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Identification of potential inhibitory peptides of enzymes involved in the metabolic syndrome obtained by simulated gastrointestinal digestion of fermented bean (*Phaseolus vulgaris* L.) seeds

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ABSTRACT

The aim of this study was to determine the conditions of bean fermentation carried out by *Lactobacillus plantarum* to obtain biologically active peptide fractions after *in vitro* digestion.

The results suggest that optimum process conditions should be selected according to the specific activity of peptides. Only a fraction with a molecular mass of 3.5–7 kDa obtained after fermentation at 22 °C for 3 h had α-amylase inhibitory activity. The optimal fermentation conditions for bean seeds to release peptide fractions with a molecular mass of 3.5–7.0 kDa and the highest lipase or ACE inhibitory activity (IC_{50} 1.19 and 0.28 mg mL^{-1}, respectively) were determined as 30 °C and 3 days. The fractions with the highest inhibitory activity were identified by LC-MS/MS and the sequences of the peptide derived from bean proteins were determined as INEGSLLLPH, FVVAEQAGNEEGFE, SGGGGGGVAGAATASR, GSGGGGGGGFGGPRR, INEGSLLLPH, GGYQGGGYGGNSGGGYGNRG, GGSGGGGGSSSGRRP, and GDTVTVEFDTFLSR.

1. Introduction

In recent years, a relationship between the lifestyle and nutrition and the prevalence of cardiovascular diseases, obesity, or diabetes has been observed, particularly in developed countries. Metabolic syndrome is associated with the co-existence of risk factors of metabolic origin, contributing to development of cardiovascular diseases, atherosclerosis, type 2 diabetes, or obesity. The diseases are connected with a glycemic index imbalance, glucose intolerance, hypertension, and chronic metabolic disorder caused by an imbalance between energy intake and expenditure (Roh & Jung, 2012; Sakulnarmrat & Konczak, 2012). Until recently, this disease entity has been characteristic only for people over 50 years of age, but now this syndrome is diagnosed in increasingly younger patients, including obese children (Pituch-Zdanowska et al., 2016). It is now known that the metabolic syndrome is associated with excessive activity of enzymes involved in glucose intolerance, hypertension, abdominal obesity, or inflammation.

Hypertension or high blood pressure is one of the long-term complications of type 2 diabetes and a major risk factor for stroke, heart attack, heart failure, and kidney disease (Bakris et al., 2010; Sowers & Epstein, 1995). This disease is associated with excessive activity of the angiotensin-converting enzyme (ACE, EC 3.4.15.1.). It is a part of the renin-angiotensin-aldosterone system (RAAS), which plays a key role in regulating blood pressure and water and electrolyte balance in the body (Brunner et al., 1972). Angiotensinogen is a substrate for renin to produce physiologically inactive angiotensin I (decapeptide), which by ACE is converted to angiotensin II (octapeptide) exhibiting strong vasoconstrictive properties. In addition, ACE inactivates bradykinin, which has a potent vasodilatory effect and influences the production of aldosterone. Thus, inhibitors of this enzyme exhibit antihypertensive properties and thus protect blood vessels and the heart. Synthetic ACE inhibitors such as captopril, enalapril, or lisinopril are commonly used in the treatment of hypertension, *e.g.* in the case of type 2 diabetes (Pfeffer & Frohlich, 2006). However, they cause serious side effects such as cough, angioedema, taste disturbance, or skin rashes.

Obesity is a multifactorial disease characterized by excessive accumulation of fat tissue caused by an imbalance between caloric intake and energy (Schrauwen & Westerterp, 2000) due to the lifestyle, low physical activity, and improper eating habits (Abete, Astrup, Martinez, Thorsdottir, & Zulet, 2010). Therefore, changing the key factors contributing to the development of obesity is a significant aspect in the treatment of the disorder. Increased physical activity and proper diet reduces the effects of obesity. Unfortunately, this approach is often long-term and recommendations are not followed by patients; therefore, it is necessary to use pharmaceutical products that are based on inhibition of the digestion of nutrients, especially fats, which are a

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major source of unwanted calories in the diet (McClendon, Riche, & Uwaifo, 2009). Drugs can also lower blood pressure, prevent the development of coronary heart disease, improve glucose tolerance, and prevent type 2 diabetes. Despite the documented positive measures to combat the occurrence of obesity, drugs also produce side effects mainly in the digestive system such as bloating, diarrhea, or improper function of the liver (Heymsfield et al., 2000). Therefore, researchers are looking for alternative compounds with ACE inhibitory activity that do not cause side effects and occur naturally in foods, Peptides, *i.e.* natural compounds in the free state (secondary metabolites) or released during the enzymatic hydrolysis of vegetable (Jakubczyk, Karaś, Baraniak, & Pietrzak, 2013) or animal (Enari, Takahashi, Kawarasaki, Tada, & Tatsuta, 2008; Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015) proteins may be one of such alternatives.

The risk of type 2 diabetes can be reduced by inhibition of enzymes involved in the release of glucose from food in the gastrointestinal tract or α-amylase produced mainly by the salivary glands and pancreas as well as α-glucosidase associated with the cell membrane of the small intestine which catalyzes the breakdown of di- and oligosaccharides to glucose, which is then transported to the bloodstream. Inhibition of these enzymes, including inhibitors derived from food, is an important adjunctive treatment of postprandial hyperglycemia (Sakulnarmrat & Konczak, 2012).

In the last few decades, researchers have discovered a number of bioactive peptides derived from food (Jakubczyk et al., 2013; Karaś et al., 2015; Suh, Whang, & Lee, 1999; Yang, Tao, Liu, & Liu, 2007). Protein hydrolysis by digestive enzymes such as pepsin, trypsin, or chymotrypsin yields peptides with different chain lengths, which may directly affect the digestive system or, delivered to the bloodstream by blood, are transported to various tissues and organs and exert a beneficial effect on the organism (Shimizu & Son, 2007; Vermeirssen, Van Camp, & Verstraete, 2004). Thus, biologically active peptides of natural origin, which tend to be more secure for patients than the commonly used drugs, may be components of the daily diet. The biological properties of peptides often become apparent only after hydrolytic release from the protein by enzymatic action, *e.g.* in the gastrointestinal tract.

Fermentation is one of the oldest biotechnological methods (Gan, Li, Gunaratne, Sui, & Corke, 2017; Zhao, Schieber, & Gänzle, 2016) in addition to technological methods such as cooking, soaking, steaming, extrusion, and blanching to enhance the digestibility of legume seeds. The using of food fermenting microorganisms changes food quality, generates taste, flavor, or contents of bioactive compounds such as peptides (Jakubczyk et al., 2013) or polyphenols (Gan, Shah, Wang, Lui, & Corke, 2016).

The aim of this study was to investigate the influence of fermentation carried out by *Lactobacillus plantarum 299*v and *in vitro* digestion on formation of metabolic syndrome-inhibitory peptides from bean proteins. The data about the interaction between fermentation conditions and bean proteins properties will enable the development the knowledge of bioactive ingredients to produce of novel food products.

2. Materials and methods

2.1. Materials

Bean seeds (*Phaseolus vulgaris* L. var. Eureka) were purchased from PNOS in Ożarów Mazowiecki, Poland.

Chemical compounds: HHL (Hippuryl-L-Histidyl-L-Leucine, PubChem CID: 94418), pepstatin A, PMSF (phenylmethanesulfonyl fluoride, PubChem CID: 4784), α-amylase from hog pancreas (50 U/mg), pepsin from porcine gastric mucosa (250 U/mg), pancreatin from porcine pancreas, bile extract, TNBS (2,4,6-trinitrobenzenesulfonic acid, PubChem CID: 11045), DNS (3,5-dinitrosalicylic acid, PubChem CID: 11873), pNPA (*p*-nitrophenyl acetate PubChem CID: 13243), DMSO (dimethyl sulfoxide, PubChem CID: 679), starch solution from Sigma-Aldrich Company, USA; protein Molecular Mass Standard, Fermentas, SM 1849, buffer samples Bio-Rad, *Lactobacillus plantarum 299v* from IPC (International Pharmaceutical Consulting, Szczecin, Poland). Any other chemicals were of analytical grade.

2.2. Preparation of fermented bean seeds

After 12 h of soaking in distilled water bean seeds without scales were milled and fermented with *L. plantarum 299*v (10^6 cfu/g fresh seeds) at different temperature (at 22, 30 and 37 °C) and time (3 h, 3 days and 7 days) conditions (Jakubczyk et al., 2013). After the process the samples were centrifuged at 8000 × *g*, 4 °C, for 20 min and supernatants were lyophilized and grounded in a laboratory mill. The powders were stored at − 18 °C until further use.

2.3. Protein assay

Total protein concentration was determined according to Lowry et al. method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine albumin as a standard.

2.4. Protein profile analysis by SDS-PAGE

The protein profile was determined by SDS-PAGE method with 5% concentrated and 12% separated polyacrylamide gel using the Mini-Protean BioRad electrophoresis system with 20 mA. The samples were mixed by buffer (1:1, v/v, Bio-Rad) and heated at 100 °C for 5 min prior to the electrophoresis run. The gels were stained with Coomassie Blue R-250. Molecular marker in range 14.4–116.0 kDa (SM 0431, Fermentas) was used as a standard. The protein mass and densitograms were determined by Photo Capt (Vilber Lourmat) version 12.6.

2.5. Hydrolysates preparation

The fermented bean products (4%, w/v) were *in vitro* hydrolyzed under gastrointestinal conditions according to the method described by Jakubczyk et al. (2013). Briefly, the samples were stirred for 5 min at 37 °C in salt solution (7 mM $NaHCO_3$ and 0.35 mM NaCl). Next, the α-amylase (50 U/mg) was added (1:10 E/S) and the samples were hydrolyzed for 10 min at 37 °C. The solution was adjusted to pH 2.5 with 1 M HCl and pepsin (≥ 250 U/mg) solution (1:100, E/S) was added. The hydrolysis was carried out for 2 h at 37 °C. Hydrolysates were neutralized with 1 M NaOH and the next step mixture containing 0.7% solution of pancreatin and 2.5% solution of bile extract (1:2.5, *v*/v) were added. The hydrolysis was carried out for 1 h at 37 °C. The reaction was stopped by heating at 100 °C for 5 min.

The peptides fractions with molecular weight < 3.5 kDa and 3.5–7.0 kDa were obtained (Karaś et al., 2015) with molecular mass cutoff membranes membrane 3.5 and 7 kDa against PBS buffer at the physiological concentration (1:4, v/v). The dialysis was carried out without light for 1 h at room temperature. The fractions were lyophilized and stored at − 18 °C until further use.

2.6. Determination of the degree of hydrolysis (DH)

The method used was according to Adler-Nissen (1979) with L-leucine as a standard. Total number of amino groups (100%) was measured in a sample after hydrolysis with 6 N HCl at 110 °C for 24 h.

2.7. Preparation of ACE from pig lung

Angiotensin converting enzyme was prepared according to the method described by Jakubczyk & Baraniak (2014). Lung tissues were homogenized in 0.1 M borate buffer pH = 8.3 containing pepstatin A (0.1 mM) and PMSF (0.1 mM) at 4 °C in ratio 1:2 (w/v) and centrifuged