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Individual contributions of Savinase and *Lactobacillus plantarum* to lentil functionalization during alkaline pH-controlled fermentation

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ABSTRACT

Legumes offer the possibility to develop multifunctional foods with benefits for metabolic syndrome. Our objective was to study the effect of alkaline fermentation by *Lactobacillus plantarum* and Savinase (FLPS) as well the individual effects of both processes on peptides, phenolics and bioactivity of lentil. FLPS increased peptides and some flavonoids and enhanced antioxidant activity, inhibition of angiotensin I-converting enzyme (ACE) and intestinal maltase activities of lentil soluble fraction. Savinase contributed to peptide release, ACE inhibitory and antioxidant activities of lentil soluble fraction. *L. plantarum* affected to phenolic composition, α-glucosidase and lipase inhibitory activities. Mass spectrometry analysis of the most active fermented lentil subfraction allowed the identification of the main bioactive compounds. Gastrointestinal digestion of fermented lentil increased bioaccessibility of peptides and phenolics as well as antioxidant activity. FLPS enhanced the overall healthy potential of lentil offering the possibility of its use as strategy for lentil functionalization.

1. Introduction

Unhealthy life style has raised the risk of population for non-communicable diseases which are the leading cause of death and disability globally (World Health Organization, 2014). In this context, regular physical activity and healthy diets combined with the intake of functional foods may help minimizing or even preventing certain chronic diseases (Jew, AbuMweis, & Jones, 2009). Functional foods are commonly designed to reach one physiological target. Nowadays, the knowledge on the multifactorial origin of many chronic diseases provides a new framework for the development of multifunctional foods.

As a legume, lentil (*Lens culinaris* Medik.) provides excellent nutritional quality and an array of functional compounds, including phenolics (Zhang et al., 2015) and bioactive peptides (Garcia-Mora et al., 2017). Lentil bioactive compounds are known to bind physiological targets to affect various signaling processes or regulatory functions that can lead to health benefits. For instance, lentil phenolic compounds have demonstrated a potential health benefit for lipid and glucose homeostasis as these compounds are able to inhibit gastrointestinal enzymes involved in carbohydrate (α-glucosidase) and lipid digestion (pancreatic lipase) (Zhang et al., 2015). Otherwise, lentil peptides derived from 11S and 7S globulins are able to bind angiotensin I-converting enzyme (ACE) and scavenge reactive oxygen species (ROS) *in vitro* (Garcia-Mora et al., 2017). All these studies position lentil on the market as a promising and reliable raw material in multifunctional food development.

Bioprocessing is a research area with potential application to exploit the functional properties of legumes. In particular, enzymatic hydrolysis with food-grade alkaline serine proteases from *Bacillus spp*. has been used to produce lentil and pinto bean functional hydrolysates rich in antioxidant and ACE-inhibitory peptides and phenolic compounds (Garcia-Mora, Peñas, Frias, & Martinez-Villaluenga, 2014; 2015). Fermentation is also a well-designed biotechnology for manufacturing functional foods (Filannino, Di Cagno, & Gobetti, 2018). Metabolic features of lactic acid bacteria may improve the bioavailability and bioactivity of phytochemicals and peptides with beneficial consequences for human health. For instance, fermentation with *Lactobacillus plantarum* (FLP) has shown to increase free phenolic compounds and the antioxidant and antihypertensive potential of lentil flour (Torino et al., 2013). More recently, it has been observed that common bean fermentation by *L. plantarum* 299v enhances the release of peptides able to bind gut enzymes including intestinal α-glucosidase, pancreatic lipase and α-amylase (Jakubczyk, Karas, Zlotek, Szymanovska, 2017). Combination of proteolytic microbial strains and commercial enzymes is an alternative strategy used to functionalize milk fermented products as reviewed by Hafeez et al. (2014); however,

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it has not been explored in legumes. Microbial and commercial enzymes may act synergistically increasing peptide content and diversifying the bioactivity of fermented products.

In this research, we aimed to further investigate the impact of fermentation with *L. plantarum* CECT 748 and enzymatic hydrolysis by Savinase 16 L (FLPS) on bioaccessibility of peptides and phenolics as well as multifunctional properties of lentil. The individual effects of FLP and hydrolysis with Savinase (HS) were also examined to evaluate their contribution when they are used in combination. As far as we know, a similar quantitative approach has not been published yet and is, therefore, one of the merits of this study. Our second objective was to identify bioactive compounds through fractionation and bioassay-guided selection of the most multiactive fraction. Gastrointestinal digestion (GD) has been shown to modify peptide and phenolic composition of foods and as consequence their health benefits (Luzardo-Ocampo et al., 2017). As such, it is important that any bioactive peptide or phenolic compound released by fermentation should be resistant to GD so as to reach the physiological targets in an active form. Thus, in this study, the impact of simulated GD on peptide and phenolic content as well as the bioactivity of lentil soluble fraction was studied.

2. Materials and methods

2.1. Materials

Lentil seeds (*Lens culinaris* Medik. var. Castellana) were purchased from Semillas Iglesias S.A. (Salamanca, Spain), milled (Moulinex, Allençon, France), passed through a 60-mesh sieve with 0.5 mm pore size and stored at 4 °C until use. A commercial food grade protease Savinase® 16 L (16 KNPU/g) was provided by Novozyme (Bagsvaerd, Denmark). *L. plantarum* CECT 748 was purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). MidiTrap™ G10 gel filtration columns were from GE Healthcare (Barcelona, Spain). Enzymes used including ACE (EC 3.4.15.1), rat intestine α-glucosidase (EC 3.2.1.20), porcine pancreatic lipase type II (EC 3.1.1.3), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9) as well as other chemicals were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated.

2.2. Techniques used for lentil processing

The following three processing techniques were implemented on lentil flour:

HS. Lentil flour was suspended in 1.5 L of sterile tap water (143 g/L final concentration), equilibrated at 37 °C and the pH adjusted to 8.5 with 1 M NaOH. Enzymatic hydrolysis was performed in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) with continuous stirring (300 rpm) and the addition of Savinase (365 mg/L final concentration) at 37 °C and pH 8.5 for 15 h.

FLP. One cryovial of *L. plantarum* CECT 748 was propagated using Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) as described previously (Limon et al., 2015). After propagation, cells were harvested by centrifugation at 8000 × *g* for 10 min, washed twice, and suspended in sterile water at the minimum cell density of 8 log CFU/mL. Fermentation was carried out in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) mixing 1.5 L of sterile tap water with lentil flour (143 g/L final concentration) and *L. plantarum* ($2x10^8$ CFU/L final cell density) under stirring conditions (300 rpm) at 37 °C, pH 8.5 for 15 h.

FLPS. Starter culture was prepared as described above. *L. plantarum* (2×10^8 CFU/L final cell density), Savinase (365 mg/L final concentration) and lentil flour (143 g/L final concentration) were used to prepare a 1.5 L fermented flour suspension in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) with a continuous speed mixer (300 rpm) at 37 °C, pH 8.5 for 15 h.

Three independent replicates were conducted for each treatment. Soluble fractions from all samples were obtained by centrifugation at 7500 × *g* at 5 °C for 15 min. Supernatants were stabilized by heating at 70 °C during 10 min for enzymatic and microbial inactivation. Soluble fraction from unfermented lentil flour was used as control. Finally, all samples were freeze-dried and stored under vacuum at −20 °C until use.

2.3. Fractionation of lentil soluble fraction by size exclusion chromatography

FLPS soluble fraction was subjected to size exclusion chromatography using MidiTrap™ G10 columns containing Sephadex G-10 in which molecules are separated on the basis of differences in size. Freeze-dried sample was dissolved in distilled water (20 mg/mL) and filtered through 0.45 μm syringe filters. Column was equilibrated with 8 mL of deionized water. A sample volume of 0.3 mL followed by 0.3 mL of deionized water were added to the column allowing entering the packed bed completely and discarding the flow-through. Elution was carried out adding 2.5 mL of deionized water to the column and collecting five fractions (F1 to F5) containing 0.5 mL of eluate. Finally, collected fractions were lyophilized and stored at −20 °C until analysis.

2.4. In vitro GD of lentil samples simulating physiological conditions

FLPS soluble fraction was subjected to a sequential gastric and duodenal digestion according to the method described by Garcia-Mora et al. (2014). Briefly, FLPS was dissolved in simulated gastric fluid (SGF, 0.15 M NaCl; pH 2.5). Samples were pre-heated for 15 min at 37 °C. Then 400 μL of a solution containing 0.59% (w/v) pepsin (3640 U/mg protein) in SGF were added and the pH was adjusted to 2.5 with 0.01 M HCl. Digestion was performed at 37 °C for 2 h and subsequently stopped by increasing the pH to 7.5 with 0.1 M NaOH. Gastric digest were adjusted to pH. 6.5 with 0.01 M HCl. In order to simulate a duodenal environment the following solutions were added: 150 μL of a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and glycodeoxycholic acid, 46 μL of 1 M $CaCl_2$, 500 μL of 0.25 M Bis-Tris (pH 6.5), and 100 μL of pancreatin in SGF at pH 7.0. Digestion was carried out at 37 °C for 2 h. Finally, pancreatin was inactivated by heating at 80 °C for 15 min.

2.5. Determination of peptide and total phenolic compounds contents

Peptide concentration was measured by Pierce Quantitative Colorimetric Peptide Assay kit (Fisher Scientific, Madrid, Spain) in permeates obtained by ultrafiltration through cellulose membranes of 10 kDa pore size (Millipore, Billerica, MA, USA). Results were expressed as mg of peptides/g of soluble fraction. Total phenolic compounds were determined using the Folin-Ciocalteau method as previously described (Garcia-Mora et al., 2015). The absorbance was read at 690 nm using a Synergy HT multi-well plate reader (BioTek, Winooski, VT, USA) and the results were expressed as mg gallic acid equivalents (GAE)/g of soluble fraction.

2.6. Determination of individual phenolic compounds

Phenolics extraction from lentil samples was performed according to a previously described method (Dueñas, Martínez-Villaluenga, Limón, Peñas, & Frias, 2015). Briefly, lentil soluble fractions (2 g) were macerated in methanol:water:trifluoroacetic acid (80:19.9:0.1, v/v/v) at 4 °C for 16 h. Subsequently, they were centrifuged at 4000 × *g* and 5 °C for 20 min in a Sorval RC 5B super-speed centrifuge (GMI, San Diego, CA, USA). The extracts were concentrated at 30 °C under vacuum for methanol evaporation. For phenolic analysis, the dry extracts were dissolved in 10 mL of water. For purification, an aliquot (4 mL) was passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), previously activated with 2 mL of methanol followed by 3 mL of