REVIEW

From: **Prof. Iskra Vitanova Ivanova, DBSc.**

Subject: evaluation of a dissertation work for the acquisition of an educational and scientific degree "Doctor" in the Field of higher education 4. Natural sciences, mathematics and informatics, Professional direction 4.3 Biological sciences, Doctoral program "Biochemistry"

By order No. PD-21-2469 of 18.12.2023. of the Rector of Plovdiv University "Paisii Hilendarski" (PU), I have been appointed as a member of the scientific jury.

Author of the dissertation: **Stanimira Angelova Angelova**

Title of the dissertation:

" INVESTIGATION OF THE PROPERTIES OF BIOENGINEERED ALPHA-D-GLUCANS SYNTHESIZED BY MUTANT GLUCANSUCHARASE URE 13-300"

Scientific supervisor: **Prof. Dr. Ilia Iliev**

1. RELEVANCE AND SIGNIFICANCE OF THE DEVELOPED PROBLEM

The group of glucansaccharases responsible for the synthesis of glucan polymers are classified in the glycoside hydrolase 70 (GH70) family. Glucan polymers differ in size, type of linkages, and degree of branching. These characteristics influence the physicochemical properties of polysaccharides. According to the type of α-glycosidic bonds in the polymers synthesized by glucansaccharases, they are distinguished: glucan, mutan, reuteran, dextran and alternan. The molecular organization of glucansaccharases is domain-based, with five different regions in the catalytic domain having complementary functions in the course of glucan or oligosaccharide synthesis - A, B, C, IV and V. In recent years, numerous studies have been done with amino acid substitutions by site-directed mutagenesis demonstrating the function of various amino acids located in or around the catalytic center of glucansaccharases that are key to the enzyme activity and the type of linkages between glucose residues in the corresponding glucans. The site-directed mutagenesis (SDM) method involves the introduction of so-called point mutations, in which an amino acid at a predetermined location in the protein molecule is replaced by one of the other 19 amino acids. A necessary condition is the availability of a sufficiently rich database for the structural features of the respective enzyme family. Also, understanding the enzyme catalytic mechanism affects the accuracy and success rate of rational design. Deep understanding of molecular aspects opens new avenues for designing strategies for metabolic engineering of microbial hosts and engineering of biocatalysts, subsequently achieving higher EPS yield and reducing production costs.

All this gives me reason to evaluate the presented scientific development as current, with the potential for scientific achievements that have a quick practical implementation.

2. SCOPE AND STRUCTURE OF THE DISSERTATION

The dissertation is set out in 157 standard pages of text. The generally accepted scheme and the recommended ratios between the individual parts of the work have been followed, as follows:

Introduction – 1 page; Literature review – 31 pages; Purpose and tasks - 1 page; Materials and methods – 14 pages; Results and discussion – 51 pages; Discussion - 1 page; Conclusions 1 page. The obtained results are illustrated with 37 figures, 5 tables, 12 appendices and 175 literary sources.

3. LITERARY AWARENESS AND STATEMENT OF THE GOAL AND TASKS

The present dissertation is complex and implies a good knowledge of the literary sources and the methods for solving it. The doctoral student has made a thorough review of the achievements of other researchers, which she was able to convey and analyze on 31 pages in the literature review. The overview presents the state of the problem in detail and proves the necessity of developing the dissertation thesis. The literature review consists of four sections. In the general characteristic of glucansaccharases (GS), also called glycosyltransferases (GTP), data are presented on extracellular enzymes that, in the presence of sucrose, catalyze the synthesis of glucose polymers called glucans. Different glucans have been characterized. Information is presented on the catalytic mechanism of the synthesis of glucan products and glucansaccharases with two catalytic domains. In the following sections, the structural and functional organization of glucansaccharases and the importance of the signal peptide and the N-terminal domain as well as the glucan-binding domain V are described sequentially and in detail. The doctoral student presents in his development the influence of mutations in the genes encoding glucansaccharases from the GH70 family. These mutations have an effect on the enzyme activity and mutations on the changes responsible for the structure of glucans and oligosaccharides. Mutations with a change in the solubility of the synthesized polysaccharide and the engineering of a polysaccharide of controlled length are discussed, as well as their influence on branching sucrases. The application of alpha-glucans and oligosaccharides is discussed. Some unsolved problems are also brought to the attention of the reader. The literature review is concrete, it is structured correctly, following the logical connection of the information. The data from the reference served for the clear and correct determination not only of the goal, but also of the formulation of the tasks. 7 well-grounded experimental tasks are set for solving. The literature (both in the overview and in the whole work) is closely related to the topic of the dissertation work. The literary list included an impressive number of 175 titles in Latin. They are mainly from recent years. This speaks of an excellent theoretical awareness of the PhD student and with the aim of finding a new scientific challenge.

4. EVALUATION OF USED METHODS AND MATERIALS

The Materials and Methods section demonstrates an impressive array of methods tailored to the specific requirements of the experiments. They are modern and adequate for the realization of the dissertation work. They are described accurately and in detail, fully covering the multifaceted areas of the work: from classical to modern studies. Microbiological and biochemical methods were used, including the determination of enzyme activity, polyacrylamide electrophoresis, etc. Enzymatic synthesis and analysis of α-glucans synthesized by mutant glucansaccharase U13M1 was carried out, as well as purification of the synthesized polysaccharide and NMR analysis of α-glucan synthesized by mutant glucansaccharase U13M1. The author used enzymatic synthesis of oligosaccharides with HPLC analysis of the obtained oligosaccharides .An analysis of enzymatic reactions was carried out in the presence

of maltose as an acceptor in the presence of organic solvents. Molecular biological methods and bioinformatic analysis included isolation of plasmid DNA from recombinant E. coli BL21 cells, site-directed mutagenesis based on bioinformatic analysis, and construction of a homology model. Site-directed mutagenesis was based on comparison of the amino acid sequence of URE 13-300 with other glucansaccharases was performed using the Clustal Omega web-based tools (https://www.ebi.ac.uk/Tools/msa/clustalo/), Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/) and ESPript v3.0. (https://espript.ibcp.fr/ESPript/ESPript/). To confirm the correct amino acid substitution, the portion of the URE 13-300 gene containing the mutation was sequenced. All this allows me to give a high assessment of the scientific level and the excellent preparation, which manages to correctly combine a variety of classical and modern methods for the purposes of the dissertation, successfully solving the set experimental tasks.

5. EVALUATION OF THE RESULTS OBTAINED

The aim of the present thesis is to investigate the relationship between the structure of synthesized glucans and the alteration of the amino acid sequence of catalytic domain 1 of glucansaccharase URE 13-300 by site-directed mutagenesis. The "Results and discussion" section is well structured, supported by tabular and graphic material, with appropriate interpretation of results obtained by foreign scientific teams. The author consistently presents evidence for her scientific thesis, thereby logically finalizing an experimental work. Extensive and varied experimental work was carried out within the framework of a complex biochemical study. Angelova studied the duration of cultivation in order to optimize the time to achieve maximum production of glucansaccharase URE 13-300 and the influence of organic solvents on the activity of glucansaccharase URE 13-300. In order to establish their effect of heterogeneous environment on the activity of glucansaccharase URE 13-300, nine organic solvents were investigated. Various organic solvents have been investigated on transferase reactions in the presence of maltose as an acceptor. Km and Vmax values were calculated by the Michaelis-Menten equation using the non-linear regression approach. Organic solvents have an effect on the degree of polymerization of glucooligosaccharides synthesized by glucansaccharase URE 13-300. The obtained results show that the synthesis of oligosaccharides as a result of the transferase reaction is not significantly affected in a heterogeneous medium with 5% or 20% DMSO, n-hexane and octanol. The influence of three monoterpenoid compounds from essential oils carvacrol, thymol and menthol on the total activity of glycosyltransferase URE 13-300 was also studied. Along with the modification of the acceptor:donor ratio, the addition of various organic solvents to the transferase reaction may prove suitable for further applications for customized products. . Some of these solvents affect the synthesis of oligosaccharides during the transferase reaction to GOS with a higher degree of polymerization without inhibiting the transferase reaction. Glycosylation of terpenoids derived from essential oils such as menthol can be included in the modeling of food additives with added value. Of particular importance are studies in connection with site-directed mutagenesis in the gene encoding glucanaccharase URE 13-300. In the first step to obtain a molecular mutant with a single amino acid substitution and a detailed bioinformatic analysis was performed. The amino acid sequence of glucansaccharase URE 13-300 was compared with that of some studied mutated glucansaccharases. Seven conserved motifs containing important amino acids for the specificity of the bonds in the synthesized products were identified. After reviewing the effects of amino acid substitution in some of these enzymes, the amino acid

glycine at position 449 was chosen. This position is not strictly conserved in them, but it appears most often in the analysis of multiple sequences. From the obtained results of the in situ analysis, it is proved that the two forms of glucansaccharase URE 13-300 show the same bands, around 300 kDa. The replacement of only one amino acid did not lead to a visible change in the molecular weight, therefore, as a result of the carried out mutation, no additional changes occurred in the amino acid sequence of the enzyme. Based on the obtained results, the conditions for obtaining mutant glucansaccharase U13M1 and the conditions for the enzyme reaction were optimized. The resulting U13M1 mutant enzyme possesses a remarkable change in kinetic parameters compared to the wild-type glucansaccharase URE 13-300. The Km values for the U13M1 mutant enzyme were almost 7-fold higher than those of the parent glucansaccharase, while maintaining an identical maximal velocity. The reduced affinity for the substrate may be due to a change in the shape of the binding site in the mutant enzyme. Consistent with the combined results, mutant forms of glucansaccharase GTF180-ΔN show an increased Km for sucrose. The pH optimum of U13M1 is shifted one unit higher than the parent type enzyme – to 6.5. After amino acid substitution with lysine, the temperature optimum of U13M1 is shifted to 20°C. Such differences in optimal conditions between the parent and mutant enzyme have not been previously reported. Subsequent studies were on the structure of α-glucan and oligosaccharides synthesized by mutant glucansaccharase U13M1. Using 20% sucrose (the optimal substrate concentration for the mutant enzyme), the polysaccharide synthesized by the U13M1 mutant enzyme showed a reduced ratio of α-(1 → 3)/α-(1 → 6) glycosidic linkages of about 30% in the main chain in compared to the parent type glucan. In turn, the modulated structure affects the specific physicochemical properties of the polymer and its application as a carrier of biologically active substances. There was an increased amount of α-(1 → 3) glycosidic linkages in the polymer when it was synthesized in the presence of 10% sucrose. The U13M1 polymer retained the property of insolubility in water, which is interesting in light of the increased amount of α-(1 → 6) linkages in its structure. This successful mutation of the glucansaccharase URE 13-300 in its entirety, without shortening the length of the gene, is a basis for studying the interaction between the two catalytic domains, acting as dextran sucrase and branching sucrase, respectively.

The obtained results presented in "Results and discussion" logically follow the course of solving the set tasks. They are summarized and discussed in the light of published data from recent years. Both the idea and the volume of research carried out on the implementation of this task and in the entire work deserve high praise. The discussion made on each experiment, the comparison of the results and the comparison with the literature data, once again emphasizes the qualities of the doctoral student in the mastery of the experimental theory. By this she proves that she has fully mastered the third degree of her training and is an accomplished experimenter.

6. CONTRIBUTIONS AND SIGNIFICANCE OF THE DEVELOPMENT FOR SCIENCE AND PRACTICE, NOTES AND QUESTIONS

I accept the contributions made.

I consider particularly significant that the data presented by the method of site-directed mutagenesis is the first time a mutation of glucansaccharase URE 13-300 has been carried out by replacing glycine at the 449th position with lysine and the first time site-directed mutagenesis of glucansaccharase has been carried out , containing two catalytic domains

without reducing the length of the gene, which was demonstrated to affect the overall 3D structure of the protein and its catalytic properties. Angelova is the author of 3 scientific publications, in two of which she is the leading researcher and six participants in scientific forums, which shows her creative and research activity in their development and shaping. The doctoral student successfully participated in 6 scientific research projects. Angelova has a specialization at the Center for Synthetic Biology at the Department of Biotechnology of the University of Ghent, Belgium, for the period 01.09. – 31/10/2022 Subject of work: Obtaining mutant libraries by site-saturation mutagenesis.

I have a few questions for the dissertation:

- ✓ What methods and techniques are used in the purification of glucansaccharases?
- ✓ What factors influence the catalytic activity of glucansaccharases and what is the role of calcium? What are the most known chemical inhibitors of this enzyme?
- ✓ What are the most used methods for analyzing the structure of exopolysaccharides?
- ✓ What new techniques of genetic manipulation contribute to advances in the mechanisms of exopolysaccharide synthesis?

CONCLUSION

The topic is current, the doctoral student has mastered modern methods, the experiments are set methodically correctly, the results obtained are reliable and are a solid basis for further scientific and applied developments. Exceptionally original scientific and applied contributions stand out. Based on the above, I can confidently state that the peer-reviewed dissertation is an original scientific work, with theoretical and applied significance. The proposed dissertation is proof that Angelova has developed competencies necessary for the award of the doctorate degree, including theoretical training, methodological knowledge, independence and experience in planning experiments and the ability to analyze the results.

The dissertation contains scientific, scientific-applied and applied results, which represent an original contribution to science and meet all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB), the Regulations for the Implementation of ZRASRB and the relevant Regulations of PU "Paisii Hilendarski" and I give my high evaluation and recommend to the members of the scientific jury to award the doctoral student Stanimira Angelova Angelova the educational and scientific degree "Doctor" in the field of higher education: 4. Natural sciences, mathematics and informatics, professional direction: 4.3. Biologically sciences PhD program Biochemistry.

15.02.2024 Reviewer:

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