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Kinetic characterization of diphenolase activity from *Streptomyces antibioticus* tyrosinase in the presence and absence of cyclodextrins

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Abstract

Streptomyces antibioticus tyrosinase was kinetically characterized after purification by PEG-8000/phosphate phase partitioning and ammonium sulfate fractionation using *tert*-butylcathechol (TBC) and dopamine. The enzyme showed an optimal pH at 6.5 and a K_M of 1.2 mM and 8.4 mM, respectively. The effect of several modulators was studied on this Gram-positive bacterium tyrosinase. In addition, previously undescribed characterization of apparent inhibition and activation of a bacterial tyrosinase using different kinds of cyclodextrins was carried out. When a hydrophobic substrate of *S. antibioticus* tyrosinase, in this case, *tert*-butylcatechol was used, a marked substrate sequestrant effect was observed in the presence of hydroxypropyl-β-cyclodextrins (OH-β-CDs) and gamma cyclodextrins (γ-CDs). This sequestrant effect was due to the complexation of TBC into the CD cavity. Moreover, the effect of some hydrophobic inhibitors in the presence of OH-β-CDs and γ-CDs was studied using dopamine, a hydrophilic substrate of *S. antibioticus* tyrosinase. Increasing concentrations of CDs in the presence of inhibitors like hexestrol or hinokitiol, were able to reactivate the inhibited enzyme to reach the non-inhibited level, as a result of the complexation of these inhibitory compounds in the hydrophobic core of the CDs. This dual effect of CDs as apparent inhibitor and activator has never before described being observed in bacteria.

Keywords: Tyrosinase; Diphenolase activity; Cyclodextrins; *Streptomyces antibioticus*; Inhibitors; Two-phase system; Hinokitiol; Hexestrol

1. Introduction

Tyrosinase (monophenol, dihydroxy-L-phenylalanine:oxygen oxidoreductase E.C. 1.14.18.1) is an enzyme that is widely distributed in nature and is responsible for the biosynthesis of melanins and other polyphenolic compounds [1]. It catalyzes both, the *o*-hydroxylation of monophenols to its corresponding *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The *o*-quinones, thus generated, polymerize to synthesize melanin or melanin-like pigments in fungi, plants, unicellular bacteria and mammalian cells.

Streptomycetes are Gram-positive soil bacteria showing mycelious growth. Members of this genus are involved in the formation and/or degradation of complex biopolymers like lignins, melanins and humic substances [2]. About 40% of *Streptomyces* species produce melanin-like exopigments on tyrosine-containing agar media, which mostly, but not always, coincide with the appearance of an extracellular tyrosinase activity [3].

Although several studies have been made of about the genetic and the molecular biology of tyrosinase from *Streptomyces antibioticus*, only a few kinetic studies on the diphenolase activity of this enzyme has been carried out [4–6], as in the case of *Streptomyces glaucescens* [7]. This is perhaps due to the difficulties associated with its purification in the *S. antibioticus* broth, which is pitch black due to large amounts of melanin [4]. This color remains bound to the enzyme preparation even after the usual ammonium sulfate fractionation step, giving rise to an unstable enzyme form unless a multistep purification procedure is followed with a two times batch treatment with Whatman DE52 cellulase, phenyl sepharose chromatography, cleaning and concentration by ultrafiltration and a final step on CI-CR CM sepharose [4]. In order to obtain a stable enzyme by a simple procedure, our group has developed a purification method based on a two steps aqueous two-phase system with PEG-8000 (5%,

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w/w)/potassium phosphate buffer (pH 7) [1] and a subsequent precipitation with ammonium sulfate. Recently, the problem has been solved by expressing the melanin-synthesizing gene operon HUT 6202 into *E. coli* BL21 (DE3) pLys-pET21a system with His Tag [8].

The presence of this enzyme has a large impact in the food industry because it is the main enzyme involved in the undesirable browning of fruits and vegetables during processing and storage. Moreover, tyrosinase is an important enzyme in biotechnology [9] due to its role in the synthesis or modification of high-value compounds like the phytoestrogen coumestrol, known for its estrogenic activity, and L-DOPA, which is used for the treatment of Parkinson's disease. Moreover, it can be used as a biosensor since it shows excellent analytical performance when immobilized in a membrane [10].

The aim of this paper was to characterize extensively the previously undescribed diphenolase activity from *S. antibioticus* using TBC and dopamine as substrates. This study was also extended to the effect of two different types of cyclodextrins (OH-β-CDs and γ-CDs) on enzymatic diphenolic oxidation.

2. Materials and methods

2.1. Reagents

Biochemicals, including inhibitors (cinnamic acid, L-mimosine, tropolone, ascorbic acid, L-cysteine and metabisulfite) were purchased from Sigma (Madrid, Spain) and used without further purification. The 2-hydroxypropyl-β-cyclodextrins (OH-β-CDs) and gamma cyclodextrins (γ-CDs) were kindly supplied by Amaizo, American Maize Products Co., Hammond, IN. Hexestrol (4,4′-1,2-diethylethylene-diphenol) and Hinokitiol were obtained from TCI (Japan).

2.2. Enzyme purification

S. antibioticus was obtained and purified using the method described by Orenes-Piñero et al. [1]. Briefly, *S. antibioticus* was grown on GYM medium [11]. After 18 h, the cultures were centrifuged at 10,000 × *g*. The medium, containing the exocellular tyrosinase, was subjected to an aqueous two-phase system based on PEG-8000 (5%, w/w)/potassium phosphate buffer (pH 7.0) [13]. After the solution had been stirred for 15 min at room temperature, it was centrifuged at 10,000 × *g* for 10 min at 25 °C. The upper black PEG-rich phase (20% of total volume) was discarded, and the clean phosphate-rich phase containing the *S. antibioticus* tyrosinase was brought to 60% saturation with $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60,000 × *g* for 30 min at 4 °C. The pellet, containing the tyrosinase activity, was collected and dissolved in a minimum volume of water.

2.3. Enzyme activity

The oxidation of the 4-*tert*-butylcatechol and dopamine was followed spectrophotometrically in an Uvikon 940 (Kontron Instruments, Italy) at their respectively absorption maxima, which are 400 nm for *tert*-butyl-*o*-quinone ($\varepsilon = 1150\ M^{-1}\ cm^{-1}$) and 475 nm for dopaminochrome ($\varepsilon = 3300\ M^{-1}\ cm^{-1}$).

The standard reaction medium at 25 °C contained, in a total volume of 1 mL, 4 μg/mL tyrosinase, 2.5 mM TBC or dopamine in 10 mM sodium phosphate buffer pH 6.5. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol *tert*-butyl-*o*-quinone/min, or 1 μmol dopaminochrome/min, respectively.

The optimum pH was measured at 25 °C, in a total volume of 1 mL, using 4 μg/mL tyrosinase and 2.5 mM TBC in 10 mM sodium acetate (pH 3.5–5.5) and in 10 mM sodium phosphate (pH 6.0–7.5).

3. Results and discussion

3.1. Effect of pH

The oxidation of *tert*-butylcatechol (TBC) by tyrosinase was practically inexistent at acidic pH but showed a clear maximum at pH 6.5. The inexistent tyrosinase activity at low pH could be the result of the rapid inactivation of the enzyme at pH below 6.0, as described by Tepper et al. [6]. The optimum pH agrees with that described for *S. antibioticus* activity towards L-DOPA [12–14], but contrasts with the higher activity shown by some plant polyphenol oxidases (PPOs) at acidic pH. This low pH optimum in plant PPOs is a result of the activation of the latent form of the enzyme by acid shock [15,16].

3.2. Effect of TBC and dopamine concentrations

The apparent kinetic parameters (V_{max} and K_M) were fitted by nonlinear regression using the Michaelis–Menten equation for the activity data obtained when the TBC and dopamine concentrations were varied from 0 to 6.5 mM and 0 to 12 mM at pH 6.5 (Fig. 2), respectively. The values obtained using TBC as substrate, were 265.7 μM/min and 1.2 mM, respectively. The K_M value was the same to the one obtained for this substrate in quince fruit pulp (1.2 mM) [17] but slightly higher than in *S. glaucescens* tyrosinase (0.9 mM) [7] and in persimmon PPO (0.68 mM) [18].

In the case of dopamine, the values obtained were 51.68 μM/min and 8.4 mM, respectively. The K_M value cannot be compared with any *Streptomyces* sp. tyrosinase, but it is higher than the values obtained for banana peel (3.9 mM) [19], for pulp PPO, which is its natural substrate (0.57 mM) [20], and *Hevea brasiliensis* (2.12 mM) [21].

3.3. Effect of inhibitors and reducing agents

To further characterize the enzyme, a detailed kinetic study of its inhibition was carried out. Table 1 shows the effect of several compounds on the diphenolase activity of *S. antibioticus* tyrosinase, using TBC as substrate, since it was the better of the two used in this paper. All of them, reducing agents and substrate analogues used in this study, reduce tyrosinase activity and the

Table 1
Inhibition percentage of *Streptomyces antibioticus* tyrosinase using TBC as substrate

	1 μM	10 μM	100 μM	1 mM
Reducing agents				
Ascorbic acid	5	6	23	100
L-Cysteine	11	14	81	100
Metabisulfite	2	10	64	100
Substrate analogues				
Tropolone	10	69	96	100
L-Mimosine	1	20	45	85
Cinnamic acid	5	6	7	17

Assayed under the standard reaction conditions with the appropriate concentration of inhibitor.

extent of the reduction was dependent on the concentration of the compound used.

Among the reducing agents, L-cysteine appeared to be the most effective (Table 1). The reduction produced by thiol compounds may be due to an addition reaction, taking place with the quinones and the formation of stable colorless products [22] and/or to the L-cysteine binding to the active center of the enzyme, as in the case of metabisulfite [23]. Ascorbate acts as an antioxidant rather than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes the secondary reactions that lead to browning. Ascorbic acid has also been reported to cause irreversible inhibition [24].

With regard to substrate analogues (Table 1), tropolone (Scheme 1) was the most effective inhibitor using TBC as substrate because it nearly inhibited the enzyme at 0.1 mM and did so completely at 1 mM. This compound was therefore selected to carry out the kinetic analysis of its inhibition. The inhibition was determined by Lineweaver–Burk plots of $1/v$ versus $1/s$ (data not shown) at three inhibitor concentrations and confirmed by a Dixon plot of $1/v$ versus inhibitor concentration (Fig. 1). The linear regression of the data defined the intersection point in the second quadrant. This means that the inhibition of tropolone on tyrosinase is competitive, and the inhibition constant, K_I, which showed a value of 4.2 μM, could be deduced from the interception points. However, the data cannot be compared since this is the first study of tyrosinase inhibition by tropolone in a Gram-positive bacteria although this value is very similar to the one obtained for quince fruit pulp PPO (4.7 μM) [17] and

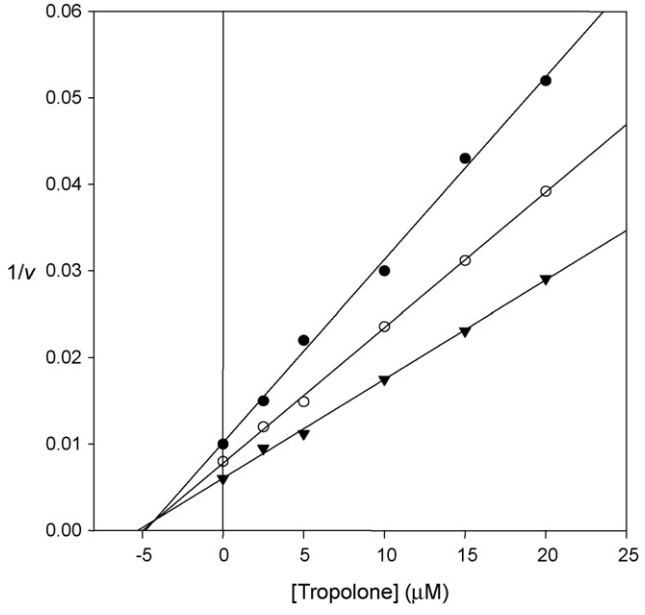


Fig. 1. Effect of tropolone inhibition (Dixon plot) in *Streptomyces antibioticus* tyrosinase. The reaction medium contained 4 μg/mL tyrosinase, tropolone (0–20 mM), and three different concentrations of TBC [0.625 mM (●), 1 mM (◯) and 2.5 mM (▼)] in 10 mM sodium phosphate buffer.

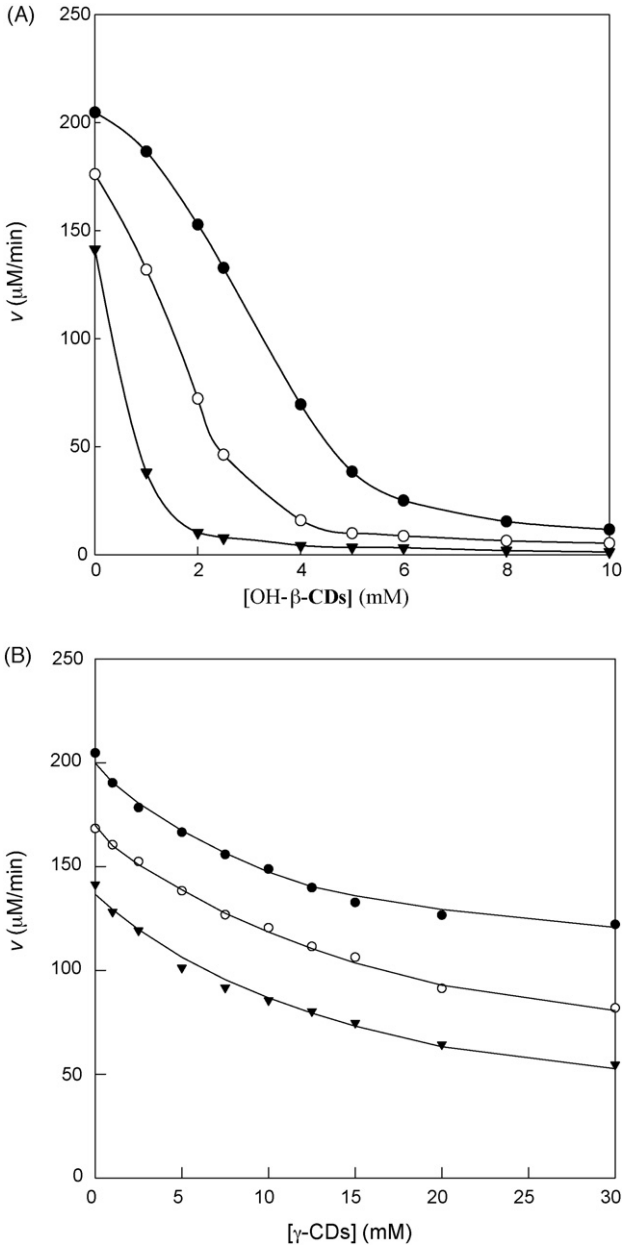
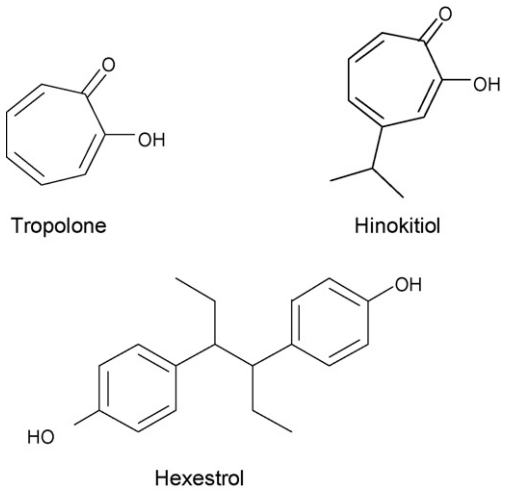


Fig. 2. (A) Effect of OH-β-CDs concentration on *S. antibioticus* tyrosinase. The reaction medium at 25 °C contained 10 mM sodium phosphate buffer pH 6.5, 4 μg/mL of partially purified tyrosinase, increasing concentrations of OH-β-CDs (0–10 mM), and 5 mM TBC (●), 2.5 mM TBC (◯) and 1 mM TBC (▼). (B) The same reaction medium conditions but with γ-CDs. Solid lines represent the adjustment of experimental data to Eq. (2).



Scheme 1. Molecular structure of the best inhibitors for *Streptomyces antibioticus* tyrosinase: tropolone, hinokitiol and hexestrol.

nearly 10 times higher than the observed for tropolone on latent persimmon PPO [18].

3.4. Effect of cyclodextrins

To extend the study of the substances affecting *S. antibioticus* tyrosinase activity, the effect of scavengers such as cyclodextrins was studied. Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by α(1 → 4) glycosidic bonds in a cylinder-shaped structure and are denominated α-, β- and γ-cyclodextrins, respectively. The central cavity of these molecules is hydrophobic, whereas the rims of the surrounding walls are hydrophilic. This hydrophobic cavity forms inclusion complexes with a wide range of organic guest molecules, including (poly)phenols [25]. It has been suggested that cyclodextrins may moderate the enzymatic browning of different fruits and vegetables [26–28] because they form inclusion complexes with the substrates of polyphenol oxidase, thereby preventing their oxidation to quinones and subsequent polymerization to brown pigments. Our group has also observed this effect during the oxidation of phenols by lipoxygenase [29], in which CDs act as secondary antioxidants in synergism with ascorbic acid.

Modified cyclodextrins, which have one or more branches of an α-D-glucopyranosil unit or a (1 → 4)-α-D-glucan at the carbon site of the glucose residues, have many advantages over the parent cyclodextrins. For example, they are highly soluble, both in water and in organic solvents, and enhance the solubility of otherwise water insoluble compounds by complexation [30]. Among them, 2-hydroxypropyl-β-cyclodextrins (OH-β-CDs) is one of the most widely used for its high solubility and low cost. These properties have been exploited commercially, especially in the pharmaceutical field [31].

When dopamine was assayed in the presence of OH-β-CDs or γ-CDs, the activity was not affected (data not shown). This clearly indicates that dopamine is a highly soluble and hydrophilic *o*-diphenol, which shows little or no complexation in CDs. To confirm this, TBC, a more hydrophobic *o*-diphenol, was used (Fig. 2). In this case, a clear decrease in activity was found at three different concentrations of TBC in the presence of increasing concentrations of OH-β-CDs (Fig. 2A), and γ-CDs (Fig. 2B).

The modified cyclodextrin was selected to carry out a kinetic analysis of the apparent inhibition because of its better complexation with TBC. This can be explained by the fact that TBC is a diphenolic compound with a hydrophobic group, which enters the hydrophobic cavity to form inclusion complexes, diminishing the free TBC concentration available for the enzyme. This concentration can be mathematically expressed as in Eq. (1) [32]:

$$[\mathrm{TBC}]_\mathrm{f} = \frac{-([\mathrm{CD}]_\mathrm{t} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1) + \sqrt{([\mathrm{CD}]_\mathrm{t}K_\mathrm{c} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1)^2 + 4K_\mathrm{c}[\mathrm{TBC}]_\mathrm{t}}}{2K_\mathrm{c}} \quad (1)$$

where subscripts 'f' and 't' refers to the concentration of the free and total compounds, respectively, in this case, TBC and CD. Thus, the complete Michaelis–Menten equation can be expressed as

$$v = \frac{V_\mathrm{m}\left[(-([\mathrm{CD}]_\mathrm{t}K_\mathrm{c} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1) + \sqrt{([\mathrm{CD}]_\mathrm{t}K_\mathrm{c} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1)^2 + 4K_\mathrm{c}[\mathrm{TBC}]_\mathrm{t}})/2K_\mathrm{c}\right]}{K_\mathrm{M} + \left[(-([\mathrm{CD}]_\mathrm{t}K_\mathrm{c} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1) + \sqrt{([\mathrm{CD}]_\mathrm{t}K_\mathrm{c} - [\mathrm{TBC}]_\mathrm{t}K_\mathrm{c} + 1)^2 + 4K_\mathrm{c}[\mathrm{TBC}]_\mathrm{t}})/2K_\mathrm{c}\right]} \quad (2)$$

Eq. (2) shows a nonlinear relationship between v and [CD], as depicted in Fig. 2. Fitting the data of this figure to Eq. (2) by nonlinear regression gives a value of 13,170 M^{-1} for the formation constant (K_c) of the inclusion complex between TBC and OH-β-CDs. This value is similar to that described for the oxidation of TBC by lipoxygenase [29] in the presence of cyclodextrins. Similarly, the K_c obtained for TBC and γ-CDs was 603 M^{-1}. This K_c has never been described before.

To complete our study of the kinetic behavior of tyrosinase from *S. antibioticus* in the presence of CDs, the effect of inhibitors was studied using dopamine as substrate to avoid any interference of the substrate with CDs. Since the inhibitors used in Table 1 are highly hydrophilic, they did not interact with the CD core. Thus, two previously undescribed inhibitors for *S. antibioticus* tyrosinase were found, hexestrol and hinokitiol (Scheme 1). These two inhibitors were able to complex with cyclodextrins, entering its central cavity.

Hinokitiol shows the greater inhibition capacity because at 10 μM it completely inhibits the enzyme in the presence of dopamine. On the other hand, hexestrol only inhibits 36% at 10 μM. When the CD concentration was increased in the presence of any of the above inhibitors, a clear activation was found

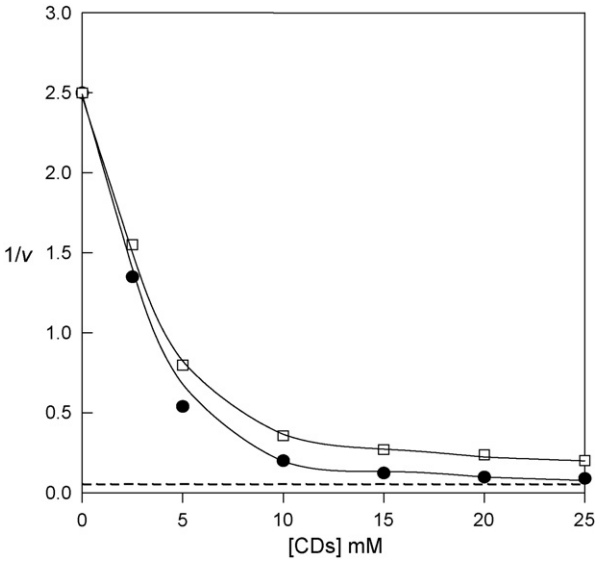


Fig. 3. Effect of OH-β-CDs (●) and γ-CDs (□) on the oxidation of dopamine by tyrosinase in the presence of hinokitiol. The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 4 μg/mL of partially purified tyrosinase, dopamine 5 mM, hinokitiol 10 μM and increasing concentrations of CDs (0–25 mM). The dotted line represents the activity level without inhibitor. Solid lines represent the adjustment of experimental data to Eq. (7).

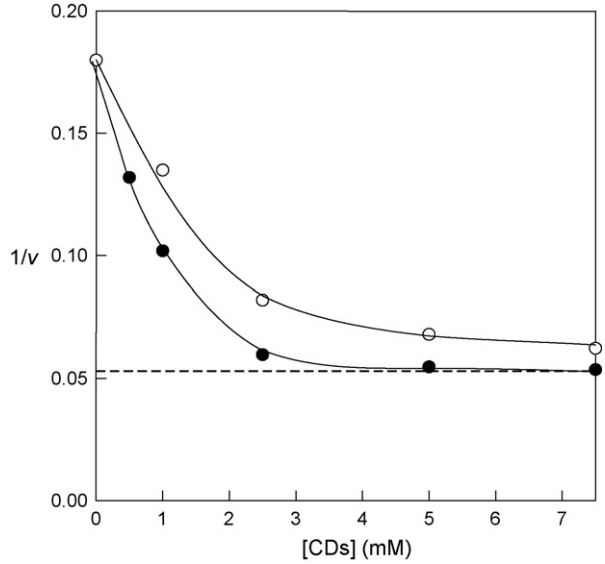


Fig. 4. Effect of OH-β-CDs (●) and γ-CDs (◯) on the oxidation of dopamine by tyrosinase in the presence of hexestrol. The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 4 μg/mL of partially purified tyrosinase, dopamine 5 mM, hexestrol 100 μM and increasing concentrations of CDs (0–7.5 mM). The dotted line represents the activity level without inhibitor. Solid lines represent the adjustment of experimental data to Eq. (7).

(Figs. 3 and 4). At increasing CD concentrations, the enzyme was clearly uninhibited by hexestrol and hinokitiol, as a result of the complexation between these inhibitors and the central core of the CDs, and the activity asymptotically approached the levels of activity obtained in the absence of inhibitors (0.053 min/mM) (Figs. 3 and 4, dotted lines). This effect is more evident in the core of OH-β-CDs (Figs. 3 and 4, solid circles).

To understand the recovery of the activity in *S. antibioticus* tyrosinase when dopamine was used as substrate (Figs. 3 and 4), a Michaelis–Menten equation for linear competitive inhibition must be obtained as a function of the known parameters, i.e., total CD concentration, total inhibitor concentration, V_{max} and K_M. For this, the following scheme was assumed:

$$\begin{array}{l} E + S \leftrightarrow ES \rightarrow E + P \\ \updownarrow \leftarrow \ I_f + CD_f \leftrightarrow I - CD \\ EI \end{array} \quad (3)$$

where subscript 'f' refers to the concentration of the free inhibitor, in this case, hinokitiol or hexestrol, and I − CD refers to the complex formed by cyclodextrins and the inhibitory compound.

Assuming the above, the defining equation for linear competitive inhibition is

$$v = \frac{V_m[S]}{K_M(1 + ([I]_f/K_I) + [S])} \quad (4)$$

and thus

$$\frac{1}{v} = \frac{K_M}{V_m[S]} + \frac{1}{V_m} + \frac{K_M}{V_m[S]K_I}[I]_f \quad (5)$$

where subscript 'f' refers to the concentration of the free inhibitors, hexestrol and hinokitiol, $[Ht]_f$. $[Ht]_f$ can be expressed as a function of $[Ht]_t$ in a similar way as described above for $[TBC]_f$ (Eq. (1)):

$$[Ht]_f = \frac{-([CD]_tK_c - [Ht]_tK_c + 1) + \sqrt{([CD]_tK_c - [Ht]_tK_c + 1)^2 + 4K_c[Ht]_t}}{2K_c} \quad (6)$$

and substituted in Eq. (6) to give

$$\frac{1}{v} = \frac{K_M}{V_m[S]} + \frac{1}{V_m} + \frac{K_M}{V_m[S]K_I}\left\{\frac{-([CD]_tK_c - [Ht]_tK_c + 1) + \sqrt{([CD]_tK_c - [Ht]K_c + 1)^2 + 4K_c[Ht]_c}}{2K_c}\right\} \quad (7)$$

Eq. (7) shows a nonlinear relationship between $1/v$ and $[CD]_t$ as in Figs. 3 and 4. Table 2 shows the data fitted by nonlinear regression.

When hinokitiol was used, the complexation constant (K_c) between this inhibitor and CDs was relatively high (12,903 M^{-1}), showing that both hinokitiol and CDs form a good and stable complex. When hexestrol was used, the K_c obtained was lower (856 M^{-1}). The latter, is very similar to the value obtained for 4-iodophenol using PPO banana pulp [32]. In both cases, the complexation between OH-β-CDs and

Table 2
Kinetic constants of complexation between CDs and inhibitors after fitting the data of Figs. 3 and 4 by nonlinear regression to Eq. (7)

	Hinokitiol (10 μM)	Hexestrol (100 μM)
OH-β-CDs		
K_M	8.4	8.4
V_{max}	62.5	48.36
K_I	0.50	0.049
K_c	12903.2	856
γ-CDs		
K_M	8.4	8.4
V_{max}	53.4	39.8
K_I	0.50	0.049
K_c	9813.5	792.6

the inhibitors was better than the one with γ-CDs; this can be explained because modified cyclodextrins enhance the solubility of otherwise water insoluble compounds and also because of the bigger cavity of γ-CDs, which reduces the hydrophobic interactions. These values cannot be compared because this is the first time that these inhibitors are used with any bacterial tyrosinase or PPO in the presence of CDs.

4. Conclusions

This paper deals with the kinetic characterization of diphenolase activity from *S. antibioticus* tyrosinase. Of the several inhibitors used, hinokitiol, hexestrol and tropolone were the best. Moreover, none of them has been described previously in bacteria. In addition, natural occurring compounds like cyclodextrins, which are used in food technology and in pharma, can release the inhibitory effect of the above inhibitors. These results, together with the simple purification and expression system described by our group for this enzyme, could lead to a low cost enzyme for pharmaceutical, food and polymer industries for transforming insoluble substrates in the presence of cyclodextrins.

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