

Proteolysis and Peptide Formation from Pea and Soy Protein by Two *Lactobacillus helveticus* Strains

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Abstract

The aim of the present study was to evaluate the ability of selected *Lactobacillus helveticus* strains to degrade plant proteins and their potential to obtain bioactive peptides. The strains included were *L. helveticus* h70 and b244. Sterile 9% reconstituted skimmed milk (RSM) was used as a reference growth medium. The plant proteins - pea and soy were used in the study. The process of proteolysis and the appearance of separate protein fractions after lactic acid fermentation was proved by SDS-PAGE (Tris-tricine electrophoresis). The detection of new bioactive peptides in media containing pea and soy protein was carried out with UHPLC-MS-system.

Keywords: lactic acid bacteria, milk, plant proteins, proteolysis

Резюме

Целта на настоящото изследване беше да се оцени способността на избрани щамове на *Lactobacillus helveticus* да разграждат растителни протеини и техният потенциал за получаване на биоактивни пептиди. Включените щамове бяха *L. helveticus* h70 и *L. helveticus* b244. Като референтна среда за растеж беше използвано 9% стерилно обезмаслено мляко (RSM). В изследването бяха използвани грахов и соев растителни протеини. Процесът на протеолиза и появата на отделни протеинови фракции след млечно-кисела ферментация бяха доказани чрез SDS-PAGE (Трис-трицинова електрофореза). Определянето на нови биоактивни пептиди в среди, съдържащи грахов и соев протеин, беше извършено чрез UHPLC-MS система.

Introduction

In recent years, the world's population has increased, reaching 8 billion people in 2022, climate and natural pollution, world military conflicts continue to be observed (UN, 2023). These complex circumstances need a greater food supply. That is why the search for new sources of food is also necessary. But food production is responsible for up to 30% of global greenhouse gas emissions (Willett *et al.*, 2019), and beef production requires an average of 60 times higher emissions per gram produced protein compared to legumes (Tilman and Clark, 2014). Stanaway *et al.* (2018) and Willett *et al.* (2019) report that consuming larger amounts of processed meat, and not enough whole grains, leads to the development of various diseases, such as coronary heart disease, stroke, diabetes. Due to the above claims, alternative and sustainable sources of proteins should be developed and implemented. Nowadays, such sources are plant-based proteins.

Some authors present in their study „planetary“ diet which is primarily used in plant-based food (legumes, seeds, soy), has a beneficial effect not only on human organism, but also on the environment (Willett *et al.*, 2019).

Recently, there have been studies on the application of plant-based proteins and peptides to various diseases and ailments. Some of these studies present that potential plant-based bioactive peptides can be used in the treatment of diabetes (Patil *et al.*, 2020), cancer disease (Vuyyuri *et al.*, 2018), improving memory (Katayama *et al.*, 2021). Various procedures are required to obtain the biologically active peptides from plant-based proteins, such as chemical and physical methods, microbiological fermentation or enzymatic hydrolysis (Nong and Hsu, 2022). Traditional extraction methods using chemicals are widely applied, but can affect both the environment and human health. In recent

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years, a wide range of alternative green extraction technologies have been practiced, including a biotechnological approach (microbial fermentation and enzymatic hydrolysis) and non-thermal techniques (high hydrostatic pressure, ultrasonic and microwave-assisted extraction methods) (Singh *et al.*, 2022; Rivero-Pino *et al.*, 2023) (Fig. 1).

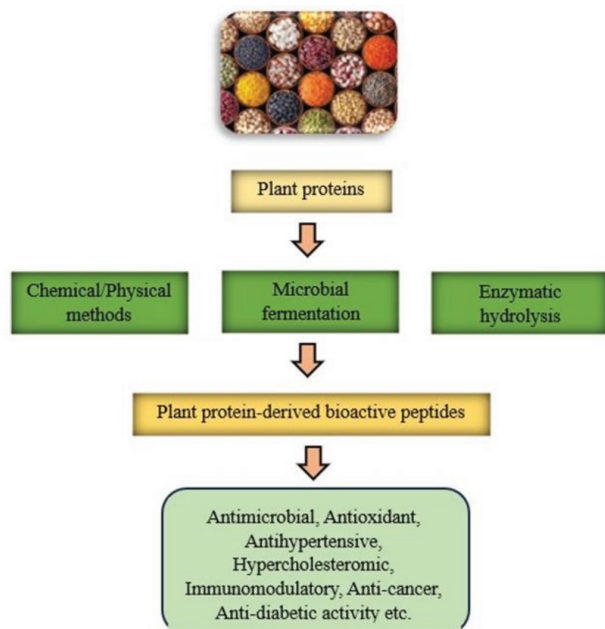


Fig. 1. Schematic representation of plant proteins, treatment and production of bioactive peptides (adapted from Nirmal N. *et al.*, 2024)

Legumes such as peas and soybeans are good sources of proteins. Several studies indicate that pea seeds contain 20–25% protein, 40–50% starch and 10–20% fiber. Peas are low in sulfur-containing amino acids, but rich in the essential amino acid lysine (Dahl *et al.*, 2012). Unlike other legumes, peas have low allergenicity. On the other hand, soy contains about 20% lipids, 40% proteins, 35% carbohydrates and 5% other substances, some of which protect against cancer and chronic diseases (Giri and Mangaraj, 2012). Chandrasekara and Kumar (2016) report that soy contains about 14 phy-

tochemicals, such as carotenoids, coumarins, flavonoids, lignans, phytic acid, triterpenes and phenols, which have a beneficial effect against cancer and cardiovascular diseases. Also, the Food and Drug Administration in the USA supports the consumption of soy products because it considers that soy protein helps in treatment of cardiovascular and chronic kidney diseases (Messina, 2016). The comparison composition of essential amino acid profiles in pea and soybean is presented on Fig. 2.

Both types of crops can be developed in a wide geographical area, without excessive seasonal requirements and costs (Asledottir *et al.*, 2023). Unlike dairy products, where the existence of bioactive peptides after lactic acid fermentation has been demonstrated, plant-based proteins have not yet been extensively researched for their presence. Therefore, the aim of the present work was to investigate the effect of proteolytic system of selected *L. helveticus* strains in plant-based media and the possibility of detection of potential bioactive peptides obtained as a result of lactic acid fermentation using pea and soy protein as sources.

Materials and Methods

Strains and culture media

Two *Lactobacillus helveticus* strains h70 and b244 both maintained in the LBB Culture Collection of LB Bulgaricum PLC, Sofia, Bulgaria were used in the study. The strains were cultivated on milk medium (9 % reconstituted skimmed milk, RSM) as a control and plant protein-based media (5% pea protein with 2% glucose and 5% soy protein with 2 % glucose). The above mentioned lactic acid bacteria were selected after a preliminary selection of 44 strains, on which the quantity of products of proteolysis products was determined by the Church method and protease activity, aminopeptidase activity and total ACE-inhibitory activity were studied (Panayotova *et al.*, unpublished results).

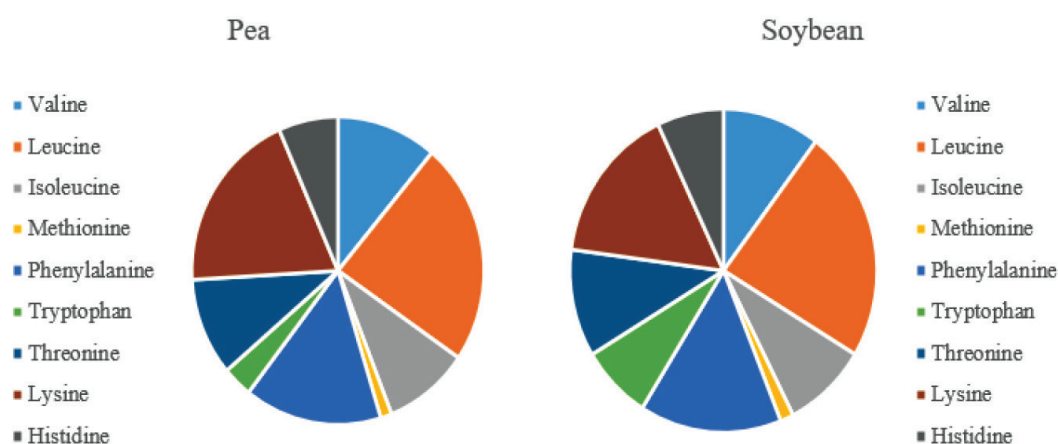


Fig. 2. Comparison composition of essential amino acid profiles in pea and soybean (Adopted from Lu *et al.*, 2020)

SDS-PAGE (Tris-tricine electrophoresis)

SDS-PAGE was performed as described by manufacturer's requirements and some modifications of Schagger and Von Jagow (1987) on vertical slab gels. For the purpose of this study 2X Tricine SDS Sample Buffer (Novex™, USA), 10X Tricine SDS Running Buffer (Novex™, USA) and 16% Tricine Gel (Novex™, USA) were used. All of this reagents were purchased from Invitrogen™ by Thermo Fisher Scientific, Waltham, MA USA. The media were inoculated with 1% of selected LAB at 37°C for 16 hours. After 16-th hour of cultivation the pH of samples was measured. 50% lactic acid was added to the samples to reach pH 3.4 and centrifugated at $6000 \times g$ for 10 min. 10 N NaOH was added to supernatants to reach pH 8.3 and centrifugated again at the same conditions. The newly obtained supernatants were ultrafiltered and concentrated 50 times. 10 µl of each sample was mixed with 15 µl sample buffer, heated at 85°C for 2 min and then applied 20 µl to the gel. Electrophoresis was at 125 V for 90 min and the samples were visualized by staining with Coomassie Brilliant Blue G-250 (Sigma Aldrich, St Louis, Missouri, USA).

UHPLC-MS

For the purpose of this study *Lactobacillus helveticus* strains h70 and *Lactobacillus helveticus* strains b244 were used again. The selected strains

were cultivated and processed in the same way as in SDS-PAGE analysis. The newly obtained supernatants were ultrafiltered using a permeability cartridge 3 kDa. The peptide samples were purified through anion exchange mini-column SOLA SAX 10 mg (Thermo Fisher Scientific, Waltham, MA USA) after adding 1N NaOH. Washed with 80% methanol and eluted with 1% formic acid in 80% methanol under cation format. Then the samples were further purified by passing through a SOLA SCX 10 mg cation exchange mini-column (Thermo Scientific, Waltham, MA USA). They were washed with 80% methanol and eluted with 1% ammonia in 80% methanol. The above eluates were evaporated at 40°C under nitrogen purging and dissolved with 1% formic acid in water. 500 µl of the supernatants were ultrafiltered through a 3 kDa centrifugal ultrafiltration cartridge. After the ultrafiltration process, 5 µl of the sample was injected into the UH-PLC-MS-system. Low molecular weight peptides (<3 kDa) were separated on a liquid chromatography system with mass spectral detection. The instrumentation used was a Thermo Fisher Scientific UHPLC-MS – Ultimate 3000 – Orbitrap LTQ XL. The chromatographic column of choice was a Synchrois aQ 100 x 2.1 mm, 1.7 µm average packed particle diameter, 175Å porosity. Peptides were reverse-phase separated with a gradient of acetonitrile.

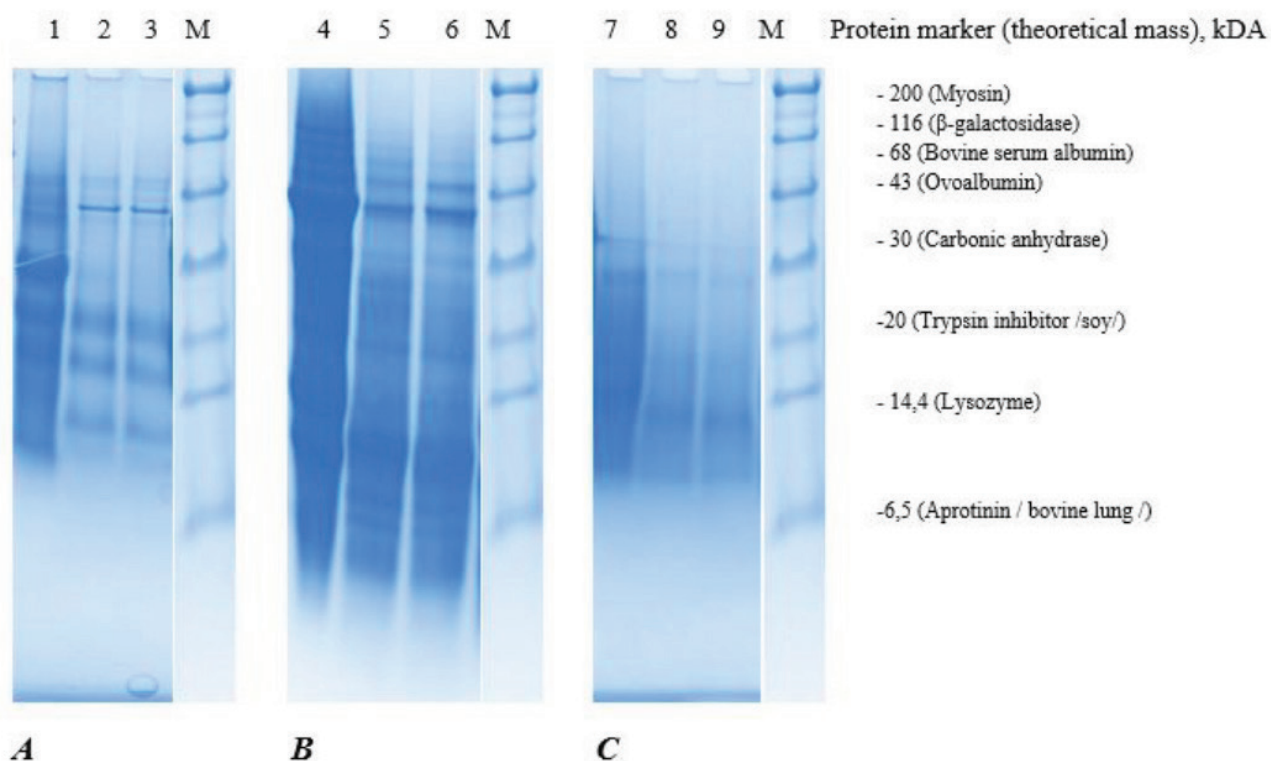


Fig. 3. Tris-Tricine PAGE. A) RSM – reconstituted skimmed milk; B) 5% Pea protein+2% Glucose; C) 5% Soy protein +2% Glucose. 1 - 9% RSM, uninoculated medium; 2 - h70 9% RSM; 3 - b244 9% RSM; 4 - 5% Pea protein+2% Glucose, uninoculated medium; 5 - h70 Pea protein+2% Glucose; 6 - b244 Pea protein+2% Glucose; 7 - 5% Soy protein +2% Glucose, uninoculated medium; 8 - h70 5% Soy protein +2% Glucose; 9 - b244 Soy protein +2% Glucose; M – marker. *RSM – reconstituted skimmed milk

trile 0-80%, column temperature - 35°C, flow rate 50 µl/min and mass spectral detection Full scan m/z = 300-2000.

Results and Discussion

Proteolysis in plant-based media

The analysis of protein fraction obtained after cultivation of selected LAB was performed by Tris-Tricine PAGE (Fig. 3 A-C).

Hydrolysis of different type of proteins by *L. helveticus* h70 and *L. helveticus* strains b244 resulted in differences in the distribution of peptides based on their molecular mass. The obtained results about milk medium – control showed that protein fragments with molecular mass of 30-43 kDa, 20-30 kDa and lower molecular fragments 6.5-14.4 kDa were found after cultivation of this strains. Unlike RSM medium, the medium with pea protein showed the presence more protein fragments (Fig. 3-B). Not inoculated medium it was much more intensely colored. Well defined hydrolysis was observed when using both strains. Similar protein fragments to those in the milk medium were also observed in the pea protein medium, with the difference that in both strains appeared low molecular protein fragments with molecular mass below 6.5 kDa. In regard to the third medium – soy protein with glucose, obtained results showed presence of protein fragments in the range 6.5-30 kDa (Fig. 3-C). The first protein fragments in soy-based medium were observed around 30 kDa in contrast to RSM medium and pea-based medium where just above 43 kDa were first seen.

In all three types of media, the same tendency was observed regarding the effect of proteolysis under the action of the two selected strains. Not inoculated media were with much more intense staining. It is clearly seen that after the lactic acid fermentation with *L. helveticus* h70 and *L. helveticus* strains b244, a proteolytic process occurs. Furthermore, the selected strains among themselves showed no visible differences compared to the different types of media.

Peptide profiles

In Figure 4, the peptide profiles of *L. helveticus* h70 and *L. helveticus* b244 strains are presented. In Fig. 4-A have showed a control data and represents the profile of the uninoculated plant medium 5% pea protein supplemented with 2% glucose. After the exposure of the selected strains, the appearance of new peptides is clearly visible due to the proteolytic effect they exerted. From the graph in Fig. 4-B it can be seen that *L. helveticus* strain h70 exerts proteolysis on low-molecular-weight proteins, and by the 28th minute of the process, the appearance of several new peptides is observed. On the other hand, the peptide profiles formed in the uninoculated medium at RT 30.71 and RT 34.60 min degraded to lower molecular weight peptides. The remarkable thing in this case is that after the 35th minute, the appearance of several new peptides, not present in the control medium until now, is observed. A similar trend was observed for *L. helveticus* strain b244 (Fig. 4C), with the peptide in the medium control at 30.71 min visibly degraded to

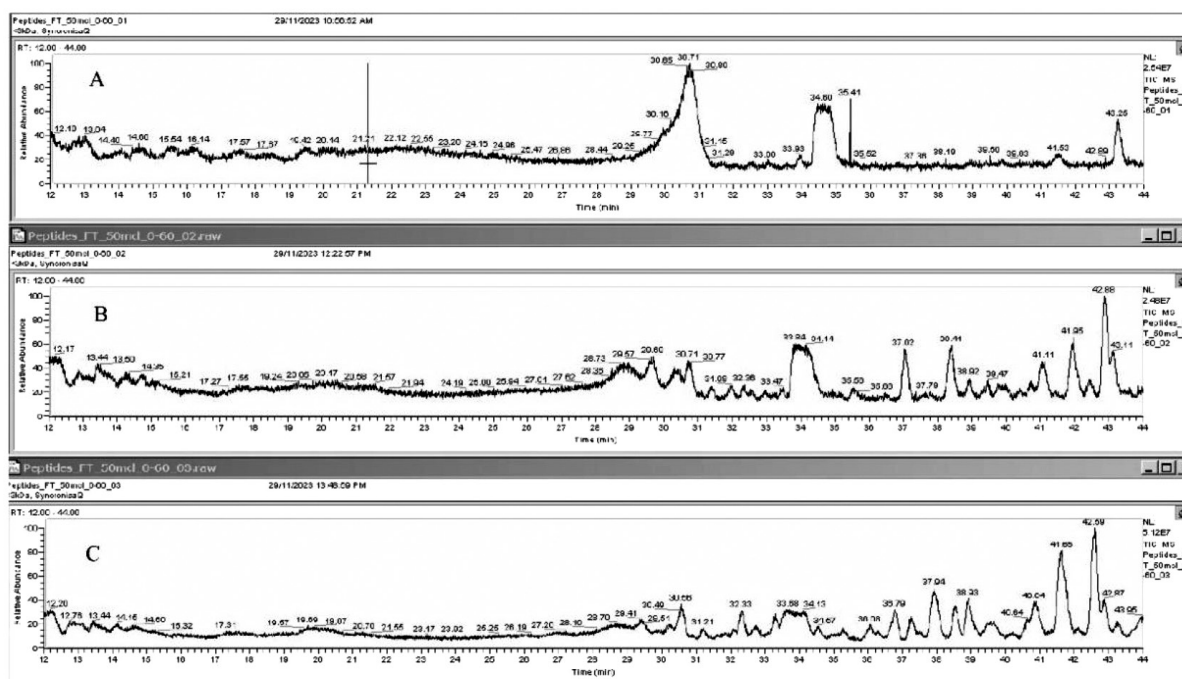


Fig. 4. Peptide profiles of *L. helveticus* h70 and *L. helveticus* b244, cultivated in: A) 9% Reconstituted skimmed milk; B) 5% Pea protein + 2% Glucose; C) 5% Soy protein + 2% Glucose

several lower molecular weight peptides. It should be noted that more pronounced peaks are observed when using this strain, i.e. the presence of more low molecular weight bioactive peptides.

Conclusions

This study demonstrated the possibility of obtaining low molecular weight peptides with a molecular weight of approximately 6.5 kDa after fermentation of 5% pea protein using strains *L. helveticus* h70 and *L. helveticus* b244. Optimization of the cultivation conditions for the studied strains for the secretion of the complex of peptidases and aminopeptidases, established in our previous studies, has been achieved.

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