Synthesis of the Antioxidant Hydroxytyrosol Using Tyrosinase as Biocatalyst

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Hydroxytyrosol (HTyr), a natural ortho-diphenolic antioxidant with health-beneficial properties that mainly occurs in virgin olive oil and olive oil mill waste waters (also known as vegetative waters), has been enzymatically synthesized using mushroom tyrosinase. This *o*-diphenol (not commercially available) was obtained from its monophenolic precursor tyrosol (commercially available) in the presence of both tyrosinase and ascorbic acid. The reaction synthesis is continuous, easy to perform, and adaptable to a bioreactor for industrial purposes. The HTyr concentration is time-predicted, and the yield of reaction can be 100%. The synthesis method reported here is an alternative approach to obtain this compound in an environmentally friendly way.

Keywords: *Enzymatic synthesis; hydroxytyrosol; olive oil; vegetative waters; tyrosinase*

INTRODUCTION

Hydroxytyrosol (3,4-dihydroxyphenylethanol, HTyr) is one of the major natural phenolic compounds present in olive fruits and virgin olive oil (*1, 2*). HTyr can be found alone or as part of other molecules such as oleuropein (*3*). The phenolic fraction of virgin olive oils is responsible for the autoxidation stability (*4, 5*) and the characteristic bitter taste of these oils (*6*). There is extensive evidence relating the intake of virgin olive oil, present in the "Mediterranean diet", to protecttion from cardiovascular diseases and risk of certain cancers (*7*). HTyr has different properties such as antibacterial activity (*8*), scavenging of free radicals (*9, 10*), protection against oxidative DNA damage and LDL oxidation (*11*), prevention of platelet aggregation (*12*), and inhibition of 5- and 12-lipoxygenases (*13*, *14*).

Recently, the good bioavailability of HTyr has been reported, which encourages its inclusion in the diet (*15*, *16*). Therefore, there is a current trend in the study of hydroxytyrosol. As this compound is not commercially available, researchers should isolate or synthesize it for further studies.

Several methods have been used to obtain HTyr including chemical synthesis (*17*, *18*) usually utilizing 3,4-dihydroxyphenylacetic acid as precursor (*19*−*21*) or hydrolysis of oleuropein (*22*). Other authors have isolated the compound after several chromatographic steps from vegetative waters (*20*, *23*, *24*).

Tyrosinase (EC 1.14.18.1) is a polyphenol oxidase that catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The *o*-quinones produced are very reactive and can tan proteins by covalent bonds or autoxidize, producing brown pigments. However, in reducing conditions (excess of either ascorbic acid or NAHD, for instance), *o*-quinones are reduced to their *o*-diphenol precursors. This approach has been previously used to enhance the sensitivity in the determination of tyrosinase activity (*25*, *26*), as well as to better understand possible tyrosinase reaction products and their possible biological activity (*27*, *28*). Recently, Mosca et al. (*29*) reported an enzymatic assay for determining olive oil polyphenol content. This spectrophotometric assay method was also based on a substrate-recycling procedure that used the enzyme tyrosinase and, in this case, NADH as reducing agent.

The main aim of the work presented here is to develop a method to synthesize enzymatically HTyr using mushroom tyrosinase in a continuous and simple approach as an alternative to chemical synthesis.

MATERIALS AND METHODS

Reagents. Ascorbic acid (AA), dehydroascorbic acid (DHA), 3-methyl-2-benzothiazolinone hydrazone (MBTH), 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), and mushroom tyrosinase (3900 units/mg) were obtained from Sigma (St. Louis, MO). Tyrosol (4-hydroxyphenylethanol) was obtained from Aldrich (Milwaukee, WI). Oleuropein was obtained from Roth (Karlsruhe, Germany). All other reagents were of analytical grade and also supplied by Sigma. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Preparation of Tyrosinase. Commercial mushroom tyrosinase from Sigma was used without further purification. To check the influence of the possible mixture of tyrosinase isoforms in the commercial extract, the enzyme was partially purified according to the method of Espín and Wichers (*30*). A main isoform (isoelectric point of 4.1) was isolated (result not shown). The same results were obtained when both commercial extract and isolated isoform were assayed.

Formation of Hydroxytyrosol. EU regulations prohibit the full description (the "know-how") of the methodology, which is currently under patent application (no. P200002073), but a brief description follows. The standard reaction medium contained 16 mM tyrosol and several tyrosinase concentrations

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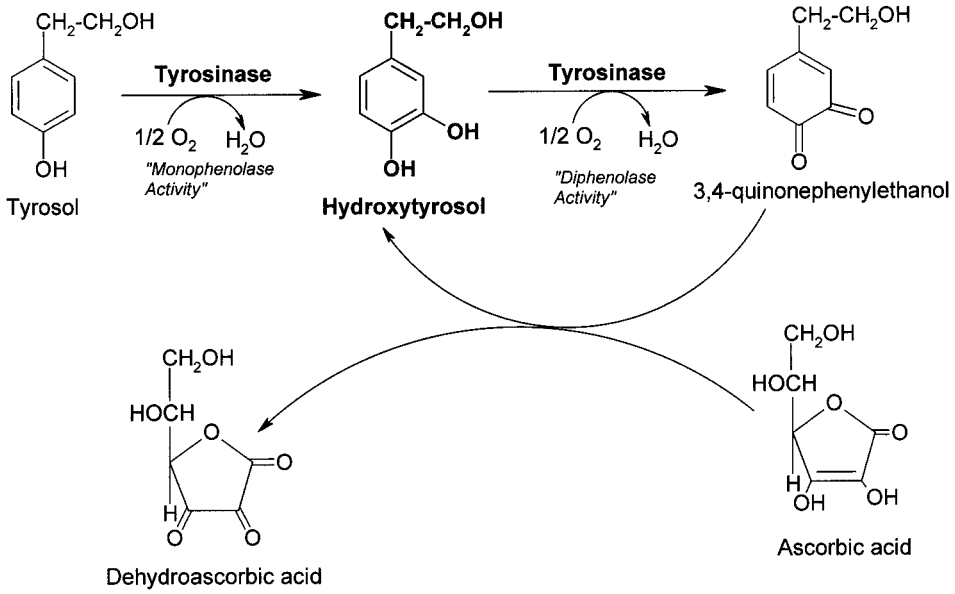

Figure 1. Formation of HTyr from its precursor tyrosol in the presence of both mushroom tyrosinase and AA.

(2.2−30 μg/mL) dissolved in 100 mM sodium phosphate buffer (PB) containing 30 mM AA with a final pH of 6.5. The reaction mixture was constantly bubbled with air. At different times, aliquots were taken and injected for monitoring the reaction by high-performance liquid chromatography (HPLC). HTyr was also isolated by using thin-layer chromatography (TLC) experiments.

Thin-Layer Chromatography. Silica gel 60 F254 TLC aluminum plates 20 × 20 cm (Merck, Darmstadt, Germany) were utilized to run TLC using as mobile phase a mixture of toluene/ethyl acetate/formic acid (50:40:10). HTyr was isolated by scratching out the corresponding band (R_f = 0.43) under UV light and extracted from the matrix with methanol. NMR analysis according to the procedure of Montedoro et al. (*31*) corroborated the purity of the band (results not shown).

A TLC sheet was stained with a 0.4 mM DPPH• solution in methanol to follow the change of initial purple color (DPPH•) to final yellow color (DPPH-H) because of the scavenging reaction between the free radical and the antiradical compounds (*10*, *32*). The R_f for HTyr was fully coincident with that determined under UV light. A standard of HTyr was prepared according to the procedure of García et al. (*22*), which was based on the alkaline hydrolysis of oleuropein.

High-Performance Liquid Chromatography Analysis. Reversed phase chromatography was carried out on an HPLC column (Novapak cartridge C18 4 μm, 8 × 100 mm, Waters) radially compressed by a special module using as mobile phase 0.1% (v/v) formic acid (A) and methanol (B) following a gradient from 0 to 10 min, 0−50% B (flow rate = 1.0 mL/min). Detection was performed by monitoring absorbance at 278 and 251 nm with a UV detector (Pharmacia, Uppsala, Sweden). AA and tyrosol peaks were identified on the basis of their retention times (RT) compared to standards (3.1 and 6.6 min, respectively).

Spectrophotometric Assays. Both spectra and kinetic assays were carried out in a UV-1603 Shimadzu spectrophotometer (Tokyo, Japan). Temperature was controlled at 25 °C with a CPS 240 Shimadzu temperature controller, checked using a precision of ±0.1 °C. The reference cuvette contained all of the components except the substrate with a final volume of 1 mL.

Monophenolase activity of tyrosinase on tyrosol and diphenolase activity of tyrosinase on HTyr were determined spectrophotometrically by using MBTH (*33*, *34*). This assay method is highly sensitive, reliable, and precise. MBTH traps the enzyme-generated *o*-quinones, rendering MBTH−quinone adducts with high molar extinction coefficients. Adduct accumulation was followed at 463 nm (ϵ = 22000 M^{-1} cm^{-1}). Two percent DMF (v/v) was added to improve adduct solubility with no effect on tyrosinase activity as previously described (*33*, *34*). The reaction medium for determining V_{max} and K_m values for both tyrosol and HTyr contained 50 mM sodium phosphate buffer, pH 6.5, 2% DMF, 4 mM MBTH, 0.1−4 mM tyrosol or HTyr, and 0.02 μg/mL mushroom tyrosinase.

Kinetic Data Analysis. The values of K_m and V_{max} for the monophenol tyrosol and the *o*-diphenol hyroxytyrosol were calculated from triplicate measurements of the steady-state rate for each initial substrate concentration. Nonlinear regression fitting of steady-state rate versus either tyrosol or HTyr concentration to the Michaelis equation (*35*) was carried out by using a Gauss−Newton algorithm (*36*) implemented in the Sigma Plot 2.01 program for Windows.

Protein Determination. Protein content was determined by using the method of Bradford (*37*) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Time Course of Hydroxytyrosol Formation. Mushroom tyrosinase catalyzed the ortho-hydroxylation of the monophenol tyrosol (monophenolase activity) to form the *o*-diphenol HTyr, which was oxidized in a reaction also catalyzed by tyrosinase (diphenolase activity) to give rise to the corresponding *o*-quinone (3,4-quinonephenylethanol) (Figure 1). This *o*-quinone was highly unstable and evolved nonenzymatically to yield brown pigments. To prevent this further evolution, ascorbic acid was added to the reaction. Under this assay condition, the system did not reach the steady state and the *o*-quinone was reduced to HTyr so that the balance of the reaction was a net accumulation of HTyr with a decrease of both tyrosol and AA concentrations (Figure 1). HTyr remained accumulated as long as AA was present in the medium. This reaction was monitored by HPLC. Figure 2 shows the evolution of the peaks at initial, intermediate, and final assay times. Both tyrosol and AA peaks decreased with the concomitant increase of HTyr peak (RT = 5.9 min). The appearance of DHA could not be simultaneously visualized because it required specific assay conditions (*38*) that interfered with the enzymatic reaction. The formation of HTyr followed a hyperbolic accumulation concomitant with the hyperbolic depletion of tyrosol (Figure 3). The hyperbolic response was observed when the initial substrate (tyrosol) became limiting in the reaction.

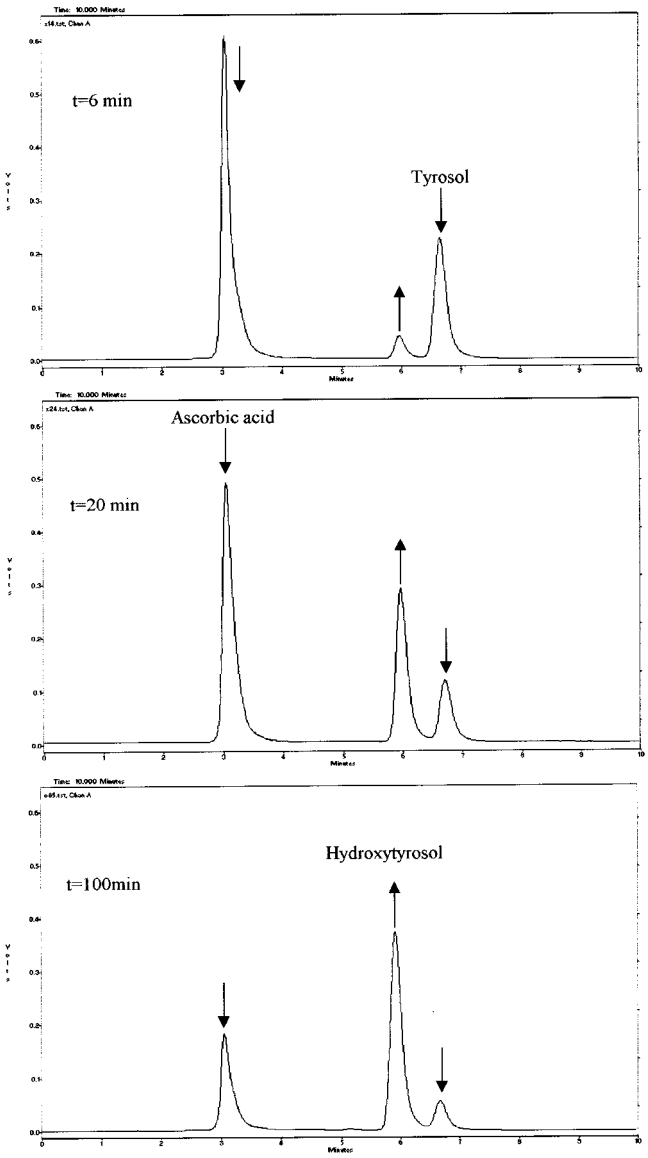

Figure 2. Detection by HPLC of HTyr formation. Conditions were as follows: 100 mM sodium phosphate buffer, 16 mM tyrosol, 30 mM AA, and 30 μg/mL mushroom tyrosinase. Arrows designate the evolution of the peak.

Dependence of Hydroxytyrosol Synthesis on Tyrosinase Concentration. The reaction rate of HTyr synthesis increased with increasing tyrosinase concentrations ([tyrosinase]) (Figure 4A). The higher [tyrosinase] assayed, the faster HTyr formation occurred. The initial synthesis rate of HTyr (V_0) was lineal before tyrosol was limiting in the medium (velocity determined in the first 15 min of the reaction) (Figure 4B). The elapsed time to generate half of the total HTyr concentration ($t_{1/2}$) uniexponentially decreased with increasing [tyrosinase], with limits at 0 and ∞ [tyrosinase]. Therefore, $t_{1/2} \rightarrow \infty$ when [tyrosinase] $\rightarrow$ 0, and $t_{1/2} \rightarrow 0$ when [tyrosinase] $\rightarrow \infty$. This means that at infinite [tyrosinase] the formation of HTyr is instantaneous (obviously, this situation is rather theoretical) and no HTyr is formed in the absence of tyrosinase. The analysis of this curve (Figure 4B) can be used to predict the time to synthesize a specific HTyr concentration under specific assay conditions (initial tyrosinase and tyrosol concentrations). The ideal system would involve the quickest possible reaction rate by balancing the amount of tyrosinase used. To increase the reaction rate as much as possible, a very large tyrosinase concentration should be used. Unfortunately, this would be unaffordable. However, this could be overcome, at least in part, by reusing the enzyme because no suicide inactivation, inhibition by product accumulation, inhibition by excess of substrate, or any other artifact occurred during the catalysis (results not shown). Tyrosinase was very stable at 25 °C with no significant loss of activity for many hours. The reutilization of the enzyme could be very interesting in the adaptation of the method for industry by means of a bioreactor and a nanofiltration system.

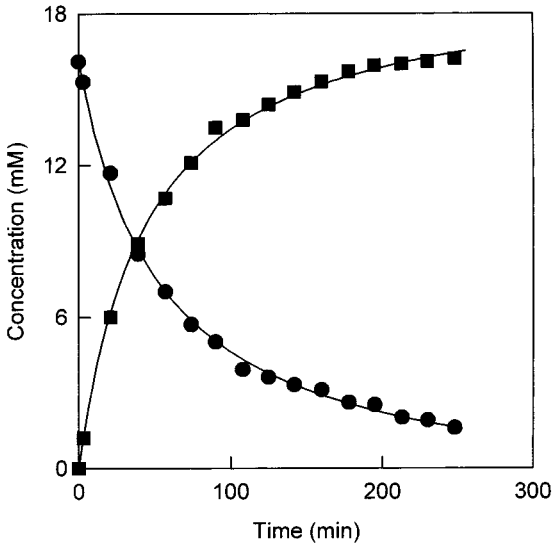

Figure 3. Time course of HTyr formation and tyrosol depletion: (●) tyrosol; (■) HTyr. Conditions were the same as in Figure 2.

Validation of the Assay Method. The reaction medium contained only three possible detectable species (tyrosol, HTyr, and AA) by HPLC in the specific assay conditions detailed under Materials and Methods (Figure 2). However, to fully validate the present enzymatic method, different tests were performed.

Detection and Isolation of Hydroxytyrosol by Thin-Layer Chromatography. TLC was another alternative way to isolate HTyr enzymatically synthesized. Aliquots of the standard reaction medium were applied to the TLC sheet, which was developed (see conditions under Materials and Methods) in the dark to avoid possible HTyr spontaneous oxidation by the presence of light. The band with R_f of 0.43 corresponding to HTyr was scratched off. The compound was extracted from the silica matrix with methanol, concentrated, and lyophilized.

The UV spectrum of HTyr obtained by enzymatic synthesis had two peaks of maximum absorbance at 218 and 278.8 nm with molar absorptivities of 4350 and 1950 M^{-1} cm^{-1}, respectively (results not shown). These results were in agreement with those previously reported elsewhere (*20*).

NMR analysis was carried out according to the method of Montedoro et al. (*31*) in chloroform-*d* and also corroborated that the compound enzymatically synthesized was pure HTyr with spectroscopic data (results not shown), in agreement with those previously reported by Capasso et al. (*20*). Therefore, the UV spectrum, RT in HPLC, and NMR analysis of the fraction indicated that it was pure HTyr.

The radical scavenger capacity of HTyr on DPPH• was used to easily detect HTyr on the TLC plate (*10*, *32*). A

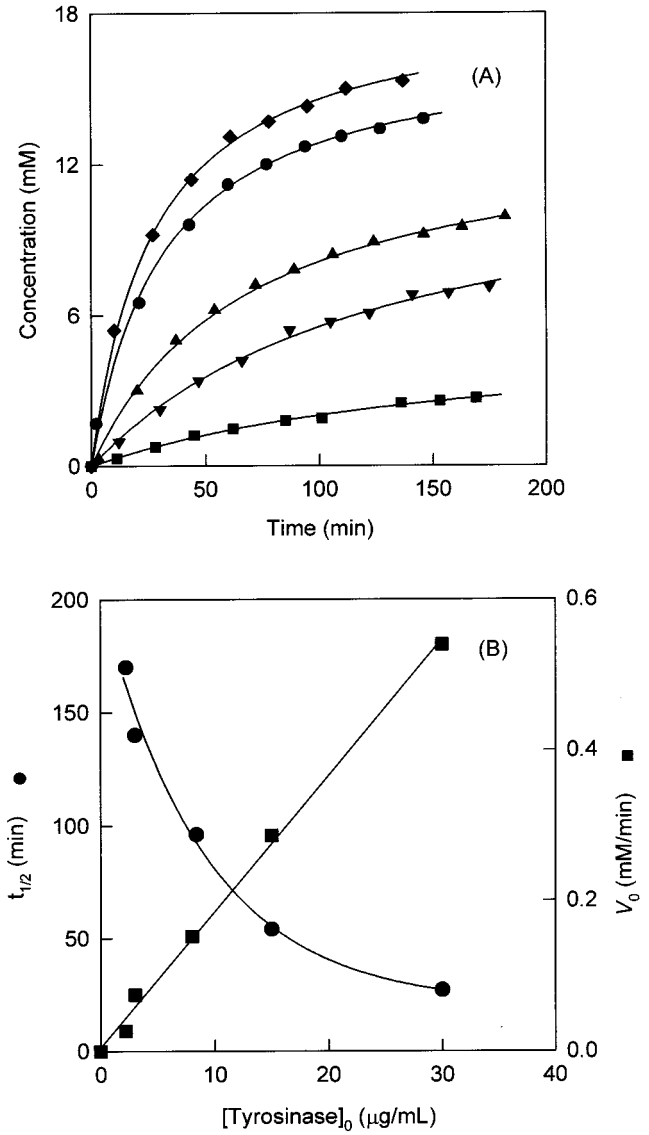

Figure 4. (A) Time course of HTyr formation with different tyrosinase concentrations. Conditions were as in Figure 2 and (■) 2.2, (▼) 3, (▲) 8, (●) 15, and (◆) 30 μg/mL mushroom tyrosinase. (B) Dependence of the elapsed time to generate half of the final HTyr concentration (●, $t_{1/2}$) and the initial velocity (■, V_0) of HTyr formation on tyrosinase concentration. Conditions were as in (A).

drop of 0.5 μL of the standard enzymatic reaction mixture (see Materials and Methods) was applied to the TLC plate every 15 min. The selected mobile phase (see Materials and Methods) separated the compounds. Afterward, TLC plates were stained with a DPPH• solution (Figure 5). The change from purple (DPPH•) to yellow color (DPPH-H) due to the antiradical capacity of the different compounds (*39*) was a very simple way to follow the reaction and to identify the bands. Tyrosol showed an R_f of 0.53, followed by HTyr (R_f = 0.43) (*10*), AA remained close to the application point with an R_f of 0.08, and DHA did not migrate in these conditions. R_f values were coincident with those detected under UV light. Tyrosol showed very low antiradical activity (*10*) so that it did not change significantly the background staining of the plate (purple color of DPPH•), and its presence was detected by a very faint yellow band on the top of the plate. HTyr had a higher antiradical capacity so that the yellow color became very evident (*10*). The intensity of the yellow color increased with time, which indicated the increasing formation of HTyr in the tyrosinase-catalyzed reaction (Figure 5). AA is also a well-known antiradical compound, so its presence was also detected. However, as AA is present in excess in the reaction, its decrease during the enzymatic reaction did not become very evident, so that no significant variation of the yellow color with time was observed (Figure 5).

MBTH Adduct Method. The oxidation of a *p*-monophenol/*o*-diphenol pair (phenol/catechol; L-tyrosine/L-dopa; tyramine/dopamine; tyrosol/hydroxytyrosol; etc.), catalyzed by tyrosinase, renders the same *o*-quinone (*o*-benzoquinone; *o*-dopaquinone; *o*-dopaminequinone; 3,4-quinonephenylethanol; respectively). If the reaction initially takes place in the presence of a *p*-monophenol (monophenolase activity), the *o*-diphenol is an intermediate with a constant concentration in the steady state of the reaction (*40*) (the velocity of formation of *o*-diphenol is equal to the velocity of its oxidation). Afterward, the *o*-diphenol is oxidized and the corresponding *o*-quinone is formed. If the reaction initially takes place in the presence of *o*-diphenol, then the reaction is faster (diphenolase activity) and tyrosinase directly catalyzes the oxidation of diphenol to form the *o*-quinone. Therefore, in both cases, the same *o*-quinone is generated (*40*) (Figure 6A). However, the usual instability of these *o*-quinones makes these assay methods, which measure *o*-quinone accumulation, unreliable (*33*, *34*). To solve this problem, the nucleophile MBTH can be used. MBTH is a potent nucleophile through its amino group, which attacks the enzyme-generated *o*-quinones to render stable adducts with high molar absorptivities (*33*, *34*). The spectrum of these adducts is characterized by specific isosbestic points, maximum wavelengths, molar absorptivities, etc. (*33*, *34*).

Therefore, in a monophenol/diphenol pair in which one of them is available (tyrosol in this case), MBTH can be used as a validating tool to demonstrate if the *o*-diphenol obtained is "its couple". In our system, tyrosol formed a specific adduct in the presence of both MBTH and tyrosinase (Figure 6B, solid line). The same adduct was formed when HTyr, enzymatically obtained, was further oxidized by tyrosinase in the presence of MBTH (Figure 6B, dotted line). This result, once again, corroborated the assumption that the peak with an RT of 5.9 in HPLC determination and an R_f of 0.43 in TLC experiments was hydroxytyrosol.

Kinetic Characterization of the Oxidation of a Tyrosol/Hydroxytyrosol Pair Catalyzed by Tyrosinase. The reaction mechanism previously proposed for tyrosinase was fully accomplished for this phenolic pair (*40*, *41*) (results not shown). Mushroom tyrosinase (as model and commercially available tyrosinase) was used for this purpose. The kinetic constants that characterized the action of mushroom tyrosinase on tyrosol and HTyr were $V_{max}^{tyrosol} = 2.7 \pm 0.1$ μM/min, $K_m^{tyrosol} = 0.15 \pm 0.01$ mM, $V_{max}^{HTyr} = 31.1 \pm 1.2$ μM/min, and $K_m^{HTyr} = 0.9 \pm 0.07$ mM (V_{max} values are related to 0.02 μg/mL mushroom tyrosinase). The V_{max} value for HTyr was >10-fold higher than that for tyrosol. However, the K_m for HTyr was 6-fold higher than that for tyrosol. Therefore, the catalytic power (V_{max}/K_m) should be considered to compare the action of mushroom tyrosinase on both substrates. V_{max}/K_m values were 0.018 ± 0.002 and 0.034 ± 0.004 min^{-1} for tyrosol and HTyr, respectively. Therefore, at least theoretically, mushroom tyrosinase will catalyze preferentially the oxidation of HTyr only

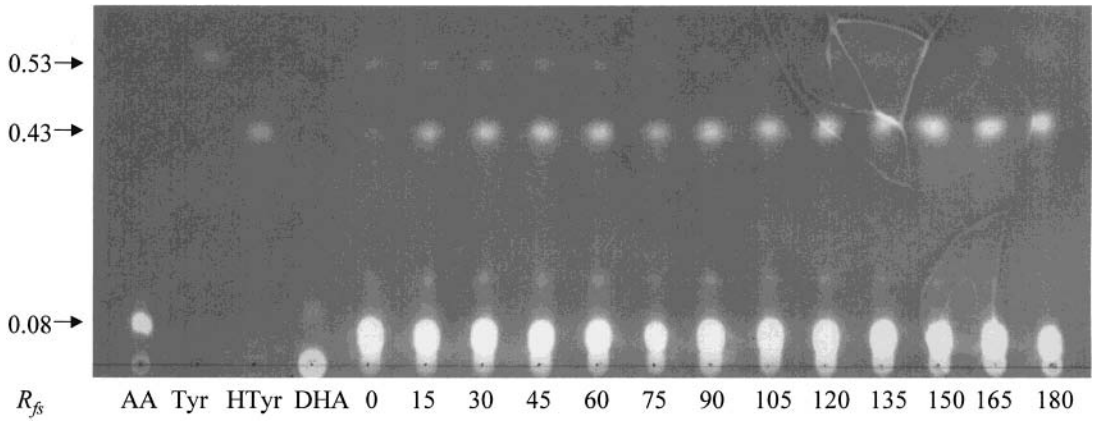

Figure 5. TLC stained with a DPPH• solution in methanol. Aliquots of 0.5 μL of the standard reaction synthesis were applied (see Materials and Methods for details). AA, ascorbic acid ($R_f = 0.08$); Tyr, tyrosol ($R_f = 0.53$); HTyr, hydroxytyrosol ($R_f = 0.43$); DHA, dehydroascorbic acid (no migration). Numbers on the bottom of the plate designate the time (minutes) after the beginning of the reaction.

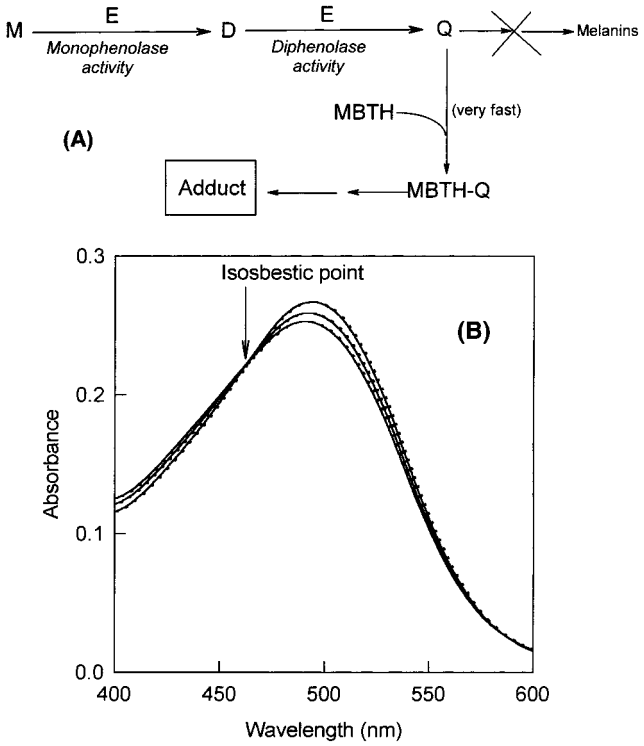

Figure 6. (A) Simplified reaction mechanism of tyrosinase in the presence of MBTH (adapted from *34*). M, *p*-monophenol (tyrosol); D, *o*-diphenol (HTyr); E, tyrosinase; Q, *o*-quinone (3,4-quinonephenylethanol). (B) Scan spectra for the oxidation of tyrosol (solid lines) and HTyr (dashed lines) catalyzed by mushroom tyrosinase in the presence of MBTH. Conditions were as follows: 50 mM sodium phosphate buffer, pH 6.5; 10 μM tyrosol or HTyr; 2% DMF; 4 mM MBTH; and 0.2 μg/mL mushroom tyrosinase. Time between recordings was 100 s.

by a factor of 2 with respect to the oxidation of tyrosol. To approach a more realistic context, the kinetic characterization of the oxidation of both tyrosol and HTyr catalyzed by olive tyrosinase is currently under study.

Enzymatic Synthesis versus Chemical Synthesis or Extraction from Olive Oil Waste Waters (ooww). The method proposed here for the synthesis of HTyr using mushroom tyrosinase as biocatalyst has several advantages over other previously published methods. There is a chemical synthesis method that uses as precursor 3,4-dihydroxyphenylacetic acid, which is reduced to give rise to HTyr (*19*, *20*). This procedure has a high yield of ~80−90% of HTyr formed. However, the process is discontinuous and involves the use of highly toxic reagents. Moreover, 3,4-dihydroxyphenylacetic acid is more expensive than tyrosol. This chemical synthesis method is fast because it is completed in only 2 h, but the compound obtained should be further purified by using chromatographic techniques to get rid of any possible contamination of either toxic reagents or byproducts formed during the reaction (*19*, *20*). Finally, the chemical method recently proposed by Tuck et al. (*21*) is also based on the reduction of 3,4-dihydroxyphenylacetic acid but includes a further tritation, which could be very interesting for metabolism and bioavailability studies about this molecule.

The alkaline hydrolysis of oleuropein to obtain HTyr (*22*) also involves the use of highly toxic reagents. Oleuropein is an expensive reagent. The protocol is discontinuous and not very reproducible. The use of active charcoal is necessary to remove the presence of byproducts, but this implies a very low yield because a high amount of HTyr is also removed by the active charcoal. However, this protocol could be useful to obtain a low amount of HTyr (to be used as "standard", for instance) because it does not require complex or expensive equipment.

The extraction of HTyr from ooww is a very interesting approach to recycle these usually discarded large volumes of water with antioxidant properties (*23*, *24*). Unfortunately, the procedure to extract and purify HTyr from ooww nowadays is cumbersome and expensive, and the final yield is rather poor (*23*).

The enzymatic synthesis of HTyr using mushroom tyrosinase is a continuous procedure, environmentally friendly, and adaptable to a bioreactor for industrial purposes. The precursor tyrosol is cheaper than both 3,4-dihydroxyphenylacetic acid and oleuropein. The yield of the protocol can be 100% and the velocity of the process controlled. The commercial mushroom tyrosinase is rather expensive, but its reutilization by a nanofiltration system can dramatically reduce the cost of the procedure. After nanofiltration and freeze-drying of the reaction medium, HTyr is obtained together with AA and sodium phosphate. This fraction could be used as a food additive to enhance both sensory and nutritional qualities of foodstuffs. Moreover, this fraction could be also further purified by preparative chromatography to obtain pure HTyr in a huge amount.

Moreover, tyrosinase could be added to ooww and other vegetative waste waters (in the presence of AA) in order to enrich the ortho-diphenolic fraction of these waters. In the case of ooww, compounds with para-monophenolic moieties such as *p*-coumaric acid and tyrosol could be hydroxylated to form their corresponding *o*-diphenols (caffeic acid and hydroxytyrosol, respectively) with antiradical properties (*10*). In other vegetative waste waters (industrial residues from artichoke, celery, etc.), the presence of any molecule (flavonoid, cinnamic acid derivative, etc.) with a para-monophenolic residue is susceptible to hydroxylation by tyrosinase because the specificity of this enzyme is rather poor (*40*).

In summary, enzymatic synthesis could be an alternative and nonpolluting procedure to obtain hydroxytyrosol, a molecule with demonstrated health-beneficial properties.

ABBREVIATIONS USED

AA, ascorbic acid; DMF, *N*,*N*′-dimethylformamide; DHA, dehydroascorbic acid; DPPH•, 2,2-diphenyl-1-picrylhydrazyl; HTyr, hydroxytyrosol; MBTH, 3-methyl-2-benzothiazolinone hydrazone; ooww, olive oil wastewater.

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