future as well.

3. Relevance and significance of the dissertation topic

The scientific problem to which Boteva's dissertation is dedicated - "DNA deglycation activity of the glycolytic enzyme phosphoglucose isomerase" - affects all aspects of modern biological science - from the potential of spontaneous chemical glycation reaction, through the metabolic transformations of their products and accumulation, to pharmacological and physiological effects of glycated biological macromolecules and appearance of an evolutionary need for their efficient elimination, which can only be achieved by enzymatic catalysis. The dissertation work is a challenge with the aim of unraveling an enzymological "enigma" and proving multifunctionality in the catalytic mechanisms and strategies of the enzyme phosphoglucose isomerase.

The spontaneous Maillard reaction and the complex network of product interconversion reactions is evolutionarily very old. It shows the possible connections between all types of biomolecules and creates the basis for thermodynamic selection of only certain pathways to final stable products. This primary metabolic pathway model interacts with metabolic pathways in the cell and triggers existing enzyme regulatory mechanisms. The study of the process of removing the products from DNA glycation makes Elitsa Boteva's dissertation work extremely relevant, especially after establishing the importance of protein glycation processes and defining them as a normal biochemical logic.

It is absolutely reasonable to ask the question whether glycation of Nucleic acids (NA) is possible? Prof. Mironova's team established (2005) that the chromosomal DNA of *E. coli* is glycated under natural physiological conditions *in vivo*. In the current dissertation, *this finding is upgraded by: demonstrating the existence* of a specific additional deglycating activity of the enzyme Phosphoglucose isomerase (PGI, EC 5.3.1.9; glucose-6-phosphate isomerase, phosphohexose isomerase, D-glucose-6-phosphate ketol-isomerase) - a conservative glycolytic/gluconeogenic enzyme catalyzing a reversible intramolecular rearrangement reaction, with different isoforms, reaction specificities, cellular localization and biological relevance for various metabolic pathways; *establishing the mutagenic nature of glycation products* in DNA; *demonstrating of deglycase activity of human PGI isoforms* and possible mechanisms for PGI involvement in glycated DNA repair. Most importantly, by *proposing a mechanistic model of the catalysis of DNA deglycation*, this integral study rises to a new and very high level.

Therefore, the topic of this doctoral thesis is highly relevant, opening opportunities for a more detailed understanding of DNA repair mechanisms and the functional plasticity of enzymes.

4. General characteristics of the dissertation and knowledge of the state of the problem by the PhD student.

Elitsa Boteva's dissertation is written extremely competently and demonstrates an excellent knowledge of the scientific search in the field, as it offers a detailed analysis and summary of the information known so far. The PhD student presents the process of glycation of biological molecules informatively, very convincingly and in a logical biochemical sequence she defines the working hypothesis, the aim of the work and the subsequent tasks. Elitsa Boteva brings out the basic biochemical principles: *identification and purification of the specific deglycase enzyme activity* expressed by glycolytic phosphoglucose isomerase (PGI) in *E.coli* and yeast used as a model; *demonstration of the localization, DNA-binding ability* and properties of the human nuclear isoform of hPGI; *evaluation of the deglycating potential* and efficiency of hPGI; *comparative structure-function interaction analysis* of all studied PGIs; *excellent scientific analysis and discussion* of the obtained experimental results and based on them, *mechanistic model of ES complex* - DNA-NH-F6P-PGI and catalytic model of deglycation by bioinformatic methods of *in silico* (molecular docking and molecular metadynamics);

involvement of PGI in DNA repair mechanisms. I can't help but notice the wonderful writing style and layout of the dissertation, which is a rarely seen case in recent years.

The *literature review* describes in detail the chemical essence of glycation (Maillard) reaction as a spontaneous process in its wholeness – consecutive stages, many variants of products and their reversible conversions, sugar concentration and structural selectivity of isoforms, biological significance of the final stable covalent glycation products of proteins and NA, and their role in the manifestation of various pathological conditions. A central part of the review is the process of glycation of DNA and the consequences for its stability, as well as the key glycomodification derivatives of guanosine and 2-deoxyguanosin, obtained *in vivo,* by glycation agents as glucose and glucose-6-phosphate glyoxal, methylglyoxal dihydroxyacetone phosphate glyceraldehyde and ascorbic acid (reactive carbonyl species of cellular metabolism). The significant structural and functional changes in DNA (single- and double-strand breaks and base modifications) that glycotoxins and their accumulation can cause are described, over and above the high mutagenic potential that these changes possess. Elitsa Boteva reveals the physiological consequences of DNA glycation and nucleotide adducts found in mouse models and observed in patients with type 2 diabetes and diabetic neuropathy, which makes the glycated nucleotides potential non-invasive diagnostic markers for these pathological conditions.

Glycation of NA from different glycated substrates (glycated AA or proteins), proves the complex network of interactions between all biological molecules in the cell, which turns the processes of removing the accumulated stable glycoproducts into an extreme challenge for the cell. The literature review also notes the process of glycation of plant DNA, which proves that the glycation of proteins and NAs is an evolutionary process common to all organisms and confirms the basic biochemical principle of the unity and diversity of life.

In the literature review, Elitsa Boteva proves the necessity and importance of the research carried out, because they aim to reveal part of the complex network of interactions between sugars, proteins and NAs, which impose the existence of another higher level of regulation by creating specific deglycating enzyme activities or additional functions to already the existing cellular enzymes, which in recent years has turned out to be also a basic biochemical principle. Elitsa Boteva finishes each section of the review with a summary and conclusion, which raises new questions and predetermines the purpose of the next logical step in the research.

The *aim of the dissertation work* is very specific - "To identify and characterize the deglycation activity in *Escherichia coli K12*, responsible for the removal of fructose-6-phosphate residues in DNA". This aim requires the initial fulfillment of 3 main tasks that define the identification of additional DNA-NH-F6P deglycase activity of the glycolytic enzyme PGI. Already established deglycase activity requires its enzymological and kinetic characterization, which involves 10 more specific sequential tasks related to demonstrate its nuclear localization, DNA-binding activity of PGI, participation of deglycation activity in reparative DNA processes, its biological functions and role.

The section *"Materials and methods"* is written extremely concisely, but sufficiently detailed and reproducible in any laboratory, taking into account the large number of methods from different scientific fields, which proves the interdisciplinary nature of the research: physicochemical and analytical (sedimentation, chromatographic, electrophoretic, spectroscopic, etc.); biochemical and enzymological; immunological and molecular-biological; proteomic analysis; statistical methods; bioinformatics analyzes for molecular modeling, molecular docking and metadynamics. Given the enormous popularity and applicability of the main analytical and biochemical methods, their literary sources are not cited, which most likely is accepted as an approach.

The most essential part of the dissertation is the **Results section**, presented in 5 separate experimentally grounded and complete chapters. The discussion of the results, the placement of the investigated PGI among others of the same family, its additional catalytic and non-catalytic functions

that may perform, the mechanism of deglycation and PGI physiological significance for the organismal world, are separated in a special section.

The *first chapter* describes the start of the scientific search for DNA-deglycating activity in *E.coli K12*, which is based on prof. Mironova's group previous discovery that the chromosomal DNA of *E.coli* is glycated under natural physiological conditions *in vivo*. The main question that opens the launch of a new stage in the research concerns the proof of the mutagenic nature of DNA glycation products and study of the possible repair mechanisms. Therefore, it is necessary to identify the specific enzyme activity designated as DNA-NH-F6P deglycase in *E. coli*. This chapter is devoted to: *the establishment and optimization of a method* for rapid and sensitive determination of enzyme activity (an absolutely necessary step subject to many requirements and experiments); *selection of an efficient scheme of chromatographic purification* of *E. coli* lysate (anion-exchange chromatography and gel-filtration by HPLC); *enzymatic and electrophoretic characterization at each purification step* (Mm of the active deglycase fractions was determined between 44.3 kDa and 66.4 kDa); *verifying the specificity of the direct reaction catalyzed by the isolated two deglycase* fractions by using a specially designed substrate - DNA-NH-F6P; *confirmation that detected* DNA-NH-F6P-*deglycase fraction* is an essential activity of the enzyme phosphoglucose isomerase (PGI) (Mm 61.433 kDa, pI 5.9, by 2D electrophoresis of purified fraction 2 and sequencing of the resulting pure protein fractions (Applied Biomics (Hayward, CA)

Elitsa Boteva analyzes and discusses the obtained results in the light of the findings of various scientific groups, which prove bioinformatically that the FrlB-deglycase of *E.coli* belongs to the class of isomerases by biochemical reaction mechanism. I believe that the results in this section and the proof of PGI deglycating activity are decisive for the implementation of the remaining tasks. In fact, the additional enzymatic activity of the glycolytic enzyme PGI is not a surprise in enzymology after the discovery of the multifunctionality of many enzymes, both in terms of substrates and reaction mechanisms, and in terms of localization in the cell, due to the structural and conformational dynamics that the AA sequence of a given polypeptide chain can provide (thermodynamically favorable conformational variants). In recent years, a new evolutionary principle has become more and more widespread, which I consider especially valid in biochemistry - "the missing law of increasing functional information". There are no more appropriate biological macromolecules that can exhibit selection for one function or another than enzymes and thus offer an evolutionary advantage.

The *second chapter* of the dissertation proves the role and kinetic properties of the isolated deglycase and its DNA-binding ability, requiring: *construction of a control E. coli strain (BW28357) with a deleted pgi*-gene (BW28358, Δ*pgi*), confirmed by genetic, immunological and physiological methods, with negligibly low isomerase activity, compared to the wild strain and prone to a higher mutation rate; *determination of direct DNA-protein interactions* to demonstrate the DNA-binding activity of PGI, which Elitsa Boteva performed by an elegant method using homogeneous commercial yeast yPGI (58% sequence homology), DNA fragments of 500bp length (from plasmid pBR322 DNA), application of optimized gel-shift electrophoresis and a specially designed and optimized protocol for immunoprecipitation. The most essential part of this section are the *enzymological studies*, which should prove the function and ability of the isolated PGI-deglycase to catalyze the reaction of F6P release from the substrate DNA-NH-F6P. The experimental challenge here is to *create a model substrate DNA-NH-F6P from a hybrid duplex of peptide nucleic acid (PNA) and DNA* with a single guanine (G) residue for glycation – 5′-CTACTAATCAGACTAATA-3′. The glycation product was examined by mass spectral analysis, purified by gel filtration, hybridized with a complementary 18-mer PNK oligonucleotide - 5′-TAT TAG TCT GAT TAG TAG-3′, and the resulting hybrid was further purified by RP-UHPLC.

This complex scheme of substrate preparation and purification is fully acceptable for carrying out *kinetic enzyme studies* requiring precisely defined specificity, selectivity and efficiency. Kinetic studies are complicated by the fact that a coupled system of sequential reactions is used - reversible isomerization of F6P to G6P and an oxidation of G6P catalyzed by the enzyme G6PDH (glucose 6-phosphate

dehydrogenase) with the cofactor NADP+, added to the reaction mixture. The kinetics of coupled reactions imposes strict requirements on the conditions of the first (zero order) and second (first order) reactions, the concentration and purity of the additional enzyme - G6PDH, the time of the *lag* period for the accumulation of the product of the first reaction, the presence of an inhibitor/ activator of the first reaction and how they relate to the components of the second to adequately determine the kinetic constants. These enzymological limitations have been taken into account by Elitsa Boteva and reflected in the detailed description of purification of the substrate from impurities. The determination of the rate of DNA-NH-F6P-deglycase (DNA-repair) enzyme activity by the glycolytic enzyme PGI isolated from lysates of *E. coli* (wild-type strain BW28357) with the two substrates (PNA-DNA(G)-NH-F6P and DNA(G)-NH-F6P) is evidence that the reaction conditions are correctly chosen. Elitsa Boteva verifies the new deglycation function of PGI also by using the competitive inhibitor of isomerase PGI activity - erythrose-4-phosphate, which also inhibits DNA deglycation up to 100%.

It is very important to accept that the native 3D structure of glycated double-stranded DNA is key for deglycase enzyme activity, which Elitsa Boteva demonstrated by using a specially created substrate of glycated high-molecular-weight DNA in attempt to bring the experimental conditions closer to natural ones. This dissertation chapter, enriched with results and confirmed hypotheses, finishes with a demonstration of the physiological and biological role of the DNA-NH-F6P deglycase activity of PGI, which makes sense of the integral study. Very convincing and biochemically logical (high metabolic concentration of G6P, F6P and G1P – 300 mM and high degree of DNA glycation) is the assumption that the deglycase activity of PGI should have a strong DNA reparative potential, removing the damage in DNA caused by G6P by Maillard reaction. Elitsa Boteva proved this hypothesis by assay of mutability rate of PGI-deficient *E. coli* strains that accumulated a significantly greater amount of G6P compared to wild-type strains, and the mutational analyzes showed a statistically significant difference in the frequency of spontaneous mutations in the *pgi*-deleted strain -gene (exponential phase mutations ~ 1.4x and stationary phase ~ 2x more, respectively). Examining glycation products (early-Amadori, AGEs and CM residues), in *E. coli* wild-type strains and with deleted *pgi*-gene, Elitsa Boteva suggests the existence of alternative compensatory and imperfect reparative mechanisms in the PGI-deficient strain (NER and TLS).

The *third chapter* of the dissertation is devoted to proving the DNA-NH-F6P-deglycase activity and the nuclear localization of human PGI, which raises the research to a higher level. Based on the high degree of sequence homology (65%) of bacterial and human PGI, similar 3D structure, identical catalytic mechanism, and their common evolutionary origin, the working hypothesis here is that hPGI acts as a DNA-NH-F6P-deglycase in all organisms, from bacteria to man. Nuclear localization of hPGI was demonstrated by immunoblotting and indirect immunofluorescence in 2 cell lines – PC3 and HEK293, cultivated in conditions with different glucose concentrations and a specially optimized protocol. The results of both methods unequivocally indicated the *presence of hPGI in the nuclear and cytoplasmic fractions of both cell lines*. The next logical step was to confirm DNA-binding activity of hPGI by ChIP-assay, for which PC3 cancer cells were used, due to the enhanced expression of hPGI in them, and application of specific, covalent and irreversible modification of the anti-hPGI antibody (magnetic beads, BS3-crosslinker and spacer of 11 Å) and the antibody against histone H3 to demonstrate the specific protein-DNA interaction. *The results prove the DNA-binding activity of hPGI*, which immediately demands sequencing of the chromatin protein immunoprecipitate by proteomic analysis and LC-MS/MS, and for reading the raw mass-spectral data, Elitsa Boteva uses software platforms of Thermo Scientific™ Proteome Discoverer and MaxQuant. The most important result obtained from the chromatin immunoprecipitation assay with anti-hPGI Ab is the detection of the glycolytic enzyme GAPDH among hPGI co-precipitated proteins (associated with DNA damage and repair processes), because GAPDH is generally associated with PGI and PKM2 in cancer cells, which is in direct support of the team's hypothesized DNA-NH-F6P-deglycase (repair) function of the enzyme hPGI. *Determination of the two*

enzyme activities of hPGI - deglycase and isomerase in fractionated lysates of PC3 cells (total, nuclear and cytoplasmic) against glycated substrate DNA-NH-F6P and F6P, *proves the higher specific activity and affinity of nuclear hPGI to glycated substrate (deglycase) and vice versa for cytoplasmic hPGI – higher activity towards F6P (isomerase), evidence of differentiation of functional activities of hPGI.*

The use of apparent value designations of kinetic constants may be explained by the use of crude enzyme forms in the lysates, but enzymology relies on a very precise mathematical definition of labels, and I believe that in this case, when using a coupled test of 2 consecutive reactions and an unknown concentration of the enzyme in the total lysate as well as presence of isoforms, the kinetic constants may not be defined as apparent because this does not allow a precise comparative analysis of these basic kinetic characteristics between the PGI fraction in eukaryotic PC3 cell lysates and those of *E.coli*. Elitsa Boteva explains in great detail and convincingly the kinetic behavior of hPGI and correctly differentiates the deglycase from the isomerase activity in the different cell fractions, because the reaction was carried out under the same reaction conditions, as she absolutely logically comes to the assumption that in the cytoplasm and nuclei of human cells function different enzyme hPGI isoforms and for two of them bioinformatic analysis showed a nuclear localization signal.

In the *next chapter*, devoted to bioinformatic analyses, primary structures of PGI and FrlB (deglycase) from *E. coli*, and FrlB from *S. enterica* (17% homology between AA sequences, but a high degree of structural similarity) are: *compared,* with an aim to establish the role of domain and functional 3D protein structure that enables new enzyme specificities; *demonstrated common sugar-isomerase domains* that indicate PGI and FrlB belonging to the sugar isomerase superfamily; *showed the potential possibilities* for dynamic domain organization, by connecting the isomerase domain with additional domains, such as DNA-binding HTH domain, which explains multifunctionality of the enzyme; *analyzed variants* (CATH algorithm) of the domain organization of *E. coli* PGI and the presence of a characteristic structural α-β-α motif, topologically classified as Rossmann fold and evolutionarily proven in about 20% of proteins from 153 homologous families; *presented 50 domains,* identified in the phosphoglucose isomerase superfamily (via the SSAP secondary structure alignment program) that are associated with transcriptional regulators and DNA binding. These comparative analyzes are indicative for the ability of PGI to perform diverse catalytic functions through organizational dynamics of its domains.

A major part of the analysis, in this chapter, is devoted to the attempt to reveal the catalytic mechanism through the structure-function relationship. Comparative bioinformatic analyzes of the catalytic sites suggest a predominant acid-base mechanism of the isomerase reaction (Glu355 (isomerization) and Arg270 and His386 (ring opening)) mediated by the coordinated action of 2 different monomers. In the deglycase reaction, the catalytic residues Glu224 and His240 also belong to 2 different monomers, which is a prerequisite for different functionality, but the reaction here requires an elementary step involving water. Next bioinformatics task, set in the dissertation, aims to prove whether the catalytic residues in the active site of *E. coli* PGI correspond to those of the FrlB deglycase of *S. enterica* (jFATCAT algorithm on the PDB platform RCSB). Moderate structural similarity is found, which relates to the recognition of the Amadori product -NH-F6P as a substrate. Elitsa Boteva suggests that similar mechanisms of DNA-NH-F6P deglycation by PGI and Lys-NH-F6P deglycation by deglycase FrlB are used. In the search for a nuclear localization signal in PGI, bioinformatics analysis (BLAST) showed that PGI-1 and PGI-3 isoforms (out of a total of 5) possess an additional region of about 40 AA at their N-terminus, and the NucPred program predicted the *presence of a strong NLS precisely in this region rich in Lys Arg residues (KRRRK)*, which classifies these PGI isoforms to proteins with a predominantly nuclear localization.

The higher level of scientific work, concerns the comparison of bacterial and human PGI with DNA binding and repair proteins using the REPAIRtoire of the Laboratory of Bioinformatics and Protein Engineering at the Warsaw International Institute of Molecular and Cell Biology. *The results revealed 25% homology of bacterial PGI to DNA glycosylases*, catalyzing the repair of modified bases. hPGI-3

shows a high degree of homology (58%) with an *E. coli* reparative methylguanine methyltransferase, which launches the mechanistic hypothesis for the DNA-NH-F6P deglycating enzyme activity of phosphoglucose isomerase in the thesis – *"PGI is not a glycosylase but a DNA a repair enzyme that directly removes the F6P-residue from the glycated base"*.

The *fifth chapter*, "Molecular Modeling", raises the entire experimental arsenal used in this integral study *to propose a mechanism for* PGI *DNA deglycating activity* through *in silico* molecular docking (using 9mG-NH-F6P = 9-methylguanine modified with F6P and attached to its exocyclic NH_2-group), which is a very ambitious task. Elitsa Boteva explains the mechanisms by which the Amadori product - NH-F6P - glycated DNA would disrupt the double-stranded structure, suggesting that the "insertion" of the F6P-residue, between the glycated base and its complementary base on the other strand, leads to a local destruption of H bonds. If the result is "*base-flipping*" of the modified base from within the double helix, local deformation and twisting of the chain is created. To confirm this working hypothesis, a *special double-stranded DNA oligonucleotide (20-mer)* with a random nucleotide sequence and a single F6P-modified G-residue "plucked" from the double helix was modeled, which was used as a ligand for the rabbit rPGI in the docking experiments. The conclusion that emerges is very significant - the *"plucked" modified base binds to the surface of rabbit rPGI* (used as a model) in a region that has a high positive electrostatic potential, and the binding of rPGI to DNA is at the surface between the two enzyme subunits, i.e. in the active site, therefore PGI could catalyze a deglycation reaction.

The molecular modeling analyzes, performed for the first time (MD simulations) for the consecutive stages of the entire process, are excellently described by Elitsa Boteva. This *last part* of the study is most impressive with the use of different programs and metadynamics for maximum approximation to the elementary catalytic steps in the enzyme's active site. They show 2 different low-energy orientations of the catalytic groups - Glu357, Lys518 of monomer A and His388 of monomer B, which are close to the G-F6P ligand upon deglycation but in an antiparallel remote position relative to the native F6P ligand, upon isomerization.

Discussion of the obtained results and their comparison with previous scientific knowledge, the existence of other enzymatic PGI functionalities in different organisms (lysyl-aminopeptidase, aldehyde-isomerase, epimerase activity), the broader substrate specificity and the molecular association with GAPDH are central to prove the multifunctional plasticity, that a polypeptide chain can provide.

5. Publications and participation in scientific forums

Elitsa Boteva presents dissertation results in 6 publications in specialized journals with a total impact factor of more than 10 (e.g. *Int.J.Biol. Macromol, IF 8.2*), for which more than 10 citations have been noticed, as well as participation in 3 international and 7 national with international participation, conferences. The PhD student is the first author in 4 of the publications, which is proof of her personal contribution. Elitsa Boteva's dissertation was funded and included in 2 research projects of the Ministry of Education, Science and Technology, a program for young scientists from the Bulgarian Academy of Sciences and a scholarship of the German Academic Exchange Service (DAAD). The scientific works meet the minimum national requirements (according to Article 2b, paragraphs 2 and 3 of the ADASRB) and the additional requirements of the IMB-BAS for the awarding of the Educational and Scientific degree "Doctor".

6. Qualities of the Abstract

The Abstract meets all the requirements for its preparation and correctly presents the results and content of the PhD Thesis.

7. Conclusion

The scientific and applied contributions of the presented extensive biochemical, molecular biological and bioinformatics research, the highly rated publications, the originality of the written work and its perfect technical layout and design, are indisputable. Elitsa Boteva demonstrated an excellent knowledge of the problem and remarkable ability to interpret the obtained results, to propose hypotheses and to compare her results with already published data.

Having read and analysed the presented PhD Thesis and the scientific papers accompanying it, as well as based on the analysis of their significance and scientific contributions, **I confirm** that Elitsa Boteva's dissertation, scientific publications, the quality and originality of the results and achievements presented in them, meet the requirements of the ADASRB and the Rules for its application for the awarding of the Educational and Scientific degree "Doctor" in the scientific field 4. Natural sciences, mathematics and informatics, professional direction 4.3. Biological Sciences (Molecular Biology).

Elitsa Boteva's dissertation fully satisfies the minimum national requirements in this professional field. Based on the above, *I recommend the Scientific Jury to award* ***Elitsa Boteva***, *the Educational and Scientific degree "Doctor"(PhD)* in the professional field 4.3. Biological Sciences (Molecular Biology).

Reviewer:

Date: 20th September, 2024

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