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Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil

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ABSTRACT

The main objective of this study was to assess the ability of polyphenol oxidase (PPO) and peroxidase (POX) enzymatic activities present in olive fruit tissues to oxidize main phenolic compounds related to the process to obtain virgin olive oil (VOO) in order to ascertain their involvement in shaping the phenolic profile of this product. In the two olive cultivars under study, Arbequina and Picual, olive PPO activity was found to be largely present in the fruit mesocarp whereas most olive POX activity is in the seed. Moreover, both enzymatic activities display relatively constant values after the onset of fruit ripening when the fruit is harvested for VOO production. Results showed that both PPO and POX activities present in olive fruit at ripening stages are able to oxidize main phenolic glycosides present in the fruit as well as those phenolic compounds arising during the industrial process to obtain the oil, especially secoiridoid compounds derived from oleuropein that mainly determine VOO nutritional and sensorial properties. Experimental data suggest a key role for endogenous olive PPO and POX enzymatic activities in determining the phenolic profile of VOO.

1. Introduction

It is well established that phenolic compounds are related to virgin olive oil (VOO) nutritional benefits and organoleptic properties (Konstantinidou et al. 2010; Mateos, Cert, Pérez-Camino, & García 2004; Servili & Montedoro 2002). VOO quality attributes are determined by the chemical composition and biochemical status of the olive fruit. Thus, the occurrence of hydrophilic phenols in VOO is related to the content of phenolic glycosides initially present in the olive tissues and the activity of various enzymes acting on these glycosides. The main phenolic glycosides identified in olive fruits from different cultivars and maturation stages (Obied et al. 2008; Gómez-Rico, Fregappane, & Salvador 2008) are oleuropein, ligstroside, demethyloleuropein, verbascoside, elenolic acid glucoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin and quercetin-3-rutinoside. The aglycon derivatives resulting from the enzymatic hydrolysis of oleuropein and ligstroside are the dialdehydic forms of decarboxymethyl oleuropein and ligstroside aglycones (3,4-DHPEA-EDA and p-HPEA-EDA, respectively), and the aldehydic forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and p-HPEA-EA, respectively). These phenolics, commonly known as secoiridoid compounds, constitute quantitatively the most significant phenolic fraction of VOO (Lerma-García et al., 2009; Mateos et al. 2001). The hydrolysis of the fruit phenolic glycosides during the olive oil extraction process is mainly carried out by an endogenous β-glucosidase that exhibits the highest substrate affinity towards oleuropein (Romero-Segura, Sanz, & Pérez 2009). On the other hand, endogenous oxidoreductases are also suggested to play an important role during the milling and kneading steps in the olive oil extraction process by promoting phenolic oxidation (Servili et al. 2008). Thus, García et al. (2001) found out that slow kneading of olive pastes in the presence of oxygen may reduce by half the concentration of orthodiphenols in oils. Besides free radicals, the main oxidizing agents of phenols in plant products are oxidoreductase activities such as polyphenol oxidase (PPO) and peroxidase (POX).

PPO is the main enzyme involved in the oxidation of phenols, both in physiological processes associated with fruit ripening and in any form of manipulation of the fruit involving tissue damage or breakage (Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaoujon 2006). Oxidation of phenols by PPO comprises two different reactions, hydroxylation of monophenols to form orthodiphenols, and oxidation of these orthodiphenols to quinones. These complex reactions have been studied in various model systems but they are not yet fully characterized in plants mainly due to the complexity of coupled oxidative reactions (Falguera, Pagan, & Ibarz 2010). Thus, some plant phenolics are not directly oxidized by PPO but are degraded by these coupled secondary reactions (Pourcel et al. 2006).

POX (Class III) oxidize phenolics as preferential substrates at the expense of peroxides, in most cases H_2O_2, producing extremely reactive free radical intermediates which, following release from the enzyme, readily condense to yield polymeric products. Although the contribution of POX to the oxidation of phenols is limited by the availability of H_2O_2, autoxidation of phenolics caused by tissue damage increases H_2O_2 pool and this may be used by POX to enhance

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oxidation of phenolics (Takahama & Oniki 2000). Both enzymes, PPO and POX seem to act synergistically, PPO generating H_2O_2 from the oxidation of certain phenols and giving rise to quinones and then POX using these two compounds as substrates.

Previous studies have focused on the role played by both PPO and POX in olive phenolics metabolism (Montedoro, Baldioli, Selvaggini, Begliomini, & Taticchi 2002). Thus, the inverse relationship between levels of PPO and oleuropein content along the ripening of the olive fruit has recently been shown (Ortega-García, Blanco, Peinado, & Peragón 2008). A central role has also been attributed to this enzyme in the enzymatic browning observed in bruised olive fruits (Segovia-Bravo, Jarén-Galán, García-García, & Garrido-Fernández 2009). On the other hand, high levels of POX activity have been found in the olive seed and the involvement of this enzymatic activity in determining VOO phenolic profile has been proved (Luaces, Romero, & Gutiérrez 2007). A POX enzyme that binds specifically to pectic polysaccharides has been purified from black ripened olives (cv. Douro) (Saraiva, Nunes, & Coimbra 2007) and more recently Tzika, Sotiroudis, Papadimitriou, and Xenakis (2009) have reported the partial purification of a POX enzyme from Koroneiki olive fruit pulp that seems to be active towards fruit phenolics such as gallic acid and protocatechuic acid but inactive towards oleuropein. None of these previous studies have focused on the enzymatic oxidation of secoiridoid compounds that constitute the most important phenolic fraction both in the olive fruit and in the VOO.

The aim of this study was to determine the ability of olive fruit POX and PPO activities to oxidize the main phenolic compounds from olive fruit and VOO and, therefore, be involved in shaping the phenolic profile of VOO.

2. Materials and methods

2.1. Plant material

Olive fruits (*Olea europaea* cv. Picual and Arbequina) were cultivated at the experimental fields of Instituto de la Grasa and collected, along two consecutive years, at the selected maturity stages. Fruit maturity was expressed in terms of weeks after flowering (WAF).

2.2. Chemicals

Reagents for enzymatic activity extraction and measurements were supplied by Sigma-Aldrich (St. Louis, MO) except for phenolic compounds (oleuropein, and verbascoside) purchased from Extrasynthese (Genay, France). Demethyloleuropein, not commercially available, was purified from olive fruit phenolic extracts by SPE using C18 cartridges (Supelco, Bellefonte, PA).

2.3. Enzyme extraction

Acetone powders were prepared from mesocarp and seed tissues of fresh harvested olive fruits. Typically, 10 g of tissue was ground in 150 mL of cold acetone (−20 °C) using a Waring blender. The residue obtained, after filtration, was re-extracted twice with 20 mL of cold acetone (−20 °C). The whitish powder obtained was finally rinsed with diethylether, dried and stored at−20 °C.

POX enzyme extracts were prepared from 0.25 g of acetone powder in 2.5 mL of a buffer consisting of 100 mM sodium phosphate buffer, pH 6.7, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 5 mM α-aminocaproic acid, and 0.1% Triton X-100 using an Ultraturrax homogenizer. The resulting homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and the clear supernatant was used as crude extract.

PPO enzyme extracts were prepared from 0.25 g of acetone powder in 10 mL of a buffer consisting of 100 mM sodium phosphate buffer, pH 6.7, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, 5 mM α-aminocaproic acid, and 1 mM sodium dodecyl sulphate (SDS) using an Ultraturrax homogenizer. The resulting homogenate was centrifuged at 27,000 *g* for 20 min at 4 °C and the clear supernatant was used as crude extract. To eliminate interferences due to other enzymatic activities, $(NH_4)_2SO_4$ was added to the crude extract and the pellet obtained at 35–60% precipitation cut was collected and redisolved in 1 mL of extraction buffer. This protein concentrate obtained was desalted through a Sephadex G-25 column (PD-10, GE Healthcare) and eluted with sodium phosphate buffer, pH 6.7, 1 mM SDS.

2.4. POX activity assay

2.4.1. Spectrophotometric method

POX activity was determined using two different substrates, ABTS and guaiacol. Oxidative activity was determined by measuring the formation of the ABTS radical cation at 414 nm ($\varepsilon = 31{,}300\ M^{-1}\ cm^{-1}$) according to the procedure described by Cantos, Tudela, Gil, and Espín (2002), and by monitoring the increase in absorbance at 420 nm related to the formation of tetraguaiacol ($\varepsilon = 26{,}000\ M^{-1}\ cm^{-1}$) by peroxidation of guaiacol (Luaces et al. 2007). Unless otherwise stated, the standard reaction medium consisted of 2.5 ml of 25 mM sodium acetate buffer pH 6.5, 5.5 mM guaiacol, 2.2 mM H_2O_2, and the appropriate amount of enzyme (5–20 μL). Blank samples with thermally denatured enzyme extracts (60 min at 100 °C) were measured in parallel to quantify and subtract non-enzymatic oxidation. One unit of POX activity was defined as the amount of enzyme oxidizing 1 μmol of substrate per min.

2.4.2. HPLC method

Analysis of POX activity towards endogenous olive phenolic compounds was based on the direct determination of the oxidative degradation of these compounds by HPLC analysis. The reaction medium consisted of 2.5 mL of 25 mM sodium acetate buffer pH 6.5, 6.5 mM olive phenolic glucoside, 2.2 mM H_2O_2 and the appropriate amount of enzyme (5–20 μL). Activity towards VOO phenolics was assayed by incubating a concentrated VOO phenolic extract in the same reaction medium. The reaction was stopped by adding 2.5 mL of methanol. HPLC analysis was performed in a Beckman Coulter liquid chromatographic system equipped with a System Gold 168 detector, a solvent module 126 and a Mediterranea Sea 18 column (4.0 mm i.d.×250 mm, particle size 5 μm) (Teknokroma, Barcelona, Spain) following a previously described methodology (Luaces et al. 2007). More details on the HPLC analysis are given below (Section 2.6). All activity measurements were carried out in triplicate.

2.5. PPO activity assay

2.5.1. Spectrophotometric method

PPO activity was determined by continuously monitoring the increase in absorbance at 400 nm related to the oxidation of tert-butylcatechol (TBC) and the formation of the corresponding quinone. Quantification of the oxidative reaction was carried out considering an extinction coefficient of $1200\ M^{-1}\ cm^{-1}$ (García-Molina, Muñoz, Varón, Rodríguez-López, & García-Cánovas 2007). The reaction medium consisted of 1.5 mL of 25 mM sodium acetate buffer pH 6.5, 1 mM SDS, 7 mM TBC, and the appropriate amount of enzyme (1–5 μL). Blank reactions with thermally denatured enzyme extracts (60 min at 100 °C) or with active extracts and 0.2 mM tropolone, a specific PPO inhibitor (Cantos et al. 2002), were measured in parallel to quantify and subtract TBC non-enzymatic oxidation or non-oxidative degradation, respectively. One unit of PPO activity was defined as the amount of enzyme forming 1 μmol of TBC-quinone per min.

2.5.2. HPLC method

The reaction medium was similar to that used for the spectrophotometric assay but using olive phenolic glucosides (6.5 mM) or a concentrated VOO phenolic extract (final concentration in reaction medium 1.5 mM) as substrates. The enzymatic reaction was stopped by