



Prof. Dr. Hab. Eng. Małgorzata Darewicz

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Faculty of Food Science

Chair of Food Biochemistry

tel. +48 895245171

e-mail: darewicz@uwm.edu.pl

## REVIEW

of the Doctoral Thesis of M.Sc. Katarzyna Kłosowska  
titled

**"The effect of phospholipids on the intestinal lipolysis and proteolysis"**

**made under the supervision of Dr. Hab. Eng. Adam Macierzanka, University Prof.,  
and Prof. Dr. Jens Brockmeyer**

**from the Faculty of Chemistry of the Gdańsk University of Technology**

The review was prepared based on an invitation letter from the Chairman of the Scientific Council of the Discipline of Chemical Sciences at the Gdańsk University of Technology, Dr. Hab. Eng. Marek Tobiszewski, University Prof., dated 14 February 2025, in accordance with the Resolution of the Scientific Council of the Discipline of Chemical Sciences at the Gdańsk University of Technology of 11 February 2025.

### Evaluation of the choice of the subject of the doctoral thesis and its purpose

Food can be a source of components that determine the correct, physiological condition of the human body, as well as regulate its psychophysical behavior. These include lipids and food proteins, and also fatty acids and peptides derived from them which show specific biological activities. Some of these compounds have already been used in the prevention and even therapy of diet-related non-communicable diseases. In turn, consumer awareness of the impact of food on health has been a strong driver of research addressing the molecular fundamentals of the activity of food biomacromolecules.

Digestion is a complex process that provides the body with nutrients, whereas the molecules released in the digestive tract can elicit a beneficial effect on human health.





Therefore, research on the molecular grounds of digestion processes is a way to expand knowledge about its impact on health and maintaining homeostasis.

*In vitro* digestion can be a viable means to acquire knowledge about the nutritional value of food because it allows process control and easy sampling. Although the *in vitro* approach to mimicking physiological processes is never perfect, it enables avoiding difficulties encountered in the *in vivo* studies, such as costs, ethical considerations or technical and organizational complexity of the experiment.

The harmonized INFOGEST digestion protocol, used by M.Sc. Kłosowska, can be harnessed to assess endpoints resulting from the digestion of components of raw material and/or food products by analyzing digestion products (e.g., peptides/amino acids, fatty acids, simple sugars) released from the food matrix. The main conclusion from the research conducted so far is that, despite their simplicity, the *in vitro* models that approximately reproduce the conditions in the human digestive system are also very useful in predicting the outcomes of *in vivo* digestion. However, the success of these predictions depends on the parameters of the *in vitro* model and its adjustment in response to specific questions related to the physiology/molecular mechanism of the human digestion process, which makes this area ripe for further research and modifications/improvements. Hence, the research problem undertaken by the PhD student regarding the influence of phospholipids (PL) and bile salts (BS) and their mutual interactions on the processes of lipolysis and proteolysis simulated *in vitro* is extremely current, important and necessary.

#### **Formal evaluation of the doctoral thesis**

The doctoral thesis titled "The effect of phospholipids on the intestinal lipolysis and proteolysis" submitted for review by M.Sc. Katarzyna Kłosowska, meets the formal requirements for this type of scientific dissertations presented in the proceedings for the scientific degree of doctor, i.e., it is experimental in nature and contains all the necessary chapters arranged in a typical sequence. The subject of the review is a 148-page thesis based on the results of the Author's research. It begins with a summary in Polish and English, has a structure typical of scientific dissertations and contains 7 chapters with subchapters: an introduction presenting an overview of the literature, a description of the study aim, description of the materials and methods used, presentation of the results and discussion of the results (providing information about the publication of the results from chapters 4.1 and 4.2 in the article by Kłosowska, K, Teresa Castillo-Santaella, Julia Maldonado-Valderrama, and Adam Macierzanka. 2024. "The Bile Salt/Phospholipid Ratio Determines the Extent of *in Vitro* Intestinal Lipolysis of Triglycerides: Interfacial and Emulsion Studies" Food Research International, 187, 114421187. The article provides statements about the contribution of the Candidate herself to the work and statements about the contribution of the other co-authors, i.e., Teresa Castillo-Santaella, Julia Maldonado-Valderrama and Adam Macierzanka. These statements clearly indicate the



leading role of Ms. Katarzyna Kłosowska, who as the first author was responsible for writing – review and editing, writing – the original draft, visualization, validation, methodology, research, formal analysis, data management), summary and conclusions, list of references and an appendix including figures and a table. The whole dissertation contains 76 figures, 17 tables, and 300 literature items. The presented illustrations and tables are characterized by a high level of graphic design, i.e., they are clear and easy to interpret. A list of abbreviations used is also provided, which allows the reader to efficiently navigate throughout the text of the dissertation. In the doctoral thesis, Ms. K. Kłosowska has not included information about her scientific achievements to date (including a list of scientific publications, conference presentations, awards or grants she was involved in). The adopted method of presenting the research results is appropriate and meets the formal statutory requirements regarding the presentation of achievements in the doctoral dissertation. The evaluated doctoral thesis constitutes a coherent whole. It was written carefully, correctly in terms of the linguistic form and well illustrated.

#### **Substantive evaluation of the doctoral thesis**

After reading the evaluated doctoral thesis, I state that its title was formulated correctly, concisely, although not fully adequately to its content. By this means, the aspects of digestion at all stages other than the intestinal one were perhaps not sufficiently expressed. The abstracts, in both Polish and English, do not raise any concerns either, where – by pointing to the limited number of experiments on digestion in the presence of phospholipids and bile salts and their mutual interactions at different concentrations, especially in the case of proteolysis but also lipolysis – the PhD student justifies the aim of the research, which was to examine the effect of phospholipids and bile salts on the digestion of triacylglycerols and proteins, including  $\beta$ -lactoglobulin, from a whey protein isolate (WPI).

In the part of the dissertation concerning the justification of the undertaken research topic, the Author analyses the addressed issue in the light of the literature findings as well as acquaints and introduces the reader to the subject of the dissertation in an interesting way.

Ms. Kłosowska introduces the subject of her research in a reliable manner, using properly selected sources and thereby underpinning its validity. This part of the dissertation was developed based on an accurate, credible, and correctly cited bibliography, indicating the great insight of the author. The selection of individual items is appropriate and includes a list of the most important literature references on the subject of research published in recent years, which indicates the understanding of the scientific problem at an international level and, thereby, confirms the great contribution of the Ph.D. student's work and preparation for the concept of the research in question. I consider the substantive selection of literature sources and the way they are used to be correct. The



part of the thesis prepared in this way fulfills its function in terms of outlining the state of knowledge and research on the scientific problems addressed in the dissertation. A properly conducted analysis of the literature sources led to a conclusion about the paucity of data on the effect of phospholipids and bile salts on the profile of peptides released after  $\beta$ -Lg digestion at different BS/PL ratios. Furthermore, Ms. Kłosowska underscored a lack of any vital findings regarding the effect of BS and PL on lipid digestion and concluded that the interactions of BS and PL as surface-active compounds at the oil-water interface remain to be elucidated.

The conclusions defined in this way from the overview of the state of knowledge allowed M.Sc. K. Kłosowska to formulate research hypotheses in the evaluated doctoral thesis, i.e., the goal and scope of the research, which involved: (i) examining the effect of phospholipids and bile salts on lipolysis in the small intestine; investigating the physicochemical aspects of the mutual interaction of these biosurfactants at the oil-water interface during the simulated intestinal lipolysis; demonstrating how phenomena observed at the interface can affect the degree of lipolysis *in vitro* in model emulsions; (ii) examining the effect of phospholipids and bile salts on the proteolysis of  $\beta$ -lactoglobulin in simulated conditions of digestion in the human gastrointestinal tract; and identifying the differences in peptide profiles obtained after *in vitro* proteolysis along with the differences in their biological activity. It should be emphasized that the aim of the study was clearly highlighted and is justified by the previously presented overview of literature findings. The formulated research hypotheses are cognitive in nature as well as innovative from the international and national perspective, which fully justified undertaking the research in question. However, I suggest to more clearly specify the research hypothesis by indicating the elements of scientific novelty in the context of the presented scientific discipline.

The methodology of the research (12 pages) is well thought out and properly selected in terms of content. It is distinguished by a large number of cutting-edge, innovative and original methods, such as the tensometric method for analyzing interfacial tension, identification of peptides by liquid chromatography coupled with LC-MS mass spectrometry, bioinformatic analysis of bioactive peptides in the aspect of the so-called integrated approach, i.e., combining *in silico* and *in vitro* analyses in the experimental strategy or a logical cascade of methods and techniques during the preparation of model emulsions from WPI, simulating intestinal lipolysis and proteolysis in the gastrointestinal tract. To make methodology description complete, it would be worthwhile to add information on the Kyte method and the author's method for using the MBPDP databases (Milk Bioactive Peptide Database) and IEDP (Immune Epitope Database) along with access dates and update dates during the elaboration of results. The experiments conducted by the Ph.D. student were planned in a logical manner and their implementation and course were described in an intelligible way. The properly selected



methodology ensured a comprehensive approach to the analyzed research problems, whereas the precisely adjusted experimental tools enabled achieving the research goals in a logical and consistent manner. Consequently, the development of conditions for the lipolysis of WPI-stabilized emulsions and proteolysis of WPI and  $\beta$ -Lg allowed accomplishing the research goal, which was to examine the effect of phospholipids and bile salts on the digestion of triacylglycerols and proteins from WPI, including  $\beta$ -lactoglobulin.

In the most extensive (63 pages) chapter of the dissertation, i.e., "Results and Discussion", the Ph.D. student described the results of particular experiments in an accessible and comprehensive way. The division into subchapters referring to the description of research stages provided in the "Materials and Methods" chapter and in the publication facilitated the analysis of the results of individual experiments.

The results of experiments concerning retrospective analysis of data from literature references addressing surfactant concentrations in bile ducts and lipolysis were included by Ms. Kłosowska in chapter 4.2. "Lipolysis" and in the publication: Kłosowska, K., Teresa Castillo-Santaella, Julia Maldonado-Valderrama, Adam Macierzanka. 2024. "The Bile Salt/Phospholipid Ratio Determines the Extent of *in Vitro* Intestinal Lipolysis of Triglycerides: Interfacial and Emulsion Studies" Food Research International, 187, 114421187; and also in the manuscript submitted for review: Kłosowska-Chomiczewska, Ilona E., Dorota Dulko, Mateusz Semborski, Mariia Yakoviv, Noel Si, Robert Staroń, Łukasz Krupa, Adam Macierzanka; "In Vitro Intestinal Lipolysis of Protein-Stabilised Emulsion: Simulating the Impact of Human Bile Using Individual Bile Salts and Phospholipids."

The candidate has demonstrated that the research results obtained are essential to the understanding of the colloidal and interface phenomena that are key to the subject of research in the field of chemical sciences as well as food and nutrition sciences. In particular, they extend the knowledge on the digestion of emulsified triacylglycerols in the context of different ratios of BS and PL. The innovative and comprehensive nature of these investigations was emphasized in an in-depth study of the interactions between BS and PL at the oil-water interface and their effect on lipolysis. M.Sc. Kłosowska has demonstrated that BS and PL synergistically reduce the oil-water interfacial tension during lipolysis; that BS desorbs much more easily from the oil-water interface compared to PLs; that the BS/PL-9:4 molar ratio proved to be optimal for effective intestinal lipolysis of triacylglycerols; and also that the presence/concentration of biliary PL should be taken into account in the *in vitro* lipolysis simulating the conditions of the human digestive system. Here a question arises for discussion during the public defense: in what area can be pointed out the possibilities of practical use of the obtained results.



The results of the experiments concerning proteolysis were included by the Candidate in Chapter 4.3. In this next stage of the research, the Ph.D. student analyzed the whey protein isolate in terms of the effect of the BS/PL ratio on its proteolysis. Ms. Kłosowska demonstrated that the rate of WPI digestion depended on the BS/PL ratio. In the case of WPI, the extent of  $\beta$ -Lg digestion without any biosurfactants was comparable to the digestion effectiveness noted at the lowest level of PL added (BS/PL-9:4). Different was the case with the digestion of isolated  $\beta$ -Lg, where the effects of biosurfactants was stronger at their physiological concentrations ( $\beta$ -Lg-BS/PL-9:4). Additionally, the Ph.D. student has indicated that further studies are necessary to elucidate the interactions between the  $\beta$ -Lg molecule, its fragments and surface-active components of bile salts in its specific regions.

This raises another question to be discussed during the public defense of the evaluated doctoral thesis: (i) where are the lists of identified peptide sequences, e.g., unique sequences of 85 peptides released after 2 h of WPI digestion at a BS/PL ratio of 9:4 or the list of peptides used for analyses conducted by means of the MBPDB database and the final list of the 39 identified peptides; (ii) in what type of studies was the biological activity demonstrated for those 39 identified peptides – *in vitro*, *in silico* or *in vivo*?

The research is summarized in Chapter 5. The statements it provides are reflected in the research results achieved. The most important findings concern, for example: determining the physiological ratio of bile biosurfactants at the level of 9 mM BS to 4 mM PL in healthy individuals; demonstrating the synergistic effect of BS and PL (in a 9:4 ratio) on the efficiency of triacylglycerol lipolysis, compared to other ratios of bile surfactants; demonstrating a correlation between the extent of *in vitro* digestion of triacylglycerol emulsion and changes in the surface tension during lipolysis, e.g., desorption, depending on the PL concentration; demonstrating the effect of PL on the rate of  $\beta$ -Lg digestion, where a correlation was found for the first time ever between the slowing down of the digestion rate and the molar ratios of biosurfactants; demonstrating after the analysis of the peptide profile that the slowed down digestion of  $\beta$ -Lg in the presence of PL may be of physiological importance for the release of bioactive peptide sequences and epitopes; and a higher number of bioactive peptide sequences and epitope sequences released during  $\beta$ -Lg digestion in the presence of BS/PL mixtures compared to BS used alone.

The high scientific level of the dissertation of M.Sc. Katarzyna Kłosowska has prompted my additional questions to be considered during the discussion: (i) what factors may influence the currently observed trends in the analysis of bioactive food components; and (ii) in what area can the research initiated by the Ph.D. student be continued?

## Final conclusion



My remarks/questions included in the review are mainly of the nature of comments and suggestions regarding possible further analyses of the rich material collected as part of the dissertation. To recapitulate, I rate the dissertation high, I appreciate its originality, the effort of the work contributed, inquisitiveness, consistency, distinctively planned methodological part, substantive discussion related to the findings reported by other authors, correct reasoning and aesthetic values. Taking into account the requirements for doctoral dissertations, in the case of the doctoral thesis of M.Sc. Katarzyna Kłosowska, it should be recapitulated that: the evaluated dissertation presents the thorough theoretical knowledge of the Ph.D. student in the field of chemistry of lipids and proteins. The Ph.D. student has a very good understanding of the national and international literature related to the conducted research. In addition, the Ph.D. student has demonstrated the ability to independently conduct scientific research. The dissertation of M.Sc. K. Kłosowska is an original solution to the problem defined in the title and in the goal of the dissertation.

To sum up the assessment of the doctoral thesis of M.Sc. K. Kłosowska, I state that the dissertation submitted for evaluation, being a fully original and valuable solution to the research problem, meets the formal requirements for doctoral theses, and also constitutes a scientific achievement that meets all statutory requirements and criteria set out in art. 187 of the Act of 20 July 2018 - Law on Higher Education and Science (Journal of Laws of 2023, item 742, as amended). Therefore, I am applying to the Scientific Council of the Discipline of Chemical Sciences at the Gdańsk University of Technology to admit Ms. Katarzyna Kłosowska to further stages of the procedure for conferring a Doctor's degree in the field of natural sciences in the discipline of chemical sciences.

H. Darewicz