

REVIEW

by Prof. Dr Velizar Kostadinov Gochev, Plovdiv University “Paisii Hilendarski”

of a doctoral thesis for the award of the academic and scientific degree of ‘Doctor’
in: field of higher education 4. Natural Sciences, Mathematics and Informatics,
professional field 4.3 Biological Sciences, doctoral programme in Biochemistry

Author: *Teodora Mincheva Panayotova*

Title: *“Investigation of the properties of biologically active peptides obtained by enzymatic*

hydrolysis with proteolytic enzymes from lactic acid bacteria”.

Supervisor: *Prof. Dr Ilia Nikolov Iliev*

1. General description of the materials presented

By Order No. PD-22-742 of 7 April 2026 issued by the Rector of Plovdiv University “Paisii Hilendarski” (PU), I have been appointed as a member of the academic jury to oversee the procedure for the defence of a doctoral thesis on the topic *‘Investigation of the properties of biologically active peptides obtained by enzymatic hydrolysis with proteolytic enzymes from lactic acid bacteria’* for the award of the academic and scientific degree of “Doctor” in: 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, doctoral programme in Biochemistry. The author of the thesis is Teodora Mincheva Panayotova – a part-time PhD student at the Department of Biochemistry and Microbiology, supervised by Prof. Dr Ilia Nikolov Iliev of Plovdiv University.

The set of materials in hard copy submitted by Teodora Mincheva Panayotova complies with Article 36(1) of the Regulations on the Development of Academic Staff at Plovdiv University and includes the following documents: an application to the Rector of Plovdiv University to initiate the procedure for the defence of the doctoral thesis; a CV in European format; minutes of the departmental council meeting regarding the report on readiness to initiate the procedure and the preliminary discussion of the doctoral thesis; the doctoral thesis; an abstract; a list of academic publications on the thesis topic; copies of the academic publications; a declaration of originality and authenticity of the attached documents; a statement confirming compliance with the minimum national scientometric requirements for the award of the academic degree of ‘Doctor’.

All administrative requirements under the procedure have been met at both university and national level

2. Brief biographical details of the doctoral candidate

Teodora Mincheva Panayotova completed a Bachelor's degree in 'Meat and Milk Technology' at the University of Chemical Technology and Metallurgy (UCTM) (2010) and a Master's degree in 'Control and Safety of Food of Animal Origin' at UCTM (2011). In 2020, she was enrolled as a part-time PhD student in the Department of Biochemistry and Microbiology at the Faculty of Biology, Plovdiv University, and in 2025 she was granted the right to defend her thesis. Since 2012, she has been working as a researcher at "LB Bulgaricum" EAD.

The PhD candidate's education and professional experience are closely linked to the subject matter of the thesis.

3. Relevance of the topic and appropriateness of the objectives and tasks

Functional foods are particularly relevant in modern nutrition because, in addition to components that ensure the plastic, energy and regulatory functions of food, they also contain nutrients with proven health benefits, such as bioactive peptides (BAPs). BAPs are formed during the technological process of producing a particular food or during digestion. The various forms of biological activity of these peptides— , antioxidant, antihypertensive, antimicrobial, immunomodulatory, and others—underpin the positive effects on human health. The growing scientific interest in them is also driven by the desire to apply the most natural alternatives to pharmacologically significant biologically active substances (BAS) obtained synthetically. Further evidence of the topicality of this subject is the fact that, over the last five years, an average of around 1,200 publications per year have been devoted to functional foods.

In this context, the topic of the thesis can unequivocally be described as topical.

The aim of the thesis is clearly and precisely formulated, and to achieve it, five logically interconnected research objectives have been set, the successful completion of which is a prerequisite for achieving the aim.

4. Understanding the problem

I assess the level of understanding of the issue based on the substance of the literature review and the scope of the literature consulted. The literature review spans 37 pages, beginning with a brief overview and, to a certain extent, limited information on the current taxonomic status of lactic acid bacteria (LAB). The genetic and biochemical bases of the production, excretion and mechanism of action of proteolytic enzymes from LAB are described. The review focuses primarily on the various BAPs with an emphasis on plant sources for their production (beans, peas, soya, rice, millet, barley, buckwheat, wheat, etc.),

which fully corresponds to the aim of the doctoral thesis, whilst not omitting traditional raw materials for their production such as milk. The PhD candidate has described various approaches to obtaining bioactive compounds, with the different forms of their biological activity – antioxidant, antihypertensive, antimicrobial, antitumour, immunomodulatory, analgesic, etc. – described in great detail.

I consider the review to be clearly focused and specific, but it could be more analytical, which would better justify the need for the present study. I believe that the PhD student is familiar with the state of the art of the research topic to a degree that allows her to adequately carry out the dissertation research.

5. Research methodology

The experimental design of the study is comprehensive and well- structured. The ‘Materials and Methods’ section spans 20 pages and provides an extremely detailed description of the microbiological, enzymatic, electrophoretic and chromatographic methods employed, which have been correctly selected for the purpose of the study.

6. Characteristics and evaluation of the thesis

The thesis is structured in the standard manner and comprises the following sections: Introduction (2 pages), Literature Review (37 pages), Materials and Methods (20 pages), Results and Discussion (72 pages), Conclusions (2 pp.), Contributions (1 p.), References (30 p) and Appendices (5 pp.). Optimal proportions have been maintained between the individual sections of the thesis. The experimental work for the thesis begins with the screening of 50 LCC strains of the species *Lactobacillus delbrüeckii subsp. bulgaricus* (30 strains), *Lactobacillus helveticus* (11 strains), *Lacticaseibacillus casei* (4 strains) and *Lactiplantibacillus plantarum* (5 strains) with regard to their proteolytic activity in a 10% skimmed milk powder (SMP) medium at two different temperatures, 37°C and 42°C for strains of the species *L. delbrüeckii subsp. bulgaricus*, *L. helveticus* and *L. plantarum*, and at 32°C and 37°C for *L. casei*. Representatives of the species

L. delbrüeckii subsp. bulgaricus and *L. helveticus* stand out as promising. For representatives of these two species, the relationship between culture growth rate, pH and the proteolytic activity of the strains was investigated. Similar experiments were conducted with 8 strains of *L. delbrüeckii subsp. bulgaricus*, 5 strains of *L. helveticus* and 3 strains of *L. casei*, but cultured in media containing pea, soya, hemp and rice protein. The highest proteolytic activity was observed in strains of the species *L. helveticus* and *L. casei*, cultured in a medium containing 10% skimmed milk powder and a 5% pea protein supplement. The specific proteolytic activity of the studied strains was determined in mMRS medium supplemented

with various plant protein hydrolysates and proteins. It was found that pea protein hydrolysate, soya protein hydrolysate and whey protein act as strain-specific activators of proteolytic activity in the *L. casei* strains studied. The strains *L. helveticus* b244 and h70 stood out with the highest specific proteolytic activity in the studied nutrient media. The production of the aminopeptidases leucine aminopeptidase, lysine aminopeptidase, arginine aminopeptidase and proline aminopeptidase in media containing 10% skimmed milk powder and mMRS medium supplemented with pea protein hydrolysate, soya protein hydrolysate and whey protein. The highest production of leucine aminopeptidase and lysine aminopeptidase was observed in strain *L. helveticus* h25, whilst the highest production of arginine aminopeptidase and proline aminopeptidase was observed in strain *L. helveticus* h48. It was found that supplements of pea protein hydrolysate, soya protein hydrolysate and whey protein do not activate enzyme production with regard to aminopeptidases. Experimental work on the thesis continues with the determination of the ACE-inhibiting activity of peptides obtained by culturing selected *L. helveticus* (5 strains) and *L. casei* (1 strain) in media containing skimmed milk powder, skimmed milk powder supplemented with pea and soya protein, and entirely plant-based media. The highest ACE-inhibiting activity was observed when culturing *L. helveticus* b244 in a wholly plant-based medium containing 5% soya protein and 2% glucose. To characterise the peptides formed during the cultivation of *L. helveticus* b244 and *L. helveticus* h70 in media containing skimmed milk and entirely plant-based nutrient media supplemented with 2% glucose, SDS-PAGE electrophoresis was performed, and the formation of low-molecular-weight peptides was demonstrated in all variants studied. In the media containing 5% pea protein + 2% glucose and 5% soya protein + 2% glucose, low-molecular-weight peptides with molecular weights of , respectively, below 6.5 kDa and below 12 kDa, were detected. In addition to SDS-PAGE electrophoresis, the PhD student demonstrated the presence of low-molecular-weight peptides in the entirely plant-based and combined media, inoculated with *L. helveticus* b244 and *L. helveticus* h70, using UPLC-MS. The results obtained by UPLC-MS correspond to those from SDS-PAGE.

The results obtained by the PhD student have been correctly interpreted and presented in 4 tables and 28 figures. Each individual stage of the experimental work concludes with a brief summary and conclusions.

Based on the results obtained, 8 conclusions have been formulated, which accurately reflect the most significant relationships identified. Four contributions have also been formulated, which I would categorise as scientific and applied in nature and original in character.

7. Assessment of publications related to the thesis

Three scientific publications are presented in connection with the thesis – one in *Bulgarian Chemical Communications* and two in *Acta Microbiologica Bulgarica*, both journals being Q4. The PhD candidate is the lead author of all three publications, which demonstrates her active participation in the production of the scientific publications.

Abstract

The abstract has been prepared in accordance with the requirements and accurately reflects the main results achieved in the thesis.

8. Critical comments, recommendations and questions.

I have the following comments on the thesis:

- ✓ In the Materials and Methods section, it is unnecessary to describe the conditions for species identification of the strains, as they are not the subject of isolation within the scope of the doctoral research. The description of the strains and their origin provided by the doctoral candidate was entirely sufficient.
- ✓ There are some terminological inaccuracies in the definition of the parameter 'enzyme activity'.
- ✓ The Materials and Methods section lacks information on the composition of the pea, soya, rice, hemp and whey proteins, as well as the pea and soya protein hydrolysates used. Had such information been available, it would likely have been possible to provide more in-depth commentary on some of the results obtained and to find appropriate explanations.
- ✓ The correlation between enzyme activities, cell density and pH is discussed in a rather descriptive manner.

I have the following questions for the PhD student:

- ✓ Why do you conduct some of the experiments in a skimmed milk base medium with or without additions of plant proteins, whilst other experiments are conducted in an mMRS medium?
- ✓ Why do you use different plant proteins, such as pea and soya, in some experiments, whilst in others you use pea protein hydrolysate and soya protein hydrolysate?
- ✓ What is the composition of the plant proteins and the corresponding plant protein hydrolysates, and how do the differences in their composition affect the proteolytic activity of the strains and the production of ACE- inhibitory peptides?

9. Personal impressions

I do not know the PhD student personally, and my only impressions of her are from her annual progress reports and her presentations to the Departmental Council of the Department of Biochemistry and Microbiology. Although limited, my personal impressions are positive.

CONCLUSION

The doctoral thesis contains scientific and applied results that are of interest to both science and practice and meet the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria (ZRASRB), the Regulations for the Implementation of ZRASRB and the relevant Regulations of Plovdiv University "Paisii Hilendarski".

The doctoral thesis demonstrates that the doctoral candidate Teodora Mincheva Panayotova possesses theoretical knowledge and professional skills in the scientific discipline of Biochemistry.

In view of the above, I give my positive assessment of the research conducted, as presented in the above-reviewed doctoral thesis, abstract, results achieved and contributions, and I propose that the distinguished academic jury award the educational and scientific degree of 'Doctor' to Teodora Mincheva Panayotova in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, doctoral programme in Biochemistry.

08.05.2026

Plovdiv

Reviewer:.....

(Prof. Velizar Gochev, PhD)