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Enzymatic tailoring of oleuropein from *Olea europaea* leaves and product identification by HRMS/MS spectrometry

Efstratios Nikolaivits[a], Aikaterini Termentzi[b,c], Alexios-Leandros Skaltsounis[b], Nikolas Fokialakis[b], Evangelos Topakas[a,*]

[a] Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 5 Iroon Polytechniou Str, Zografou Campus, 15700, Athens, Greece
[b] Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimioupolis Zografou 15 771, Athens, Greece
[c] Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, St. Delta 8, 14561, Kifissia, Greece

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ABSTRACT

Oleuropein, a bioactive compound found in all parts of olive tree, especially in leaves and branches, presents numerous health promoting properties that increase research and market interest the last few years. In addition, oleuropein degradation products, such as hydroxytyrosol, elenolic acid, and the aglycones also exhibit biological activities with different properties compared to the starting compound. Under this view, a commercial lipase preparation Lipolase 100L and a thermophilic β-glucosidase from *Myceliophthora thermophila* were used for the regioselective hydrolysis of oleuropein towards the production of the corresponding biologically active compounds. The enzymatic degradation products of oleuropein, such as hydroxytyrosol, elenolic acid and its glucoside, and oleuropein aglycones were identified by LC-HRMS/MS and NMR spectroscopy. The latter, was found as a mix of diastereomers of the monoaldehydic form of oleuropein aglycone, identified as (5*S*, 8*R*, 9*S*)-, (5*S*, 8*S*, 9*S*)- and (5*S*, 8*R*, 9*R*). The high substrate specificity exhibited by both lipase and β-glucosidase allows the successful tailoring of oleuropein towards the production of different biologically active compounds with significant potential in the cosmeceutical and food industry.

1. Introduction

Oleuropein, the bitter-tasting main phenolic compound found in all parts of the olive tree, *Olea europaea*, can reach concentrations of up to 500 mg/g dry extract in the leaves (Le Tutour and Guedon, 1992). Oleuropein is also abundant in olive mill wastewaters among other phenolics (Balasundram et al., 2006). It is a glucosylated ester of hydroxytyrosol (HT) with the secoiridoid elenolic acid. Other natural sources of oleuropein except the *Oleaceae* family are the *Fraximus*, *Syringa*, *Philyrea* and *Ligustrum* genera (Omar, 2010). Similar to other secoiridoids of the *Oleaceae* family (ligstroside, dimethyl-oleuropein, nuezhenide), oleuropein is a secoiridoid glucoside comprising of the oleosidic skeleton glycosylated with glucose (oleoside) and esterified with HT (Soler-Rivas et al., 2000). Oleuropein's various pharmacological properties have been unveiled, including antioxidant, anti-inflammatory, anti-cancer, cardioprotective, neuroprotective and hypolipidemic (Hassen et al., 2015). The main degradation product of oleuropein, during the maturation of the olive fruit, is HT, which is a strong antioxidant showing a plethora of biological activities (Fernández-Bolaños et al., 2012), in cases higher than oleuropein itself (Visioli et al., 1998). In addition, HT is the only olive molecule with an approved health claim from European Food Safety Authority (EFSA) (Panel on Dietetic Products Nutrition and Allergies, 2011).

Apart from oleuropein and HT, oleuropein aglycone is also gaining increasing attention due to its several biological properties that mostly resemble those of oleuropein, such as antioxidant, antimicrobial anti-inflammatory, hypolipidemic and cardioprotective properties (Fleming et al., 1973; Masella et al., 1999; Rigacci and Berti, 2010). In addition, oleuropein aglycone has a proven potential against Alzheimer disease (Grossi et al., 2013; Rigacci et al., 2011). This compound is formed from oleuropein after the detachment of the glucose moiety. Since it is a major constituent of extra virgin olive oil (Kanakis et al., 2013), it is considered responsible for several of its positive effects in human health. It can be found in several isomers due to differentiated configuration of the iridoid moiety (Pérez-Trujillo et al., 2010), however, the most abudant isomer in olive oil is always its monoaldehydic form.

Another hydrolysis product of oleuropein present in extra virgin olive oil is the secoiridoid elenolic acid. It has been mentioned in the

* Corresponding author.
E-mail addresses: vtopakas@chemeng.ntua.gr, vtopakas@central.ntua.gr (E. Topakas).

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past as an effective anti-viral agent (Heinze et al., 1975; Hirschman, 1972). There have been several efforts towards HT production through oleuropein hydrolysis using chemical procedures (Walter et al., 1973). However, the enzymatic approach is ideal, since it results to environmentally friendly and safe products through selective hydrolysis. In literature, the use of lactic acid bacteria (*Lactobacillus* genus) has been tested with satisfactory yields reaching a 30% conversion to HT (Ciafardini et al., 1994; Santos et al., 2012). In addition, the single-enzyme hydrolysis reaction has proven to be efficient and easier, since bacteria's growth rate and activity are inhibited by high concentrations of oleuropein and its hydrolysis products. So far, a crude commercial preparation of *β*-glucosidase from sweet almonds has been used for the hydrolysis of oleuropein to glucose and aglycone, as well as a hyperthermophilic *β*-glucosidase, which catalyzed the hydrolysis of the glycosidic bond, releasing HT by the chemical hydrolysis of the ester bond due to the reaction conditions (Briante et al., 2000; Capasso et al., 1996).

In the present study, the enzymatic tailoring of oleuropein, purified from *O. europaea* leaves is reported. Utilization of a commercial lipase (Lipolase, Novozymes) and a recombinant GH3 *β*-glucosidase from *Myceliophthora thermophila*, resulted to the hydrolysis of either the glycosidic bond between the glucose and the elenolic moiety or of the ester bond between the elenolic moiety and HT. The identification of all the hydrolysis products was performed by LC-HRMS/MS and 1D & 2D NMR experiments. The enzymatic tailoring of oleuropein utilizing purified fungal enzymes towards the production of HT and/or non commercially available oleuropein aglycones has never been mentioned before.

2. Materials and methods

2.1. Oleuropein isolation

Oleuropein was isolated from olive leaves according to the procedure previously described (Xynos et al., 2012). In brief, dried and pulverized olive leaves were extracted with ethanol by pressurized liquid extraction. The extract obtained was concentrated to dryness and was subjected to open column chromatography using silica gel as a static phase and methanol as eluent. The fractions that contained oleuropein were merged and concentrated to dryness. Further purification was assessed by preparative HPLC. Totally, 50 mg of pure oleuropein (purity > 98%) were collected from 1 g dry leaves.

2.2. Enzymes

The commercial lipase preparation from *Thermomyces lanuginosus* Lipolase 100L, was provided by Novozymes A/S (Denmark). The recombinant *β*-glucosidase *Mt*bgl3a from *M. thermophila* was expressed in *P. pastoris* X-33 and purified, as previously described (Karnaouri et al., 2013). In the case of the Lipolase preparation, protein content was calculated by the Bradford (1976) method, while for the purified *β*-glucosidase by the absorbance at 280 nm using a molar extinction coefficient of 115655 M^{-1} cm^{-1} (Stoscheck, 1990).

Enzymatic solution of *β*-glucosidase contained 97.7 U $mg_{protein}^{-1}$ measured on *p*-nitrophenyl β-D-glucopyranoside by the method described in Karnaouri et al. (2013). The activity of the commercial lipase preparation was measured to be 2818 U $mg_{protein}^{-1}$ applying a *p*-nitrophenyl laurate (0.4 mM) assay at 40 °C and pH 6.

2.3. Enzymatic tailoring of oleuropein

Reactions took place in a final volume of 1 mL in 1.5 mL Eppendorf tubes containing 0.8 mg mL^{-1} oleuropein. Oleuropein was first diluted in 0.1 M phosphate-citrate buffer pH 6.0 prior to enzyme addition. All reactions were performed in an Eppendorf ThermoMixer® Comfort (Eppendorf, Germany), at 30 °C for 20 h. The enzymes, *Mt*bgl3a *β*-glucosidase (0.032 mg) and Lipolase 100L lipase (4.4 mg), were used as biocatalysts either separate or in the same reaction aiming in tailoring the oleuropein structure.

2.4. Production and isolation of oleuropein aglycone

In preparative scale, the hydrolysis reaction using *Mt*bgl3a *β*-glucosidase in the reaction medium (1 mL) was carried out in sealed flask at 30 °C without stirring. Oleuropein of 80% purity (20 mg) was diluted in 0.1 M phosphate-citrate buffer pH 6.0, and 36 μg of *Mt*bgl3a was added. The isolation of oleuropein aglycone was made by preparative HPLC on a μBondapak™ C18 10 μm 125 Å (7.8 mm × 300 mm) Prep Column (Waters, Ireland). Detection was achieved by the photodiode array detector Varian ProStar set at 280 and 210 nm. The isolation was executed with a linear gradient method using double distilled water (A) and acetonitrile (B) as eluents at a flow rate of 2.5 mL min^{-1}. The total running time was 60 min during which the following proportions of the solvent B were used: 0–20 min 5–20%, 20–25 min 20–30%, 25–30 min 30%, 30–35 min 30–35%, 35–40 min 35–50%, 40–45 min 50–95%, 45–50 min 95–5%, 50–60 min 5%. Fractions containing oleuropein aglycone were merged and evaporated under reduced pressure.

2.5. Products identification

2.5.1. Thin layer chromatography (TLC)

Reaction mixtures were qualitatively analyzed by TLC on aluminum sheets coated with Silica gel 60 F_{254} (Merck, Germany). Chloroform/methanol (8:2 v/v) was used as a solvent system for the resolution of oleuropein and its hydrolysis products. Oleuropein, glucose and elenolic acid glucoside were detected on dried chromatograms, using the *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent (Bounias, 1980). The chromatograms treated with the detection reagents were heated at 105 °C in an oven for a few minutes prior to analysis. Furthermore, universal staining procedures were performed: a potassium permanganate stain (0.75% (w/v) $KMnO_4$, 5% (w/v) K_2CO_3, 0.625% (v/v) of 10% (w/v) NaOH diluted in distilled water) or visualization under iodine vapors.

2.5.2. LC-HRMS/MS analysis

The analyses of the reaction mixtures were performed on an HPLC-HRMS LTQ-Orbitrap platform (Thermo Scientific) at negative ESI ionization mode. The analytical methodology was based on the one previous developed on the same system by Kanakis et al. and is optimized for the chromatography and identification of olive-derived molecules (Kanakis et al., 2013). In brief, a C18 column was used (Supelco, 150 × 2.1 mm i.d., 3 μm) at a flow rate of 400 μL/min, using the solvent system (A) H_2O, AA (0.1% v/v) and (B) ACN. The following elution gradient program was followed: 5% B for 2 min; 10% B in 2.5 min; 25% B in 11.5 min; 95% B in 13 min; 95% B for 2 min; 5% B in 0.5 min; 5% B for 2.5 min; injection volume: 5 μL. The HRMS data were acquired at a full scan mode, with mass range of 100–1500 *m/z*. ESI conditions: capillary temperature 350 °C; capillary voltage −3 V; tube lens −43.46 V. Nitrogen was used as sheath gas (30 arb) and auxiliary gas (10 arb).

Oleuropein and HT were quantified using calibration curves constructed by known concentrations of these substances obtained commercially from Sigma-Aldrich (USA).

2.5.3. NMR analysis

The structure elucidation of the purified molecules was performed by 1 & 2D-NMR analyses on an Avance III 600 spectrometer (Bruker, Billerica, MA, USA). For all experiments deuterated chloroform ($CDCl_3$) was used as the dilution solvent.