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Inhibitory effect of microwaved thinned nectarine extracts on polyphenol oxidase activity

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ABSTRACT

By-products from agricultural practices or from the fruit processing industry are a source of bioactive compounds that could be used in the food industry. Such by-products include thinned fruits, which are expected to contain high quantities of interesting compounds. One possible application of this fruits is the prevention of the enzymatic browning suffered by fruits and vegetables after minimal processing. The aim of this study is to determine the *in vitro* and *in vivo* activity of microwaved extracts obtained from thinned nectarines. It has been observed that *in vitro* the extracts obtained after the application of high microwave power levels (500, 1000 and 1500 W) are mixed type inhibitors of polyphenoloxidase enzyme, showing an irreversible inactivation. This inhibition could be attributed to the Maillard reaction products formed during the microwave treatment. *In vivo*, a solution of 2% of the extract obtained at 1500 W inhibited the enzymatic browning in minimally processed peaches for 8 days of storage.

1. Introduction

Fruit browning is a serious problem in the fruit and vegetable processing industry since it causes an undesirable appearance that reduces the product quality. Moreover, this problem is the main factor that limits fruit and vegetable shelf-life. It has been estimated that over 50% of fruit industry losses occur for this reason (Arias, Gónzalez, Peiró, Oria, & López-Buesa, 2007; Luna, Tudela, Mártinez-Sánchez, Allende, & Gil, 2013; Matmaroh, Benjakul, & Tanaka, 2006). The main enzyme responsible for browning enzymatic reactions is polyphenoloxidase (PPO) (Gomes, Vieira, Fundo, & Almeida, 2014). PPO is a copper-containing enzyme which catalyses two distinct reactions of melanin synthesis involving molecular oxygen, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone which evolves spontaneously to form brown melanoidins (Queiroz, Mendes, Fialho, & Valente-Mesquita, 2008).

Prevention of enzymatic browning can avoid many of the losses in the vegetable processing industry. Various physical and chemical methods have been used to inhibit PPO activity. However, due to the increasing demand by consumers for substituting synthetic compounds with natural substances as food ingredients (Amado, Franco, Sánchez, Zapata, & Vázquez, 2014), many studies have been carried out to find new natural inhibitors of enzymatic browning such as the peptide components of honey (de la Rosa et al., 2011), sulphur containing amino acids and their derivatives (Ding, Chackin, Ueda, & Wang, 2002), thiol containing compounds (Roux, Billaud, Maraschin, Brun-Mérimee, & Nicolas, 2003) and caramelisation products (Lee & Lee, 1997). Many of these natural compounds have been obtained from by-products of the fruit and vegetable processing industries (Amado et al., 2014; Lee et al., 2002; Roldán, Sánchez-Moreno, de Ancos, & Cano, 2008). However, some by-products generate during agricultural practices could also be a source of bioactive compounds. Such by-products include thinned fruits. These are small fruits that are thinned from the tree in order to increase the size of the remaining fruit, reduce the hazard of limb breakage and avoid an alternate bearing cycle. This agricultural practice has economical and time costs of 3.4–4.1 €/tree and 25–30 min/tree (Martín, Torregrosa, & García-Brunton, 2010). Moreover, if thinned fruits have a large amount of interesting compounds (Zheng, Kim, & Chung, 2012), they might be considered a rich source of extracts for use in the food industry that can be used to inhibit lipid oxidation, fungal growth or enzymatic browning. Consequently, their re-use could generate a profit that is currently lacking.

For the case of enzymatic browning, Lee et al. (2002) have demonstrated that the addition of a heated onion extract to potato exhibited a marked inhibitory effect on potato polyphenol oxidase and the formation of a brown colour, attributing this to the presence of Maillard reaction products (MRPs). Several authors have also found that MRPs might inhibit enzymatic browning initiated

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by PPO (Billaud, Brun-Mérimée, Louarme, & Nicolas, 2004; Tan & Harris, 1995). The formation of these complex compounds is due to the Maillard reaction, a kind of non-enzymatic reaction initiated during the heating of reducing sugars and amino acids or proteins that is influenced by multiple factors such as reaction time, pH and solvents (Martins, Jongen, & Van Boekel, 2001). In the first steps of the Maillard reaction, the reducing sugar condenses with a free amino group of amino acids or proteins, forming a Schiff's base and then an Amadori or Heyns product, depending of the sugar involved in the Maillard reaction. Subsequently, several reactions take place in the advanced stages in order to degrade the Amadori product, including cyclisations, dehydrations, retroaldolisations, rearrangements, isomerisations and further condensations, which lead to the formation of brown nitrogenous polymers known as melanoidins (Martins et al., 2001). These MRPs have the ability to inhibit certain oxidoreductases such as PPO with different mechanisms including antioxidant activity (Brun-Mérimée, Billaud, Louarme, & Nicolas, 2004), free radical-scavenging action (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007), metal ion-chelation (Matmaroh et al., 2006) and hydrogen/electron donation (Tan & Harris, 1995). Microwave radiation where the sample is directly heated using different power levels and times has been applied by various authors (Ahmad & Langrish, 2012; Zhang, Yang, & Wang, 2011). Moreover, the use of microwave radiation for extraction has increased significantly as a result of its inherent advantages (reduction in extraction time, solvent volume and energy) over conventional techniques (Jang, Sanada, Ushio, Tanaka, & Ohshima, 2002).

The aim of this study is to determine the potential of the MRPs originated during microwave treatment of thinned nectarines to inhibit the browning enzymatic reaction catalysed by PPO, and to establish their mechanism of inhibition. For this purpose, various assays of enzymatic inhibition were carried out both *in vitro* with mushroom PPO and *in vivo* with a shelf-life study of fresh-cut peach.

2. Materials and methods

2.1. Materials

Thinned nectarine fruits (*Prunus persica* (L.) Batsch var. nectarine Laura) were hand-harvested 46 days after blooming on an orchard in La Almunia de Doña Godina (Zaragoza, Spain). The fruits were immediately transported to our laboratory, selected and stored at 1 °C until processing.

2.2. Obtaining of Maillard reaction products (MRPs)

Thinned nectarines were cut into slices and air-dried in a drying oven (Selecta, Madrid, Spain) at a temperature of 50 °C during 24 h. A fine powder (20 mesh) was obtained using a refrigerated hammer stirrer (IKA, Staufer, Germany). The samples of dried nectarines (0.5 g) were mixed with 15 mL of distilled water and microwaved during 90 s at 3 different power levels (500, 1000 and 1500 W) using a laboratory scale Ethos One microwave (Milestone; Sorisole, Italy). The homogenates were centrifuged at 4000 rpm for 10 min and filtered through Whatman No. 4 filter paper. The supernatants were used directly for analyses or stored at −25 °C until use.

2.3. Extract colour changes

The possible formation of MRPs was determined by a quantitative analysis of the colour extract obtained at different microwave power levels, measuring the absorbance at 420 nm.

2.4. Acetic and formic acids quantification

The presence of acetic and formic acids in the extracts was determined by HPLC using the method described by Ahmad and Langrish (2012). A Hewlett–Packard Series 1100 chromatograph (Agilent Technologies; Santa Clara, USA) coupled with a photodiode array detector (DAD) (Series 1100) and an autosampler (Series 1100) operated by HP ChemStation 3365 software was used for sample analysis. The column was a Zorbax SB-C18 (3.5 μm, 150 × 4.6 mm I.D., Agilent Technologies). Standard solutions (0–1000 ppm) of acetic and formic acid (Panreac, Barcelona, Spain) were dissolved in water acidified with HCl 0.1 N (pH 3.5). The mobile phase was 90% acid water (pH 3.5) and 10% acetonitrile solution. The acid water was used to avoid dissociation of the acids. The flow rate of the pump was adjusted to 0.6 mL/min, the injection volume was 20 μL and the wavelength selected was 215 nm. Prior to the analysis, the samples were filtered with a 0.45 μm nylon filter.

2.5. In vitro assays

2.5.1. Determination of PPO activity

The enzymatic activity of mushroom PPO (Sigma, St Louis, USA) was determined by measuring the increase in absorbance over time at a wavelength of 475 nm with a spectrophotometer (Unicam, Waltham, USA). The enzyme was dissolved in 0.05 M phosphate buffer (pH 7.0) at a concentration of 10 μg/mL. DL-DOPA (Sigma, St Louis, USA) was also dissolved in the same buffer at different concentrations (0–1.5 mM) and used as substrate. The reaction mixture consisted of 5 μL of enzymatic solution, different amounts (25–150 μL) of MRPs and a certain volume of substrate in order to obtain a total volume of 1.0 mL. The assay was conducted at 30 °C using a Peltier system. PPO activity was calculated as the slope in the linear part of the activity curve obtained. One unit of enzyme activity (ua) was defined as the increase in absorbance of 0.1 units/min at 475 nm.

2.5.2. Effect of MRPs volume on the PPO activity

This assay was carried out mixing to a final volume of 1 mL, the appropriate amount of 1.5 mM DL-DOPA substrate, different volumes of MRPs (0, 25, 50, 75, 100 and 150 μL) obtained at different microwave power levels (0, 500, 1000 and 1500 W) and 5 μL of PPO solution.

2.5.3. Determination of kinetics parameters

The Km and Vmax kinetics parameters were determined by using Lineweaver–Burk plots with GraphPad Prisma 3.03 software (La Jolla, USA). For this, 25 μL of MRPs obtained in different microwave treatments (500, 1000 and 1500 W) were mixed with 970 μL of different DL-DOPA concentrations (0–1.5 mM). Finally, 5 μL of PPO solution was added.

2.5.4. Characterization of the mode of action of MRPs

In order to study and understand the mode of action by which these MRPs could act on the enzymatic browning reaction, different types of *in vitro* tests were conducted. The aim of these tests was to ascertain whether the MRPs act on the enzyme, the substrate or both, and whether inactivation is reversible or irreversible.

2.5.4.1. Pre-incubation of PPO and MRPs. A PPO solution was pre-incubated with MRPs obtained after a 1500 W microwave treatment at different PPO:inhibitor ratios (1:1, 1:2 and 1:3, v:v). The total incubation time was 180 min. All the incubation was carried out at 30 °C using a thermostatic bath (Selecta; Barcelona, Spain). Aliquots (10 μL) of the medium were withdrawn at several time