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Effects of polyphenol enzymatic-oxidation on the oxidative stability of virgin olive oil

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ABSTRACT

Activity of laccase from *Trametes versicolor* was assayed directly in virgin olive oil (VOO) samples. Laccase-treated oils led to the formation of insoluble precipitate and to significant qualitative and quantitative changes of their polyphenol composition. At the extreme condition of oil/laccase incubation (60 °C for 1 h) depletion of oleuropein and *o*-diphenols was estimated up to 90% and 77%, respectively. Results of Rancimat test (130 °C and 20 L h^{-1}) and oven-test (60 °C) evidenced controversial effects on the VOO oxidative stability. At high temperature, induction time of laccase-treated oils was found to be similar or even lower than the one recorded in the chemically-dephenolized oils; therefore, enzyme treatment induced a pro-oxidant effect. Conversely, during the storage at 60 °C, all the laccase-treated oils showed an improvement on their oxidative stability compared to the fresh oil counterparts. Experimental data suggest that polyphenol enzymatic-oxidation generated oil by-products which manifest higher radical scavenging or conversely pro-oxidant property, depending on the treatment temperature and storage conditions of the oils.

1. Introduction

Virgin olive oil (VOO) is an important component of the so-called 'Mediterranean diet'. Unlike any other vegetable oils, VOO contains numerous phenolic substances, deriving from olives during oil production and characterized by well-known healthy, organoleptic and antioxidant properties (Bianco & Uccella, 2000; Gallina-Toschi, Cerretani, Bendini, Bonoli-Carbognin, & Lercker, 2005; Gutfinger, 1981). In the major producer countries, such as Italy, Greece and Spain, VOO is widely preferred not only as salad dressing, but also as cooking fat, as ingredient in various sauces and bakery foods and, finally, as cover-oil in the preparation of vegetables and fish preserves. However, among the various factors that can modify both the content and chemical stability of healthy natural olive oil antioxidants (polyphenols), food-processing procedures as well as the direct exposure to several food-reactive components and even to water emulsified in veiled oils are constantly under investigation (Ambrosone, Angelico, Cinelli, Di Lorenzo, & Ceglie, 2002; Mosca, Cuomo, & Ceglie, 2013; Nicoli, Anese, & Parpinel, 1999).

For instance, copper-containing oxidoreductive enzymes, generally classified as polyphenoloxidase (PPO), can oxidize the phenolic substances of edible material in the presence of molecular oxygen. The whole process, which is known as 'enzymatic browning', is responsible not only for the color-change in food but also for the rapid enzymatic depletion of phenols, which in turn is reflected in the loss of nutritional and quality value. Indeed, PPO catalyzes the oxidation of phenols by converting them into reactive oxygen species (ROS) and quinones. Both these undergo spontaneous no-enzymatic polymerization in water, giving rise to the formation of surface discoloration due to dark brown and stable polymers named melanin (Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaoujon, 2006). Depending on their source, PPO manifests different specificities of action (Mayer, 1986). Some of them are specific for the oxidation of *o*-diphenols to *o*-quinones (catecholase activity, EC 1.10.3.1), while others, such as laccase (benzenediol or oxygen oxidoreductase, EC 1.10.3.2) and tyrosinase (polyphenol oxidase, EC 1.14.18.1) are also able to catalyze the hydroxylation of mono-phenols to *o*-diphenols (cresolase activity) and then, to oxidize the latter to *o*-quinones.

Therefore, to control the PPO activity, depending on the type of food processing procedure and in the preparation of preserves or sauces, vegetables are preventively blanched and/or acidified, salted, fermented, or subjected to thermal processing.

Enzymatic browning is documented abundantly for olive leaves and fruits (Ben-Shalom, Harel, & Mayer, 1978; Goupy, Fleuriet, Amiot, & Macheix, 1991; Ortega-García, Blanco, Peinado, & Peragón, 2008). In olive fruit, oleuropein and other *o*-diphenols are the most important substrates of endogenous PPO and their oxidation is involved in the

Abbreviations: EOP, enzyme-oxidation products; IT, induction time; LAC, laccase; OSC, oxidation stability coefficient; OR, oxidative resistance; PBS, phosphate buffer solution; PPO, polyphenoloxidase; ROS, reactive oxygen species; VOOn, virgin olive oil (n = number of oil sample); VOOn-PBS, oil treated with phosphate buffered at pH 9; VOOn-LAC(20), oil treated with laccase at 60 °C for 20 min; VOOn-LAC(60), oil treated with laccase at 60 °C for 60 min; VOOn-DEPH, oil chemically dephenolized.

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fruit color development during harvesting or during subsequent processing treatments (Charoenprasert & Mitchell, 2012; García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011; Segovia-Bravo, Jarén-Galán, García-García, & Garrido-Fernández, 2009).

Activity of different PPOs was also investigated in the detoxification of olive-mill wastewater effluents (Minussi et al., 2007; Sampedro et al., 2009), and to set up specific analytical biosensors (Torrecilla, Mena, Yáñez-Sedeño, & García, 2007).

The present investigation aims to extend the knowledge on the enzyme-catalyzed oxidation of VOO phenolic compounds, which is still not yet well documented comparing to a variety of studies on VOO chemical oxidation. Indeed, to our knowledge, there are few reports about the enzymatic-oxidation occurring on the polyphenolic fraction usually present in VOO. Georgalaki, Sotiroudis, and Xenakis (1998) evidenced in VOO the presence of endogenous lipoxygenase and PPO (deriving from the olives), able to modify the phenol compounds during the oil storage. Depletion of intrinsic VOO phenols, catalyzed by exogenous enzymes from vegetables in contact with the oil, was also reported (De Leonardis & Macciola, 2011; De Leonardis, Macciola, & De Felice, 2001; Nicoli, Anese, & Manzocco, 1999; Silva, Garcia, & Paiva-Martins, 2010). PPO activity toward the typical VOO polyphenols was assayed either on water-in-olive oil microemulsions stabilized by lecithin as emulsifier or in other model systems (Papadimitriou, Sotiroudis, & Xenakis, 2005; Tzika, Papadimitriou, Sotiroudis, & Xenakis, 2008).

With this aim, in the present study we assayed two types of virgin olive oils, and the oxidative activity of a commercial mushroom laccase (*p*-diphenol oxidase, EC 1.10.3.2) from *Trametes versicolor*, which can mimic the action of the polyphenoloxidase present in several vegetables preserved in VOO. Basidiomycete *T. versicolor* is a very common type of *white-rot fungi*, which is known to be the most efficient lignin degraders (Boyley, Kropp, & Reid, 1992). This fungus secretes significant amounts of laccase that has been widely investigated in various contexts, mostly emphasizing its important biotechnological potentialities (Minussi, Pastore, & Duràn, 2002). In this study, laccase changed both qualitatively and quantitatively the oil polyphenol composition by exerting its activity directly within the oil fat matrix. Finally, depending on the different methods used to investigate the reactions in accelerated conditions, i.e., Rancimat or oven test, a peculiar and at same time controversial influence of laccase activity on the VOO oxidative stability was also evidenced.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade. Pure phenolic compounds caffeic, chlorogenic, ferulic, gallic, *o*-coumaric and *p*-coumaric acids, tyrosol and oleuropein, and finally, the enzyme laccase (*p*-diphenol oxidase, EC 1.10.3.2) from *T. versicolor* were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). A powdered olive pulp extract (OleaSelect®), kindly offered by Indena S.p.A. (Milan, Italy), composed by standardized amounts of hydroxytyrosol and verbascoside, was used to control the enzymatic activity toward both the polyphenols, respectively. All phenolic standards and the powdered olive pulp extract were dissolved in 50% ethanol:water (v/v) solution at 2 mM and 20 mg mL^{-1} concentration, respectively.

Two types of extra virgin olive oils (hereafter classified as VOO1 and VOO2) were taken directly from local mills, both of them being of recent production; untreated VOO samples were hereafter called 'Fresh' (FR). Phosphate buffered saline (PBS) was prepared by mixing monobasic sodium phosphate NaH_2PO_4 (0.01 g/L) and dibasic sodium phosphate Na_2HPO_4 (2.66 g/L), adjusting the final pH 9 with HCl 2 N or NaOH 2 N.

2.2. Oil analysis

Peroxide value (PV) and K232 were determined using the analytical methods described in the European Union standard methods (European Union Commission, 1991). PV was expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg); K232 was the extinction coefficient of diene conjugates calculated on the absorption at 232 nm wavelength by using a 1% solution of oil in isooctane and a path length of 1 cm. Extractions of phenolic compounds were carried out through a liquid–liquid extraction. Each oil sample was mixed with 80% methanol/water (v/v) in the ratio 3:2 (w/v) and centrifuged at 4000 *g* for 15 min. The extraction process was repeated four times. The collected hydroalcoholic supernatants were dried in a rotary evaporator and the residue dispersed in a fixed volume of same methanolic mixture. The resulting oily mixture was dried again in rotary evaporator, thus obtaining the chemically-dephenolized oil (hereafter indicated with suffix "DEPH"). Total phenols (Folin–Ciocalteu's method) and *o*-diphenols (molybdate method) were determined spectrophotometrically by using an independent 4-methyl-cathecol calibration curve as reference (Gutfinger, 1981). HPLC analyses were performed with a Varian ProStar 230 instrument (Mulgrave, AUS), equipped with a column Kinetex 2.6u PFP (100 × 4.6 mm) (Phenomenex, USA) and supplied with UV–VIS detector set up at a wavelength of 280 nm. Chromatographic separation was performed using a mixture of acetic acid–bidistilled water 1% v/v (eluent A) and methanol (eluent B); the gradient for eluent B was as follows: 0 min 5%; 5 min 25%; 10 min 50%; 15 min 100%.

2.3. Measurement of laccase activity

Laccase solution was prepared immediately before use by dispersing 5 mg mL^{-1} of enzyme in PBS. For enzymatic assays in aqueous solution, 0.7 mL of distilled water and 0.1 mL of each phenolic solution were mixed with either 0.2 mL of enzyme solution or 0.2 mL of PBS in the Test and Control samples, respectively. The test tubes with reaction solutions were rapidly closed, shaken and incubated in a thermostat at 60 °C for 15 min; then, 2 mL of pure methanol was added to stop the reaction, giving a final volume of 3 mL. After filtration through a 0.45 mm PVDF syringe filter, the reaction solutions were injected into HPLC column. Laccase selectivity was calculated by measuring the concentration change of standard phenols in the Test solution versus Control. For the enzymatic assay in oil, an equal volume of laccase solution (oil samples hereafter indicated with suffix "LAC") or PBS (oil samples hereafter indicated with suffix "PBS") was added in separate aliquots of each VOO sample, in order to have a total water content of 5% (v/w) and a final concentration of laccase of 0.25 mg g^{-1}. The beakers containing oil samples were covered with laboratory-plastic film and put in a sonicator thermostatic water-bath heated at 60 °C; after an ultrasonic mixing for 5 min, oil samples were left to stand for another 15 or 55 min, stirring them with a glass rod from time to time. At the end of the incubation time, oil samples were filtered onto hydrophilic absorbent cotton and stored at +4 °C until analysis was performed.

2.4. Accelerated oil oxidation

For the Rancimat test, a Rancimat Mod.730 from Methrom Instrument (AG, Herisau, Switzerland) was used at 130 °C and 20 L h^{-1} air flow; induction time (IT) was measured in hours (h). For the Schaal oven-test, 10 g per oil sample was weighed in 20 mL closed test-tubes (14 mm diameter × 160 mm height) and stored for 28 days in a static oven at the constant temperature of 60 °C. Then, on a weekly basis each battery of samples (FR; DEPH; LAC) was used to determine PV and K232. For each oil sample the slope of the best linear fit of PV or K232 versus oven-test period (in days) was calculated. Specifically, the reciprocal of the slope was associated to the oxidative resistance (OR), which