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Modulating oxidoreductase activity modifies the phenolic content of virgin olive oil

Rosa García-Rodríguez, Carmen Romero-Segura, Carlos Sanz, Ana G. Pérez *

Department of Biochemistry and Molecular Biology of Plant Products, Instituto de la Grasa, CSIC, Padre García Tejero 4, 41012 Seville, Spain

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ABSTRACT

The effect of modifying polyphenol oxidase (PPO) and peroxidase (POX) activity during the extraction of virgin olive oil has been assessed in terms of its influence on the phenolic profile of the oil produced. These enzymes were modified by adding exogenous enzyme or specific inhibitors during the milling and subsequent kneading step, studying the effect on specific phenolic compounds in the oils. PPO is the main enzyme involved in phenolic oxidation at the milling step whereas POX activity seems to be the main influence during the kneading step. The data obtained suggest it is possible to increase the nutritional and organoleptic quality of virgin olive oil by inhibiting these enzymes during olive fruit processing. Treatment with the PPO inhibitor tropolone produced a twofold increase in the phenolic fraction, which would therefore seem to be an interesting strategy to improve the nutritional and organoleptic properties of virgin olive oil.

1. Introduction

Virgin olive oil (VOO) is the primary source of lipids in the Mediterranean diet and has been linked with positive health benefits (Konstantinidou et al., 2010; Visioli, 2012). It is well established that phenolic compounds are directly related to the nutritional benefits and organoleptic properties of VOO. The long term dietary consumption of VOO represents the delivery of phenolic compounds over time, which may attenuate the inflammatory responses associated with eating, and reduce the associated risk of chronic inflammatory diseases (Lucas, Russell, & Keast, 2011). Phenolic compounds are also responsible for the bitter and pungent sensory tones of VOO (Andrewes, Busch, de Joode, Groenewegen, & Alexandre, 2003; Inarejos-García, Androulaki, Salvador, Fregapane, & Tsimidou, 2009; Mateos, Cert, Pérez-Camino, & García, 2004), common and desirable attributes when present at low to moderate intensity.

In most oils, the main phenolic components found are the dialdehyde forms of decarboxymethyl oleuropein and ligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively), as well as the aldehyde forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively). The accumulation of hydrophilic phenols in VOO is related to the initial phenolic glycoside content of the olive fruit, and to the activity of hydrolytic and oxidative enzymes on these glycosides during the oil extraction process (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011; Romero-Segura, Sanz, & Pérez, 2009). However,

* Corresponding author. Tel.: +34 95 611550; fax: +34 95 616790.
E-mail address: agracia@cica.es (A.G. Pérez).

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there is evidence that the phenolic glycoside content should not be regarded as a limiting factor for the phenolic content of VOO, given that the total secoiridoid compounds in VOOs represent an average of 1–4% of the secoiridoid glycosides present in the fruit (Gómez-Rico, Fregapane, & Salvador, 2008). Experimental evidence suggests that endogenous enzymes such as β-glucosidase (which hydrolyses phenolic glycosides) and oxidoreductases like polyphenol oxidase (PPO) and peroxidase (POX) (which oxidase phenolic compounds) may be the main biochemical factors affecting the phenolic content of VOO. Indeed, olive β-glucosidase has been shown to play a critical role in shaping the phenolic profile of VOO (Romero-Segura, García-Rodríguez, Sanz, & Pérez, 2011; Romero-Segura, García-Rodríguez, Sánchez Ortiz, Sanz, & Pérez, 2012). However, most kinetic studies into the formation and degradation of phenolic compounds during the extraction of VOO suggest that olive oxidoreductase enzymes, that catalyse the oxidative degradation of these compounds during fruit processing, are the key factors limiting the phenolic content of the oils (Fregapane & Salvador, 2013; Taticchi et al., 2013). PPO activity is involved in the oxidation of phenols associated with olive fruit ripening and processing (Ortega-García, Blanco, Peinado, & Peragón, 2008; Segovia-Bravo, Jarén-Galán, García-García, & Garrido-Fernández, 2009). On the other hand, the influence of the olive seed on the phenolic composition of VOO has been related to the high levels of POX activity found in this tissue (Luaces, Romero, Gutiérrez, Sanz, & Pérez, 2007). The oxidation of phenolic compounds by POX requires H_2O_2 which may be produced as a consequence of secondary PPO coupled reactions (Takahama & Oniki, 2000). Thus, PPO and POX might act synergistically on the oxidative degradation of olive phenolic compounds during the oil extraction process (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011).

In recent years, a number of studies have focused on improving the phenolic composition of VOO by means of technological innovations that substantially modify the operational parameters of the oil extraction process (Frankel, Bakhouche, Lozano-Sánchez, Segura-Carretero, & Fernández-Gutiérrez, 2013; Inarejos-García, Fregapane, & Salvador, 2011; Servili et al., 2008). Although it is clear that changes in the phenolic profile of the oil induced by any such innovations will be mostly based on the effect of these parameters on the activity of oxidative enzymes, no specific studies have been carried out on the selective modulation of their activities. A few studies have been carried out into the use of enzyme complexes to increase the oil extraction yield, yet very little is known about the specific role of the various constituents present in these formulations, or their implications in the organoleptic and nutritional quality of VOO (Clodoveo, 2012).

We previously showed that partially purified olive PPO and POX can oxidise the main phenolic glycosides present in olive fruit in vitro, as well as their secoiridoid derivatives present in the oil (García-Rodríguez et al., 2011). However, there is little information about the specific roles played by PPO and POX during the disruption of fruit tissue in the milling step and the subsequent kneading of the olive paste. A better understanding of these biochemical processes could help in designing new biotechnological approaches to improve VOO quality. Therefore, the aim of this study was to evaluate how olive PPO and POX activities shape the phenolic profile of VOO, and to assess the possibility of tailoring this phenolic profile by modulating PPO and POX activities during the industrial extraction of VOO.

2. Materials and methods

2.1. Plant material

Olive fruit (*Olea europaea*, cv. Picual and cv. Arbequina) was cultivated at the experimental fields of the Instituto de la Grasa and collected during the 2010–2011 and 2011–2012 olive fruit seasons (October–December) with a maturity index (MI) of 1 (fruit with green skin) and 5 (fruit with black skin and <50% purple flesh), according to García and Yousfi (2005).

2.2. Chemicals

Reagents for enzymatic extraction and activity measurements were supplied by Sigma–Aldrich (St. Louis, MO), except for some phenolic compounds, such as oleuropein, hydroxytyrosol, luteolin and apigenin, which were purchased from Extrasynthese (Genay, France). The diol-bonded phase cartridges used to isolate the VOO phenolic compounds were supplied by Supelco (Bellefonte, PA).

2.3. Olive oil extraction

Olive oil extraction was performed using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO production on a laboratory scale (Martínez, Muñoz, Alba, & Lanzón, 1975). The milling of olive fruit (1 kg) was performed using a stainless steel hammer mill operating at 3000 rpm and equipped with a 5 mm sieve. The resulting olive paste was immediately kneaded in a mixer at 50 rpm for 30 min at 30 °C. The kneaded olive paste was then centrifuged in a basket centrifuge at 3500 rpm for 1 min, after which the oil was decanted and paper-filtered. Samples were stored under nitrogen at −18 °C prior to analysis.

To modulate the PPO and POX activity during the process to obtain the oil, different amounts of enzymes were added to the olive fruit during the milling or kneading steps, such as horseradish peroxidase (HRP, 400 mg enzyme/kg of olive fruit: Sigma–Aldrich, St. Louis, MO), olive seed POX (in the range of 650–850 units/kg of olive fruit) and olive PPO (in the range of 60,000–200,000 units/kg of olive fruit), as well as co-substrates (H_2O_2, 100–500 μl/kg of olive fruit) or specific inhibitors like sodium azide (66–264 mg/kg of olive fruit) and tropolone (20–480 mg/kg of olive fruit). Olive seeds were used as a source of olive POX (35–45 g seeds/kg of olive fruit), added at the milling step, or the corresponding amount of olive seed acetone powder was added during the kneading step. Olive pulp acetone powder was used as a source of olive-PPO. All the substances added were previously dissolved in 20 ml of water and the same volume of pure water was added to the control samples. Duplicate experiments were carried out for each cultivar and MI.

2.4. Extraction and analysis of phenolic compounds from virgin olive oil

VOO phenolic compounds were isolated by Solid Phase Extraction (SPE) in a diol-bonded phase cartridge following a previously described protocol (Mateos et al., 2001). A solution of *p*-hydroxyphenyl acetic acid (46.4 μg/ml) and *o*-coumaric acid (9.6 μg/ml) in methanol was used as the internal standard in this extraction procedure. An aliquot (0.5 ml) of standard solution was added to each oil sample (2.5 g) prior to phenolic extraction. VOO and fruit phenolic extracts were further analysed by HPLC in a Beckman Coulter liquid chromatography 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 method (Luaces et al., 2007). Phenols (except ferulic acid), cinnamic acid and lignans were quantified at 280 nm using *p*-hydroxyphenyl acetic acid as the internal standard, while flavones and ferulic acid were quantified at 335 nm using *o*-coumaric acid as the internal standard. The nature of the compounds was confirmed by