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Stereospecificity of Mushroom Tyrosinase Immobilized on a Chiral and a Nonchiral Support

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Mushroom tyrosinase was immobilized from an extract onto glass beads covered with the cross-linked totally cinnamoylated derivates of D-sorbitol (sorbitol cinnamate) and glycerine (glycerine cinnamate). The enzyme was immobilized onto the support by direct adsorption, and the quantity of immobilized tyrosinase was higher for sorbitol cinnamate, the support with the higher number of esterified hydroxyls per unit of monosacharide, than for glycerine cinnamate. The results obtained from the stereospecificity study of the monophenolase and diphenolase activity of immobilized mushroom tyrosinase are reported. The enantiomers L-tyrosine, DL-tyrosine, D-tyrosine, L-dopa, DL-dopa, D-dopa, L-α-methyldopa, DL-α-methyldopa, L-isoprenaline, DL-isoprenaline, L-adrenaline, DL-adrenaline, L-noradrenaline, and D-noradrenaline were assayed with tyrosinase immobilized on a chiral support (sorbitol cinnamate), whereas L-tyrosine, DL-tyrosine, D-tyrosine, L-dopa, DL-dopa, D-dopa, L-α-methyldopa, and DL-α-methyldopa were assayed with tyrosinase immobilized on a nonchiral support (glycerine cinnamate). The same V_{max}^{app} values for each series of enantiomers were obtained. However, the K_m^{app} values were different, the L isomers showing lower values than the DL isomers, whereas the highest K_m^{app} value was obtained with D isomers. No difference was observed in the stereospecificity of tyrosinase immobilized on a chiral (sorbitol cinnamate) or nonchiral (glycerine cinnamate) support.

KEYWORDS: Mushroom tyrosinase; immobilization; cinnamic carbohydrate esters; tyrosinase extraction; enzyme kinetics; stereospecificity

INTRODUCTION

Tyrosinase or polyphenol oxidase is a copper enzyme that catalyzes the ortho-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity), using oxygen (*1*). It is of central importance in such processes as vertebrate pigmentation and browning of fruits and vegetables. Because tyrosinase is an expensive enzyme, it is considered worthwhile to immobilize it, so that it can be reused. Several authors have endeavored to immobilize tyrosinase on different solid supports (*2*−*5*), using several immobilization methods (*6*, *7*), and have studied its behavior, stability, and kinetic properties on different supports using a variety of reaction substrates (*3*, *4*, *8*, *9*).

Catalytic stereospecificity has been reported for mushroom tyrosinase in aqueous solution (*10*), for free pear and strawberry tyrosinase (*11*), and for other free enzymes including horseradish peroxidase (*12*). However, no reference was found in the literature to characterizing the stereospecificity of tyrosinase immobilized on chiral or nonchiral supports. Furthermore, because the way in which tyrosinase acts on its natural sources might be influenced by chiral environnements, we considered it to be of interest to determine whether immobilization on chiral supports may affect its stereospecificity. Another reason for determining the stereospecificity of immobilized tyrosinase was because previous works (*2*, *8*) had pointed to substantial changes in the K_m^{app} values upon immobilization, and we wished to know how this affected the K_m^{app} values of the enantiomers and the stereospecificity. The aim of the present work was to systematically study the stereospecificity of mushroom tyrosinase immobilized on a chiral (sorbitol cinnamate) and a nonchiral (glycerine cinnamate) support in its action on several chiral monophenolic and ortho-diphenolic substrates.

MATERIALS AND METHODS

Natural mushrooms (*Agaricus bisporus*) supplied by Mercadona (Murcia, Spain) were used to obtain fresh tyrosinase. L-Dopa, DL-dopa, D-dopa, L-α-methyldopa, DL-α-methyldopa, L-isoprenaline, dl-isopre-

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naline, L-adrenaline, DL-adrenaline, L-noradrenaline, D-noradrenaline, L-tyrosine, dl-tyrosine, D-tyrosine, *p*-nitrophenol, and 4-*tert*-butylcatechol were purchased from Sigma (Madrid, Spain). A stock solution of the phenolic substrate was prepared in 0.15 mM phosphoric acid to prevent autoxidation. All other chemicals were of analytical grade and supplied by Fluka (Madrid, Spain), Panreac (Barcelona, Spain), J. T. Baker (Paris, France), and Sigma (Madrid, Spain). Ultrapure water from a Milli-Q system (Millipore Corp., Madrid, Spain) was used throughout this research.

Preparation of Photoreactive Prepolymers. The preparation of carbohydrate derivatives followed a modified version of the method proposed by Van Cleve (*13*), in which 0.02 mol of carbohydrate was dissolved in 100 mL of pyridine. The mixture was heated at 60 °C for 1 h to ensure complete dissolution. After the mixture had cooled to room temperature, 0.15 mol of cinnamic acid chloride was added for sorbitol cinnamate and 0.07 mol for glycerine cinnamate. The reaction was allowed to proceed at room temperature for 4 h, after which the resulting mixture was poured into vigorously stirred water. The precipitate obtained, after decanting and filtering of this mixture, was dissolved in chloroform and purified by adding it, one drop at a time, to vigorously shaken hexane. The solid obtained was redissolved and reprecipitated before being dried on P_2O_5 at reduced pressure.

All hydroxyl groups were esterified with cinnamoyl groups, as can be deduced from various experimental analyses: ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) spectra, different two-dimensional experiments (COSY and C/H ratio), and infrared spectra of the prepared compound. All of these methods pointed to full esterification (*2*).

Tyrosinase Extraction. Mushroom tyrosinase was extracted as previously described (*8*). Briefly, before use, the natural mushrooms were lyophilized, ground mechanically, and stored at −18 °C. To extract the fresh tyrosinase enzyme, 600 mg of lyophilized ground mushroom was added to 16 mL of a 30 mM aqueous solution of *p*-nitrophenol (pH 7.0), to ensure minimal deterioration of the enzyme due to the transformation of other substrates present in the extract (*8*), magnetically stirred for 30 min at 4 °C, and finally centrifuged at 4000 rpm for 5 min. The supernatant (9 mL) containing the tyrosinase activity was collected and equilibrated to pH 5.5 by adding 1 mL of a 0.9 M aqueous solution of NaH_2PO_4 and 0.1 M of H_3PO_4. The solids were totally eliminated by means of a second centrifugation.

Tyrosinase Immobilization. JM-50 (1.7−2.4 mm diameter) and Microperl Industrial (type A, 0.6−1.0 mm diameter) glass beads, both manufactured by Sovitec Iberica S.A. (Barcelona, Spain) and supplied by Jaque (Murcia, Spain), were used as inert matrix for tyrosinase immobilization.

To determine diphenolase activity, the JM-50 glass beads were washed and degreased (*14*) before use. A chloroform solution of the corresponding immobilization support at 5 g/L was prepared, in which the glass beads were immersed. A prepolymer film was formed on the beads (0.2 mg per gram of glass beads) when the solvent was eliminated by evaporation (*8*, *15*). After drying, the prepolymer film was polymerized by irradiation in the ultraviolet zone for 15 min using an Osram HOL-125 W mercury vapor lamp providing a power of 1.6 mW/cm^2, as determined by a Nover-Laser power/energy monitor (Ophir Optronics Ltd.). To immobilize fresh tyrosinase, 3.5 mL of tyrosinase extract (pH 5.5) was added to a syringe containing 1.5 g of glass beads covered with the immobilization support, and the immobilization was allowed to proceed for 1 h at 4 °C. After immobilization, the enzyme solution was withdrawn and the immobilized enzyme was thoroughly rinsed in distilled water.

To improve the spectrophotometric detection of monophenolase activity, we used smaller glass beads than those used to detect diphenolase activity. The 11 g of Microperl Industrial glass beads used in this case improved the activity values obtained by a factor of 21.2 ± 0.5 with respect to 1.5 g of JM-50 glass beads (1.7−2.4 mm diameter) (results not shown). The immobilization process was the same as that described above.

Tyrosinase Activity Assay. Spectrophotometric measurements were made with a Perkin-Elmer Lambda 35 UV−vis spectrophotometer controlled by a PC running the software Lambda 35, KinLab, after adjustment to the desired wavelength. A variable flow peristaltic pump (DINKO D25V) was used to pump (50−55 mL min^{-1}) the reaction medium through the spectrophotometer, which contained a quartz cuvette (1 cm). Syringes containing glass beads covered with the support and bound tyrosinase were used as small packed-bed continuous reactors with recirculation (8 and 10.5 mL in diphenol and monophenol assays, respectively) and descending flow. Spectrophotometric measurements were made as in a previous work (*2*) using as substrates l-tyrosine, DL-tyrosine, D-tyrosine, L-dopa, DL-dopa, D-dopa, L-α-methyldopa, DL-α-methyldopa, l-isoprenaline, DL-isoprenaline, L-adrenaline, DL-adrenaline, L-noradrenaline, and D-noradrenaline at the desired concentration at room temperature in 50 mM sodium phosphate buffer (pH 6.8). When tyrosine, dopa, α-methyldopa, isoprenaline, adrenaline, noradrenaline, and 4-*tert*-butylcatechol were used as substrates, an increase in absorbance was monitored at 302 nm (ϵ_{302nm} = 9360/M cm), 475 nm (ϵ_{475nm} = 3600/M cm), 475 nm (ϵ_{475nm} = 3200/M cm), 500 nm (ϵ_{500nm} = 4300/M cm), 475 nm (ϵ_{475nm} = 4000/M cm), 490 nm (ϵ_{490nm} = 3580/M cm), 400 nm (ϵ_{400nm} = 1150/M cm), respectively. In the case of diphenols the initial rate was measured between the first 10 and 70 s of the reaction. In the case of the monophenol, tyrosine, the steady-state reaction rate was measured after the initial lag period, which preceded the steady state (*16*). In the case of 4-*tert*-butylcatechol, the absorbent species was the quinone generated, whereas in all other cases the absorbent species was the aminochrome derivate of the quinone generated (*17*).

Determination of Immobilized Tyrosinase. Fresh tyrosinase immobilized on both types of glass bead (14 g) was released after a treatment in which the immobilization support was dissolved. To each sample of immobilized tyrosinase were added 10 mL of chloroform and 5 mL of 400 mM sodium phosphate buffer, pH 6.8, before stirring for 3 min. To assay the tyrosinase activity, 0.5 mL of 20 mM 4-*tert*-butylcatechol ([4-*tert*-butylcatechol]$_{final}$ = 4 mM) and 0.5 mL of 250 mM sodium phosphate buffer, pH 6.8, were added to a 1.5 mL aliquot of the aqueous phase.

The quantity of immobilized enzyme was determined by extrapolation from a straight line calibrated from the known concentrations of purified enzyme (*18*) submitted to the same treatment; that is, 5 mL of a solution of tyrosinase purified at a known concentration in 400 mM sodium phosphate buffer, pH 6.8, 10 mL chloroform, and 14 g of beads coated with the support (without enzyme) were stirred for 3 min, taking a 1.5 mL aliquot of the aqueous phase to measure the activity.

Steady-State Kinetics and Kinetic Data Analysis. The steady-state kinetic constants, V_{max}^{app} (apparent maximum steady-state rate) and K_m^{app} (apparent Michaelis constant) of the fresh immobilized mushroom tyrosinase were obtained by measuring the initial rates of the reaction with L-tyrosine (0.05−2 mM), DL-tyrosine (0.05−1.5 mM), D-tyrosine (0.05−2 mM), L-dopa (0.1−7 mM), DL-dopa (0.1−18 mM), D-dopa (0.1−7 mM), L-α-methyldopa (0.1−20 mM), DL-α-methyldopa (0.1−20 mM), L-isoprenaline (0.1−35 mM), DL-isoprenaline (0.1−35 mM), L-adrenaline (0.1−10 mM), DL-adrenaline (0.1−12 mM), L-noradrenaline (0.1−20 mM), and D-noradrenaline (0.1−10 mM) in 50 mM sodium phosphate buffer, pH 6.8, at room temperature from triplicate measurements of v_0, as indicated above. To avoid the effect of a second substrate, oxygen, on the enzyme activity, the concentration of oxygen was kept constant in the assay medium (0.26 mM). The reciprocals of the variances of v_0 were used as weighting factors in the nonlinear regression fitting of v_0 versus [substrate] to the Michaelis equation. The fitting was carried out using the Sigma Plot 8.0 program for Windows.

NMR Assays. ^{13}C NMR spectra of several substrates were obtained in a Varian Unity spectrometer at 300 MHz. The spectra were obtained by using D_2O as solvent for the substrates. Chemical displacement (δ) values were measured relative to those for tetramethylsilane (δ = 0). The maximum line width accepted in the NMR spectra was 0.06 Hz. Therefore, the maximum error for each spectrum peak was ±0.1 ppm.

RESULTS AND DISCUSSION

Quantification of Immobilized Enzyme. To calculate the apparent catalytic constant, k_{cat}^{app}, the amount of tyrosinase immobilized on glass beads covered with the cross-linked sorbitol cinamate and glycerine cinnamate derivates (**Figure 1**)

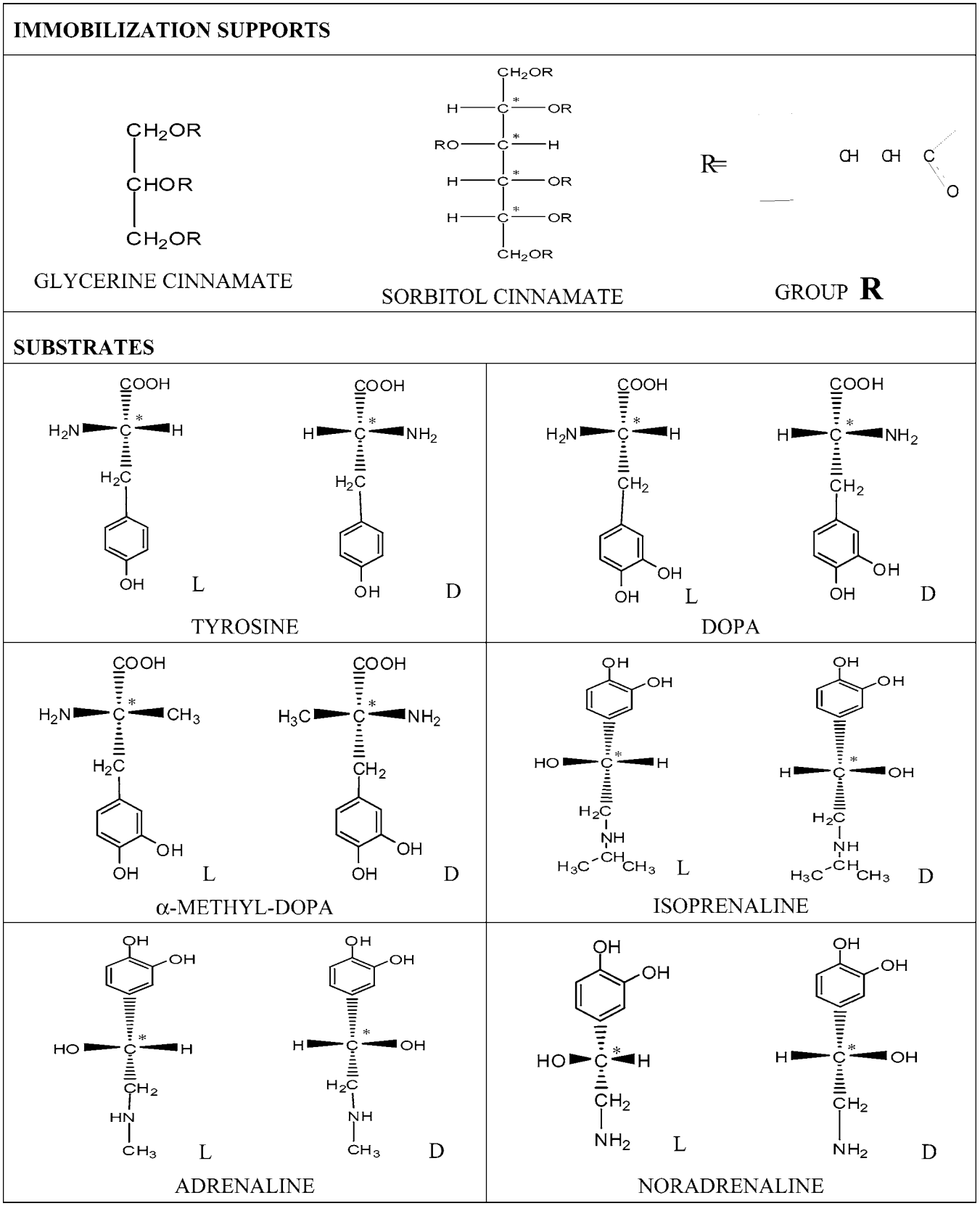

Figure 1. Schematic representation of the structure of the immobilization supports of the enzyme and the substrates of the catalytic reaction.

was determined by measuring the activity of the enzyme released after each support had been redissolved as described. These activity values were interpolated in the calibration straight line made for purified enzyme submitted to the same treatment. The resulting equation was $v_0 = 0.195x[\text{Tyr}] \pm 0.0016$ (v_0 in ΔA min^{-1} and [Tyr] in μg/mL). The quantity of enzyme immobilized on each of the supports was 1.18 $\mu g_{Tyr}/mg_{support}$ in the case of sorbitol cinamate derivate and 0.72 $\mu g_{Tyr}/mg_{support}$ in the case of glycerine cinnamate derivate. The quantity of enzyme immobilized on the cross-linked support derived from a hexafunctional monomer (sorbitol cinnamate) was greater than that obtained on the support derived from a trifunctional

Table 1. Kinetic Constants for the Diphenolase Activity of Mushroom Tyrosinase Immobilized on Sorbitol Cinnamate (*) and Glycerine Cinnamate (**) Using Several Ortho-diphenolic Enantiomers as Enzymatic Reaction Substrates

o-diphenol	V_{max}^{app} ($\times 10^3$ mM min^{-1})	k_{cat}^{app} (s^{-1})	K_m^{app} (mM)	V_{max}^{app}/K_m^{app} ($\times 10^2$ min^{-1})	k_{cat}^{app}/K_m^{app} (s^{-1} mM^{-1})
*L-dopa	276 ± 3	6252 ± 118	7.4 ± 0.6	3.73 ± 0.3	844.86 ± 70
*DL-dopa	278 ± 3	6275 ± 118	9.3 ± 0.3	3.01 ± 0.1	674.73 ± 25
*D-dopa	276 ± 3	6256 ± 118	15.2 ± 1.5	1.82 ± 0.2	411.57 ± 41
**L-dopa	276 ± 5	10226 ± 673	9.5 ± 0.6	2.90 ± 0.2	1076 ± 98
**DL-dopa	275 ± 5	10190 ± 671	11.0 ± 0.3	2.50 ± 0.1	926 ± 66
**D-dopa	275 ± 5	10190 ± 671	16.7 ± 1.5	1.64 ± 0.2	610 ± 68
*L-α-methyldopa	279 ± 2	6312 ± 108	4.37 ± 0.10	6.38 ± 0.2	1444.39 ± 41
*DL-α-methyldopa	279 ± 2	6323 ± 108	5.12 ± 0.10	5.45 ± 0.1	1234.96 ± 32
**L-α-methyldopa	284 ± 5	10518 ± 691	4.7 ± 0.3	6.04 ± 0.4	2237 ± 205
**DL-α-methyldopa	280 ± 5	10370 ± 681	6.8 ± 0.4	4.12 ± 0.3	1525 ± 134
*L-isoprenaline	265 ± 5	5980 ± 146	7.5 ± 0.3	3.53 ± 0.2	797.33 ± 37
*DL-isoprenaline	261 ± 5	5930 ± 145	7.5 ± 0.3	3.48 ± 0.2	790.66 ± 37
*L-adrenaline	299 ± 6	6782 ± 171	2.89 ± 0.10	10.35 ± 0.4	2347 ± 100
*DL-adrenaline	295 ± 4	6669 ± 137	3.59 ± 0.10	8.22 ± 0.3	1857 ± 64
*L-noradrenaline	200 ± 10	4516 ± 236	4.75 ± 0.3	4.21 ± 0.3	950 ± 78
*D-noradrenaline	210 ± 6	4770 ± 154	5.0 ± 0.3	4.20 ± 0.3	954 ± 64

Table 2. Kinetic Constants Published by Espín et al. (*10*) for the Diphenolase and Monophenolase Activity of Free Mushroom Tyrosinase Using Several Diphenolic and Monophenolic Enantiomers[a]

substrate	V_{max}^{app} (μM min^{-1})	k_{cat}^{app} (s^{-1})	K_m^{app} (mM)	V_{max}^{app}/K_m^{app} ($\times 10^2$ min^{-1})	k_{cat}^{app}/K_m^{app} (s^{-1} mM^{-1})
L-dopa	24.5 ± 1	107.4 ± 44	0.8 ± 0.04	3.1 ± 0.28	134.25
DL-dopa	24.5 ± 1	107.0 ± 4.4	1.4 ± 0.06	1.7 ± 0.14	76.43
D-dopa	24.5 ± 1	107.4 ± 4.4	4.5 ± 0.15	0.54 ± 0.04	23.86
L-α-methyldopa	10.1 ± 0.5	44.3 ± 1.8	6.8 ± 0.28	0.15 ± 0.014	6.51
DL-α-methyldopa	10.1 ± 0.5	44.3 ± 2.2	8.0 ± 0.41	0.13 ± 0.013	5.53
L-isoprenaline	6.7 ± 0.3	29.4 ± 1.2	6.6 ± 0.31	0.10 ± 0.0092	4.45
DL-isoprenaline	6.7 ± 0.3	29.4 ± 1.2	9.7 ± 0.61	0.069 ± 0.0074	3.03
L-tyrosine	1.80 ± 0.05	7.9 ± 0.2	0.27 ± 0.01	0.67 ± 0.045	29.26
DL-tyrosine	1.85 ± 0.06	8.1 ± 0.3	0.90 ± 0.01	0.2 ± 0.0074	9.00
D-tyrosine	1.82 ± 0.06	8.0 ± 0.3	1.86 ± 0.02	0.098 ± 0.0043	4.30

[a] Conditions: 50 mM phosphate buffer, pH 6.8, 2% (v/v) DMF, saturating MBTH concentration, differing monophenol concentrations, and 3.8 nM mushroom tyrosinases. Results are means ± SD for three separate experiments.

monomer (glycerine cinnamate), probably due to the lower molecular size of the latter and the different structure arising from the cross-linking (*14*).

Kinetic Assay. In all cases (*o*-diphenols and monophenols) a Michaelis−Menten behavior was observed. With regard to the kinetic constants for each series of isomers, the V_{max}^{app} and k_{cat}^{app} values were the same, but the K_m^{app} values differed. The sequence of K_m^{app} values was D isomers > DL isomers > L isomers (demostrating the greater affinity for the natural L isomer), the inverse sequence from the V_{max}^{app}/K_m^{app} and k_{cat}^{app}/K_m^{app} values.

Diphenolase Activity. Enantiomers of several diphenols (dopa, α-methyldopa, isoprenaline, adrenaline, and noradrenaline, **Figure 1**) were assayed with tyrosinase immobilized on glass beads covered with the chiral support sorbitol cinnamate (**Table 1**). To ascertain whether the chirality of the immobilization support affects the stereospecificity of immobilized mushroom tyrosinase, enantiomers of dopa and α-methyldopa were assayed with tyrosinase immobilized on glass beads covered with the nonchiral support glycerine cinnamate (**Table 1**).

Maximum Steady-State Rate (V_{max}^{app}). The results (**Table 1**) showed that the apparent maximum steady-state rate values, V_{max}^{app}, of mushroom tyrosinase immobilized on a chiral (sorbitol cinnamate) or a nonchiral (glycerine cinnamate) support were the same for each series of isomers. The V_{max}^{app} values of free mushroom tyrosinase (**Table 2**) were also the same for each series of isomers and were related with the same nucleophilic power of the oxygen from the aromatic hydroxy group of the isomers to attack the copper atoms of the enzyme active site (*10*, *11*). This nucleophilic power can be measured by ^{13}C NMR assays and is related with the highest electron donor capacity of the side chain of the substrate, which corresponds to the highest electronic charge and the lowest δ values for C-3 (δ_3) and C-4 (δ_4) in *o*-diphenols. As the substituent is the same for enantiomers, the δ values were similar for the whole series (L, DL, and D isomers) of each *o*-diphenol (**Table 3**). Therefore, the corresponding V_{max}^{app} values for the three enantiomers must be the same, as can be seen for immobilized enzyme (**Table 1**) and for free enzyme (**Table 2**).

Table 3. δ Values for C-3 and C-4 of Several Phenolic Compounds at pH 6.8

substrate	δ_3	δ_4	substrate	δ_3	δ_4
L-dopa	146.9	146.1	L-adrenaline	143.7	143.7
DL-dopa	146.9	146.1	DL-adrenaline	143.7	143.7
D-dopa	146.9	146.1	L-noradrenaline	144.0	144.0
L-α-methyldopa	146.7	146.2	D-noradrenaline	144.0	144.0
DL-α-methyldopa	146.7	146.2	L-tyrosine		158.8
L-isoprenaline	146.9	146.8	DL-tyrosine		158.8
DL-isoprenaline	146.9	146.8	D-tyrosine		158.8

For immobilized mushroom tyrosinase we observed similar V_{max}^{app} values when the *o*-diphenol δ_4 values increased, perhaps because these δ_4 values were very similar and not sufficiently different from to observe differences in immobilized mushroom tyrosinase and due to the influence of other factors, such as enzyme−support interactions, which do not exist in the case of the free enzyme (*10*, *11*). Of note are the higher apparent catalytic constant values of the immobilized enzyme (**Table 1**)

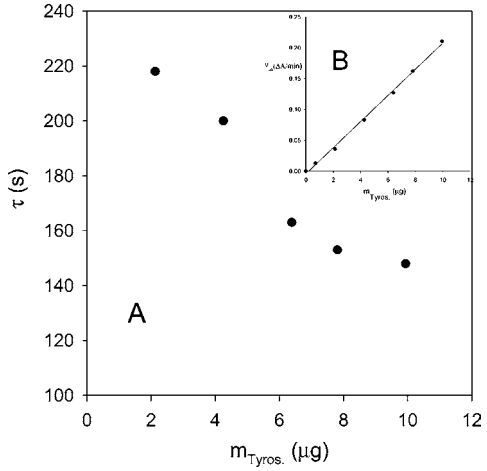

Figure 2. Effect of enzyme quantity on (**A**) lag time and (**B**) tyrosinase activity. Support, sorbitol cinnamate; substrate, L-tyrosine, 2 mM.

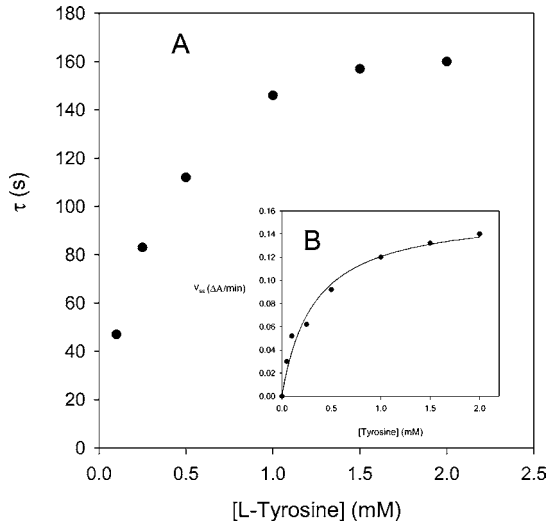

Figure 3. Effect of substrate concentration on (**A**) lag time and (**B**) tyrosinase activity. Support, sorbitol cinnamate.

compared with those of the free enzyme (**Table 2**), which may have been due to conformational changes to more unfolded states (*19*) provoked by the strong hydrophobic interactions taking place during immobilization, although perhaps other factors derived from the use of a crude mushroom material could also have had some influence. In an aqueous solution, the enzyme would find itself in a state in which the ionic groups are in contact with the solution and the innermost hydrophobic part would remain protected; when immobilized on the hydrophobic supports, the enzyme would open out, binding to the support by means of the hydrophobic part, whereas the ionic part would remain in contact with the solution. The substrate would then be able to reach the active center of the immobilized tyrosinase more easily than the active center of free enzyme, thus explaining the higher k_{cat}^{app} values obtained for immobilized tyrosinase and the similar values obtained for substrates with similar δ_4.

Michaelis Constant. The apparent Michaelis constant values, K_m^{app}, of immobilized mushroom tyrosinase differed between isomers of the same series of *o*-diphenols (**Table 1**). When immobilized tyrosinase acted on L isomers, K_m^{app} was lower than when immobilized tyrosinase acted on DL isomers, which was lower than in the case of D isomers, like free mushroom tyrosinase (**Table 2**). The same sequence of K_m^{app} values was observed for mushroom tyrosinase immobilized on sorbitol

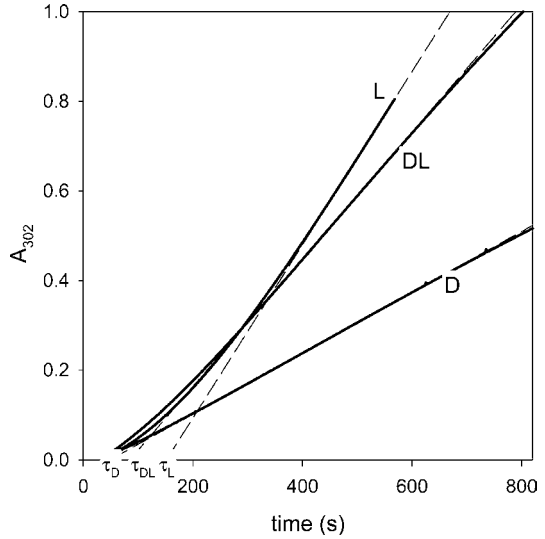

Figure 4. Effect of nature of monophenol enantiomers on lag time. Spectrophotometric recordings were taken of the monophenolase activity of immobilized fresh mushroom tyrosinase (7.81 μg) in 50 mM phosphate buffer (pH 6.8). L-Tyrosine (L, 1 mM), DL-tyrosine (DL, 1 mM), and D-tyrosine (D, 1 mM) were used as substrates.

cinnamate (chiral support) and glycerine cinnamate (nonchiral support). However, the differences between the K_m^{app} values of the enantiomers were smaller for immobilized tyrosinase than for free tyrosinase, and even substrates such as isoprenaline and noradrenaline showed very similar K_m^{app} values between isomers in the case of immobilized tyrosinase (**Table 1**). The sequence of K_m^{app} values obtained for the different enantiomers (L < DL < D) could be explained by the effect of the spatial orientation of the side chain, which would affect one of several rate constants in the Michaelis constant expression for the diphenolase activity of tyrosinase (*10*, *11*).

The effect of introducing a methyl group in the side chain of the substrate had the opposite effect on the K_m^{app} values in immobilized enzyme than in free enzyme. In the case of free enzyme, K_m^{app} values increased when the molecular mass of the substituent side chain of *o*-diphenols was raised by adding a methyl group (*10*, *11*). The immobilized enzyme showed lower K_m^{app} values for methyldopa than for dopa and lower values for adrenaline than for noradrenaline (**Table 1**). This effect could be due to the increase in substrate hydrophobicity caused by adding a methyl group, because the more hydrophobic the substrate, the greater the affinity for the hydrophobic immobilization support, and the higher the concentration of substrate in the microenvironnement of the enzyme, the lower the K_m^{app} values (*20*). However, we found that when the molecular mass of the substituent was quite high (introduction of two methyl groups), the K_m^{app} values of the immobilized enzyme increased, as in the case of free enzyme; for example, isoprenaline showed a higher K_m^{app} value than noradrenaline (**Table 1**). The absolute values of K_m^{app} were higher for tyrosinase immobilized on glycerine cinnamate than for tyrosinase immobilized on sorbitol cinnamate, perhaps due to different support−substrate interactions resulting from the structural differences in this immobilization support. There are two kinds of factors that affect K_m^{app} values for immobilized tyrosinase. The first is the hydrophobicity of the substrate and the support, which is responsible for substrate−support and enzyme−support interactions, and conformational changes in the enzyme; the second is the steric effect produced by the

Table 4. Kinetic Constants for the Monophenolase Activity of Mushroom Tyrosinase Immobilized on Sorbitol Cinnamate (*) and Glycerine Cinnamate (**) Using Several Monophenolic Enantiomers as Enzymatic Reaction Substrates

monophenol	V_{max}^{app} ($\times 10^3$ mM min^{-1})	k_{cat}^{app} (s^{-1})	K_m^{app} (mM)	V_{max}^{app}/K_m^{app} ($\times 10^2$ mM min^{-1})	k_{cat}^{app}/K_m^{app} (s^{-1} mM^{-1})
*L-tyrosine	34.08 ± 0.80	45.94 ± 1.35	0.32 ± 0.02	10.65 ± 0.71	143.6 ± 9.9
*DL-tyrosine	33.97 ± 1.29	45.79 ± 1.95	0.51 ± 0.02	6.66 ± 0.36	89.8 ± 5.2
*D-tyrosine	34.12 ± 1.61	46.00 ± 2.37	1.5 ± 0.10	2.27 ± 0.19	30.6 ± 2.6
**L-tyrosine	30.07 ± 0.95	68.01 ± 4.85	0.55 ± 0.05	5.46 ± 0.52	123.6 ± 14.3
**DL-tyrosine	30.98 ± 1.45	68.48 ± 5.42	1.06 ± 0.15	2.92 ± 0.44	64.6 ± 10.9
**D-tyrosine	31.83 ± 2.15	70.37 ± 6.54	1.71 ± 0.20	1.86 ± 0.25	37.83 ± 5.6

different spatial orientations of different isomers or by big substituents in the substrate.

Monophenolase Activity. The monophenolase reaction mechanism of tyrosinase is coupled to diphenolase activity (*1*, *21*). When tyrosine was used as substrate (**Figure 1**), immobilized mushroom tyrosinase showed a lengthened transient phase before reaching the steady state (*22*), as did free tyrosinase (*16*). When the product is plotted versus the time of reaction, the lag time (τ) can be estimated by extrapolation of the linear portion of the curve to the abscissa. When we determined the behavior of τ versus immobilized enzyme quantity and versus substrate concentration (**Figures 2A** and **3A**), τ decreased with increasing quantity of enzyme and increased with increasing concentrations of substrate, as has been described for free mushroom tyrosinase (*23*). In the range of enzyme quantity assayed, the activity versus the quantity of immobilized mushroom tyrosinase acting on L-tyrosine as substrate showed a linear relationship, the activity increasing with the enzyme quantity (**Figure 2B**). The activity of immobilized mushroom tyrosinase versus substrate concentration also increased with higher L-tyrosine concentrations (**Figure 3B**). The chirality of the support did not affect the behavior of τ or activity versus immobilized enzyme quantity or versus substrate concentration, and the same behavior was obtained for tyrosinase immobilized on a chiral support (sorbitol cinnamate) (**Figures 2** and **3**) as for tyrosinase immobilized on a nonchiral support (glycerine cinnamate) (results not shown).

Maximum Steady-State Rate (V_{max}^{app}). The results showed that the V_{max}^{app} of mushroom tyrosinase immobilized on a chiral (sorbitol cinnamate) or a nonchiral (glycerine cinnamate) support was the same for the three isomers of tyrosine (**Table 4**), as in the case of *o*-diphenols (**Table 1**) and free tyrosinase (**Table 2**). The reason of this behavior of immobilized mushroom tyrosinase with monophenols, was the same as with *o*-diphenols and explained in a previous section. The δ_4 values in ^{13}C NMR experiments (**Table 3**) were the same for the three isomers of tyrosine, which would explain the same value of V_{max}^{app}. These δ_4 values were much lower for *o*-diphenols than for monophenols, which means that the oxygen from the aromatic hydroxy group of monophenols has less nucleophilic power than that of the *o*-diphenols, so the nucleophilic attack on the active center would be more difficult for monophenols than for *o*-diphenols, resulting in lower V_{max}^{app} and k_{cat}^{app} values in the first case. Note the higher k_{cat}^{app} values obtained for immobilized enzyme (**Table 4**) than for free enzyme (**Table 2**), as occurred with the *o*-diphenols.

Michaelis−Menten Constant. With regard to the K_m^{app} values (**Table 4**), the behavior of immobilized mushroom tyrosinase acting on monophenols was the same as that previously found for *o*-diphenols. The sequence of K_m^{app} values was D isomers > DL isomers > L isomers, the same as for free tyrosinase (**Table 2**), and was not affected by the chirality of the support. The spatial orientation of the substituent of the substrate affected enzyme−substrate affinity and altered K_m^{app} values. Furthermore, the spectrophotometric recordings of the monophenolase activity of immobilized fresh mushroom tyrosinase on a chiral support (sorbitol cinnamate) with L-, DL-, and D-tyrosine (**Figure 4**) showed different lag periods (τ). The largest τ corresponded to the isomer having the lowest K_m^{app}, and the smallest τ corresponded to the isomer having the hightest K_m^{app} (**Table 4**). The same behavior was obtained for tyrosinase immobilized on a nonchiral support (glycerine cinnamate) (results not shown). That the substrate with the highest catalytic power (V_{max}^{app}/K_m^{app}) showed a longer lag period might seem to be contradictory, but it could be explained in terms of the reaction mechanism (*10*, *11*).

To sum up, the results concerning the diphenolase and monophenolase activity of immobilized mushroom tyrosinase suggest that this enzyme shows stereospecificity in its affinity toward the substrates (K_m^{app}), but does not show stereospecificity in the maximum steady-state rate (V_{max}^{app}) of these substrates. This behavior is the same for mushroom tyrosinase immobilized on a chiral or nonchiral support and for free tyrosinase, demonstrating that the process of immobilization has no effect on the stereospecificity of mushroom tyrosinase. These results suggest that when tyrosinase is adsorbed on natural supports, such as cell menbranes, its stereospecificity will be maintained, as it is on the hydrophobic supports described above. Moreover, knowledge of this property could facilitate the use of immobilized tyrosinase in the food industry as a sensor of phenols in food solutions and for their possible elimination.

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