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Purification and Characterization of an Olive Fruit β-Glucosidase Involved in the Biosynthesis of Virgin Olive Oil Phenolics

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An olive β-glucosidase was purified to apparent homogeneity from mature fruits (*Olea europaea* cv. Picual) by selective extraction and successive anion exchange and hydrophobic interaction chromatographic procedures. The enzyme was shown to be a homodimer made up of two identical subunits of 65.4 kDa. Optimum activity was recorded at pH 5.5 and 45 °C. The enzyme was active on the main olive phenolic glycosides, with maximum activity toward oleuropein (100%), followed by ligstroside (65%) and demethyloleuropein (21%). The enzyme showed very low activity with apigenin and luteolin glucosides and was not active on verbascoside and rutin. Kinetic values show that olive β-glucosidase is 200-fold more active against oleuropein than against the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG). According to its catalytic properties, the implication of the purified olive β-glucosidase on the synthesis of virgin olive oil phenolics is discussed.

KEYWORDS: β-Glucosidase; phenolics; olive fruit; virgin olive oil

INTRODUCTION

The large increase in the demand for high-quality virgin olive oil (VOO) observed during the past few years may be attributed to its potential health benefits related to protection against cancer and cardiovascular diseases (*1*−*3*) and also to its extraordinary organoleptic properties. It is well established that phenolic compounds have a direct influence on both factors; as antioxidants they provide important nutritional benefits, and additionally some of them are associated with the bitter and pungent sensory notes of the oil (*4*, *5*). The VOO phenolic profile may be affected by multiple preharvest and postharvest factors reviewed by Servilli et al. (*6*). In this sense, although processing technology may severely affect VOO quality, it is clear that most quality attributes of the oil are determined by the chemical composition and biochemical status of the olive fruit. Thus, the content of the main hydrophilic phenols found in VOO is closely related to the content of phenolic glycosides initially present in the olive tissue and the pool of enzymes associated with phenolic metabolism available in the fruit.

The main phenolic glycosides found in olive fruit are oleuropein, ligstroside, and demethyloleuropein, although many others such as verbascoside, an elenolic acid glucoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin, and quercetin-3-rutinoside have also been identified in fruits from different cultivars and maturation stages (*7*, *8*). The aglycon derivatives resulting from the enzymatic hydrolysis (*9*) of oleuropein, ligstroside, and demethyloleuropein, identified as dialdehydic forms of decarboxymethyl oleuropein and ligstroside aglycones (DGO and DGL, respectively) and aldehydic forms of oleuropein and ligstroside aglycones (AGO and AGL, respectively), are commonly known as secoiridoid compounds and constitute the most significant phenolic components found in VOO. The key enzymatic system controlling the hydrolysis of phenolic glycosides when olive tissues are mechanically or pathogen damaged is the endogenous pool of β-glucosidases. The importance of plant β-glucosidases to food quality is in fact derived from their ability to hydrolyze flavor precursors, liberating aglycone moieties that provide desirable organoleptic properties to plant-derived foods (*10*).

Previous studies have proved that the final concentration of phenolic compounds in the oil is greatly affected by processing parameters such as temperature, time, or exposure to air. These factors probably modulate the activity of key enzymes within the metabolism of phenolic compounds during the crushing and malaxation steps in the industrial process to obtain VOO (*6*). Thus, modulation of phenolic content by olive fruit heat treatments, probably mediated through a β-glucosidase inactivation, have been described (*11*), and in a similar way an improvement of VOO phenolic content has been achieved by means of stone removal that eliminates the important pool of oxidative enzymes located in the olive seed (*12*). Nevertheless, sometimes conflicting results have been reported on the effect of some of these new technological procedures that may indeed be explained by the poor knowledge on catalytic properties of the enzymes controlling metabolic processes affecting VOO phenolic content. In this sense, there is a need for characterizing the specific enzyme or enzymes that hydrolyze olive glycosides and gives rise to VOO secoiridoid compounds.

Several authors have previously studied the enzymatic hydrolysis of oleuropein by microbial recombinant β-glucosidases or crude olive extracts, and the intermediate and final reaction products have been tentatively characterized in model reactions (*13*, *14*).

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Most of these previous studies have focused on the physiological role of this enzymatic hydrolysis as a specific defense mechanism in the Oleacea family that produces oleuropein-derived compounds with demonstrated antimicrobial activities (*15*). Despite the importance of olive β-glucosidase from this physiological point of view and as a key biological factor closely related to olive fruit and VOO quality, the enzyme has never been purified, and there is no information on its catalytic characteristics. The aim of this study was to purify and characterize proteins with β-glucosidase activity present in olive fruit which hydrolyze the main phenolic glycosides accumulated in the olive fruit.

MATERIALS AND METHODS

Plant Material. Olive fruits (*Olea europaea* cv. Picual) used in this study were cultivated at the experimental fields of the Instituto de la Grasa and collected, during two consecutive years, at the appropriate maturity stage. Fresh olive pulp tissue was used to assay β-glucosidase activity along ripening, and acetone powders obtained from fruits collected 35 weeks after flowering were used as starting material for purification purposes.

Chemicals. Reagents for enzymatic activity extraction and measurements were supplied by Sigma-Aldrich (St. Louis, MO) except for phenolic compounds (oleuropein, verbascoside, rutin, apigenin glucoside, and lutein glucoside) purchased from Extrasynthese (Genay, France). Ligstroside and demethyloleuropein, not commercially available, were purified from olive fruit phenolic extracts. Hitrap Q HP, Hitrap butyl HP, and Superose 12 10/300 GL columns were from GE Healthcare Amersham Biosciences (Barcelona, Spain).

Milling of Olive Fruits. Milling of olive fruits (1 kg) was performed using a stainless steal hammer mill operating at 3000 rpm provided with a 5 mm sieve. This mill is the currently used (*12*) as the first step in the process of VOO extraction at laboratory scale (Abencor analyzer, commercial Abengoa, S.A., Sevilla, Spain). Olive pastes were immediately sampled for phenolic analysis.

Analysis of Fruit and Paste Phenolics. Longitudinal pieces of mesocarp were cut from 20 olive fruits, finely chopped, and used to prepare phenolic extracts in triplicate. Representative samples of fruits and pastes (1 g) were homogenized at 4 °C with dimethyl sulfoxide (6 mL) by means of an Ultraturrax homogenizer (2 × 30 s at maximum speed). After centrifugation at 3000*g* for 2 min, supernatant was filtered thorough a 0.22 μm filter and analyzed by HPLC as described by Luaces et al. (*12*).

β-Glucosidase Activity Assays. Two methods for assaying β-glucosidase activity were used in this study. For a rapid localization of activity during the purification process, a spectrophotometric method was utilized. In this method β-glucosidase activity was determined by continuously monitoring the increase in absorbance at 405 nm related to the increasing amount of *p*-nitrophenol liberated from the synthetic glucoside *p*-nitrophenyl-β-D-glucopyranoside (pNPG). Quantification of the hydrolytic reaction was done by using a calculated molar extinction coefficient of 552.8 M^{-1} cm^{-1}. The reaction medium consisted of 1.5 mL of 50 mM sodium acetate buffer, pH 5.5, 15 mM pNPG, and the appropriate amount of enzyme (5−20 μL). One unit of β-glucosidase activity was defined as the amount of enzyme able to release 1 μmol of *p*-nitrophenol per minute at 45 °C.

The second method to assay β-glucosidase activity was based on the direct determination of the hydrolyzed natural olive glucoside, oleuropein, by HPLC analysis. The reaction medium, consisting of 1.5 mL of 50 mM sodium acetate buffer, pH 5.5, 6.5 mM oleuropein, and the appropriate amount of enzyme (5−20 μL), was incubated at 45 °C. After 1 min, the reaction was stopped by adding 1.5 mL of methanol. Remaining oleuropein was quantified by HPLC analysis in an AKTA Basic 10/100 (Pharmacia) liquid chromatographic system equipped with a UV-900 detector and a Mediterranea Sea 18 column (4.0 mm i.d. × 250 mm, particle size = 5 μm) (Teknokroma, Barcelona, Spain) following a previously described methodology (*12*). One unit of β-glucosidase activity was defined as the amount of enzyme hydrolyzing 1 μmol of oleuropein per minute at 45 °C. For assaying enzyme activity with other phenolic glucosides present in olive fruits, the same procedure was used. All activity measurements were carried out in triplicate.

Protein Determination. Protein was measured according to the method described by Bradford (*16*) using the Bradford reagent (Sigma-Aldrich, St. Louis, MO) with crystalline bovine serum albumin (BSA) as the standard protein.

Enzyme Extraction and Purification. *Extraction*. Acetone powders were prepared from the pulp tissue of freshly harvested olive fruits. Typically, 100 g of pulp tissue was ground in 1500 mL of cold acetone (−20 °C) using a Waring blender. The residue obtained after filtration was re-extracted twice with 200 mL of cold acetone (−20 °C). The whitish powder obtained was finally rinsed with diethyl ether, dried, and stored at −20 °C.

Enzyme extracts were prepared from 2 g of acetone powder in 140 mL of a buffer consisting of 100 mM borate buffer, pH 9, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.25% (w/v) ditiothreitol (DTT) using an Ultraturrax homogenizer. The resulting homogenate was centrifuged at 27000*g* for 20 min at 4 °C, and the clear supernatant was used as crude extract.

Purification. The whole purification procedure was carried out with an FPLC system (AKTA Basic 10/100, Pharmacia) at 0−4 °C. Proteins were recorded in column effluents at 280 nm.

Step 1: $(NH_4)_2SO_4$ was added to the crude extract, and the pellet obtained at 50% saturation was collected. The protein concentrate obtained was desalted through a PD-10 column (Sephadex G-25, Pharmacia) and eluted with 50 mM HEPES buffer, pH 7.2, for further chromatographic procedures.

Step 2: Protein extract was submitted to anion exchange chromatography on a Hitrap Q HP 5 mL column (1.6 × 2.5 cm) (GE Healthcare Amersham Biosciences), pre-equilibrated with 50 mM HEPES buffer, pH 7.2. The column was washed with the same buffer, and the bound proteins were eluted with a linear 0−1 M NaCl gradient at flow of 2 mL/min. One milliliter fractions were collected and assayed for β-glucosidase activity. The active fractions were pooled and concentrated in 50 kDa microcentrifuge filters (Amicon ultra-15 centrifugal filter device, Millipore).

Step 3: The pooled active fractions were conditioned to 50 mM sodium phosphate buffer, pH 6.8, 1.5 M NaCl and loaded onto a HiTrap butyl Sepharose HP 5 mL column (1.6 × 2.5 cm) (GE Healthcare Amersham Biosciences), equilibrated with the same buffer. Washing of unbound proteins was performed with equilibrium buffer, and elution of bound proteins was carried out by using a linear decreasing gradient of NaCl (1.5−0 M).

Molecular Mass Determination. The molecular weight of the native enzyme was determined using a size exclusion chromatography Superose 12 10/300 GL column eluted with 50 mM phosphate buffer, pH 6.8, 0.15 M NaCl. The column was calibrated with lactate dehydrogenase (140 kDa), phosphorylase *b* (94 kDa), serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). The subunit molecular mass was determined by SDS-PAGE as described below.

Electrophoresis. SDS-PAGE was performed by means of a Hoefer mini-gel apparatus (Pharmacia Biotech). Proteins were resolved on 10% (w/v) polyacrylamide 0.75 mm thick slab gels containing 1% SDS according to the method of Laemmli (*17*). A 4% (w/v) polyacrylamide was used as stacking gel. All samples were preincubated (1:1) in the presence of sampling buffer [125 mM Tris, pH 6.8, 0.14 M SDS, 20% glycerol (v/v), 2% (v/v) mercaptoethanol, 0.03 mM bromophenol blue] for 2 min at 100 °C before being loaded onto the gels. For the determination of the subunit molecular mass by SDS-PAGE, a plot of relative mobility versus log(molecular mass) was constructed with the following standard proteins: phosphorilase *b* (94 kDa), bovine serum albumin (67 kDa), catalase (60 kDa), ovoalbumin (43 kDa), lactate dehydrogenase (36 kDa), and carbonic anhydrase (30 kDa). Proteins were stained within the gel with Coomassie blue.

Effect of pH. The effect of pH on olive β-glucosidase activity was assesed in the range 4−8. The optimum pH was determined by measuring β-glucosidase activity under standard assay conditions using sodium acetate, phosphate, and borate buffers (50 mM).

Effect of Temperature. The optimum temperature for olive β-glucosidase was determined by measuring activity under standard conditions in the temperature range of 20−70 °C. Thermal stability of olive β-glucosidase was determined under standard assay conditions after incubation of enzyme crude extracts for 60 min at different temperatures.

Effect of Inhibitors. The purified β-glucosidase was incubated for 1 and 24 h at 4 °C in the presence of different metal ions (1 mM). The

activity was then measured under standard conditions. The effect of three previously described β-glucosidase competitive inhibitors, glucose, trehalose, and δ-gluconolactone, was also studied by adding increasing amounts of these inhibitors to the standard reaction medium.

Substrate Specificity. The activity of the purified protein was measured with various substrates. Three different synthetic glycosides, pNPG, *p*-nitrophenyl-α-glucopyranoside, and *p*-nitrophenyl-β-galactopiranoside, were assayed by the standard spectrophotometric method. The activity against the main natural olive glycosides, oleuropein, demethyloleuropein, luteolin-7-glucoside, apigenin-7-glucoside, ligstroside, verbascoside, and rutin, was assayed by means of the HPLC method. Although most natural glycosides were tested at a concentration of 6.5 mM in the reaction medium, in some cases the final concentration of specific substrates was limited by its low water solubility.

Kinetic Parameters. The activity of the purified olive β-glucosidase was measured under standard assay conditions in a final concentration of pNPG and oleuropein in the ranges of 2.5−60 and 0.025−9.5 mM, respectively.

RESULTS AND DISCUSSION

We have developed a simple purification procedure for the selective isolation of an olive β-glucosidase active against oleuropein from acetone powder using a high-pH extraction buffer. Preliminary studies were carried out to assay β-glucosidase activity in protein extracts obtained from fresh fruits at different ripening stages and their corresponding acetone powders. Acetone powders from Picual fruits collected 35 weeks after flowering were selected as optimum material (data not shown) and a mixture of 0.1 M borate, pH 9, 5 mM AEDT, 1 mM PMSF, and 0.25% (w/v) DTT as the optimum extraction buffer.

The use of acetone powder has proved to be very useful for olive protein purification given that it permits the isolation of protein samples free of pigments, phenolics, and lipids, which would interfere with further purification steps. Different buffers with different pH values and different ionic strengths have been used for differential solubility fractionation of plant glycosidases. The high-pH extraction buffer used in this study greatly differs

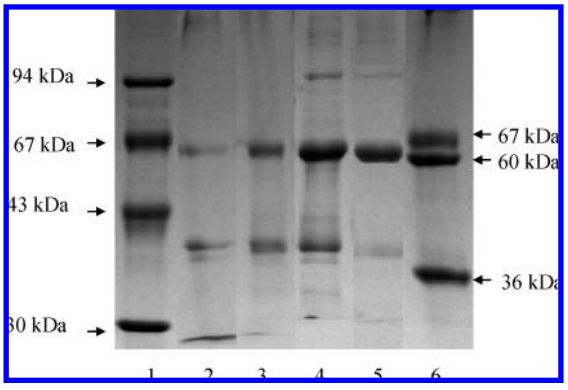

Figure 1. SDS-PAGE analysis of total proteins (10 μg) extracted from olive acetone powder with different extraction buffers. Lanes: 1 and 6, molecular weight markers; 2, acetate buffer, pH 4.5; 3, phosphate buffer, pH 6.8; 4, borate buffer, pH 8.0; 5, borate buffer, pH 9.0.

from the acidic buffers previously used to extract other plant glycosidases such as those from cherry (*18*), maize (*19*), or olive (*20*) but coincides with that used by Briante et al. (*21*) to assay β-glucosidase and esterase activities during olive fruit ripening. As shown in **Figure 1** and **Table 1**, the extraction buffer selected provided crude extracts with high specific activity and low level of contaminant proteins. Because the addition of detergents or the increase of ionic strength did not improve the level of activity in the crude extract (data not shown), it may be assumed that the purified β-glucosidase is a soluble protein. Similar cytosolic β-glucosidases have been described in other fruits (*10*). A recent study carrying out in situ localization of β-glucosidase activity in the mesocarp tissue of olive fruit revealed the presence of the enzyme in different cell compartments (*22*). Initial characterization of olive glycosidases (*23*) concluded that in green mature fruits a significant proportion of most hydrolytic activities was ionically or covalently associated to the cell wall, whereas only a small proportion was soluble forms.

Olive β-glucosidase was purified from crude extracts by means of ammonium sulfate precipitation, anion exchange chromatography, and hydrophobic chromatography. Protein precipitation with 50% ammonium sulfate proved to be a very simple but efficient step to selectively increase the specific activity of olive β-glucosidase active on oleuropein. The high purification factor obtained at this initial step (4.3) seems to indicate that besides other contaminant proteins crude extracts obtained from olive acetone powders contained unspecific glucosidases active toward pNPG but not able to hydrolyze oleuropein. Thus, as shown in **Table 1** the ratio between total activity measured against oleuropein and pNPG in the crude extracts was 6.7 and increased to 16 in the ammonium sulfate precipitate. A single broad peak of activity was obtained after anion exchange chromatography through a Hi-Trap Q column (**Figure 2A**). Active fractions started to elute at 0.8 M NaCl, whereas a small peak of nonactive protein remained strongly bound to the phase. Active fractions were pooled, concentrated with 50 kDa centrifugal cartridges, conditioned to 50 mM sodium phosphate buffer, pH 6.8, 1.5 M NaCl, by means of a PD-10 column, and loaded onto a butyl Sepharose column. As shown in **Figure 2B**, an initial peak of inactive protein was eluted as nonretained protein, whereas olive β-glucosidase was strongly adsorbed to the column phase and eluted only after the initial NaCl concentration had been decreased to 0.5 M. At this stage of purification the β-glucosidase appeared to be electrophoretically homogeneous, giving rise to a single protein band in SDS-PAGE with an apparent molecular mass of 65.4 kDa (**Figure 3**). This estimated molecular mass seems to coincide with most plant β-glucosidase monomers described so far ranging from 55 to 65 kDa (*10*). Although the active fraction collected after hydrophobic chromatography seems to contain a single electrophoretic band, the overall purification factor increased only 8-fold (**Table 1**). This result, quite similar to that found in the purification of cherry (*18*) and vanilla β-glucosidases (*24*), could be explained by the already mentioned high specific activity obtained in the crude extracts from acetone powders. A similar abundance of β-glucosidase activity has been previously described

Table 1. Purification of β-Glucosidase from Olive Fruit (*Olea europaea* Cv. Picual)

purification step	protein (mg)	total activity pNPG (U)[a]	total activity oleuropein (U)[b]	specific activity (U/mg prot)	purification factor	yield (%)
crude extract	46.83	3003.5	20153.1	430.3	1	100.0
50% $(NH_4)_2SO_4$ precipitation	12.74	1411.0	23672.8	1858.1	4.3	100.0
ion exchange chromatography	3.36	553.5	10922.3	3250.7	7.6	54.2
hydrophobic chromatography	0.48	104.8	1680.1	3500.2	8.1	8.3

[a] One unit of activity was defined as the amount of enzyme hydrolyzing 1 μmol of pNPG per minute at 45 °C. [b] One unit of activity was defined as the amount of enzyme hydrolyzing 1 μmol of oleuropein per minute at 45 °C. Specific activity, purification factors, and yields were calculated from these activity data.

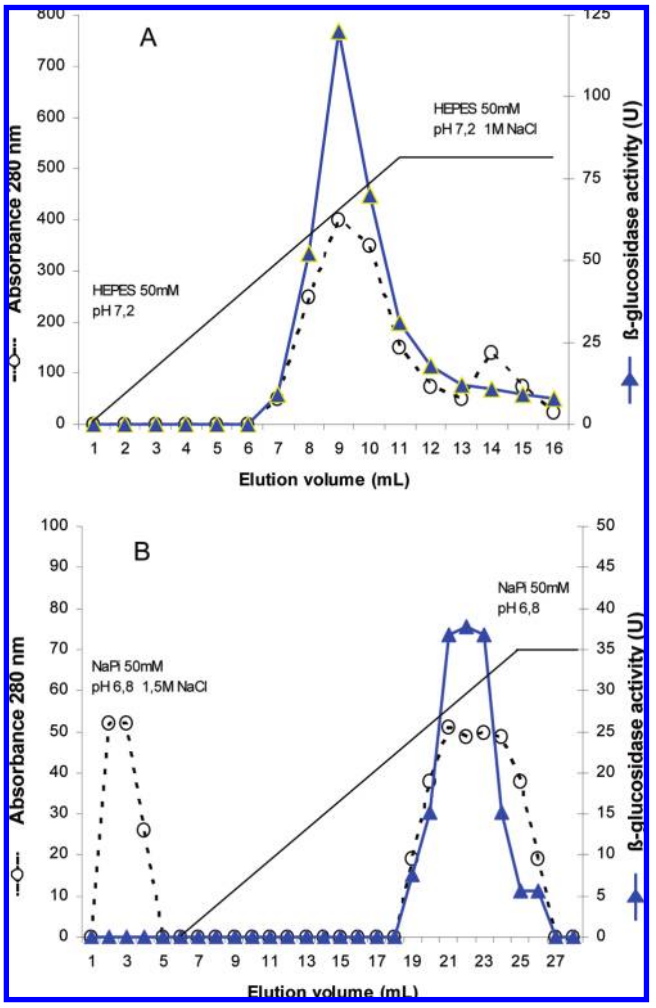

Figure 2. Purification of olive β-glucosidase by anion exchange chromatography on Hitrap Q HP 5 (**A**) and hydrophobic chromatography on Hitrap butyl Sepharose HP 5 (**B**).

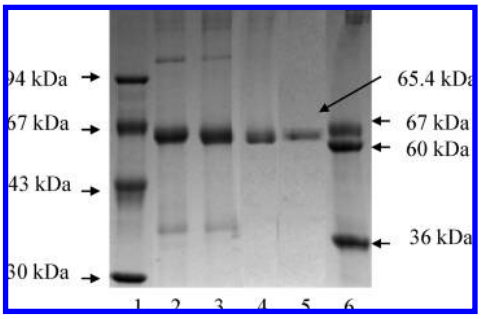

Figure 3. SDS-PAGE analysis of β-glucosidase purification steps. Lanes: 1 and 6, molecular weight markers; 2, 10 μg of total proteins extracted from acetone powder; 3, 10 μg of $(NH_4)_2SO_4$ precipitated proteins; 4, 10 μg of active fraction recovered after anion exchange chromatography; 5, 10 μg of active fraction recovered after hydrophobic chromatography.

in other plant crude extracts such as white clover or maize (*19*). Such abundance may be due to either the low turnover of the enzyme or the high demand for its catalytic function.

The purified olive β-glucosidase exhibited a broad optimum pH curve with a maximum at pH 5.5 and a rapid decline of activity above it (**Figure 4A**). This maximum pH is higher than those previously used to assay olive glycosidases (*22*, *23*) but similar to those of maize or orange (*19*, *25*). The purified enzyme

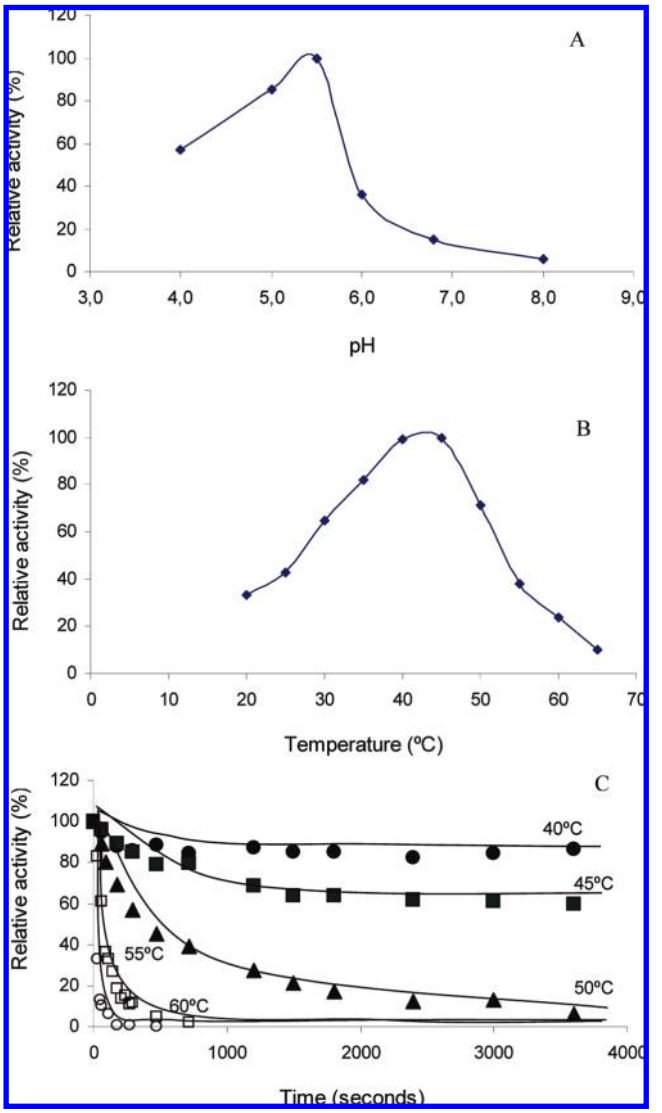

Figure 4. (**A**) Effect of pH on olive β-glucosidase activity; (**B**) effect of temperature on olive β-glucosidase activity; (**C**) thermal stability of olive β-glucosidase.

showed the highest activity when assayed at 45 °C (**Figure 4B**) and, as most plant glycosidases, was quite stable up to 40 °C with a dramatic decrease of activity above this temperature (**Figure 4C**). Data obtained on the effect of temperature on the catalytic activity are quite similar to those reported for cherry, maize, or orange β-glucosidases, showing optimum temperatures around 40−45 °C (*18*, *19*, *25*).

The molecular mass of the native protein calculated by size exclusion chromatography in the conditions described under Materials and Methods (data not shown) corresponded to a molecular mass of around 130 kDa, consistent with a dimeric protein with two identical subunits of a molecular mass of around 65 kDa such as that estimated by SDS-PAGE. Although, as suggested in some plants, the monomer form may be catalytically active (*26*), in most cases catalytic active forms are homodimers with a molecular mass of around 120 kDa (*10*). High molecular weight aggregates of β-glucosidases formed by interaction with specific binding proteins have also been described in maize, oat, flax, and cabbage (*10*). In fact, some of those aggregates may reach native molecular masses that exceed 1500 kDa and therefore cannot be detected in native electrophoresis gels (*27*).

Table 2. Relative Activity of Purified Olive β-Glucosidase on Various Natural Glycosides

substrate	relative activity (%)
oleuropein (6.5 mM)	100
ligstroside (6.5 mM)	65.4
demethyloleuropein (6.5 mM)	21
apigenin glucoside (0.06 mM)	1
luteolin glucoside (0.06 mM)	0.2
verbascoside (6.5 mM)	nd[a]
rutin (0.06 mM)	nd

[a] No activity detected.

The main goal of this study was to isolate an olive β-glucosidase form able to hydrolyze olive phenolic glycosides to establish its contribution to the synthesis of VOO phenolic components. The activity of the purified olive β-glucosidase was assayed with three different artificial substrates, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-α-D-glucopyranoside, and the most significant phenolic glycosides identified in olive fruit (oleuropein, demethyloleuropein, ligstroside, verbascoside, rutin, apigenin-7-glucoside, and luteolin-7-glucoside). The results obtained with the artificial substrates showed that the purified olive enzyme has certain β-galactosidase activity (33%) but no α-glucosidase activity (data not shown). This fact is quite characteristic of most members of the family 1 glycosyl hydrolases such as the sweet almond β-glucosidase (*10*). The broad substrate specificity often attributed to plant β-glucosidases, usually assessed by means of nonphysiological substrates such as pNPG, does not necessarily mean that these enzymes catalyze in vivo the hydrolysis of a large number of natural substrates. The reason could be that artificial substrates sometimes may have better shapes and complementarities with respect to the enzyme active site, and their aglycone moieties may be better leaving groups in the final reaction steps than those of the physiological substrates. Substrate specificity experiments confirmed the ability of the enzyme to hydrolyze olive natural glycosides (**Table 2**). The maximum activity was obtained with oleuropein, followed by ligstroside (65.4%) and demethyloleuropein (21%). The very low levels of activity obtained with luteolin and apigenin glucosides could be partially explained by the low solubility of these substrates in water. These data of β-glucosidase activity on phenolic glycosides present in olive fruit are in good agreement with phenolic profiles found in the olive pastes resulting from the milling of olive fruits, the first step in the process to obtain VOO. As displayed in **Figure 5**, inmediatelly after the milling step, most of the oleuropein and ligstroside initially present in the olive fruit were metabolized. On the contrary, demethyloleuropein was still present in olive pastes, and verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside were metabolized at a lesser rate.

Previous studies on phenolic compound biotransformation do not fully elucidate the formation of the main secoiridoid compounds found in VOO. Thus, the enzymatic formation of DGO, quantitatively the most important secoiridoid in VOO and byproducts, from demethyloleuropein is well-known in model systems (*8*). However, the scarce content of demethyloleuropein in most olive varieties supports the possible formation of DGO from oleuropein. Taking into account the higher selectivity of the isolated olive β-glucosidase toward oleuropein and the higher abundance of oleuropein compared to that of demethyloleuropein in most olive varieties, it is possible to assume that in vivo hydrolysis of oleuropein could render DGO by means of an endogenous methylesterase acting on the initial products of the hydrolysis.

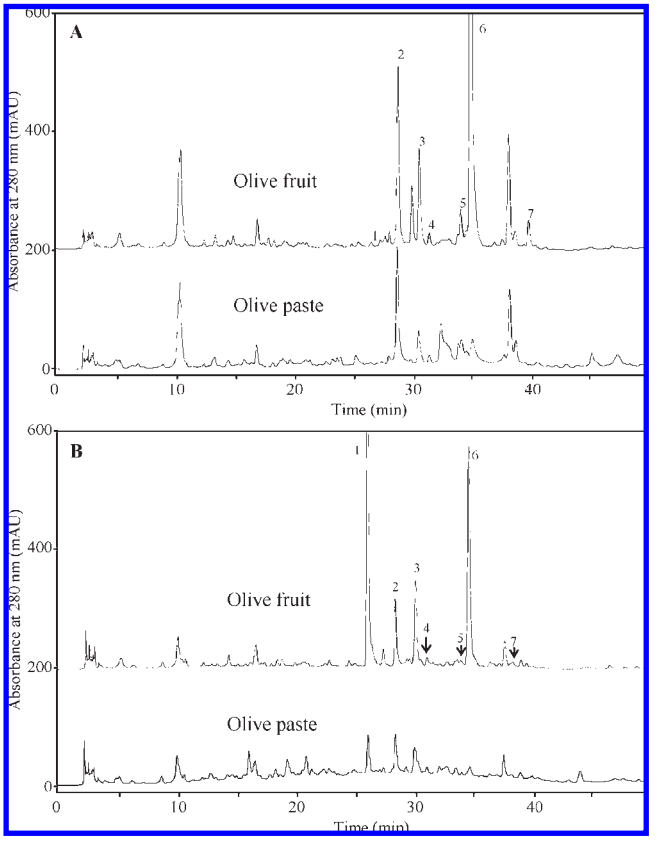

Figure 5. Phenolic profiles of Picual (**A**) and Arbequina (**B**) olive fruits and the pastes obtained immediately after milling of the fruits in the process to obtain virgin olive oil. Peaks: 1, demethyloleuropein; 2, verbascoside; 3, luteolin-7glucoside; 4, rutin; 5, apigenin-7-glucoside; 6, oleuropein; 7, ligstroside.

Because pNPG and oleuropein were the best substrates among synthetic and natural glycosides tested, they were selected for kinetic studies with the purified enzyme. The calculated K_m for pNPG was 61.5 mM, a value higher than those reported for other plant β-glucosidases (*24*, *25*, *28*), and the V_{max} 172.4 U/mg. When kinetic studies were carried out with the natural substrate oleuropein, the K_m value was 3.8 mM and the V_{max} was 2500 U/mg. Therefore, olive β-glucosidase specificity on oleuropein resulted in >200-fold higher compared to pNPG, calculated from V_{max}/K_m ratios of respective substrates. In a similar way, the catalytic efficiency of olive β-glucosidase on oleuropein hydrolysis seems to be significantly higher than that of commercial almond β-glucosidase, with calculated K_m and V_{max} values of 4.6 mM and 0.003 U/mg, respectively (*29*). The K_m value obtained with oleuropein, lower than those found for vainilla or blood orange β-glucosidases on their natural substrates (*24*, *28*), points out the in vivo role of the purified β-glucosidase as a key enzyme involved in the hydrolysis of olive phenolic glucosides during the process to obtain VOO.

The effects of metal ions and typical β-glucosidase inhibitors on the purified olive enzyme were also assessed (**Table 3**). Among the metal ions tested, Hg^{2+} and Ag^{+} were those causing the most potent inhibition. No β-glucosidase activity was detected after 1 h of incubation at Hg^{2+} and Ag^{+} ion concentrations of 1 mM. Both ions have been described as powerful inhibitors of most studied plant β-glucosidases (*10*), presumably due to chelation by the active site carboxylate groups. A certain inhibition was also found when the enzyme was preincubated with Cu^{+} (1 mM), showing a 21% residual activity after 24 h of incubation. Little effect was

Table 3. Effects of the Addition of Various Substances on the Relative Activity of β-Glucosidase from Olive Fruit (*Olea europaea* Cv. Picual)

substance	relative activity (%) after 1 h	relative activity (%) after 24 h
1 mM Hg^{2+}	0	0
1 mM Ag^{+}	0	0
1 mM Cu^{2+}	100	21
1 mM K^{+}	100	105
1 mM Mg^{2+}	100	106
1 mM Ca^{2+}	99	96
1 mM Zn^{2+}	99	97
15 mM glucose	100	100
15 mM trealose	100	100
15 mM δ-gluconolactone	100	100

observed with other ions tested. Olive β-glucosidase activity was not affected after incubation with inhibitors such as glucose, trehalose, and δ-gluconolactone (**Table 3**). However, olive β-glucosidase was inhibited by δ-gluconolactone by 50% when this sugar analogue was present in the reaction medium at a concentration of 15 mM. On the contrary, no inhibition was observed in similar experiments with glucose or trehalose.

Although further technological and molecular studies will be required, preliminary data on the characterization of the purified olive β-glucosidase point to this enzyme as the main biochemical factor affecting the metabolism of phenolic compounds during olive fruit processing. A deeper knowledge of specific aspects such as the enzyme catalytic mechanism, its turnover, or genetic variability will provide critical information to fully understand VOO phenolic biosynthesis and may allow new technological applications to modulate phenolic metabolism during olive fruit processing to obtain VOO.

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