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Isolation and identification by high resolution liquid chromatography tandem mass spectrometry of novel peptides with multifunctional lipid-lowering capacity

Isabel M. Prados, M. Luisa Marina, M. Concepción García*

Departamento de Química Analítica, Química Física e Ingeniería Química, Facultad de Biología, Ciencias Ambientales y Química, Instituto de Investigación Química "Andrés del Rio" (IQAR), Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain

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ABSTRACT

This work describes the isolation, characterization, and identification by RP-HPLC-ESI-Q-TOF of novel peptides that interfere in the fat digestion and absorption mechanisms by multiple pathways. Peptides were ultrafiltrated and peptides in the most active fraction were further separated by semipreparative RP-HPLC. Nine different subfractions were obtained observing a high amount of peptides in subfraction F3. Peptides in subfraction F3 could simultaneously reduce the solubility of cholesterol in micelles and inhibit pancreatic cholesterol esterase and pancreatic lipase, even after a simulated gastrointestinal digestion. The identification of lipid-lowering peptides has been scarcely performed and when done, low selectivity or sensitivity of employed identification techniques or conditions did not yield reliable results. Separation and detection of peptides by RP-HPLC-ESI-Q-TOF-MS was optimized and most favorable conditions were employed for the identification of peptides using *de novo* sequencing. Ten different peptides with 4-9 amino acids were identified. Main feature of identified peptides was the high acidity derived from a high presence of amino acids glutamic acid and aspartic acid in their sequences.

1. Introduction

Hypercholesterolemia and hyperglycemia are metabolic disorders characterized by high levels of cholesterol and triglycerides in blood. Hyperlipidemia has increased worldwide due to the popularity of high-fat diets and non healthy lifestyles (Jacobson, Miller, & Schaefer, 2007). Prevalence of hyperlipidemia is a risk factor in cardiovascular diseases such as coronary heart disease and atherosclerosis. Different synthetic drugs for treating this disease have been developed such as inhibitors of cholesterol endogenous synthesis, inhibitors of membrane proteins that promote the intestinal absorption of cholesterol, bile acid sequestrants, *etc*. (Descamps, Sutter, De Guillaume, & Missault, 2011). Nevertheless, diverse side effects (increase of hepatic transaminases and creatine kinase, muscle weakness, headache, sleep disorders, *etc.*) have been described derived from the long-term consumption of these synthetic drugs (Heidrich, Contos, Hunsaker, Deck, & Vander Jagt, 2004). Moreover, these synthetic drugs are usually limited to fight hyperlipidemia by following a single mechanism. Therefore, and especially in cases where cholesterol and triglycerides levels are moderate, the consumption of foods that contain or are enriched with lipid-lowering substances is advisable.

Bioactive peptides are natural molecules that have demonstrated a wide range of activities although hypolipidemic peptides have been much less reported than others. Peptides with capacity to reduce cholesterol and/or triglyceride levels have been obtained from different foods (Alhaj, Kanekanian, Peters, & Tatham, 2010; Lammi et al., 2016; Liyanage et al., 2010; Marques, Fontanari, Pimenta, Soares-Freitas, & Areas, 2015; Rho, Park, Ahn, Shin, & Lee, 2007; Yust, Millán-Linares, Alcaide-Hidalgo, Milán, & Pedroche, 2012; Zhang, Yokoyama, & Zhang, 2012). In addition to these products, some byproducts derived from the food industry are also considered sources of bioactive peptides although, again, not much attention has been paid to hypolipidemic peptides (García, Orellana, & Marina, 2016). Nakade et al. (2009) described the hypocholesterolemic capacity of a protein hydrolysate obtained from a meat industry byproduct that enabled the reduction of the micellar cholesterol solubility, the suppression of the cholesterol uptake by Caco-2 cells, and the cholesterol reduction in the serum of rats fed with the hydrolysate. More recently, our research group has published a work describing antihypertensive, antioxidant, and hypocholesterolemic activities in seed hydrolysates obtained from *Prunus* genus fruits and olives. We observed that the hydrolysate obtained with Alcalase enzyme from the *Picual* olive seeds showed a high capability to reduce

* Corresponding author.
E-mail address: concepcion.garcia@uah.es (M.C. García).

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micellar cholesterol solubility (García, González-García, Vásquez-Villanueva, & Marina, 2016). Nevertheless, further studies are required to confirm these first results and to characterize these peptides.

Identification of peptides and proteins is mainly performed using reversed-phase (RP)-HPLC coupled to tandem mass spectrometry. Despite there is a wide bibliography devoted to the characterization and identification of bioactive peptides (García, Orellana, & Marina, 2016), a few works have been addressed in the case of lipid-lowering peptides. Moreover, in most cases no peptide identification was carried out or only amino acid composition was detailed (Zhang et al., 2012). The first identification of an hypolipidemic peptide was carried out using a protein sequencer which enabled to identify the peptide IIAEK in bovine milk β-lactoglobulin (Nagaoka et al., 2001). Few years later, Zhong, Zhang, Ma, and Shoemaker (2007) could identify the hypolipidemic peptide WGAPSL in soy using RP-HPLC-MS. Alhaj et al. (2010) used RP-HPLC-MS/MS with mobile phases containing 0.1% (v/v) trifluoroacetic acid (TFA) for the detection of peptides with hypocholesterolemic activity but, as expected, no sequencing and only information on molecular weight of peptides was obtained. Marques et al. (2015) also identified hypocholesterolemic peptides from cowpea using RP-HPLC-MS/MS but reliability of data is questioned since only peptides with ALC between 50-80% were identified.

The aim of this work was to evaluate the capacity of olive seed peptides to reduce the amount of exogenous cholesterol and triglycerides, to isolate peptides with lipid-lowering capacity from the olive seed, and to optimize a RP-HPLC-tandem mass spectrometry (MS/MS) method enabling the reliable identification of novel peptides.

2. Materials and methods

2.1. Chemical and samples

All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Supergradient HPLC grade acetonitrile (ACN), acetic acid (AA), acetone, methanol (MeOH), and hexane were purchased from Scharlau Chemie (Barcelona, Spain). Tris (hydroxymethyl) aminomethane (Tris), hydrochloric acid (HCl), sodium dihydrogen phosphate, di- sodium tetraborate, and sodium dodecyl sulphate (SDS) were from Merck (Darmstadt, Germany). Sodium hydroxide, dithiothreitol (DTT), trifluoroacetic acid (TFA), bovine pancreatic cholesterol esterase, porcine pancreatic lipase, *p*-nitrophenylbutylrate (*p*-NPB), *p*-nitrophenylpalmitate (*p*-NPP), taurocholic acid, taurodeoxycholic acid, glycodeoxycholic acid, oleic acid, phosphatidylcholine, simvastatin, rutin trihydrate, cholestyramine, β-sitosterol, o-phthaldialdehyde (OPA), L-glutathione (GSH), pepsin, pancreatin, taurocholate, cholesterol, β-mercaptoethanol, sodium chloride (NaCl), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified eagle's medium (DMEM), antibiotics (penicillin, streptomycin, and amphotericin), fetal bovine serum, and phosphate buffered (PB) were all from Sigma-Aldrich (Saint Louis, MO, USA). Cholesterol oxidase kit was purchased from BioAssay Systems (Hayward, CA, USA). Total bile acid kit was from Bio-Quant (San Diego, CA, USA). Alcalase 2.4 L FG, produced by fermentation of a selected strain of *Bacillus licheniformis*, mainly composed by Subtilisin A, with catalytic activity on serine, and with an activity of 2.4 Anson units per gram, was kindly donated by Novozymes Spain S.A. (Madrid, Spain). All cell lines (HeLa, HT-29, and HK-2) were from the American Type Culture Collection ATCC (Rockwell, MD, USA). Raw olives of 'Manzanilla' variety were kindly donated by the olive company FARO-LIVA S.L. (Murcia, Spain).

2.2. Peptide production

Olive stones were crushed to release the seed and seeds were ground in a domestic mill. Olive seeds were defatted with hexane for 30 min (four times). Extraction of proteins from defatted olive seeds was performed following the method developed by Esteve, Del Río, Marina, and García (2010). Briefly, 0.03 g of milled and defatted olive seeds were treated with 5 mL of an extracting buffer (100 mM Tris-HCl (pH 7.5), 0.5% (w/v) SDS, 0.5% (w/v) DTT) using a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonic Vibra-Cell, Hartford, CT, USA) at 30% of amplitude for 5 min. After centrifugation (4000 *g*, 10 min), proteins in the supernatant were precipitated with cold acetone (10 mL) in the fridge for 24 h, followed by centrifugation and drying at room temperature to obtain a protein isolate. The protein isolate was dissolved in a 5 mM borate buffer (pH 8.5) and hydrolyzed with Alcalase (0.15 UA/g protein) by incubation in the Thermomixer Compact (Eppendorf, Hamburg, Germany) at 50 °C during 4 h. The digestion was stopped by increasing the temperature to 100 °C for 10 min and centrifuging for 10 min at 6000 *g*. Finally, the supernatant, containing peptides, was stored at −20 °C.

2.3. Semipreparative RP-HPLC fractionation

The hydrolysate solution was firstly fractionated by ultrafiltration using molecular weight (Mw) cut-off filters Amicon® Ultra of 5 kDa and 3 kDa from Merck Millipore (Tullagreen, Ireland) to obtain three fractions: a fraction with peptides bigger than 5 kDa, a fraction with peptides with Mw between 5 and 3 kDa, and a fraction with peptides below 3 kDa.

Peptide separation by RP-HPLC was performed using a HPLC equipment from Agilent Technologies (Pittsburgh, PA, USA) model 1100, equipped with a vacuum degasser, a quaternary pump, an automatic injection system, a thermostatic column compartment, a diode array detector, and a fluorescence detector. Control of the equipment and data acquisition were performed with the HP ChemStation software. The separation was carried out in a Jupiter 4u Proteo (250 × 10 mm id) from Phenomenex (Torrance, CA, USA). Peptides were separated using an elution gradient from 25-54% B in 45 min, where mobile phase A was water with 0.1% (v/v) TFA and mobile phase B was ACN with 0.1% (v/v) TFA. Other conditions were: flow-rate, 1 mL/min; temperature, 25 °C; injection volume, 600 μL; fluorescence detection at λexc = 280 nm and λem = 360 nm. Sample was injected two times and nine fractions (F1-F9) were collected at 5 min intervals in every injection. Every fraction was evaporated using a centrifugal evaporator (Eppendorf, Hamburg, Germany) and the remaining pellet was dissolved in 600 μL of digestion buffer (5 mM borate buffer, pH 8.5). Solutions corresponding to every fraction, injected two times, were pooled and used for future analysis.

2.4. Peptide content

The content of peptides was determined according to the method described by Wang et al. (2008) with some modifications. A 40 mg/mL solution of OPA reagent in MeOH was employed to prepare a fresh 5 mL mixture consisting of 2.5 mL of 100 mM disodium tetraborate, 1.0 mL of 5% (v/v) SDS, 1.39 mL of water, 10 μL of β-mercaptoethanol, and 100 μL of previous OPA solution. Next, 2.5 μL of sample was incubated with 100 μL of that mixture for 8 min at room temperature and the absorbance corresponding to the compounds formed by the reaction of OPA reagent with α-amino groups of peptides was measured at 340 nm using a spectrophotometer Lambda 35 from Perkin-Elmer (Waltham, MA, USA). The peptide content was calculated by interpolation in a calibration curve obtained when using the tripeptide GSH (0–5 mg/mL) as standard.

2.5. Evaluation of hypolipidemic activity of peptides

Hypolipidemic activity of hydrolysates and fractions was evaluated using four different *in vitro* assays to measure the ability of peptides to reduce absorption of dietary cholesterol (ability to reduce micellar