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Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts

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ABSTRACT

Hydrolysis of oleuropein, the main phenol in olive (*Olea europaea* L.) leaf extracts, to oleuropein aglycon and other subsequent products in the hydrolytic pathway can be catalyzed by different enzymes. Three of the most used hydrolases were assayed to catalyze the process, and β-glucosidase from *Aspergillus niger* was selected. Acceleration of the enzymatic hydrolysis by ultrasound (US) was studied using a Box-Behnken design (duty cycle, amplitude, cycle time) and an oleuropein standard, and the optimum US conditions for achieving maximum yield of oleuropein aglycon were 0.5 s/s duty cycle, 50% amplitude and 45 s cycle. The method was applied to obtain oleuropein aglycon from commercial and laboratory extracts from olive leaves, which may have a pharmacological use as deduced by its healthy properties. The kinetics of the US-assisted enzymatic hydrolysis was monitored by analysis of the target compounds using liquid chromatography–tandem mass spectrometry.

1. Introduction

Oleuropein and oleuropein aglycone, present in virgin olive oil (VOO), could be used as nutraceutical or as component of new functional foods (Van der Stelt et al., 2015). Oleuropein and its aglycon possess beneficial pharmacological properties such as cardioprotective (Manna et al., 2004), antioxidant (Andreadou et al., 2006), anti-inflammatory (Khalatbary & Zarrinjoei, 2012), anti-cancer (Hamdi & Castellon, 2005), and neuroprotective effects (Bazoti, Bergquist, Markides, & Tsarbopoulos, 2006), among the most important.

The use of VOO as a source of oleuropein and/or oleuropein aglycone for preparation of functional foods is not recommended owing to the high price of VOO and relatively low concentration of these minor components. An almost ideal raw material for extraction of these and other valuable compounds are olive leaves: a residue generated from olive pruning and VOO production.

Olive leaves may account for up to 5% of the weight of collected olives removed during the process of cleaning olive drupes in the oil mill prior to VOO production (Zoiopoulos, 1983). Adding up the leaves from this process to those from pruning olive trees, the total amount of twigs and leaf residues may reach 12–30 kg/tree (Nefzaoui, Hellings, & Vanbelle, 1983). There are almost 900 million olive trees over 10 million hectares worldwide, 98% of which are in the Mediterranean countries (Sesli & Yegenoglu, 2009); therefore, the management of these residues should be properly exploited taking into account the highly valuable compounds they contain (Xie, Huang, Zhang, & Zhang, 2016), and the scant applications developed so far. The present main uses of olive leaves are animal feed, production of thermal energy, extraction of useful components (Joint Ministerial

Abbreviations: BBD, Box-Behnken design; ESI, electrospray ionization source; FA, formic acid; LC, liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; SRM, selected reaction monitoring; US, ultrasound; USAEH, ultrasound-assisted enzymatic hydrolysis; VOO, virgin olive oil.

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Decision, 2012), or composting (the last probably because the high C/N ratio of this residue) (Manios, 2004).

The aglycon form of oleuropein is obtained by enzymatic hydrolysis of oleuropein, the β-glucosidase being the key enzyme involved in the process, as shown in Fig. S1, which also shows the possible subsequent evolution of oleuropein aglycon to hydroxytyrosol and/or decarboxymethyloleuropein aglycon depending on the working conditions.

The different sources of β-glucosidase to catalyze the hydrolysis of oleuropein to obtain hydroxytyrosol and/or oleuropein aglycon have been almonds (Briante et al., 2000; Jemai, Bouaziz, Fki, Feki, & Sayadi, 2008), *Sulfolobus solfatarius* (Briante et al., 2000; Briante, La Cara, Febbraio, Patumi, & Nucci, 2002; Briante, Patumi, Febbraio, & Nucci, 2004), or *Aspergillus niger* (Hamza & Sayadi, 2015; Khoufi, Hamza, & Sayadi, 2011), which require intervals from 2 to 16 h to complete hydrolysis (Briante et al., 2004; Jemai et al., 2008). Also hemicellulase, a glycolytic enzyme that decomposes hemicellulose of plant cell walls, has been proposed to hydrolyze oleuropein, to hydroxytyrosol in this case (De Faveri, Aliakbarian, Avogadro, Perego, & Converti, 2008; Yuan, Wang, Ye, Tao, & Zhang, 2015).

Ultrasound (US) as modifier of enzyme activity has provided very controversial results (Delgado-Povedano & Luque de Castro, 2014). While oxidases seem to suffer deactivation under US application, an activity enhancement on hydrolytic enzymes is experienced in the case of sugarcane bagasse for the production of fermentable sugars, with yield of 0.26 g sugar/g dry sugarcane bagasse, around twice the value obtained without US assistance (Lunelli et al., 2014). Also in the enzymatic hydrolysis for extraction of luteolin and apigenin from celery, US-assistance provided yield increases of 26.1-fold and 32.2-fold, respectively, as compared with no ultrasonicated systems (Zhang, Zhou, Chen, Cao, & Tan, 2011).

With these premises, and with the aim of developing a fast method for enzymatic hydrolysis of oleuropein to its aglycon with minimum production of more degraded products, the following steps were planned: (i) selection of the most suitable hydrolase among the three more frequently used in the literature; (ii) application of US to shorten the enzymatic hydrolysis step as much as possible; (iii) use of a Box-Behnken design (BBD) to optimize the US variables duty cycle, amplitude, and cycle time for maximum acceleration of the hydrolysis of an oleuropein standard; (iv) application of the method to different olive leaf extracts.

2. Materials and methods

2.1. Olive leaf samples

Olive leaves collected from January to March 2015 from different cultivars located in the Denomination of Origin "Baena" in the South of Spain were used to obtain phenols extracts.

Commercial olive leaf extract powder enriched in oleuropein (40% oleuropein), from Ferrer HealthTech (Barcelona, Spain), was also used in this work for comparison with the behavior of fresh extracts.

2.2. Chemicals and reagents

Standards of oleuropein (purity ⩾90%) and hydroxytyrosol (purity >98.0%) for quantitative analysis of these metabolites were from Extrasynthese (Genay, France). Standards of oleuropein aglycon, decarboxymethyl oleuropein aglycon, decarboxymethyl ligstroside aglycon and ligstroside aglycon were donated by *P. Magiatis* (Athens, Greece). The three tested enzymes (hemicellulase and β-glucosidase from *A. niger*, and β-glucosidase from almonds, the characteristics of which are listed in Table S1) were all from Sigma–Aldrich (Madrid, Spain). MS (mass spectrometry) grade glacial acetic acid from Scharlab (Barcelona, Spain) and anhydrous sodium acetate from Panreac (Barcelona, Spain) were used to prepare buffers at different pHs. MS grade formic acid (FA) from Scharlab and methanol from Sigma–Aldrich were used to prepare the chromatographic mobile phases. Methanol was also used for sample preparation. Deionized water (18 mΩ·cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare aqueous solutions.

2.3. Extraction of oleuropein from olive leaves

Olive leaves (1 g) were dried and chopped, and 30 mL of 4:1 (v/v) methanol–water was added to the chopped olive leaves and the heterogeneous system thus formed was kept under agitation for 24 h in a Vibromatic rocking mixer from JP Selecta S.A. (Barcelona, Spain) for transfer of the polar compounds to the liquid phase. Then, the system was centrifuged at 1500×*g* for 15 min, the extract was filtered through 0.2 μm filter and divided into 8 aliquots before evaporation to dryness in a Concentrator Plus speed-vac from Eppendorf (Hamburg, Germany). This approach has been widely validated in previous research and it is accepted as sample preparation strategy prior to quantitative analysis of oleuropein in olive leaves (Jemai et al., 2008).

2.4. Enzymatic hydrolysis

The hydrolysis step catalyzed by each of the three enzymes under study was performed by locating 10 mg of oleuropein in each of three 12-mL glass bottles, and adding to them the same units (2.5 U/mg oleuropein substrate) of one of the enzymes and 10 mL of 0.05 M sodium acetate buffer. The amount of enzyme to be added depended on its activity: 21.6 mg of β-glucosidase from *A. niger* (1.2 U/mg), 3.5 mg of β-glucosidase from almonds (7.7 U/mg) and 16.7 mg of hemicellulase from *A. niger* (1.5 U/mg). The bottle was placed in a digital block heater, model SBH130D, from Stuart (Staffordshire, ST15 OSA, UK) to maintain constant temperature during the enzymatic hydrolysis. The enzyme–substrate ratio had been optimized in a previous study (Yuan et al., 2015), and other enzymatic conditions (the best temperature and pH) for each enzyme were those commercially recommended (37 °C for both β-glucosidases and 40 °C for hemicellulase, and pH 4.0 for β-glucosidase from *A. niger*, pH 5.0 for β-glucosidase from almonds and pH 4.5 for hemicellulase).

Sampling at different enzymatic hydrolysis times (0, 0.5, 2, 4, 6, 8, 10 and 12 h) was performed. The samples were immediately frozen and stored at −20 °C to stop the catalyzed reaction (Molinari & Silva, 1998), then analyzed to monitor the development of the reaction at each preset time. Also the evolution of the no catalyzed reaction was tested by developing the experiment in the absence of biocatalyst.

The hydrolysis of oleuropein was calculated by the following expression: [(concentration of oleuropein in the sample before hydrolysis − concentration after hydrolysis)/concentration in the sample before hydrolysis] × 100.

2.5. US-assisted enzymatic hydrolysis (USAEH) of oleuropein standard

USAEH of oleuropein by β-glucosidase from *A. niger* was performed under the conditions described in Section 2.4. The mixture was immersed in a water bath to maintain the temperature constant during hydrolysis. The ultrasonic probe—a Branson 450 digital sonifier (20 kHz, 400 W) with tunable amplitude, corresponding 100% of amplitude to 400 W, and duty cycle, equipped with a cylindrical titanium alloy probe microtip (3 mm in diameter)—was