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Identification by hydrophilic interaction and reversed-phase liquid chromatography–tandem mass spectrometry of peptides with antioxidant capacity in food residues

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ABSTRACT

HILIC- and RP-HPLC-ESI-Q-TOF identification of bioactive peptides with antioxidant capacity in peach by-products was carried out. Peach seeds contain more than 40% of proteins (as dried and defatted basis) and could constitute a cheap source of bioactive peptides. Extracted proteins were digested using four different commercial enzymes. Five assays based on different antioxidant mechanisms were employed for a reliable evaluation of the antioxidant capacity of the extracts. Thermolysin enzyme originated the extract with the most favorable antioxidant capacity. Probably due to a synergic effect among antioxidant peptides, it was not possible to find a peptide fraction with a higher antioxidant capacity than the whole extract. Eighteen peptides were identified in the whole hydrolysate when combining HILIC- and RP-HPLC-ESI-Q-TOF. A high percentage of hydrophobic amino acids were observed within their sequences which is a characteristic feature of the antioxidant nature of peptides.

1. Introduction

Bioactive peptides have many advantages against synthetic drugs since they have low toxicity and different bioactivities, they exhibit less side effects, and they are more bioavailable [1]. Nevertheless, the complexity and the high cost of the production and purification of natural peptides are the main factors limiting the large scale production and commercialization of these peptides. An approach to reduce these costs is the use of food by-products for that purpose. Peptides with biological activities can be released from protein sequences. The enzymatic hydrolysis of protein by-products is the most efficient technology to recover bioactive peptides [2,3]. Bioactive peptides normally show short amino acid chains (approximately 2–30 amino acids) [4,5]. Moreover, antioxidant peptides possess a high level of hydrophobic amino acid residues within their sequence [6].

Peptides exerting antioxidant capacity can prevent oxidative processes occurring in both organisms and foodstuff. Accumulation of reactive oxygen species (ROS) in the organism plays a key role in the development of some pathologies and aging and they have been related with the development of degenerative diseases [7]. ROS are constituted by a large amount of reactive molecules derived from molecular oxygen and free radicals formed in organisms by oxygen consumption, water reduction, lipid oxidation, glycosylation, and environmental causes such as smoking and exposition to irradiation and air pollutants [7,8]. On the other hand, food oxidation processes, especially lipid oxidation, leads to food degradation and reduction of food quality. Synthetic antioxidants preventing lipid autooxidation (*e.g.*, tert-butylhydroquinone, tert-butyllatedhydroxyanisole, butylatedhydroxytoluene, and propyl galate) are allowed in food industry within certain regulation limits but there is a negative consumer perception against them [9]. Natural antioxidants constitute an alternative to prevent oxidation in foodstuff. Most natural antioxidant compounds that have been extracted from residual sources are polyphenols [10]. Antioxidant peptides from animal, egg or seaweed by-products have also been found [4,11].

Peach (*Prunus persica* (L.) *Batsch*) processing industry leads to a large amount of waste (around 22–28% of raw material) mainly constituted by the peel and the stone [12]. Taking into account that peach seeds have 43 g of protein per 100 g of dried and defatted milled seeds [13], this by-product could be a potential source of bioactive peptides. Our research group has obtained peptides extracts with antioxidant capacity from olive, plum and cherry seeds [14–16] but this is the first time that antioxidant peptides have been recovered from peach seeds.

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Identification of peptides and proteins is mainly performed using reversed-phase (RP)-HPLC coupled to tandem mass spectrometry. Nevertheless, RP sometimes results usefulness for the separation of most polar molecules that elute very early or even in the dead volume. Bioactive peptides are characterized by having short chains and their comprehensive identification would require the use of a chromatographic mode complementary to RP-HPLC. Hydrophilic interaction liquid chromatography (HILIC) is suitable for the separation of small size peptides [17]. Unlike RP mode, gradient elution in HILIC is performed by increasing the hydrophilicity of mobile phase. In fact, elution in HILIC involves the use of a high concentration of a low polarity organic solvent (usually acetonitrile) at the beginning followed by an increasing water content. These mobile phases are highly compatible with electrospray mass spectrometry [18].

The objective of this work was the comprehensive identification of bioactive peptides with antioxidant capacity in peach by-products by tandem mass spectrometry (Q-TOF-MS/MS) coupled to reversed-phase chromatography (RP-HPLC) and hydrophilic chromatography (HILIC).

2. Materials and methods

2.1. Chemical and samples

All reagents were of analytical grade. Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid, sodium phosphate, and sodium dodecyl sulfate (SDS) were purchased in Merck (Darmstadt, Germany). Ammonium bicarbonate (ABC), sodium hydroxide, dithiothreitol (DTT), o-phthaldialdehyde (OPA), methanol (MeOH), sodium tetraborate, 2-mercaptoethanol (2-ME), glutathione (GSH), acetic acid (AA), Thermolysin, bovine serum albumin (BSA), pepsin, pancreatin, 2,2′-azino-bis(3-ethylbenzothiazonile-6-sulphonic) acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethanol (EtOH), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,10-phenantroline, ferrous sulfate, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), linoleic acid, ammonium thiocyanide (NH_4SCN), and ferrous chloride ($FeCl_2$) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Alcalase 2.4 L FG and Flavourzyme 1000 L were kindly donated by Novozymes Spain S.A. (Madrid, Spain). Protease P was kindly donated by Amano Enzyme Inc. (Nagoya, Japan). *Sencha* green tea and peaches were purchased in a local market (Alcalá de Henares, Madrid, Spain).

2.2. Protein extraction

Peaches were manually pitted and seeds were extracted from stones using a nutcracker. Ground seeds were defatted with hexane for 30 min (three times) before protein extraction. The protein extraction procedure was developed in a previous work by Vásquez-Villanueva et al. [13]. Briefly, 30 mg of defatted peach seed was treated with 5 mL of extracting buffer (100 mM Tris–HCl (pH 7.5), 0.5% (w/v) SDS, and 0.5% (w/v) DTT) with a high intensity focused ultrasound (HIFU) probe (Sonic Vibra-Cell, Hartford, CT, USA) during 1 min at 30% of amplitude. After centrifugation (4000 × *g* for 10 min), proteins in the supernatant were precipitated with cold acetone (10 mL) in the fridge for 15 min.

2.3. Protein hydrolysis

Four different enzymes (Alcalase, Thermolysin, Flavourzyme, and Protease P) were employed for the digestion of peach seed proteins. Buffer, substrate concentration, enzyme:protein ratio, temperature, and time employed in every case have been grouped in Table 1. Incubation was carried out in a Thermomixer Compact (Eppendorf, Hamburg, Germany) with shaking at 600 rpm. The digestion was stopped by boiling at 100 °C for 10 min. After centrifugation (4500 × *g* for 5 min), the supernatant was collected and the degree of hydrolysis (DH, %) and antioxidant capacity were estimated. Enzyme autolysis blanks in absence of sample were also measured. The DH was measured following the method described by Vásquez-Villanueva et al. [13].

2.4. Fractionation

Whole peptide extracts were fractionated by ultrafiltration using molecular weight cut-off (MWCO) filters Amicon® Ultra of 5 kDa and 3 kDa from Merck Millipore (Tullagreen, Ireland). Whole extracts sequentially passed through each filter in decreasing size order, for 1.30 h by centrifugation at 10,000 rpm. Fractions with molecular weights above 5 kDa, from 3 to 5 kDa, and below 3 kDa were obtained. Every fraction was dissolved in the corresponding digestion buffer up to the initial volume.

2.5. Evaluation of antioxidant capacity

Antioxidant capacity of peptides seed extracts was estimated by measuring the ability to scavenge free radicals as DPPH•, $ABTS^{\bullet+}$, and hydroxyl and by measuring the capacity to inhibit the lipid peroxidation of linoleic acid (all methods based on hydrogen atom transfer). Furthermore, the ability to reduce Fe (III) (method based on electron transfer) was also assayed. All measurements were carried out in three individual samples measured, at least, by triplicate.

2.5.1. DPPH radical assay

The capacity of extracted peptides to scavenge DPPH radicals was measured following the method described by You et al. [19] with some modifications. A 50 μL volume of a DPPH• solution (0.1 mM DPPH• in 95% (v/v) MeOH) was mixed with 50 μL of sample and kept in the dark. After 30 min, the absorbance corresponding to the DPPH radical was measured at 517 nm. GSH (0–5 mg/mL) was employed as positive control. DPPH• scavenging capacity was calculated using the equation:

$$\textit{DPPH radical scavenging capacity}\,(\%) = \left(1 - \frac{Abs_{sample} - Abs_{control}}{Abs_{blank}}\right) \times 100 \quad (1)$$

where Abs_{sample} is the absorbance of the sample with DPPH• solution, $Abs_{control}$ is the absorbance of the sample with 95% (v/v) MeOH and without DPPH•, and the Abs_{blank} is the digestion buffer with DPPH• solution.

2.5.2. ABTS radical assay

The capacity to scavenge $ABTS^{\bullet+}$ was measured according to Wiriyaphan et al. [20]. Briefly, an $ABTS^{\bullet+}$ stock solution was prepared by mixing 7.4 mM $ABTS^{\bullet+}$ and 2.6 mM potassium persulfate in 10 mM phosphate buffer (PB) (pH 7.4). This solution was kept in the dark for 16 h at room temperature. An $ABTS^{\bullet+}$ working solution was daily prepared, just before measuring, by dilution of the $ABTS^{\bullet+}$ stock solution (with 10 mM PB pH 7.4) and adjustment of the absorbance at 0.70 ± 0.01 UA. Next, 1 μL of sample was mixed with 100 μL of $ABTS^{\bullet+}$ working solution, incubated in the dark for 6 min, and the absorbance corresponding to ABTS radicals was measured at 734 nm. Trolox (synthetic analog of vitamin E) from 0 to 1.5 mM was employed as positive control. Scavenge capacity of