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Evidence of oleuropein degradation by olive leaf protein extract

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ABSTRACT

The enzymatic activity of raw protein olive leaf extract has been investigated *in vivo*, on olive leaf homogenate and, *in vitro* with pure oleuropein and other phenolic substrates. At least two types of enzymes were found to be involved in the degradation of endogenous oleuropein in olive leaves. As for the *in vitro* experiments, the presence of active polyphenoloxidase and β-glucosidase was determined by HPLC and UV–Visible spectroscopy. Interestingly, both the enzymatic activities were found to change during the storage of olive leaves. Specifically, the protein extracts obtained from fresh leaves showed the presence of both the enzymatic activities, because oleuropein depletion occurred simultaneously with the formation of the oleuropein aglycon, 3,4-DHPEA-EA. In comparison leaves subjected to the drying process showed a polyphenoloxidase activity leading exclusively to the formation of oxidation products responsible for the typical brown coloration of the reaction solution.

1. Introduction

It is well known that olive fruits and the derived virgin oils are good sources of polyphenols and other antioxidants that recently received growing interest (Bendini et al., 2007; Brewer, 2011). The *o*-dihydroxyphenolic glycoside oleuropein is one of the main phenolic compounds identified in olive fruits and leaves (Ryan & Robards, 1998; Soler-Rivas, Espin, & Wichers, 2000). Oleuropein has demonstrated antioxidant properties and provides important nutritional benefits. Moreover its derivatives are responsible for the bitter and pungent taste of olive green fruits and virgin olive oil (Servili, Baldioli, Selvaggini, Macchioni, & Montedoro, 1999). In the olive plant physiology, the enzymatic biotransformation of oleuropein is related with the fruit maturation and with tissue specific defense mechanism guaranteed by the oleuropein-derivatives having antimicrobial activity (Briante, Patumi, Limongelli, et al., 2002; Mazzuca, Spadafora, & Innocenti, 2006; Romero-Segura, Sanz, & Perez, 2009).

A considerable number of studies has been carried out to elucidate the biochemical pathways of oleuropein in the olive leaf and fruit (Capozzi, Piperno, & Uccella, 2000). An enzymatic endogenous pool of enzymes, including β-glucosidase, esterases and oxidoreductases, controls the hydrolysis and the oxidation of oleuropein (Konno, Hirayama, Yasui, & Nakamura, 1999). Different products can be derived from oleuropein according to the type of the enzyme. The β-glucosidase (EC 3.2.1.21) releases glucose forming principally the aldehydic form of oleuropein aglycon (3,4-DHPEA-EA) and the dialdehydic form of decarboxymethyl oleuropein aglycon (3,4-DHPEA-EDA), while esterases hydrolyze the ester bonds of oleuropein, producing glucosyl derivates, hydroxytyrosol (3,4-DHPEA) and elenolic acid (Briante, Patumi, Febbraio, & Nucci, 2004; Mazzuca et al., 2006; Wang, Li, & Hu, 2009). Among the oxidoreductase enzymes, the most relevant are certainly peroxidase and polyphenoloxidase (PPO); the latter is known to catalyze the oxidation of *o*-dihydroxyphenols to *o*-quinones that successively condense to form the dark pigments typical of the enzymatic browning (Garcia-Garcia, Segovia-Bravo, Lopez-Lopez, Jaren-Galan, & Garrido-Fernandez, 2009; Segovia-Bravo, Jaren-Galan, Garcia-Garcia, & Garrido-Fernandez, 2009).

In the food industry, depending on the cases, enzymatic degradation of oleuropein may be a desirable reaction or not. For instance, it is unambiguous that during the preparation and malaxation of the olive paste, the activity of endogenous hydrolytic and oxidative enzymes of olive fruit significantly influence the phenolic profile and their content in virgin olive oil (Clodoveo, Hbaieb, Kotti, Mugnozza, & Gargouri, 2014). Indeed, the most abundant phenolic compounds found in the virgin olive oil are secoiridoids resulting from the enzymatic hydrolysis of oleuropein, ligstroside and demethyloleuropein. Endogenous and exogenous PPO, whereas, may cause a significant decrease of the oleuropein

Abbreviations: 3,4-DHPEA-EA, aldehydic form of oleuropein aglycon; DL, dried leaves; FL, fresh leaves; p-NPG, p-nitrophenyl-β-D-glucopyronoside; p-NP, p-nitrophenol; PPO, polyphenoloxidase; RPE-FL, raw protein extract from fresh leaves; RPE-DL, raw protein extract from dried leaves.

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derivatives content in the oil, thus affecting its oxidative stability (De Leonardis, Angelico, Macciola, & Ceglie, 2013; De Leonardis & Macciola, 2011; Garcia-Rodriguez, Romero-Segura, Sanz, Sanchez-Ortiz, & Perez, 2011). On the other hand, degradation of oleuropein is fundamental to provide desirable organoleptic properties for the preparation of table olives (Boskou et al., 2006). Finally, biotransformation of oleuropein catalyzed by specific enzymes was also investigated in the treatment of olive-mill waste (Khoufi, Hamza, & Sayadi, 2011; Paraskeva & Diamadopoulos, 2006) and to set up specific analytical biosensors (Torrecilla, Mena, Yanez-Sedeno, & Garcia, 2007).

The demand of food industry and customers to replace synthetic additives leads to search plants, herbs and processed foods as suitable candidates for supplying functional molecules to food industry (Hochkoeppler, Kofod, & Zannoni, 1995; Mosca, Cuomo, Lopez, & Ceglie, 2013). Olive leaves are a copious by-product deriving from olive tree cultivation and olive oil mills that currently are burned, let rot on the ground and only marginally used as animal feed or for phytotherapy (El & Karakaya, 2009). Several researches have revealed the high potential of olive leaves as source of phytochemicals (Briante, Patumi, Terenziani, et al., 2002; De Leonardis, Aretini, Alfano, Macciola, & Ranalli, 2008; Jemai, Bouaziz, Fki, El Feki, & Sayadi, 2008; Pereira et al., 2007), while the recovery of their enzymes has been poorly investigated, although the presence of key enzymatic activities in the plant leaves has been repeatedly evidenced (Briante et al., 2004; Dignum, Kerler, & Verpoorte, 2001; Li, Jiang, Wan, Zhang, & Li, 2005; Wang et al., 2009).

The main goal of this study is to evidence that olive leaves could be a low cost source of substances for interesting biotechnological application. At this regard, the naturally occurring enzymes involved in the biotransformation of oleuropein have been investigated. Preliminarily, presence and activity of endogenous enzymes were observed directly in olive leaf homogenate. Successively, an acetone powder from a high-pH extraction medium was produced and assayed *in vitro* towards pure oleuropein and other phenolic substrates.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Caffeic acid (PubChem CID: 689043), oleuropein (PubChem CID: 5281544) and p-nitrophenyl-β-D-glucopyronoside (p-NPG) (PubChem CID: 259380), p-nitrophenol (p-NP) (PubChem CID: 980) were purchased from Sigma–Aldrich Co (St. Louis, MO, USA). Hydroxytyrosol (PubChem CID: 82755) was synthesized in the laboratory by acid/heat hydrolysis of pure oleuropein (De Leonardis et al., 2008).

2.2. Olive leaves

Olive leaves (*Olea europaea*) were randomly collected in late May 2014 from one olive tree of *Gentile di Larino* cultivar locally cultivated (Larino, Italy). Part of the leaves was immediately analyzed (indicated in the text as FL: fresh leaves), while the remainder was air-dried at ambient temperature for at least 15 days (indicated in the text as DL: dried leaves). For the *in vivo* experiments, both the FL and DL were homogenized with distillated water in ratio 1:2 (FL)–1:4 (DL) w/v to form an homogenate that was immediately analyzed after the preparation and then subjected to heat treatment in closed jars at 60 °C for 24 h. At the fixed time, three-gram of leaf homogenate were centrifuged at 4000*g* for 20 min and the supernatant was filtered through a 0.45 mm PVDF syringe filter and directly injected in the HPLC.

2.3. Raw olive leaf protein extract (RPE)

The protein extracts for *in vitro* experiments were prepared as follows: according to the method reported by (De Leonardis, Albanese, & Macciola, 2006) about 200 g of FL and DL were homogenized with 400 mL of a medium at pH 9. Homogenate was centrifuged at 4000*g* for 20 min by filtering the supernatant through a large fluted paper to remove traces of suspended material. Solid residues were then homogenized over again with aqueous solution. Whole supernatant was gently mixed with a peer volume of frozen acetone (−20 °C) and refrigerated at 4 °C for 1 h. Subsequently, the sample was centrifuged at 4000*g* for 30 min and the precipitate was recovered. After overnight dialysis in cellulose tubing with molecular mass cutoff of 12,000 Da (Sigma–Aldrich Co, USA), protein suspension was lyophilized and stored at 4 °C. Immediately before use the extract powder were dissolved in the above mentioned medium and centrifuged at 4000*g* for 10 min to remove the insoluble residues. Protein was measured by Kjeldhal method, using 6.25 as protein-coefficient.

2.4. Enzymatic activity assay

Reaction solution was prepared by adding water, substrates and finally, the raw olive leaf protein extract solution (RPE-FL or RPE-DL) in order to have a final volume of 1 mL. Reactions were carried out for variable times at the temperature of 25 and 60 °C. The reaction solution at zero time was immediately analyzed after preparation, and the progress of the reaction was compared with the one without enzymes (no RPE). Enzyme activity was monitored by both spectrophotometric and HPLC methods. β-glucosidase activity was determined by monitoring the absorbance at 405 nm related to the amount of p-NP liberated from p-NPG. All standards were prepared with a concentration of 5 mg/mL: p-NPG and oleuropein in distillated water, while caffeic acid in 30% ethanol:water v/v solution, respectively. The p-NPG hydrolysis was determined by recording the spectra at different times using a Varian spectrophotometer equipped with thermostatted cell holder. The final pH of for the reaction solutions was about 6.

Products of enzyme activity were determined by HPLC analysis by withdrawing and injecting directly reaction medium (20 μL). A Varian ProStar 230 instrument (Mulgrave, AUS) was used, equipped with a column Kinetex 5u C18 100A (160 × 4.6 mm) (Phenomenex, USA) and supplied with UV–VIS detector set up at a wavelength of 280 or 325 nm. Chromatographic separation was performed according to the IOC method ("Determination of biophenols in olive oils by HPLC", 2009) using a mixture of H_3PO_4–bidistilled water 0.2% v/v (eluent A), methanol (eluent B), acetonitrile (eluent C); the gradient for the A/B/C eluent mixture was as follows: 0 min 96/2/2%; 24 min 50/25/25%; 27 min 40/30/30%; 36 min 0/50/50%; 49 min 96/2/2%. Individual phenolic compounds were identified by comparing retention times and absorption spectra with those of pure standards, with exception of 3,4-DHPEA-EA identified by indication of IOC method ("Determination of biophenols in olive oils by HPLC", 2009). Each phenol was quantified through a corresponding calibration curve derived from a plot of area counts versus concentration. Hydroxytyrosol and 3,4-DHPEA-EA were expressed as oleuropein equivalent. Analytical assay were carried out at least in three replicates.

3. Results and discussion

3.1. Enzymatic activity of olive leaf homogenate

Olive leaves are a seasonal by-product of olive-oil industry that are commonly dried to make their transport, storage and processing