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# Production of Glucose and Bioactive Aglycone by Chemical and Enzymatic Hydrolysis of Purified Oleuropein from *Olea Europea*

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## ABSTRACT

Pure-grade oleuropein, a bitter, hypotensive, phenolic glucoside, was obtained from organic extracts of olive plant leaves by two chromatographic steps. The purified compound was characterized by spectroscopic NMR and FAB-MS methods. The glucoside underwent chemical and enzymatic hydrolysis. Aglycone was characterized by spectroscopic methods ($^1$H-NMR and FAB-MS). Glucose was measured by enzymatic methods. The enzymatic hydrolysis of oleuropein was carried out by a soluble β-glucosidase. The reaction was characterized in terms of kinetic parameters, optimal pH value, activation energy, inhibition constant by glucose, and thermal stability. Preliminary experiments were also performed in a continuous-flow ultrafiltration membrane reactor. The cut-off of the membrane was lower than the molecular-weight of the enzyme, thus determining β-glucosidase confinement within the reactor. Under these conditions, β-glucosidase had a good long-term stability. This is an encouraging result in view of possible industrial applications.

**Index Entries:** oleuropein, aglycone, chromatography, chemical and enzymatic hydrolysis, spectroscopy, immobilized β-glucosidase.

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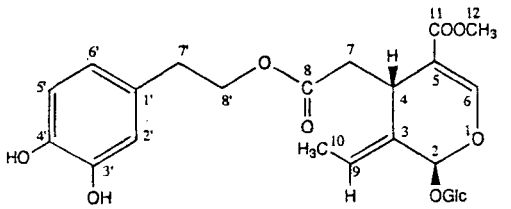
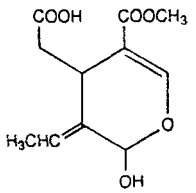
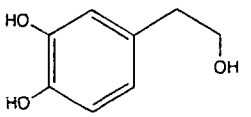


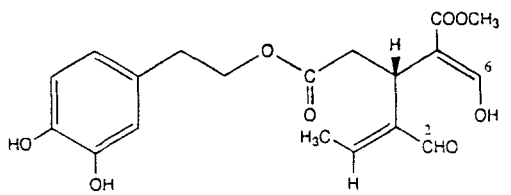
1 Oleuropein



2 Elenolic acid



3 Hydroxytyrosol



4 Aglycone

Fig. 1. Structures of oleuropein (**1**) and its derivatives elenolic acid (**2**), hydroxytyrosol (**3**), and aglycone (**4**).

## INTRODUCTION

Oleuropein (**1**, Fig. 1) is a phenolic compound which accumulates in olive (*Olea europea*) fruits and leaves (*1,2*). As elucidated by Panizzi et al. (*1*), **1** is an hydroxytyrosol (3,4-dihydroxyphenylethanol) ester with a β-glucosilated elenolic acid. It has an intense bitter taste and high browning capacity (*2*). Relevant hypotensive dose-dependent effects and protection against induced arrhythmias, shown in rats by various macerates and tinctures of *Olea europea*, were correlated with oleuropein content in bud and leaf preparations (*3*). Furthermore, high concentrations of oleuropein and its derivative compounds seem to confer to *Olea europea* part of its resistance to microbe and insect attack (*4*). The inhibitory effect of oleuropein to lactic acid bacteria and to several other microorganisms involved in olive fermentation was speculated by Vaughn (*5*) and subsequently demonstrated by Juven et al. (*6*). Further studies (*7,8*) indicate that ole-

uropein hydrolysis products have a much more marked inhibitory effect than oleuropein.

Chemical hydrolysis of **1** produces glucose, elenolic acid (**2**), hydroxytyrosol (**3**), and the aglycone (**4**) (Fig. 1). On the contrary, enzymatic hydrolysis by β-glucosidase (β-D-Glucoside glucohydrolase, EC 3.2.1.21) gives rise to glucose and aglycone (*9*). (**4**), as bitter as oleuropein, shows antibiotic properties against *Pseudomonas syringae* pv. *savastanoi* (Cristinzio G., private communication), the causal agent of olive knot (*10*). As suggested by Cruess and Alsberg (*11*), it could be naturally produced by an endogenous β-glucosidase.

The chemical hydrolysis of oleuropein, however, must be carried out under severe physico-chemical conditions as described in the literature (*1,9*) and is associated with low yields (*9*). Side products other than glucose and aglycone are also present, thus reducing process selectivity. On the contrary, enzymatic hydrolysis of **1** occurs under mild conditions with complete conversion into glucose and **4**.

Possible technological and biotechnological developments of the hydrolysis reaction deserve specific consideration. Indeed, the aglycone (**4**) of **1** is interesting for its antimicrobial activity. Glucose is of obvious appeal, mainly in view of alimentary applications. Furthermore, the natural source of oleuropein, i.e., olive leaves, is abundant, easily available, and is regarded as a waste material.

In the present work, β-glucosidase hydrolysis of oleuropein is afforded. A simple chromatographic purification procedure of oleuropein is also reported. Moreover, new spectroscopic data of the glucoside and its aglycone are described.

Preliminary results on the use of the immobilized enzyme in an ultrafiltration reactor for the continuous production of glucose and aglycone are shown.

## EXPERIMENTAL

### Extraction, Purification and Spectroscopic Characterization of Oleuropein

Solvents and reagents were of analytical grade and purchased from Carlo Erba (Milan, Italy), Fluka (Buchs, Switzerland), and Merck (Milan, Italy).

Crude oleuropein was extracted from leaves of olive plant (*Olea europea*) according to the four-step procedure described by Panizzi et al. (*1*).

Crude oleuropein (2.5 g) were chromatographed through a column (6 × 48 cm) packed with silica-gel (Merck, Kieselgel 60, 0.040-0.063 μm) eluted under medium pressure (20 bar) with a mixture of *n*-hexane-ethyl acetate-methanol 20:60:20, at a flow rate of 14 mL/min.

Table 1
FAB Mass Spectrometry Data in Positive Ions of Oleuropein (1) and Its Aglycone (4)

| 1 (*m/z*) | 4 (*m/z*) |
|---|---|
| 541 $[MH]^+$ | 379 $[MH]^+$ |
| 379 $[AglyconeH]^+ \equiv [MH\text{-}(glucose\text{-}H_2O)]^+$ | 361 $[MH\text{-}H_2O]^+$ |
| | 225 $[(Elenolic\ acid\text{-}H_2O)H]^+ \equiv [MH\text{-}hydroxytyrosol]^+$ |
| 361 $[(Aglycone\text{-}H_2O)H]^+$ | 207 $[(Elenolic\ acid\text{-}2H_2O)H]^+$ |
| 225 $[(Elenolic\ acid\text{-}H_2O)H]^+$ | |
| 207 $[(Elenolic\ acid\text{-}2H_2O)H]^+$ | |
| 165 $[(Elenolic\ acid\text{-}AcOH)H]^+$ | |
| 137 $[(Hydroxytyrosol\text{-}H_2O)H]^+$ | |

Fractions (7-mL) were collected and analyzed by means of TLC (Merck, Kieselgel $F_{254}$, 0.25 mm; eluents: *n*-hexane-ethyl acetate-methanol, 30:60:10; detectors: UV light selected at 254 nm, 10% sulphuric acid in MeOH, and 5% phosphomolybdic acid in EtOH or 1% $FeCl_3$ in MeOH followed by heating at 105°C for 10 min). Homogeneous fractions showing the same $R_f$ as a control sample of pure oleuropein, were pooled and the residue was rechromatographed in the conditions described above, yielding 0.7 g of pure oleuropein.

The purity and identity of the product were determined by reverse-phase TLC (Whatman, $KC_{18}$ $F_{254}$, 0.2 mm, eluent: water-acetonitrile, 70:30, detectors: same as already described), by infrared spectroscopy (FT-IR, Perkin Elmer, 1760x in neat or chloroform), and ultraviolet spectroscopy (UV, Perkin Elmer 550S in methanol), by electronic ionization (EI-MS, Fisons, Trio 2000 quadrupole, 70 eV), fast atom-bombardment mass-spectrometry (FAB—MS, VG ZAB 2SE, Cs atoms at 8 kV; glyc. + thioglyc.) and by magnetic resonance spectrometry ($^1H$- and $^{13}C$-NMR at 270 or 400 MHz and 67.92 or 100 MHz respectively; Bruker AC 270 or AM 400 WHz). MS-, $^1H$- and $^{13}C$-NMR data are summarized in Table 1, Table 2, and Table 3, respectively.

## Chemical Hydrolysis: Identification of Aglycone (4), Hydroxytyrosol (3), and Glucose

Ten milliliters of 18.5 m*M* oleuropein in 1N $H_2SO_4$ was kept at 55°C under stirring. The course of chemical hydrolysis was monitored by TLC every 30 min, using oleuropein obtained as described above, aglycone, and hydroxytyrosol as pure, reference compounds. Aglycone was obtained from β-glucosidase hydrolysis of oleuropein; hydroxytyrosol had been isolated previously from olive vegetation waters (*12*). After 3 h oleuropein was completely hydrolyzed into glucose, elenolic acid, and hydroxytyrosol.

Table 2
$^1$H-NMR Data of Oleuropein (**1**) and Its Aglycone (**4**)[a]

| | **1** ($DMSO_{d6}$)[b] | | | **4** ($CDCl_3$) | | |
|---|---|---|---|---|---|---|
| | $\delta$ | | $J$ (Hz) | $\delta$ | | $J$ (Hz) |
| H-2 | 5.87 | s | | 9.53 | d | 2.9=6.7 |
| H-4 | 3.85 | dd | 4,7$_A$=4.0; 4,7$_B$=9.0 | 3.40 | dd | 4.7$_A$=3.2; 4.7$_B$=9.6 |
| H-6 | 7.53 | s | | 7.59 | s | |
| H-7$_A$ | 2.62 | dd | 4,7$_A$=4.0; 7$_A$, 7$_B$=14.0 | 2.88 | dd | 4.7$_A$=3.2; 7$_A$, 7$_B$=16.2 |
| H-7$_B$ | 2.40 | dd | 4,7$_B$=9.0; 7$_A$, 7$_B$=14.0 | 2.55 | dd | 4.7$_B$=9.6; 7$_A$, 7$_B$=16.2 |
| H-9 | 5.97 | q | 9,10=6.5 | 4.47 | dq | 2.9=9, 10=6.7 |
| 3H-10 | 1.64 | d | 9,10=6.5 | 1.41 | d | 9,10=6.7 |
| 3H-12 | 3.64 | s | | 3.75 | s | |
| H-2′ | 6.60 | d | 2′,6′=1.8 | 6.78 | d | 2′,6′=1.9 |
| H-5′ | 6.63 | d | 5′,6′=7.9 | 6.81 | d | 5′,6′=8.0 |
| H-6′ | 6.47 | dd | 5′,6′=7.9; 2′,6′=1.8 | 6.62 | dd | 2′,6′=1.9; 5′,6′=8.0 |
| 2H-7′ | 2.68 | t | 7′,8′=7.0 | 2.82 | t | 7′,8′=6.3 |
| 2H-8′ | 4.03 | m | | 4.27 | m | |
| OH | 8.73 | br s | | | | |
| H anomeric | 4.64 | d | 7.7 | | | |

[a]The chemical shifts are in $\delta$-values (ppm) from TMS.
[b]In agreement with literature data (**4**).

Table 3
$^{13}$C-NMR Data of Oleuropein in $DMSO_{d6}$, Using the Same Solvent as Internal Standard[a]

| | $\delta$ | m[b] | | $\delta$ | m[b] |
|---|---|---|---|---|---|
| C-2 | 92.9 | d | C-1′[c] | 128.4 | s |
| C-3[a] | 129.1 | s | C-2′[c] | 116.2 | d |
| C-4 | 30.2 | d | C-3′[c] | 145.2 | s |
| C-5 | 107 | s | C-4′[c] | 143.8 | s |
| C-6 | 153.5 | d | C-5′[c] | 115.6 | d |
| C-7 | 33.1 | t | C-6′ | 119.6 | d |
| C-8 | 170.7 | s | C-7′[b] | 39.7 | t |
| C-9 | 123.1 | d | C-8′ | 65.1 | t |
| C-10 | 13.1 | q | 1-Glc[d] | 99.0 | d |
| C-11 | 166.2 | s | 2-Glc | 73.3 | d |
| C-12 | 51.3 | q | 3-Glc | 76.5 | d |
| | | | 4-Glc | 69.9 | d |
| | | | 5-Glc | 77.4 | d |
| | | | 6-Glc | 61.1 | t |

[a]The attributions were in agreement with the data reported for **1** (**4**), in which the chemical shift values were not assigned to each carbon, and with literature data (*14*).
[b]Multiplicity was determined by DEPT (Distortionless Enchancement by Polarization Transfer) spectrum (*14*) in which the signals of C-7, that was masked in the PND spectrm from the $DMSO_{d6}$ signal, appeared.
[c]These attributions may be exchanged.
[d]The carbon shifts of the sugar moiety were in full agreement with those observed for the methyl-*O*-$\beta$-glucoside when the $^{13}$C-NMR spectrum of this compound was recorded in the same solvent (*15*).

## Enzymatic Hydrolysis

In the enzymatic tests, a commercial β-glucosidase from sweet almonds was used without further purification (Sigma Chimica Divisione Italia, Milan, Italy). The synthetic substrate, *p*-nitrophenyl-β-D-glucopyranoside (PNBG), was also supplied by Sigma.

### *Preliminary Experiments: Isolation and Characterization of Aglycone (4) and Enzymatic Determination of Glucose*

For this experiment, 18.5 m*M* oleuropein in 1 mL 0.1*M* acetate buffer, pH 4.2 was incubated at 37°C with different enzyme amounts, ranging from 1 to 2.2 mg. After a suitable incubation time, the reaction mixture was extracted with 1 mL of chloroform. The latter procedure was iterated six times. The extract was evaporated to dryness, yielding a homogeneous residue (6 mg). This was identified and characterized as the aglycone (**4**) by means of spectroscopic and spectrometric analyses, using the same methods described above for **1**. The corresponding FAB-MS and $^1$H-NMR data are reported in Table 1 and 2, respectively. Glucose was measured by an enzymatic kit (Hexokinase + Glucose-6-phosphate dehydrogenase, Boehringer Mannheim, Germany).

### *Free-Enzyme Characterization*

Soluble enzyme assays were performed at 37°C in a reaction vessel containing 300 mL of substrate dissolved in 0.1*M* sodium-acetate buffer at pH 5.5. Substrate concentrations were 18.5 m*M* (maximum concentration achievable in buffer) and 10 m*M* for oleuropein and PNBG, respectively. In all experimental runs a suitable protocol (in terms of enzyme amounts and reaction time) was followed, in order to keep conversion levels within 5%. Therefore, all the data points within the same experimental run refer to the same product and substrate concentrations. The reaction was stopped by adding $Na_2CO_3$ (1*M*) in the ratio of 1:2. In the case of oleuropein, glucose concentration was determined as described above. For the synthetic substrate assays, product concentration was determined by reading the absorbance of *p*-nitrophenol at 405 nm ($\epsilon$ = 17.2 m$M^{-1}$ cm$^{-1}$) with an Ultrospec II spectrophotometer (Pharmacia, LKB, Denmark). One unit of enzymatic activity was defined as the enzyme amount producing one millimole of product per minute at 37°C and pH 5.5.

Kinetic tests were carried out at 37°C at different substrate concentrations, in 0.1*M* sodium acetate buffer, pH 5.5. The kinetic parameters $K_M$ and $V_{max}$ were calculated by nonlinear regression, according to the Michaelis Menten relationship.

The effect of temperature on enzyme activity was determined by carrying out enzymatic runs with 18.5 m*M* oleuropein in 0.1*M* sodium acetate buffer, pH 5.5, at reaction temperatures ranging from 15 to 37°C. This range was chosen in order to rule out thermal deactivation effects.

The activity-pH profile was obtained at 37°C with 18.5 m*M* oleuropein at different pH values in the range 3–7. Different 0.1*M* buffers were used. Namely: glycine/HCI (pH 3–3.5), sodium acetate (pH 3.6–5.5) and sodium phosphate (pH 5.6–7.5).

In order to check for possible glucose inhibition, experiments were carried out at 37°C at different PNBG concentrations in 0.1*M* sodium/acetate buffer, pH 5.5. Runs were performed with different, initial glucose concentrations, ranging from 20 to 200 m*M*. It should be noted that the synthetic substrate (PNBG) was used in place of oleuropein. This is due to the determination of product concentration being much easier than that of aglycone. The absolute specifity of β-glucosidase action towards the β-glucosidic bond, independently of the nonglucosidic moiety of the substrate, allows the results to be quite pertinent for oleuropein, as well.

### *Immobilized Enzyme Characterization*

The experimental apparatus consisted of an unstirred ultrafiltration membrane reactor (of approximately 4 mL volume). It is described in detail elsewhere (*13*). The reactor was equipped with an ultrafiltration membrane (RC70, DDS Filtration, Denmark) whose molecular weight cut-off was such as to reject the protein completely. The reactor was immersed in a thermostatic bath at 37°C and connected to a nitrogen-pressurized vessel containing the substrate solution.

Enzyme immobilization onto the membrane was achieved by injecting 1 mL of 1 mg/mL β-glucosidase solution in 0.1*M* sodium acetate buffer, pH 5.5, into the reactor.

Experiments were performed by feeding the system with 10 m*M* oleuropein solution in 0.1*M* sodium acetate buffer at pH 5.5 at a flow rate of 10 mL/h. Permeate samples were collected at predetermined time-intervals and assayed for product concentration by means of enzymatic analyses.

The long-term stability of β-glucosidase was investigated within the ultrafiltration membrane reactor, in the absence of substrate. The cell was loaded with 1 mg of enzyme, kept at 37°C, and continuously fed with 0.1*M* sodium acetate buffer at pH 5.5 at a flow rate of 15 mL/h. The feed stream was switched periodically to 10 m*M* PNGB in the same buffer. The synthetic substrate was used in place of oleuropein for obvious economic reasons.

## RESULTS AND DISCUSSION

### Oleuropein Purification

Oleuropein extraction, according to the procedure described by Panizzi et al. (*1*), produced 12.6 g per kg of leaves. Purification by a two-step medium pressure chromatographic method, yielded 0.28 g of pure **1** per g

of crude product from leaves. This method is faster and simpler than those described in the literature. Indeed, Kubo et al. (*4*) and Walter et al. (*9*) purified **1** by four chromatographic steps, using drop countercurrent and thin layer chromatography (DCCC and TLC).

The purity and identity of **1** were assessed by TLC control and, mainly, by spectroscopic analyses. In fact, UV, IR EI-MS, $^1H$– and $^{13}C$-NMR data, agreed with those described in the literature (*4,9*) for **1**. In addition, Table 1 lists the positive ions of fundamental structural components of the oleuropein molecule observed in the FAB-MS spectrum, and Table 2 reports the assignments of $^{13}C$ chemical shift values to each carbon. In fact, the FAB mass spectrum shows a peak at *m/z* 541 (corresponding to the protonated molecular ion of oleuropein) and two peaks at *m/z* 379 and 361 (corresponding to the protonated aglycone ion and to the same ion minus $H_2O$, respectively). Moreover, the peaks at *m/z* 225, 207, and 165 corresponding to the protonated ion of elenolic acid minus $H_2O$, to the same ion minus two $H_2O$ molecules and to the same ion minus acetic acid, respectively, were present. A further peak at *m/z* 137, corresponding to the protonated ion of hydroxytyrosol minus $H_2O$, was also present. Finally, the presence of glucose in the molecule was inferred by evaluating the mass of the neutral molecule (162 amu) that was lost in the fragmentation of the molecular protonated ion at *m/z* 541 to yield the ion at *m/z* 379.

These data indicate that this ionization technique could be used as a simple, highly sensitive, and rapid analytical method to check the purity and identity of oleuropein.

## Oleuropein Hydrolysis

The chemical transformation of **1** into **4** and glucose by acid hydrolysis was performed in milder conditions than those described in the literature (*1,11*). In these conditions, after 1.5 h, **1** was only partially transformed into **4** and glucose. Correspondingly, **4** was partially transformed into hydroxytyrosol (**3**) and elenolic acid (**2**). After 3 h the complete transformation of **1** into **2, 3,** and glucose took place.

The enzymatic transformation of **1** by β-glucosidase had to be taken into consideration, since the chemical hydrolysis can only give rise to a partial conversion of oleuropein in aglycone and glucose, associated with the formation of side products such as hydroxytyrosol and elenolic acid.

The enzymatic hydrolysis produced only **4** and glucose. The aglycone was identified and characterized by $^1H$-NMR and FAB mass spectrometry analyses. In Table 2, $^1H$-NMR data of **4** are listed in comparison with those of **1**. The latter data are fully consistent with the structure assigned to **4**.

In particular, the presence of the doublet at $\delta$ 9.53, typical of an aldehyde group and the appearance of H-9 as a double quartet, while H-4 remained a double doublet as in **1**, allowed to attribute the structure **4** to the aglycone as reported in Fig. 1, and to rule out the two possible alter-

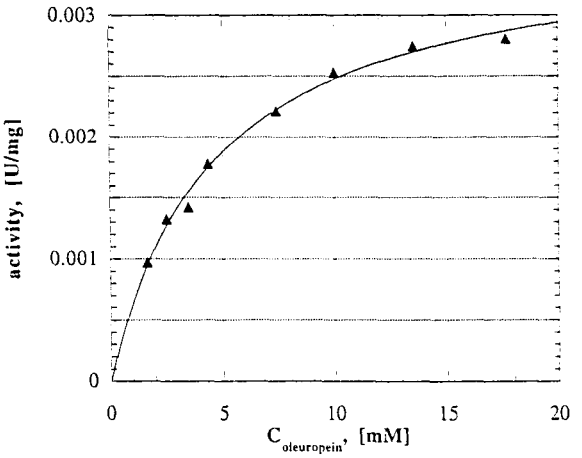



Fig. 2. Kinetics of oleuropein hydrolysis by β-glucosidase. Experimental conditions: T = 37°C, pH = 5.50 in 0.1*M* Na-acetate buffer.

Table 4
Characterisation of Native β-glucosidase

| | |
|---|---|
| $V_{max}$ (U/mg) | $3.6 \cdot 10^{-3}$ |
| $K_m$, (m*M*) | 4.66 |
| Optimal pH | 5.5 |
| Activation Energy (kJ/mole) | 80 |
| $K_i$ (m*M*) | 390 |

native structures such as the emiacetal and the dialdehyde forms (*17*). **4** was also characterized by positive ion FAB-MS. As shown in Table 1, the data are consistent with the protonated molecular ion of **4** and the fundamental structural components: elenolic acid (**2**) and hydroxytyrosol (**3**). In fact, two peaks were present at *m/z* 379 and 361, corresponding to the protonated molecular ion of oleuropein aglycone and to the same minus $H_2O$, respectively. Further peaks were registered at *m/z* 225 and 207. These correspond to the protonated ion of elenolic acid minus $H_2O$ and to the same ion minus two $H_2O$ molecules, respectively. The presence of the hydroxytyrosol moiety was inferred as a neutral molecule loss of 154 amu, arising from the fragmentation of the protonated molecular ion at 379 *m/z* to yield the ion at *m/z* 225.

## Kinetic Characterization of the Enzymatic Hydrolysis

A first set of runs was devoted to the determination of the kinetic parameters of oleuropein enzymatic hydrolysis. Enzyme activity was measured at 37°C, at different substrate concentrations. Figure 2 shows the results, in terms of enzyme activity vs oleuropein concentration.

The kinetic parameters, calculated by nonlinear regression following the Michaelis-Menten model, are reported in Table 4.

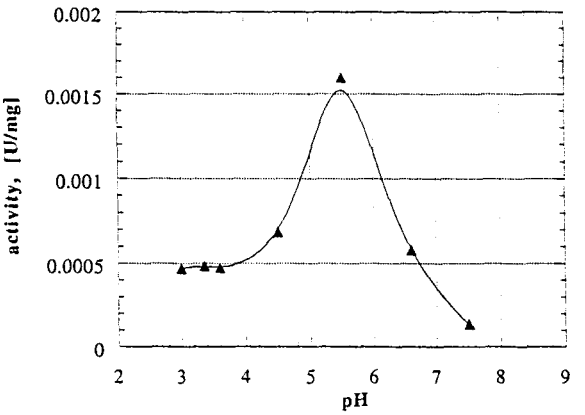



Fig. 3. Activity-pH profile of oleuropein hydrolysis by β-glucosidase. Experimental conditions: T = 37°C, oleuropein concentration = 18.5 m*M*.

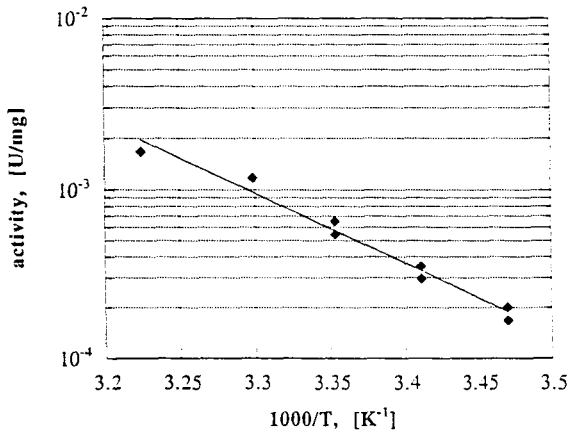



Fig. 4. Arrhenius plot of oleuropein hydrolysis by β-glucosidase. Experimental conditions: pH = 5.50 in 0.1*M* Na-acetate buffer, oleuropein concentration = 18.5 m*M*.

The $K_M$ value was lower than that found by Iborra et al. (*16*) using picrocrocin (the glycosilate precursor of safranal) as a substrate, and indicated a higher affinity of β-glucosidase towards oleuropein. The results reported in Fig. 3 and Table 4 show an optimal pH value of 5.5 in good agreement with that determined by Iborra et al. (*16*).

Experiments were carried out in order to calculate the activation energy of the hydrolysis reaction. The results, arranged in terms of Arrhenius plot, are shown in Fig. 4. The activation energy, calculated by nonlinear regression, is 80 kJ $mole^{-1}$ (Table 4). It is in good agreement with the value calculated by other authors (*16*).

A set of experimental runs was performed in order to analyse glucose inhibition effect on enzyme activity. The results are reported in Fig. 5, in terms of activity vs substrate concentration.

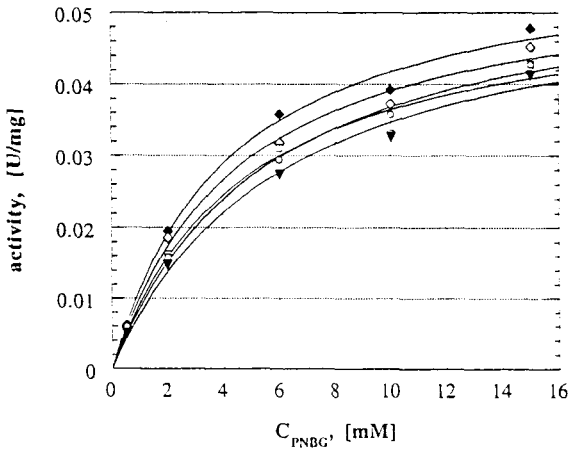



Fig. 5. Glucose inhibition on PNBG hydrolysis by β-glucosidase. Experimental conditions: T = 37°C, pH = 5.50 in 0.1*M* Na-acetate buffer. Glucose concentrations (m*M*): ■ (0), ✧ (20), □ (50), ○ (80) and ▼ (200).

The value of $V_{max}$ does not depend on glucose concentrations tested, whereas the apparent $K_m$ increases with increasing glucose concentration. This indicates competitive inhibition. The inhibition constant, $K_i$, was estimated as 390 m*M*. This is in good, general agreement with the results by Iborra et al. (*16*). The authors found a $K_i$ value of 175 m*M* for glucose inhibition in picrocrocin hydrolysis by the same β-glucosidase.

These results suggest that glucose inhibition might be negligible for oleuropein hydrolysis, under the experimental conditions discussed in the present work. Indeed, it should be remembered that oleuropein concentration was 18.5 m*M* at most, due to solubility problems.

As regards immobilized enzyme performance, an oleuropein conversion of approx 60% was achieved in the ultrafiltration membrane reactor. Experimental conditions were: enzyme amount 1 mg, reaction temperature 37°C, 10 m*M* oleuropein concentration, pH 5.5, volumetric flow rate of 10 mL/h.

The long-term stability of the enzyme was evaluated, in view of possible industrial applications of the process. Figure 6 shows the experimental results corresponding to 40 d of continuous operation with PNBG feed at 37°C. In the time-range explored, the data follow irreversible first order deactivation kinetics. The deactivation constant is 0.0614 $d^{-1}$, corresponding to an enzyme half-life time of 11 d.

## CONCLUSIONS

Oleuropein was purified from olive leaves by a simple and rapid purification process. A fast and reliable identification of the glucoside and

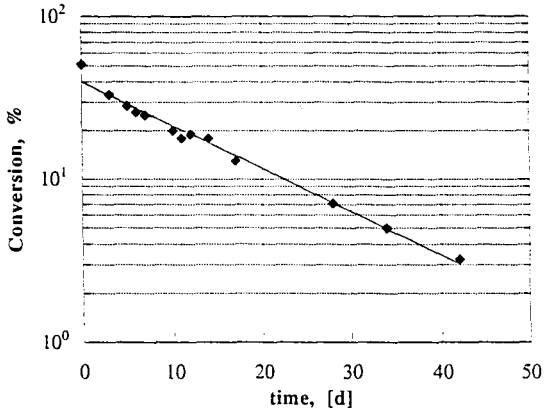



Fig. 6. Long-term stability of β-glucosidase within an UF-membrane reactor. Experimental conditions: T = 37°C, pH = 5.50 in 0.1*M* Na-acetate buffer, PNBG concentration = 10 m*M*.

its aglycone, obtainable by both chemical and enzymatic hydrolysis, was achieved by TLC and spectroscopic analyses. The enzymatic hydrolysis of the glucoside by a free β-glucosidase was characterized in terms of Michaelis-Menten parameters, optimal pH value, activation energy, inhibition constant by glucose and thermal stability.

Immobilization of the enzyme within an ultrafiltration device yielded satisfying oleuropein conversion levels and the immobilized enzyme showed a good long-term stability.

In conclusion, the straightforward oleuropein production from olive leaves, the fast and reliable identification of the glucoside and its aglycone by TLC and spectroscopic analyses, the good immobilised β-glucosidase long-term stability suggest reasonable possibilities for further, biotechnological developments of the process.

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