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Identification of plum and peach seed proteins by nLC-MS/MS via combinatorial peptide ligand libraries

Estefanía González-García [a], María Luisa Marina [a], María Concepción García [a], Pier Giorgio Righetti [b], Elisa Fasoli [b,*]

[a] Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares Madrid, Spain
[b] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, 20131 Milan, Italy

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ABSTRACT

Plum (*Prunus domestica* L.) and peach (*Prunus persica* (L.) Batsch) seed proteins are a source of bioactive peptides. These seeds, though, are usual residues produced during canning and beverage preparation that, in most cases, are irreversibly lost. The recovery and identification of these proteins might be of importance in human nutrition. This work employs the combinatorial peptide ligand libraries (CPLLs) technology as a tool to reduce the proteins dynamic concentration range. The most suitable extraction and CPLL capture conditions have been obtained and applied for the comprehensive identification of seed proteins. The analysis of recovered species by nLC-MS/MS has allowed the identification of 141 and 97 unique gene products from plum and peach seeds, respectively. It was possible to identify 16 proteins belonging to the *Prunus* genus. Moreover, a high number of histones and seed storage proteins were identified. Additionally, 21 and 14 bioactive peptides previously identified were found within protein sequences in plum and peach seeds, respectively.

Significance: Plums and peaches seeds are cheap sources of proteins that are irretrievably lost after canning and beverage production. Although this kind of residues has been used in animal feed or production of biofuel, they are usually incinerated or sent to landfills, wasting their huge potential. In order to exploit this, it is important to comprehensively study proteins present in plum and peach seeds. Nevertheless, since proteomics analysis is in most cases handicapped by the presence of high-abundance proteins masking the detection of the low-abundance ones, it is important to overcome this challenge. In this sense, combinatorial peptide ligand libraries (CPLLs) have been used in this work to reduce the dynamic protein concentration range to enable the identification of a higher amount of proteins than employing conventional methods. In this work, the better extracting conditions have been optimized and up to 141 and 97 unique gene products from plum and peach seeds have been found, respectively. Moreover, 21 and 14 peptides previously identified as bioactive peptides were ascertained within protein sequences in plum and peach seeds, respectively. For that reason, this research takes the first step in the recovery of these valuable proteins and in the extraction of bioactive peptides, which could be successfully adopted in human nutrition.

1. Introduction

Plums (*Prunus domestica* L.) and peaches (*Prunus persica* (L.) Batsch) are among the most produced fruits in the world, with 11,530 and 21,640 thousand tons in 2013, respectively [1]. The processing of plums and peaches for the canning and beverage industries generates a great amount of residues, where peel and stones comprise 10–25% of the raw material in the case of plums and 22–38% in the case of peaches [2]. It has been previously reported that those stones contain seeds inside with about 40% of proteins [3,4], able to release bioactive (antioxidant and Angiotensin-converting enzyme (ACE)-inhibitory) peptides after enzymatic digestion [3–6]. Nevertheless, no information about the proteins present in these seeds has been published.

Reliability of proteomic analysis depends largely on the protein sample preparation process [7–9]. This issue becomes even more significant in the case of vegetable samples, which contain high levels of proteases and non-protein compounds such as phenolic, lipids or secondary metabolites, able to interfere with the protein extraction and separation [7–12]. Moreover, protein extraction represents a great challenge considering the presence of low-abundance proteins and the difficulty to solubilize them due to the presence of vacuoles and rigid cell walls [8,10,12,13]. Additionally, identification of proteins is another limiting step in proteomics due to: (1) the presence of high-abundance proteins which mask the detection of the low-abundance species, and (2) the lack of databases available for plant organisms since the vast majority of genomes is still not sequenced [14,15].

* Corresponding author.
E-mail address: elisa.fasoli@polimi.it (E. Fasoli).

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The combinatorial ligand libraries (CPLLs) technology, emerged in 2005 [16], is an excellent tool to reduce the dynamic concentration range of proteins and to allow the detection and identification of low-abundance proteins ("hidden" proteome) [17–20]. Due to its extraordinary advantages, this technology has been widely employed in proteomic studies to identify proteins and peptides with either positive (antioxidant, antimicrobial…) or negative (allergens) effects [21], or to detect possible frauds in food products [22,23]. Some proteomes studied by using this technology have been those in olive [14,24], avocado [25], banana [15], mango [26], and also goat milk [27].

The aim of this work was to comprehensively identify proteins in plum and peach seeds by nLC-MS/MS and to evaluate their potential for obtaining bioactive peptides, after the application of a suitable method for the protein extraction and a CPLLs treatment for reduction of dynamic range in the samples.

2. Materials and methods

2.1. Chemicals and samples

All reagents used were of analytical grade. Hexane, acetone, methanol (MeOH), Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), sodium chloride (NaCl), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, glycine, ammonium sulphate, phosphoric acid, acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid (FA), sodium hydroxide (NaOH), trichloroacetic acid (TCA), ammonium persulphate and ammonium bicarbonate were acquired at Sigma-Aldrich (Saint Louis, MO, USA). Blue Coomassie, Laemmli buffer, 40% acrylamide/Bis solution, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), Precision Plus Protein Standards (recombinant proteins expressed by *E. Coli* with molecular mass values of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) and ProteoMiner™ (PM) (combinatorial hexapeptide ligand library beads) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Water and acetonitrile (ACN) (OPTIMA® LC/MS grade) for LC/MS analyses were acquired at Fisher Scientific (Leicestershire, UK). Home-made-CPLLs (HM-CPLLs) were synthetized in our laboratory. Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics (Basel, Switzerland). Seeds were obtained from plums and peaches from a local supermarket (Madrid, Spain).

2.2. Plum and peach protein extraction

Protein extraction from plum and peach seeds was carried out following the procedure of González-García et al. and Vásquez-Villanueva et al. [3,4] with some modifications.

Plums and peaches were cut, stones were open with a nutcracker and seeds were ground and kept at −20 °C until use. In order to defat seeds, approximately 20 mL of hexane were used per half gram of milled seeds followed by shaking three times for 30 min. Afterwards, 200 mg of defatted seeds were washed twice with 4 mL MeOH/H_2O (80:20) and twice more with 4 mL acetone/H_2O (80:20), both solutions at −20 °C. Two different extracting buffers (10 mL) were employed: a native buffer and a denaturing one. The native buffer consisted of 50 mM Tris-HCl pH 7.4, 15 mM NaCl and protease inhibitor cocktail. The denaturing buffer was constituted, in addition, by 1% SDS and 25 mM DTT. Extraction was made by sonication for 10 min and gentle shaking overnight. Then, protein precipitation with acetone was carried out by employing an extract:acetone ratio of 1:2 and storing in the freezer overnight. Afterwards, the solution was centrifuged (30 min, 13,400 rpm) and the resultant pellet evaporated until dried.

2.3. Protein capture with CPLLs

The precipitated proteins were redissolved by employing 10 mL of a solubilizing buffer consisting of 50 mM Tris-HCl pH 7.4, 15 mM NaCl, 0.5% CHAPS, and 1 mM EDTA. Afterwards, the extract was divided into two parts and pH adjusted to optimal pHs for CPLLs: pH 7.0 for PM-CPLLs and 2.2 for HM-CPLLs. To each half, 100 μL of the corresponding libraries were added and gently shaken overnight. The CPLLs beads were recovered by filtering 3 min at 13,400 rpm through Micro Bio-Spin chromatographic columns (Bio-Rad). Before protein desorption, the beads were washed with water to remove all possible contaminants and unbound proteins. Protein desorption was performed by elution with 100 μL of 4% SDS containing 20 mM DTT, under boiling conditions.

2.4. SDS-PAGE

The proteins in control samples and CPLL eluates were separated by SDS-PAGE. Control samples were prepared by dissolving the pellet in 50 μL of Laemmli buffer containing 5% (v/v) β-mercaptoethanol. The CPLL eluates were prepared by mixing 5 μL of desorbed proteins with 5 μL of Laemmli buffer. Samples were boiled for 5 min and loaded onto a home-made gel composed by a stacking gel (4% polyacrylamide, 125 mM Tris-HCl pH 6.8, 0.1% (m/v) SDS, TEMED, and APS) cast over a running gel (12% polyacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% (m/v) SDS, TEMED, and APS). The gels were run in a Bio-Rad Mini-Protean system employing a Tris-glycine buffer (pH 8.3) containing 0.1% (m/v) SDS and a Tris buffer (pH 8.8) as cathodic and anodic buffers, respectively. Electrophoresis was carried out by applying 50 V until the dye front entered the running gel, followed by 150 V until complete separation. For the estimation of molecular mass values, a marker ladder was used. Gels were stained with Colloidal Coomassie Blue followed by destaining with a 7% acetic acid solution. Scanning of gels was performed by a VersaDoc imaging system (Bio-Rad) and the images were treated with the software Quantity One (Bio-Rad).

2.5. Mass spectrometry and data analysis

The sample bands obtained by SDS-PAGE were cut out and destaining performed by washing with acetonitrile and 50 mM ammonium bicarbonate (AmBic) at 56 °C. Afterwards, the gel pieces were reduced and alkylated with 1.5 mg/mL DTT (in 50 mM AmBic) at 56 °C and 10 mg/mL iodoacetamide (in 50 mM AmBic) at room temperature, respectively. Finally, proteins were digested with 0.02 μg/μL trypsin (in 25 mM AmBic) at 37 °C overnight.

The tryptic digests were acidified with FA up to a final concentration of 10% (v/v) and 8 μL of this mixture were loaded on a nano chromatographic system, UltiMate 3000 RSLCnano System (Dionex, Sunnyvale, CA, USA). Prior to the chromatographic separation, samples were cleaned up and pre-concentrated using a reversed-phase trap column Acclaim® PEPMap100 (C18, 100 Å, 10 μm i.d. × 2 cm) from Dionex. Next, the trap column was located in series with the separation column by switching the 2-position valve. The separation column was a fused silica reversed-phase PicoFrit (C18, 2.7 μm) from New Objective (Woburn, MA, USA). Peptide elution was performed with the following chromatographic conditions: mobile phase A, H_2O/ACN (98/2) with 0.1% FA; mobile phase B, H_2O/ACN (2/98) with 0.1% FA; elution gradient, 4–60% B in 30 min; flow rate, 300 nL/min; and temperature, 25 °C. The liquid chromatographic system was connected to a LTQ-XL mass spectrometer (Thermo Scientific) equipped with a nano spray ion source. Full scan mass spectra were acquired in the mass range from 350 to 1800 *m/z* and the five most intense ions were automatically selected and fragmented in the ion trap. The targeted ions already selected for mass spectrometry (MS/MS) and fragmented were dynamically excluded for 30 s. The mass runs were performed in triplicate for the final search and protein identification. MS/MS data was processed by the Proteome Discoverer software (v. 1.2.0 Thermo) and by using Mascot