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Inhibitory effects of substrate analogues on enzyme activity and substrate specificities of mushroom tyrosinase

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Abstract

Tyrosinase can catalyze the oxidation of *o*-diphenols to *o*-quinones. In this paper, some *o*-diphenols were used as mushroom tyrosinase substrates to study the catalyzed specificity of the enzyme. The enzyme kinetic analysis of substrate specificities and the substrate analogues towards mushroom tyrosinase has been investigated. Taking L-3,4-dihydroxyphenylalanine (**I**), 3,4-dihydroxyhydrocinnamic acid (**II**), 3,4-dihydroxycinnamic acid (**III**) and 1,2,4-benzenetriol (**IV**) as substrates, the results of specificity studies showed that the oxidation reaction of tested *o*-diphenols by mushroom tyrosinase followed Michaelis–Menten kinetics. The Michaelis–Menten constants for these four substrates were determined to be 0.615, 1.238, 0.331 and 1.886 mM, respectively. The values of V_m/K_m, which denotes the affinity of the enzyme to the substrate, were determined and compared, and the results showed that the affinity of the enzyme to these substrates followed the order: compound **IV** > **III** > **I** > **II**. Furthermore, mushroom tyrosinase cannot catalyze the oxidation of 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**). On the contrary, compounds **a**, **b** and **c** can inhibit the activity of tyrosinase for the oxidation of DOPA, while compound **d** had no effects on enzyme activity. The results show that compounds **a** and **b** are reversible non-competitive inhibitors.

Keywords: Mushroom tyrosinase; Substrate specificity; Substrate analogues; Inhibition; Kinetics

1. Introduction

Tyrosinase (EC 1.14.18.1) is a polyphenolase with broad selectivity. It displays two enzymatic activities which are located on the same active sites: cresolase activity, which is thought to be selective towards monophenols (the hydroxylation of L-tyrosine), and catechol oxidase activity, which is determined to be selective towards diphenols (the oxidation of diphenols into corresponding quinones) (Espín, Jolivet, Overeem, & Wichers, 1999). The active site of tyrosinase consists of two copper atoms and three states: *met, deoxy* and *oxy* (Espín, Varón, & Fenoll, 2000; Jiménez & García-Carmona, 2000). Study of the active site of tyrosinase has been the subject of numerous studies. Different methods such as IR and NMR have been employed for this purpose (Bubacco, Salgado, Tepper, Vijgenboom, & Canters, 1999; Gentschev, Lüken, Möller, Rompel, & Krebs, 2001). Structural models for the active site of these three forms of tyrosinase have been proposed (Fenoll et al., 2001), but the crystal structure of tyrosinase has not been obtained. The reaction kinetic assay based on a spectrophotometric technique for the study of the activity of tyrosinase has attracted attention, mainly because it is convenient, sensitive and inexpensive.

Specificity studies can not only help to unravel the reaction pathway or catalytic mechanism, but also guide

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substrate structure-based inhibitors development and drug design. We are involved in a systematic study of the specificity of mushroom tyrosinase towards different substrates, aimed at the understanding of the factors that govern the interaction between the enzyme and the substrate or some substrate analogues.

Due to the decrease in pigmentation caused by the enzyme action, tyrosinase inhibitors are supposed to have broad applications in medicinal and cosmetic whitening agents (Fenoll et al., 2001; Friedman, 1996; Likhitwitayawuid & Sritularak, 2001). Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. Substrate analogues, such as cinnamic acid derivatives and 4-substituted resorcinols, could be potent enzyme inhibitors (Jiménez & García-Carmona, 1997; Shi, Chen, Wang, Song, & Qiu, 2005).

In this paper, four kinds of substrate, L-3,4-dihydroxyphenylalanine (L-DOPA) (**I**); 3,4-dihydroxyhydrocinnamic acid (**II**); 3,4-dihydroxycinnamic acid (**III**) and 1,2,4- benzenetriol (**IV**), were tested and compared. The effects of substrate analogues, 3,4-dihydroxybenzonitrile (**a**); 3,4-dihydroxybenzaldehyde (**b**); 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**) were also tested for tyrosinase inhibitory ability (Fig. 1). Despite their close structural similarity, these analogues showed many differences in inhibition towards mushroom tyrosinase. The aim of this present experiment is, therefore, to carry out a kinetic study on the inhibition of tyrosinase by these *o*-diphenol derivatives and to evaluate the kinetic parameters and inhibition constants characterizing the system, as well as investigate the inhibition mechanism involved.

2. Materials and methods

2.1. Reagents

Mushroom tyrosinase (EC 1.14.18.1), L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyhydrocinnamic acid, 3,4-dihydroxycinnamic acid and 1,2,4-benzenetriol were purchased from Sigma. 3,4-Dihydroxybenzonitrile, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid were the products of Aldrich (St. Louis, MO, USA).

2.2. Enzyme activity assay

Tyrosinase catalyzes the reaction between two substrates, a phenolic compound and oxygen, so the assay was carried out in air-saturated solutions. The diphenolase activity of mushroom tyrosinase was measured spectrophotometrically at 30 °C, by following the oxidation of substrates as previously reported (Chen et al., 2005). The enzyme activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) (Jiménez, Chazarra, Escribano, Cabanes, & García-Carmona, 2001). The reaction media (3 ml) for *o*-diphenolase activity contained 0.5 mM of substrate in 50 mM sodium phosphate buffer (pH 6.8). The final concentration of mushroom tyrosinase was 6.67 μg/ml. This solution was immediately monitored for 1 min, after a lag period of 5 s, for the formation of dopachrome, by measuring the linear increase in optical density at 475 nm. Absorption was recorded using a UV-6000 spectrophotometer.

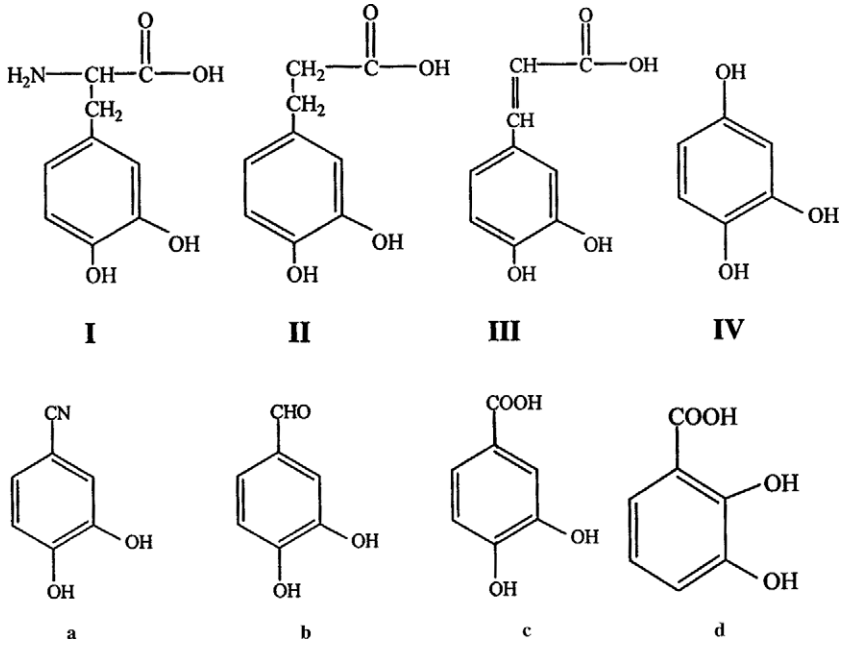

Fig. 1. Chemical structures of L-3,4-dihydroxyphenylalanine (**I**), 3,4-dihydroxyhydrocinnamic acid (**II**), 3,4-dihydroxycinnamic acid (**III**), 1,2,4-benzenetriol (**IV**) as substrates and 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**), 2,3-dihydroxybenzoic acid (**d**) as substrate analogues.