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A kinetic model describing melanin formation by means of mushroom tyrosinase

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ABSTRACT

Melanins are an heterogeneous group of polymers formed by enzymatic reactions in vegetable tissues containing phenolic or polyphenolic molecules. Recent studies have discovered some beneficial properties of melanins on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumor properties, so not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation. Then, a further knowledge about the kinetic mechanism of melanogenesis is required prior to its possible industrial utilization. In this work, an autocatalytic kinetic model to explain melanin formation from L-tyrosine using mushroom tyrosinase and measuring the absorbance of the solution has been developed and fitted to experimental data. This expression allows to describe melanin formation as a function of time of reaction, including some important parameters such as the extinction coefficient. Absorbance will start growing after a lag period in which colorless intermediates are produced. The extinction coefficient of the resulting products is not a constant value, because it depends on the conditions of each experiment. Tyrosinase seemed to have a lower catalytic effect on L-tyrosine than on L-DOPA.

1. Introduction

Tyrosinase (polyphenoloxidase, PPO, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Duckworth & Coleman, 1970; Muñoz-Muñoz et al., 2007; Ziyan & Pekyardimci, 2003).

Tyrosinase converts L-tyrosine, a monophenol, firstly to L-DOPA (an *o*-diphenol) and this to *o*-dopaquinone, which is spontaneously cyclated in form of leukodopachrome and quickly converted to dopachrome, which polymerizes forming melanins (De Faria, Moure, Lopes, Krieger, & Mitchell, 2007). These reactions are shown in Fig. 1.

Initial studies of tyrosinases were motivated by a desire to understand and prevent the enzymatic browning that occurs in the presence of air when mushrooms, fruits or vegetables are cut or bruised. These melanins, formed by these enzymatic reactions in vegetable tissues containing phenolic or polyphenolic molecules, have been considered up to the present substances that produce deterioration in many foods, specially in fruit juices and other fruit derivatives, and decrease its market value. Nevertheless, recent discoveries on beneficial properties on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumor properties, have done that not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation. In addition, some intermediate products have been used as medicines, like L-DOPA which has been the preferred drug for treatment of Parkinson's diseases since 1967 (De Faria et al., 2007).

The wide range of substrate specificity of polyphenoloxidase has led to many methods being proposed to measure its activity: radiometric, electrometric, chronometric and especially spectrophotometric, which are fast and affordable by most laboratories (García-Molina et al., 2007). To implement these benefits that have recently been discovered, it is fundamental to have a mathematical expression to easily predict the quantity and characteristics of the melanin that can be synthesized. The aim of this work has been to develop and check the validity of a kinetic expression that allows describing and predicting melanin formation as a function of the reaction time using a fast and cheap analysis method like spectrophotometric measurements.

2. Kinetic considerations

As it has been explained, the first step to apply this enzymatic process is to obtain a kinetic characterization of melanin formation. In this work, a kinetic model to explain this reaction as a spectrophotometrical function has been developed. According to the reaction mechanism that leads L-tyrosine to melanins (De Faria

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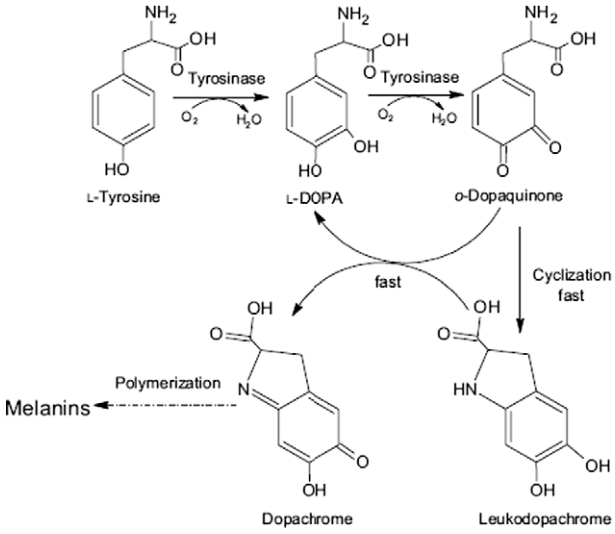

Fig. 1. Mechanism by which tyrosinase converts L-tyrosine firstly to L-DOPA and then to *o*-dopaquinone, and the following steps that lead to melanin formation (Adapted from De Faria et al., 2007).

et al., 2007), an autocatalytical reaction in two-steps, each one defined by a kinetic constant, has been assumed:

$$L\text{-Tyrosine} \rightarrow \overset{k_1}{\rightarrow} \rightarrow \text{Intermediate}$$

$$L\text{-Tyrosine} + \text{Intermediate} \rightarrow \overset{k_2}{\rightarrow} \rightarrow 2\ \text{Melanin}$$

The mass balance for the global reaction in a discontinuous stirred reactor would be (Levenspiel, 1986):

$$\frac{dC_A}{dt} = -(k_1 \cdot C_A + k_2 \cdot C_A \cdot C_M) \quad (1)$$

where C_A is the tyrosine concentration and C_M the melanin concentration.

For each time of reaction, melanin concentration (C_M) can be expressed as the difference between the initial tyrosine concentration (C_A^0) and the concentration at this moment (C_A):

$$C_A^0 - C_A = C_M$$

Then, using this expression in the mass balance and rearranging it:

$$\frac{dC_A}{k_1 \cdot C_A + k_2 \cdot C_A\ (C_A^0 - C_A)} = -dt$$

Now, the integration can be done with the boundary conditions:

$$\text{For} \quad t = 0 \rightarrow \quad C_A = C_A^0$$
$$\text{For} \quad t = t \rightarrow \quad C_A = C_A$$

So then:

$$\int_0^t dt = \int_{C_A}^{C_A^0} \frac{dC_A}{k_2 \cdot C_A\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A\right]} \quad (2)$$

The result of this integration leads to:

$$\ln\left[\frac{C_A^0\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A\right]}{C_A\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A^0\right]}\right] = (k_1 + k_2 C_A^0)(t - t_0) \quad (3)$$

Being X_A the fractional conversion of tyrosine, the substrate concentration in each moment can be expressed in the following way:

$$C_A = C_A^0(1 - X_A) = C_A^0 - C_A^0 X_A \quad (4)$$

Then, using expression (4) in (3):

$$\ln\left(\frac{1 + \frac{k_2}{k_1} C_A^0 X_A}{1 - X_A}\right) = (k_1 + k_2 C_A^0)(t - t_0) \quad (5)$$

Rearranging this expression and using the Lambert–Beer law in order to obtain the absorbance as a function of the reaction time it is obtaining:

$$A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot [\exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)] - 1]}{k_2 \cdot C_A^0 + k_1 \cdot \exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)]} \quad (6)$$

where ε_λ is the extinction coefficient at a λ wavelength, l is the cell width, C_A^0 the initial tyrosine concentration and t_0 the induction time.

3. Materials and methods

Experimental data was obtained following the absorbance of the solutions containing tyrosinase and L-tyrosine at eight different concentrations in order to obtain a different curve for each one, so the kinetic expression (6) can be tested. *Agaricus bisporus* tyrosinase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at −12 °C until use. This solution was stored at 4 °C since 12 h before experiments started, and then pre-incubated at room temperature for 1 h. L-Tyrosine (Sigma Chemical, St. Louis, MO) was prepared in aqueous solution in a concentration range from 0.5 mM up to 5.5 mM (0.5, 1.0, 1.75, 2.5, 3.25, 4.0, 5.0 and 5.5 mM).

Three hundred μL of the tyrosinase preparation (150 U) were added to 15 mL of tyrosine solution of each concentration (final enzyme content: 10 U/mL). The evolution of absorbance at 480 nm was taken with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width cell. A data point was taken every 120 s with Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). All experiments were carried out at room temperature by duplicate. The shown results are the average of these two series for each concentration.

The experimental results obtained were fitted to the developed kinetic expression using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level.

4. Results and discussion

As indicated above, tyrosinase exhibits an unusual kinetic behavior due to its autocatalytical process depending on the generation of a dihydric phenol substrate, which acts an activator of the enzyme (Cooksey et al., 1997; Rodríguez-López, Tudela, Varón, García-Carmona, & García-Cánovas, 1992). These reactions lead to a sigmoid absorbance curve preceded by a lag period, as it is

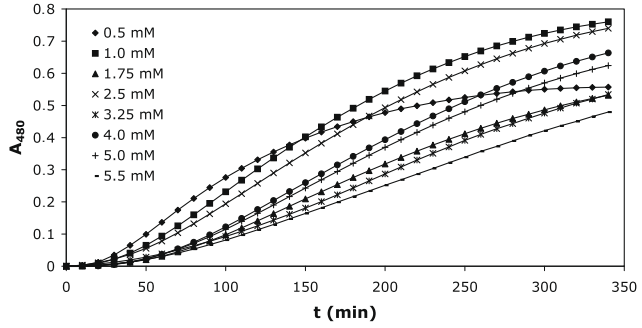

Fig. 2. Experimental data fitted to the developed kinetic model.