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The Tyrosinase Produced by *Lentinula boryana* (Berk. & Mont.) Pegler Suffers Substrate Inhibition by L-DOPA

Rodrigo Otávio de Faria[1], *Vivian Rotuno Moure*[1], *Wellington Balmant*[1], *Maria Angela Lopes de Almeida Amazonas*[2], *Nadia Krieger*[3] *and David Alexander Mitchell*[1*]

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx. P. 19046, Centro Politécnico, Curitiba 81531-990, Paraná, Brazil

[2]Centro Nacional de Pesquisa de Florestas, Empresa Brasileira de Pesquisa Agropecuária – Embrapa Florestas, Cx. P. 319 Colombo 83411-000, Paraná, Brazil

[3]Departamento de Química, Universidade Federal do Paraná, Cx. P. 19081, Centro Politécnico, Curitiba 81531-990, Paraná, Brazil

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Summary

We undertook a preliminary characterization of the tyrosinase produced by a strain of *Lentinula boryana* from Brazil, with a view to evaluate its potential for biotechnological applications. The enzyme was similar to other fungal tyrosinases in many respects. When the crude extract was characterized, the tyrosinase activity was optimal at pH=6 and was not particularly thermostable, with half-lives of about 10 min and 1 min at 50 and 60 °C, respectively. We purified the enzyme with ammonium sulfate precipitation followed by ion exchange chromatography on a DEAE Sepharose column, obtaining a yield of 33 % and a 5.3-fold enrichment. The purified preparation gave three bands on SDS-PAGE, with molecular masses of 20, 27 and 47 kDa. This preparation showed substrate inhibition kinetics with L-DOPA (3,4-dihydroxy-L-phenylalanine), with a K_M of 1.9 mM and a K_I of 72 mM. Under the same reaction conditions, a commercial mushroom tyrosinase followed Michaelis-Menten kinetics, with a K_M of 0.51 mM. Although the present study did not identify properties that would make the tyrosinase of *L. boryana* more suitable in biotechnological applications than tyrosinases from other mushrooms, it has made a contribution by showing that the enzyme suffers substrate inhibition by L-DOPA, something that has not previously been reported for mushroom tyrosinases.

Key words: tyrosinase, *Lentinula boryana*, substrate inhibition, 3,4-dihydroxy-L-phenylalanine, L-DOPA

Introduction

Lentinula boryana is an edible fungus, often referred to as American shiitake, that grows in tropical and subtropical regions of the American continent, from the southeast of the United States of America to South America (*1*). During recent studies of a strain of *L. boryana* from Brazil, we noted the production of a dark pigment, which was characterized as a DOPA-melanin (*2*). Since the production of DOPA-melanin is a strong indication that the producing organism also produces the enzyme tyrosinase, we tested the mycelium for tyrosinase activity. The test result was positive.

Tyrosinase (EC 1.14.18.1; tyrosine, L-DOPA:oxygen oxidoreductase; catecholase; diphenol oxidase; polyphe-

*Corresponding author; Phone: ++55 41 3361 1658; Fax: ++55 41 3266 2042; E-mail: davidmitchell@ufpr.br

nol oxidase) is a bifunctional copper-containing enzyme. In its first reaction with a phenolic substrate, it introduces a hydroxyl group into the *ortho* position of the aromatic ring. This activity is referred to as the monooxygenase or cresolase activity. In its second reaction, tyrosinase oxidizes the *o*-dihydroxy compound produced in the first reaction to an *o*-quinone. This activity is referred to as the diphenolase or catecholase activity (*3*). These activities have recently become of interest for use in several biotechnological applications (*4–6*). For example, the cresolase activity of tyrosinase has been used to produce L-DOPA (3,4-dihydroxy-L-phenylalanine) from tyrosine, L-DOPA being important in the treatment of Parkinson's disease. There has also been recent interest in the development of biosensors for phenolic compounds that work on the basis of the reaction of these compounds with immobilized tyrosinase (*4,5*). This idea has been extended to the development of systems in which immobilized tyrosinase is used to remove phenolic compounds from wastewaters (*6*).

In the present work, as an initial step in evaluating the biotechnological potential of the tyrosinase of *L. boryana*, we undertook a preliminary characterization of the enzyme. Although the enzyme did not have characteristics that would make it more suitable for biotechnological applications than other mushroom tyrosinases, it did show an interesting difference with respect to its kinetics, namely the fact that it suffered substrate inhibition with L-DOPA.

Materials and Methods

Microorganism

The strain used in this work, *Lentinula boryana* CNPF 24, originally isolated from a collection made in the Atlantic Forest in the Brazilian State of Paraná, came from the macrofungi culture collection of Embrapa Florestas, Paraná, Brazil, where it was inoculated into logs of *Eucalyptus grandis* and then re-isolated from a fruiting body. The mycelium used in this work was maintained on potato dextrose agar (PDA) plates and stored at 4 °C.

Production and extraction of tyrosinase

Mycelium of *L. boryana* was scraped from a plate with a wire loop and inoculated into 250-mL conical flasks containing 50 mL of a medium, modified from Fang and Zhong (*7*), which contained, in g/L of distilled water: glucose 35, peptone 5, malt extract 5, $NaH_2PO_4 \cdot H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 0.5 and thiamin chloride 0.05. Flasks were incubated in an orbital shaker at 120 rpm and 21 °C for 21 days. The mycelium from each flask was then recovered by filtration through a metal sieve and transferred into a 500-mL conical flask containing 100 mL of the same medium. These flasks were incubated in an orbital shaker at 120 rpm and 30 °C until the mycelium became dark (approximately 15 days). The dark mycelium was recovered by filtration through Qualy filters of 14-μm pore size under vacuum and then lyophilized. The lyophilized mycelium was resuspended in phosphate buffer (0.05 M, pH=6.8) and then passed three times through a French press at 1000 psi. The extract obtained in this step was centrifuged at 5000×*g* in a Hitachi Himac (CR 21E) centrifuge. The supernatant after centrifugation represented the crude extract.

Purification of the crude extract

The crude extract was maintained on ice with agitation and ammonium sulfate was added slowly until 60 % of saturation. The solution was agitated overnight at 4 °C and then centrifuged at 10 000×*g*. The supernatant was discarded. The precipitate was resuspended in phosphate buffer (0.05 M, pH=6.8) and then dialyzed against the same buffer with a 6 kDa molecular mass cut-off dialysis membrane. The resulting preparation will be hereafter referred to as the dialyzed resuspended precipitate. A 5-mL sample of this dialyzed resuspended precipitate was applied to a 5×15 cm column containing DEAE Sepharose, equilibrated with phosphate buffer (0.05 M, pH=6.8) and then eluted with a stepwise gradient of NaCl (0 to 1.0 M in the same buffer). Fractions of 5 mL were collected. The fractions with the highest activity were pooled and then concentrated at 4000×*g* in Centricon tubes (exclusion limit of 5 kDa).

Analytical methods

The catecholase activities of the tyrosinase purified from *L. boryana* and of that purchased from Sigma-Aldrich Co. (Steinheim, Germany, hereafter referred to as commercial mushroom tyrosinase) were determined based on the method of Masamoto *et al.* (*8*), in which the formation of the final dopachrome product was monitored spectrophotometrically at 475 nm. The assay mixture contained an L-DOPA (3,4-dihydroxy-L-phenylalanine, Sigma-Aldrich Co., Steinheim, Germany) concentration of 10 mM. The standard assay was done in 0.05 M phosphate buffer at pH=6.8 and 25 °C. In all cases initial velocities were calculated, the values being determined by adjusting a polynomial to the data and determining the tangent at zero time. A unit of activity (U) was defined as a variation of 0.01 in A_{475} per minute. In some cases activities were expressed as percentages of the highest enzyme activity obtained in the assay. The kinetic constants were determined using L-DOPA concentrations ranging from 0.25 to 30 mM.

SDS-PAGE was performed according to Laemmli (*9*), with 4.5 % (mass per volume) polyacrylamide in the stacking gel and 12.5 % in the separation gel. Molecular mass markers from 14.4 to 97 kDa (Amersham Pharmacia Biotech) were used. The gels were stained with Coomassie Brilliant Blue R250.

Protein concentrations in fractions were determined by the Bradford method (*10*). Bovine serum albumin was used to construct the standard curve.

Numerical methods

Nonlinear regression was undertaken using the subroutine NLREG of TKSolver (Universal Technical Systems). This subroutine uses the Levenberg-Marquardt algorithm of nonlinear optimization, which begins by using the steepest descent method but later switches to the Newton method to ensure rapid convergence (*11*).

Two equations were adjusted to the data, namely the classical Michaelis-Menten equation:

$$v_o = \frac{V_{max}[S]}{K_M + [S]} \qquad /1/$$

and the equation for substrate inhibition,

$$v_o = \frac{V_{max}[S]}{K_M + [S] + \frac{[S]^2}{K_I}} \qquad /2/$$

The set of ordinary differential equations that constituted the mathematical model (presented later) was solved using the FORTRAN subroutine DASSL, which uses the backward differentiation method (*12*). The initial velocities were determined for the simulated reaction profiles in the same manner as described above for the experimental profiles.

Results

Characterization of the crude extract

Initially, we determined the effect of temperature and pH on the stability and activity of tyrosinase in the crude extract. Temperature had relatively little effect on the initial velocity over the range of 20 to 45 °C (Fig. 1). In the case of pH, the maximal activity occurred at pH=6 (Fig. 2). The relative activity remained high as the pH was decreased to 4.6; at this pH it was still above 70 %. However, at still lower pH values the relative activity fell sharply. Above pH=6, it fell relatively slowly, being approximately 70 % at pH=8.

The tyrosinase activity was not particularly thermostable. Fig. 3 shows the residual activity after a 10-minute incubation in 0.05 M phosphate buffer at pH=6.8, at temperatures ranging from 25 to 90 °C. At 25 °C the residual activity was 100 %. At 40 °C the residual activity was still above 90 %, but for higher temperatures the residual activity fell sharply, being around 10 % at 60 °C.

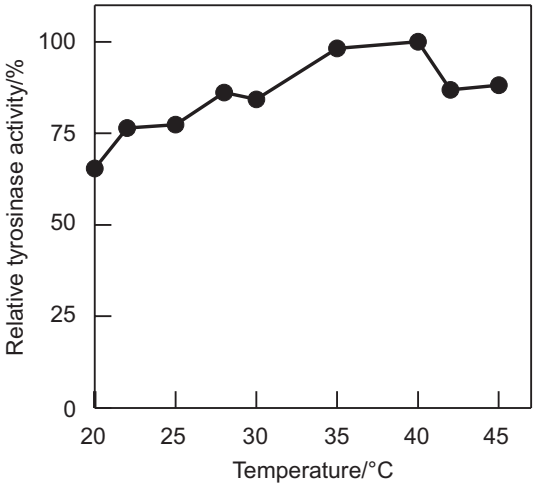

Fig. 1. Effect of temperature on the tyrosinase activity within the crude extract prepared from *L. boryana* CNPF 24. Assays were done in phosphate buffer (0.05 M, pH=6.8). The activity of the sample incubated at 40 °C was taken as 100 %

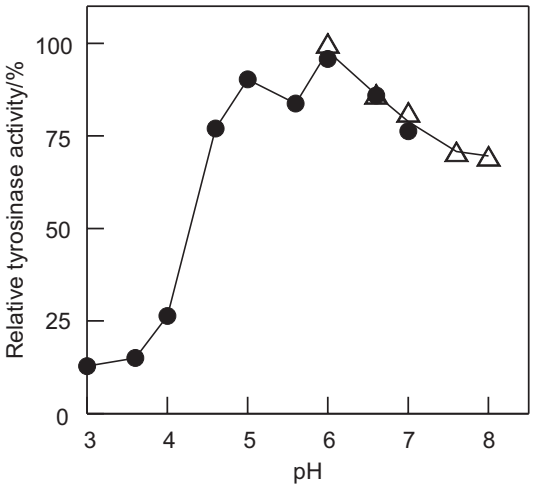

Fig. 2. Effect of pH on the tyrosinase activity within the crude extract prepared from *L. boryana* CNPF 24. Assays were done at 25 °C. The activity of the sample incubated at pH=6 in phosphate buffer was taken as 100 %. Key: (Δ) phosphate buffer (50 mM) and (•) citrate-phosphate buffer (0.05 M)

Fig. 4 shows the kinetics of denaturation at 60 °C. Over the first two minutes the denaturation followed first order kinetics, with a half-life of 1.1 min, however, at longer times the residual activity was higher than a first order decay model would predict. A temperature of 25 °C was chosen for future assays since the enzyme shows good stability at this temperature over the duration of activity assays.

Partial purification of tyrosinase

We decided to purify the enzyme further before characterizing its kinetics. To purify the enzyme, we resuspended the precipitate obtained after ammonium sulfate fractionation and dialyzed it. This dialyzed re-

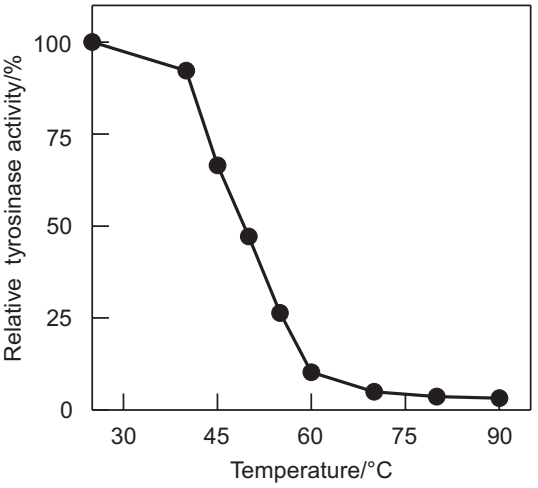

Fig. 3. Stability of the tyrosinase activity within the crude extract prepared from *L. boryana* CNPF 24 as a function of temperature. Samples of the enzyme were incubated at pH=6.8 in phosphate buffer (0.05 M) at the given temperature for 10 min before the residual activity was determined. The activity of the original sample was taken as 100 %

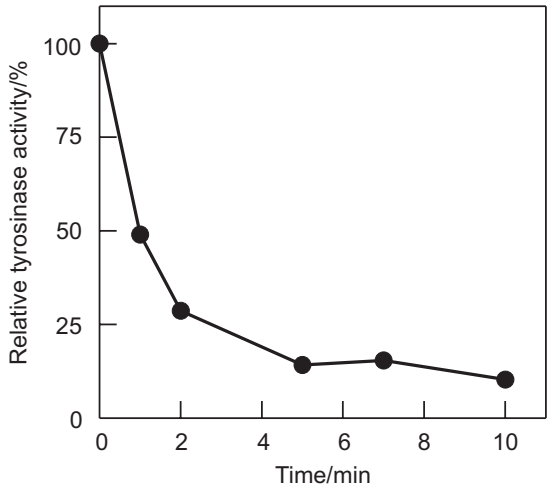

Fig. 4. Denaturation kinetics at 60 °C of the tyrosinase activity within the crude extract prepared from *L. boryana* CNPF 24. The enzyme was incubated in phosphate buffer (0.05 M, pH=6.8)

suspended precipitate still contained melanin. It was applied to a DEAE-Sepharose column and eluted with a stepwise-increasing NaCl gradient. The enzyme was eluted at 0.3 M NaCl (Fig. 5). This step separated the tyrosinase from the melanin, since the melanin remained in the column, not being eluted even at 1 M NaCl. In fact, the melanin was only eluted when 1 M NaOH was passed through the column.

This two-step purification scheme, involving ammonium sulfate precipitation and ion-exchange chromatography, resulted in a partially purified tyrosinase preparation, obtained by pooling fractions 9, 10 and 11 of Fig. 5 (*i.e.* the eluate between 40 and 55 mL). The total activity in this preparation was 33 % of that present in the crude extract. The specific activity was improved 5.3--fold, from 20.8 to 110.7 U/mg of protein. SDS-PAGE of this preparation gave three bands, with approximate molecular masses of 20, 27 and 47 kDa (Fig. 6).

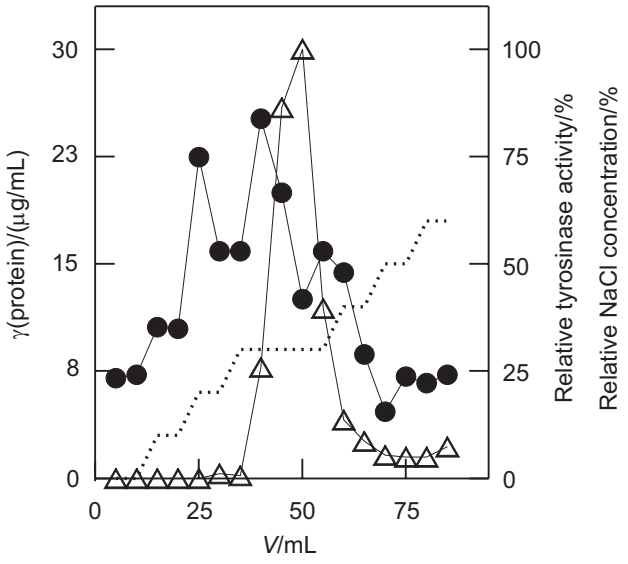

Fig. 5. Elution profile obtained after ion-exchange chromatography of the dialyzed resuspended precipitate obtained from *L. boryana* CNPF 24. Key: (Δ) tyrosinase activity, expressed as a percentage of the fraction with the highest activity; (•) protein concentration; (···) NaCl gradient expressed as a percentage, with 100 % of the gradient corresponding to an NaCl concentration of 1 M

Effect of substrate concentration on the partially purified tyrosinase

The effect of substrate concentration on initial velocity was tested for the partially purified tyrosinase and the commercial mushroom tyrosinase. In the case of the tyrosinase from *L. boryana*, the Michaelis-Menten equation (Eq. 1) was not able to fit adequately to the data (Fig. 7A). In fact, at the highest substrate concentrations the activity decreased, suggesting substrate inhibition. The equation for substrate inhibition adjusted well to the data, with a K_M of 1.9 mM and a K_I of 72 mM. On the other hand, the commercial enzyme followed classical enzyme kinetics, with a K_M of 0.51 mM (Fig. 7B).

Kinetic modeling of the substrate inhibition of the tyrosinase of L. boryana

Cabanes *et al.* (*13*) proposed a kinetic model for the action of frog epidermis tyrosinase on tyrosine and L-DOPA and provided estimates of the parameters of the model. We extended the part of their model that describes oxidation of L-DOPA by the inclusion of the classical mechanism that underlies the substrate inhibition equation, namely the ability of a second substrate molecule to bind to the enzyme-substrate complex to produce a ternary complex that is unable to catalyze the reaction. Note that tyrosinase can be in either of two forms, namely

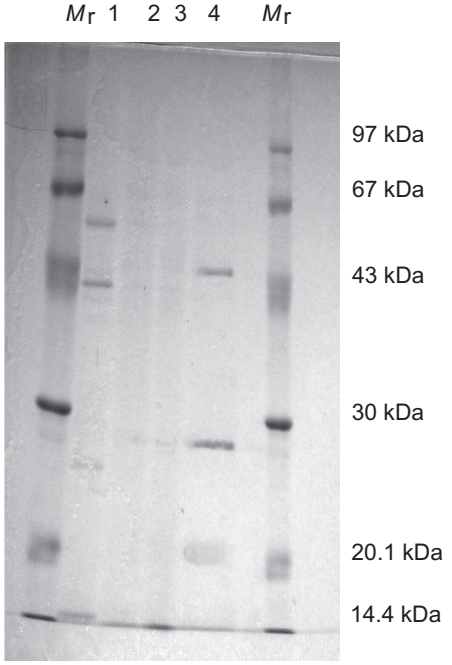

Fig. 6. SDS-PAGE of partially purified *L. boryana* CNPF 24 tyrosinase. Key: lane 1 is the commercial mushroom tyrosinase (Sigma Co.); lane 2 is the crude extract obtained from *Lentinula boryana* CNPF 24; lane 3 is the resuspended precipitate; lane 4 is the pool of fractions 9, 10 and 11 obtained after ion-exchange chromatography. The two lanes marked M_r correspond to molecular mass markers (Amersham Pharmacia Biotech)

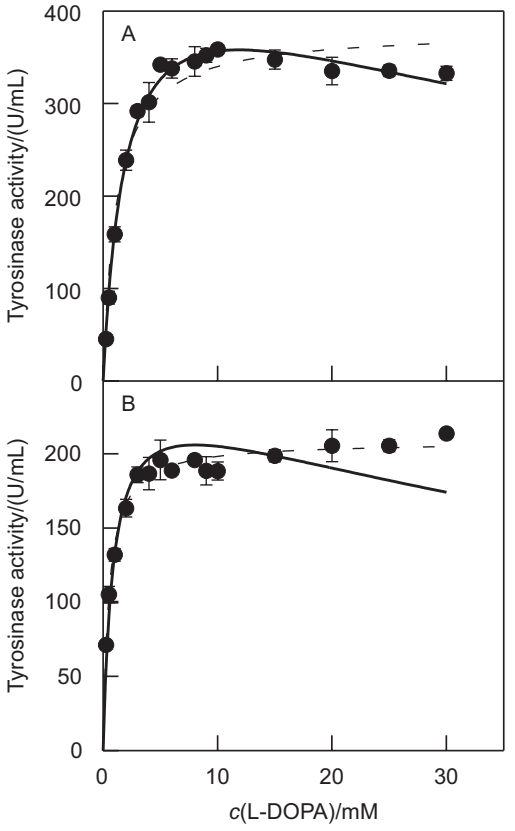

Fig. 7. Effect of substrate concentration on the initial velocities for reaction with L-DOPA of (A) the partially purified *L. boryana* CNPF 24 tyrosinase and (B) the commercial mushroom tyrosinase. Assays were done at pH=6.8 and 25 °C. In both cases the symbols represent the experimental data, the solid line represents the best fit of the substrate inhibition equation (Eq. 2) and the dashed line represents the best fit of the Michaelis-Menten equation (Eq. 1)

E_{met} and E_{oxy}, both of which can bind L-DOPA (represented by D in this scheme). We assumed that both the $E_{met}D$ and the $E_{oxy}D$ complexes were able to bind an extra molecule of D, which results in the reaction scheme shown in Fig. 8.

This scheme is described by the following set of differential equations:

$$\frac{d[D]}{dt} = k_3[E_{oxy}D] - k_4[E_{oxy}][D] - k_{10}[E_{met}][D] + k_9[E_{met}D] + 0.5k_{12}[Q] - k_{13}[E_{oxy}D][D] + k_{14}[E_{oxy}D_2] - k_{15}[E_{met}D][D] + k_{16}[E_{met}D_2] \quad /3/$$

$$\frac{d[E_{oxy}]}{dt} = k_3[E_{oxy}D] + k_{11}[E_{met}D] - k_4[E_{oxy}][D] \quad /4/$$

$$\frac{d[E_{met}]}{dt} = k_9[E_{met}D] - k_{10}[E_{met}][D] + k_5[E_{oxy}D] \quad /5/$$

$$\frac{d[E_{oxy}D]}{dt} = k_4[E_{oxy}][D] - k_3[E_{oxy}D] - k_5[E_{oxy}D] - k_{13}[E_{oxy}D][D] + k_{14}[E_{oxy}D_2] \quad /6/$$

$$\frac{d[E_{met}D]}{dt} = k_{10}[E_{met}][D] - k_9[E_{met}D] - k_{11}[E_{met}D] - k_{15}[E_{met}D][D] + k_{16}[E_{met}D_2] \quad /7/$$

$$\frac{d[E_{oxy}D_2]}{dt} = k_{13}[E_{oxy}D][D] - k_{14}[E_{oxy}D_2] \quad /8/$$

$$\frac{d[E_{met}D_2]}{dt} = k_{15}[E_{met}D][D] - k_{16}[E_{met}D_2] \quad /9/$$

$$\frac{d[Q]}{dt} = k_{11}[E_{met}D] + k_5[E_{oxy}D] - k_{12}[Q] \quad /10/$$

$$\frac{d[P]}{dt} = 0.5k_{12}[Q] \quad /11/$$

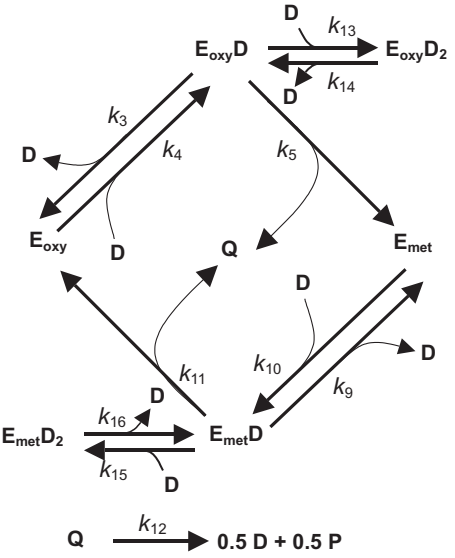

Fig. 8. The scheme for reaction of tyrosinase with L-DOPA, modified from Cabanes *et al.* (*13*) in order to include substrate inhibition. Note that the enzyme can be in either of two forms, E_{met} or E_{oxy}, both of which are capable of binding L-DOPA. Key: D is L-DOPA, P is the final product, dopachrome, Q is the intermediate product, dopaquinone; $E_{oxy}D$ and $E_{met}D$ are the catalytic enzyme-substrate complexes; $E_{oxy}D_2$ and $E_{met}D_2$ are the complexes that result from the binding of a second L-DOPA molecule to the enzyme-substrate complexes. These ternary complexes cannot catalyze the reaction

These equations were solved numerically, using the parameter values listed in Table 1. In the case of the constants associated with the formation of the $E_{met}D_2$ and $E_{oxy}D_2$ complexes, it was assumed that the fundamental velocity constants were the same, independently of the enzyme being in the met or oxy form (*i.e.* $k_{13}=k_{15}$ and $k_{14}=k_{16}$), and that in both cases the ratio of the dissociation constant to the complex formation constant was equal to 72 (*i.e.* $k_{14}/k_{13}=k_{16}/k_{15}=K_I=72$ mM). When this model was used to simulate the reactions, the predicted initial velocities were very close to the experimental values (Fig. 9).

Discussion

Many of the characteristics of *L. boryana* tyrosinase are similar to those of other mushroom tyrosinases and

Table 1. Values of the kinetic constants in the model used to describe substrate inhibition (see Fig. 8)

Kinetic constant*	Value and units	Source
k_3	10 s^{-1}	(*13*)
k_4	10^5 $M^{-1}{\cdot}s^{-1}$	(*13*)
k_5	10^2 s^{-1}	(*13*)
k_9	5 s^{-1}	(*13*)
k_{10}	10^6 $M^{-1}{\cdot}s^{-1}$	(*13*)
k_{11}	10^2 s^{-1}	(*13*)
k_{12}	0.41 s^{-1}	(*13*)
k_{13}	0.001 $M^{-1}{\cdot}s^{-1}$	this work
k_{14}	0.072 s^{-1}	this work
k_{15}	0.001 $M^{-1}{\cdot}s^{-1}$	this work
k_{16}	0.072 s^{-1}	this work

*The same numbering system is used as that used by Cabanes *et al.* (*13*). Since they also included the reaction with L-tyrosine in their model, they had kinetic constants that are not included in the current model (namely k_1, k_2, k_6, k_7 and k_8)

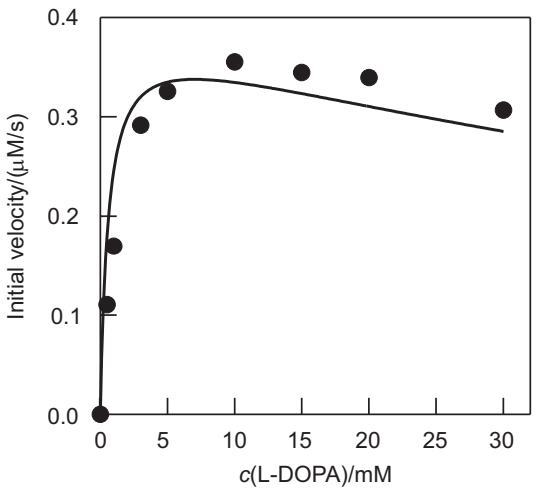

Fig. 9. Simulation of substrate inhibition of the *L. boryana* tyrosinase. Key: (•) experimental results; (—) predictions of the model of Cabanes *et al.* (*13*), modified to describe substrate inhibition

therefore we did not identify properties that would make it more suitable for biotechnological applications than other tyrosinases. A pH optimum in the vicinity of 6 to 7 was also found for the tyrosinases of *Pycnoporus sanguineus* and Portabella mushrooms (a large, brown variety of *Agaricus bisporus*) (*14*,*15*). The tyrosinase from *L. boryana* is less thermostable than that from *P. sanguineus*. The *P. sanguineus* enzyme had a half-life of 120 min at 50 °C (*14*), whereas Fig. 3 suggests that the 10-minute incubation time represented the half-life at 50 °C for the *L. boryana* enzyme.

The most interesting feature of the *L. boryana* tyrosinase identified in the current work is the occurrence of substrate inhibition in the reaction with L-DOPA. Although many mushroom tyrosinases have been kinetically characterized, substrate inhibition of the catecholase activity with L-DOPA has not previously been reported. Typically, studies with L-DOPA as the substrate have shown that mushroom tyrosinases follow Michaelis-Menten kinetics, with K_M values varying from about 1 to 10 mM (*14*,*16–18*). On the other hand, the cresolase and catecholase activities of commercial mushroom tyrosinase have been reported to suffer substrate inhibition by L-tyrosine and 4-methylcatechol, respectively (*19*,*20*).

We were able to modify the model of tyrosinase kinetics proposed by Cabanes *et al.* (*13*) to describe substrate inhibition. In fact, the modified model fitted reasonably well to our data when we used the same values for the fundamental constants as they did, at least for those reaction steps that are common. This may appear surprising, since the two tyrosinases are from widely different sources, ours being from a mushroom while that of Cabanes *et al.* was from frog epidermis. However, the good fit is a coincidence that owes itself to the fact that our enzyme had a similar K_M to that of the frog epidermis enzyme.

It is interesting that the *L. boryana* tyrosinase shows substrate inhibition by L-DOPA whereas the commercial mushroom tyrosinase does not. An understanding of the reasons underlying this difference would only be possible with studies of substrate binding, such as that undertaken by Gamage *et al.* (*21*) with the enzyme SULT1A1; they obtained crystal structures in which a second molecule of the substrate, in this case *p*-nitrophenol, had bound to the active site. Such a study would identify the differences in the structures of the active sites of the two enzymes that are responsible for the different kinetic behavior. However, first it would be necessary to identify in which polypeptide or polypeptides the tyrosinase activity resides. Note that our finding of three bands in the SDS-PAGE after ion-exchange chromatography is not without precedent and that it is possible that more than one will show tyrosinase activity. Kanda *et al.* (*22*) obtained two activity peaks after ion exchange chromatography of an extract from *Lentinula edodes*. When the fractions corresponding to each peak were analyzed by partially denaturing SDS-PAGE, both had three bands that showed tyrosinase activity. Fully denaturing SDS-PAGE of the same fractions gave bands at 15, 49 and 54 kDa for one fraction and 15, 50 and 55 kDa for the other.

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