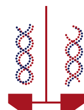
Journal of Bioscience and Bioengineering
VOL. 117 No. 2, 142–146, 2014

www.elsevier.com/locate/jbiosc

Inhibitory kinetics of chlorocinnamic acids on mushroom tyrosinase

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Received 22 April 2013; accepted 2 July 2013
Available online 16 August 2013

Tyrosinase (EC 1.14.18.1) is the key enzyme of most food enzymatic oxidation. Tyrosinase inhibitors are important in food industry. In the present paper, 2-chlorcinnamic acid and 2,4-dichlorocinnamic acid were synthesized and the inhibitory kinetics on mushroom tyrosinase were investigated. The results showed that both compounds synthesized could inhibit tyrosinase activity. For monophenolase activity, both chlorocinnamic acids could extended the lag time and decrease the steady-state activities, 2-chlorcinnamic acid extended the lag time just by 5%, and 2,4-dichlorcinnamic acid extended the lag time more than by 30.4%. For diphenolase activity, the IC_{50} values of 2-chlorcinnamic acid and 2,4-dichlorocinnamic acid were determined to be 0.765 mM and 0.295 mM, respectively. The inhibition kinetics showed that 2-chlorcinnamic acid and 2,4-dichlorocinnamic acid displayed a reversible and uncompetitive mechanism. The inhibition constants were determined to be 0.348 mM and 0.159 mM, respectively. The research may supply the basis for designing new tyrosinase inhibitors.

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[**Key words:** Chlorcinnamic acid; Synthesis; Mushroom tyrosinase; Inhibition; Kinetics]

Tyrosinase (EC 1.14.18.1) is known to be a multifunctional copper-containing enzyme from the oxidase superfamily (1). It is ubiquitously distributed in organisms and has multi-catalytic functions: the hydroxylation of tyrosine to L-DOPA (monophenolase activity), the oxidation of L-DOPA to dopaquinone (diphenolase activity), and the oxidation of 5,6-dihydroxyindole to 5,6-dihydroxyquinone (2,3). In most food, the browning process has two components: enzymatic and nonenzymatic oxidation. The enzymatic oxidation can be contributed by tyrosinase (4), which is responsible for browning in certain fruits and vegetables at the time of postharvest handling, and tyrosinase is considered to be deleterious to the color quality of their derived food and beverages. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and economic values and has been of great concern (5–7). However, Tyrosinase is not only the key enzyme in the browning of fruits and vegetables, but also the key enzyme of the darkening of skin, hair and eyes in animals (8). Hence, the discovery of new and safe tyrosinase inhibitors should have broad applications.

Many efforts have been spent searching tyrosinase inhibitors which are feasible and effective. A large number of tyrosinase inhibitors have already been reported, such as 4-substituted benzaldehydes (9), α-cyano-4-hydroxycinnamic acid (10), cardol triene (6), 4-chlorosalicylic acid (11), tetrahydrofolic acid (12). These compounds showed inhibitory effects on the activity of tyrosinase. Cinnamic acid is an organic acid occurring naturally in plants that has low toxicity and a broad spectrum of biological activities (13). Cinnamic acid and its derivatives are widely used in food additives, pharmaceutical industry including hepatoprotective and anti-oxidant (14). Shi et al. (15) reported that cinnamic acid strongly inhibited the diphenolase activity of mushroom tyrosinase and the inhibitory was reversible, the IC_{50} was 2.10 mM. In this present work, 2-chlorcinnamic acid and 2,4-dichlorocinnamic acid were synthesized, which are derivatives of cinnamic acid. In the present paper, we studied series of the inhibitory kinetics of the compounds on the mushroom tyrosinase, and investigated the kinetic parameters and the inhibition mechanisms to supply the basis for designing new tyrosinase inhibitors.

MATERIALS AND METHODS

Reagents Tyrosinase from mushroom was the product of Sigma (St. Louis, MO, USA). The specific activity of the enzyme is 6680 U/mg. Dimethyl sulfoxide (DMSO), L-tyrosine (L-Tyr) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Aldrich (St. Louis, MO, USA). All other reagents were local analytical grade. The water used was re-distilled and ion-free.

Synthesis Compounds were prepared by the method mentioned as below: 5 mM furfural mixing with 16 ml 170 mM acetic anhydride, mixing 7 g anhydrous potassium carbonate, adding zeolite, refluxing 1.5 h, pouring into 100 ml distilled water, adding sodium carbonate to slightly alkaline, mixing activated charcoal, boiling 5 min, then filtering. The filtrate was added hydrochloric acid until complete precipitation of the product. The products were purified by recrystallization

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Abbreviations: DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; IC_{50}, the inhibitor concentrations leading to 50% activity lost; K_{IS}, equilibrium constant of the inhibitor combining with the enzyme-substrate complex; L-Tyr, L-tyrosine.

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http://dx.doi.org/10.1016/j.jbiosc.2013.07.002

from ethanol/water = 4:1 (v/v). They were identified by ^{1}H NMR, ^{13}C NMR and IR analysis. ^{1}H NMR and ^{13}C NMR data were acquired on a 400 MHz NMR spectrometer (Bruker AV400).

Enzyme activity assay Monophenolase and diphenolase activities of tyrosinase were determined at 475 nm as previously reported (16). In this investigation, L-Tyr was used as substrate for monophenolase activity assay and L-DOPA was used as substrate for diphenolase activity assay. The activity assay used 3 ml of reaction medium containing 2 mM L-Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentrations of tyrosinase were 13.33 μg/ml for monophenolase activity and 3.33 μg/ml for diphenolase activity. The substrate reaction progress curve was analyzed to obtain the reaction rate constants. The reaction was carried out at 30°C and pH 6.8. The kinetic and inhibition constants were obtained by the method previously described (17,18).

RESULTS

Chemical synthesis of 2-chlorocinnamic acid and 2,4-dichlorocinnamic acid 2-Chlorocinnamic acid is a white needle-shaped crystal, yield: 30.59%. ^{1}H NMR (DMSO-D6, MS, 400 MHz): δ (ppm) 7.93 (1H, m), 7.55 (1H, d), 7.45 (1H, m), 7.39 (1H, m), 6.63 (1H, d); ^{13}C NMR (DMSO-D6, TMS, 400 MHz): δ (ppm) 168.02 (s), 150.99 (s), 146.22 (s), 131.68 (s), 117.97 (s), 116.72 (s), 113.19 (s); IR (KBr): 3131.48–2589.42, 1684.73, 1622.02, 1470.59, 1338.60, 1277.78, 984.70. 2,4-Dichlorcinnamic acid is also a white needle-shaped crystal, yield: 14.2%. ^{1}H NMR (DMSO-D6, TMS, 400 MHz): δ (ppm) 12.74 (1H, COOH, s), 7.96 (1H, d), 7.81 (1H, d), 7.71 (1H, d), 7.47 (1H, m), 6.65 (1H, d); ^{13}C NMR (DMSO-D6, TMS, 400 MHz): δ (ppm) 167.55 (s), 137.98 (s), 135.77 (s), 134.80 (s), 131.41 (s), 129.98 (d), 128.42 (s), 123.51 (s); IR (KBr): 2978.87–2607.37, 1688.41, 1618.41, 1416.95, 1279.21, 984.22. The structures are shown in Fig. 1.

Inhibitory effects of chlorocinnamic acids on monophenolase activity of tyrosinase The inhibitory effects of the different concentrations of 2-chloricinnamic acid on the oxidation of L-Tyr by the enzyme were studied. A marked lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of dopachrome which is the first stable product. The system reached a steady-state rate after the lag period, which was estimated by extrapolation of the curve to the abscissa. The kinetics course of the oxidation of the substrate in the presence of different concentrations of 2-chlorocinnamic acid is shown in Fig. 2. The lag period was estimated to be 100 s in the absence of the inhibitor, and extended to 105 s in the presence of 3 mM of 2-chlorcinnamic acid, which lengthened only 5%. The steady-state rate decreased with increasing the concentration of 2-chlorocinnamic acid. When the concentration of the inhibitor is 3 mM, the remaining enzyme decreased 41.5%.

The inhibitory kinetic courses of 2,4-dichloricinnamic acid on the oxidation of L-Tyr by mushroom tyrosinase were tested. The results (Fig. 3) indicated that the lag time increased with increasing inhibitor concentration from 100 s in its absence to 130.4 s in the presence of 3 mM, which lengthened 30.4%. The steady-state rate of monophenolase activity decreased by 47.2% at 3 mM of 2,4-dichlorcinnamic acid.

Inhibitory effects of chlorocinnamic acids on diphenolase activity of tyrosinase We used L-DOPA as substrate for the assay of the diphenolase activity. The progress curve of the enzyme reaction was a straight line passing through the origin without lag period. The formation of the product was in proportion to reaction time. The value of the slope of line indicated the diphenolase activity. 2-Chlorocinnamic acid and 2,4-dichlorocinnamic acid were tested for the effect on the oxidation of L-DOPA by tyrosinase. The result showed that both compounds could inhibit the diphenolase activity of tyrosinase in a dose-dependent manner (Fig. 4). With increasing concentrations of inhibitors, the remaining enzyme activity decreased exponentially. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 0.765 mM and 0.295 mM, respectively.

The inhibitory mechanism of chlorocinnamic acids on tyrosinase The inhibitory mechanism of 2-chlorcinnamic acid and 2,4-dichlorcinnamic acid on tyrosinase for oxidation of L-DOPA was studied. Both inhibitors were in the same behavior. Fig. 5 shows the relationship between enzyme activity and its concentration in the presence of 2-chlorcinnamic acid and 2,4-dichlorcinnamic acid. The plots of the remaining enzyme activity versus the concentration

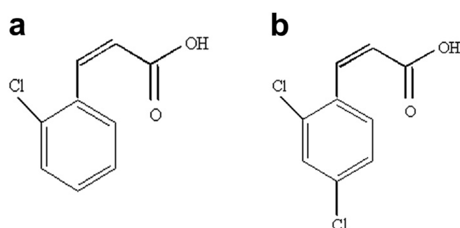

FIG. 1. Chemical structures of 2-chlorocinnamic acid (a) and 2,4-dichlorocinnamic acid (b).

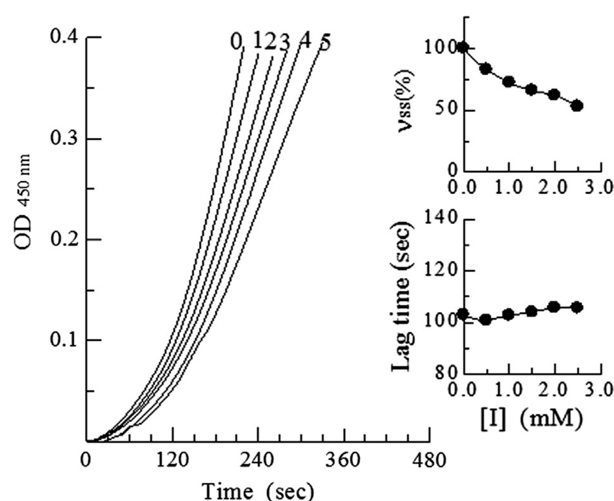

FIG. 2. Progress curves of the activity of monophenlase inhibited by 2-chlorocinnamic acid, inhibition effect on the steady-state activity and the lag time, the curves of 0–5 represent the concentration of 2-chlorocinnamic acid were 0, 0.6, 1.2, 1.8, 2.4, and 3.0 mM, respectively.

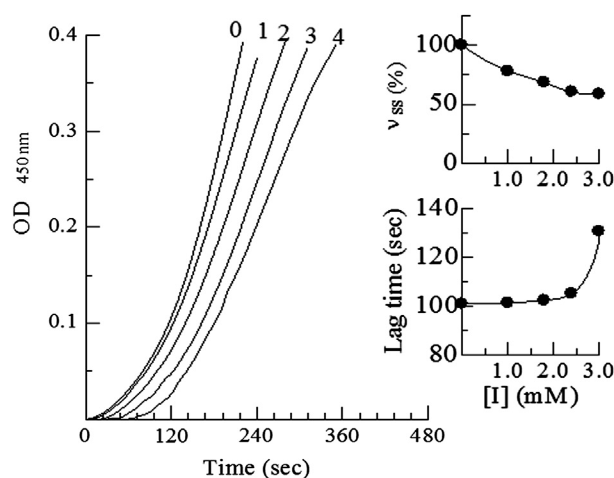

FIG. 3. Progress curves of the activity of monophenlase inhibited by 2,4-dichlorocinnamic acid, inhibition effect on the steady-state activity and the lag time, the curves of 0–4 represent the concentration of 2,4-dichlorocinnamic acid were 0, 1.0, 1.8, 2.4, and 3.0 mM, respectively.

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