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Thylakoid peroxidase activity responsible for oxidized chlorophyll accumulation during ripening of olive fruits (*Olea europaea* L.)

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ABSTRACT

Type III peroxidase (EC 1.11.1.7) (POX) is the enzyme direct responsible of the 13^2 OH chlorophyll formation on oxidative catabolism of chlorophylls (chls). Despite the higher content of oxidized derivatives of chlorophylls (ox-chls) in fruits of the Arbequina variety compared to Hojiblanca, the evolution of total chlorophyll oxidative peroxidase activity (POX-chl) showed that this activity levels were higher in fruits of Hojiblanca compared with Arbequina variety. Subsequently, a deepened study on the subcellular distribution of POX-chl activity from mesocarp and epicarp cells of olive fruit of both varieties was made, finding that the POX-chl activity located in thylakoid fraction (the only fraction in direct contact with chls *in vivo*) was in Arbequina fruits higher than in Hojiblanca ones and involved more than 50% of the membranous POX-Chl activity. It has been demonstrated also that the evolution of the POX-chl activity in thylakoid membranes enriched fraction throughout the whole life cycle was parallel with the formation and accumulation of ox-chls in olive fruits. Data allowed to conclude that the formation of ox-chls during the chl catabolism is mediated by a POX-chl activity localized in thylakoid fraction and allow to hypothesize that the high percentage of POX activity found in the soluble cell fraction, estimated at 99.8%, may be involved in the loss of pigmentation by oxidation occurring during fruit processing for obtaining olive oil.

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1. Introduction

Chlorophyll pigments are highly appreciated as functional components in both fruits and vegetables for its green coloring properties as its health benefits for the human consumption derived from their biological properties (Ferruzzi & Blakeslee, 2007). In addition, the ripening process of fruits or technological treatment for food production is associated with chemical and/or enzymatic specific transformations of these pigments making them quality indicators of end products and demonstrating a potential applicability as a tool for traceability the processing (Gandul-Rojas, Roca López-Cepero, & Mínguez-Mosquera, 2000).

The fruit of the olive tree (*Olea europaea* L.) is mainly used to obtain olive oil, a natural food that is obtained from the olive fruit solely by mechanical or physical processes. The fundamental steps of the process are crushing, malaxation and phase separation by solid/liquid centrifugation systems, being malaxation the step in oil extraction that especially modifies their qualitative and quantitative composition. After the crushing step, with the broken plant tissue, and released chloroplasts and thylakoid membranes, the lipophylic chlorophyll pigments will transfer to the oil phase during malaxation, giving different colors to olive oil. In this process the chlorophylls undergo a series of structural changes mainly due to breakage of cellular structures that allow the release of acid as well as greater accessibility of their natural substrates for endogenous enzymes. Different reactions can occur as pheophytinization, phytol hydrolysis via chlorophyllase and chemical and enzymatic oxidative reactions that lead to the formation of colored oxidized intermediates and noncolored final products. Chlorophyllic pigment content of olive fruits is cultivar dependent and negatively correlated to fruit ripening (Mínguez-Mosquera & Garrido-Fernández, 1989; Roca & Mínguez-Mosquera, 2003) and the occurrence of lipophylic chlorophyll compounds in virgin olive oil can be related to the activities of several endogenous enzymes present in the olive fruit such as POX promoting chlorophyll oxidation (Gandul-Rojas, Roca, & Mínguez-Mosquera, 2004). So that the study of catabolic processes involved in degradation of these pigments is particularly relevant, this field of food technology aims to know biochemical fundamentals that define the profile of these intrinsic minor components of virgin olive oil, potentially applicable as a tool for olive oil traceability. There currently exists a general consensus regarding the succession of enzymatic reactions implicated in the catabolism of the chlorophyll *a* (Chl) molecule which takes place during leaf senescence. First step is the removal of Mg by Mg-dechelating substance (MCS) (Shioi, Tomita, Tsuchiya, & Takamiya, 1996), producing pheophytin *a* followed by the removal of phytol and the formation of pheophorbide *a* in a reaction catalyzed by pheophytin pheophorbide hydrolase (PPH) (Schelbert et al., 2009). Then pheophorbide is degraded to fluorescent chl catabolites (FCCs),

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which are primarily colorless catabolites, via the enzymatic system pheophorbide *a* oxygenase (PaO) and red Chl catabolite reductase (RCCR). Finally, outside the chloroplast, the FCCs are exported to the cytosol where they are modified with different functional groups and inside the vacuole, isomerized into no colored chl catabolites (NCCs), which are considered as the final catabolites of chls (Hörtensteiner & Kräutler, 2011).

There is much less research regarding the degradation systems which are involved in the chlorophyllic catabolism of the fruit. It could not be demonstrated the functionality of PPH, checking (Shemer et al., 2008) that the pathway starts with sequential action of chlorophyllase (CHLASE) that catalyzes the phytol hydrolysis in chl *a* resulting to chlorophyllide *a* (Amir-Shapira, Goldschmidt, & Altman, 1987) and MCS that becomes pheophorbide *a* (Suzuki & Shioi, 2002). Besides these main steps of the PaO pathway, additional/alternative reactions of Chl breakdown have been described. These were (mostly) inferred from the identification of different types of Chl degradation products, such as pyropheophorbide *a* (Suzuki, Amano, & Shioi, 2006), Chl-derived monopyrroles (Suzuki & Shioi, 1999), urobilinogenoidic catabolites (Losey & Engel, 2001), and various oxidized chl derivatives with an intact porphyrin ring, that they could not be located within PaO route.

There is evidence (Maunders, Brown, & Woolhouse, 1983; Roca, Gandul-Rojas, & Mínguez-Mosquera, 2007; Takahashi et al., 2001; Yamauchi, Akiyama, Kako, & Hashinaga, 1997) which suggests the hypothesis of the coexistence of a specifically oxidative metabolism in parallel to the general catabolic route (Janave, 1997), peroxidase (POX) being the enzyme which has been most clearly linked with this metabolism (Gandul-Rojas et al., 2004; Huff, 1982; Matile, 1980; Roca et al., 2007).

Aljuburi, Huff, and Hshieh (1979) were the first to describe an enzyme which exhibits an oxidizing and decolorizing action on the chlorophyll in the flavedo of oranges in the presence of H_2O_2 and a phenol. However, Matile (1980) was the first to suggest that POX is responsible for that activity by observing an *in vitro* modification to chl in the presence of H_2O_2, 2,4-dichlorophenol (2,4-DCP) and horseradish peroxidase (HRP). These results were confirmed by Huff (1982), ascertaining that the chl *a* molecule was degraded by POX extracted from oranges (*Citrus sinensis* L.), in presence of various phenols, even determining the activity with various chl substrates and estimating the corresponding kinetic parameters (K_m and V_{max}).

Until recently it was assumed that peroxidase mediates *in vitro* Chl degradation only in the presence of phenolic compounds, being the highest enzyme activities in plant systems found with *p*-coumaric acid, apigenin and naringenin, which have a hydroxyl group at the *p*-position (Kato & Shimizu, 1985; Yamauchi & Eguchi, 2002; Yamauchi & Minamide, 1985). Hynninen, Kaartinen, and Kolehmainen (2010) have defended the opposing hypothesis demonstrating that, *in vitro*, HRP can oxidize chl *a* in the absence of phenols, and for this reason propose a direct reaction between the chlorophyll molecule and POD, in which chl *a* would act as a reducer substrate, rendering the presence of a second electron donor substrate totally unnecessary. Recently, Vergara-Domínguez, Roca, and Gandul-Rojas (2013) demonstrated that POX is capable of directly oxidizing chl *a* in the absence of another electron donor; however the presence of a cosubstrate donor like 2,4-DCP accelerates the oxidation rate of the chlorophyllic substrate catalyzed by POX.

Due to the multitude of isoforms of POX described in the bibliography (Akiyama & Yamauchi, 2001; Funamoto, Yamauchi, & Shigyo, 2003; Gandul-Rojas et al., 2004; Saraiva, Nunes, & Coimbra, 2007), the study of the intracellular distribution of the POX-chl activity was approached from different perspectives. Some papers demonstrate the existence of this activity in thylakoid membranes (Gandul-Rojas et al., 2004; Johnson-Flanagan & Spencer, 1996; Martinoia, Dalling, & Matile, 1982); however, data exist to demonstrate the presence of this activity in other subcellular fractions (Akiyama & Yamauchi, 2001; Funamoto et al., 2003).

Akiyama and Yamauchi (2001), studying the distribution of different enzymes involved in the degradation of chlorophylls and applying differential centrifugal techniques demonstrated that, of all the POX-chl activity present in the cotyledon cells of the radish (*Raphanus sativus* L.), only a percentage of less than 1% were located in the chloroplasts, whereas the rest was found in other cellular organelles. This fact was understood as evidence that the majority of the POX-chl present in the cell is found on the exterior of the chloroplast and that only a minority fraction is found in the interior of this organelle, which would be the part potentially involved in the oxidative degradation of chlorophyll that takes part *in vivo*. Similar results were obtained by Funamoto et al. (2003) on measuring POX-chl activity in different subcellular fractions of broccoli florets (*Brassica oleracea* L.). It was established that the microsomal and cytosolic fraction made up approximately 95% of the entire POX-chl activity present in the cell, whereas the activity measured in the chloroplast only represented 1%.

To date, there is no information regarding the distribution of POX-chl activity in fruit. Nevertheless, the bibliography contains studies of the distribution of the POX activity relating to the oxidation of phenols (POX-phe), although the data obtained are contradictory. On the one hand, Civello, Martínez, Chaves, and Añón (1995), studying the distribution of the POX activity against guaiacol in *Fragaria ananassa* (Duch.), found that the specific POX-phe activity of the fraction linked to membranes was far higher than that measured in the soluble fraction and was equivalent to approximately 95% of the total POX-phe activity. On the other hand, Estrada, Bernal, Díaz, Pomar, and Merino (2000), studying the distribution of this activity in *Capsicum annuum* (L.) from an organelle homogenate, found that the POX activity measured over 4-methoxy-R-naphthol (4MN) in the soluble fraction was always higher than that obtained in the rest of the subcellular fractions (ionic bonding to membranes, ionic bonding to cellular wall and covalent bonding to cellular wall) and that it increased from 77% to 96% with ripening.

An interesting model for the study of the physiological involvement of POX in chl metabolism is provided by certain varieties of olive given that differences in the oxidized chl profile for this fruit based on the variety have already been detected (Roca et al., 2007). In general, in olive fruit varieties the qualitative composition of pigments is basically the same and is not subject to modification with the advancement of maturity in the fruit (Roca & Mínguez-Mosquera, 2001). In contrast, in the variety Arbequina an accumulation of 13^2 OH chl *a* has been detected during the transition from the periods of growth through to maturity, reaching up to 15% of the chl fraction (Roca & Mínguez-Mosquera, 2003), while in other varieties such as Hojiblanca and Picual the presence of this compound represents 1% of the total chl fraction during the complete fruit life cycle.

The study of the involvement of POX-chl activity in the metabolism of chls in the Hojiblanca variety has revealed that there is a correlation between the formation and/or build-up of certain oxidized chl derivatives and the total POX-chl activity levels measured from protein precipitated (Vergara-Domínguez, Gandul-Rojas, & Roca, 2011). However, in varieties with a high level of chlorophyllase (CHLASE) activity, such as Arbequina, it was not possible to establish this correlation, and an *in vitro* competence between POX and CHLASE was revealed towards the chl *a* substrate. Subsequently, Vergara-Domínguez et al. (2013), in an optimization of the *in vitro* measurement of the POX-chl activity, established new measurement conditions that permitted the displacement of the competence between CHLASE and POX towards the chl *a* substrate, in favor of the latter.

These new measurement conditions permitted the study of the involvement of POX-chl activity in the oxidation metabolism for chls in fruits with high CHLASE activity to be studied, in order to establish distinctions between varieties with different oxidative chl profiles (Arbequina vs. Hojiblanca). Consequently, in this paper a study has been made of the subcellular distribution of the POX-chl activity in olives from both varieties and this compared with the total measured for this activity, based on a protein precipitated. Subsequently, the

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