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Inhibitory Effects of Fatty Acids on the Activity of Mushroom Tyrosinase

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Abstract The effects of fatty acids, octanoic acid, (2*E*, 4*E*)-hexa-2,4-dienoic acid, hexanoic acid, (2*E*)-but-2-enoic acid, and butyric acid on the activities of mushroom tyrosinase have been investigated. The results showed that the fatty acids can potently inhibit both monophenolase activity and diphenolase activity of tyrosinase, and that the unsaturated fatty acids exhibited stronger inhibitory effect against tyrosinase than the corresponding saturated fatty acids, and the inhibitory effects were enhanced with the extendability of the fatty acid chain. For the monophenolase activity, the fatty acids could not only lengthen the lag period, but also decrease the steady-state activities. For the diphenolase activity, fatty acids displayed reversible inhibition. Kinetic analyses showed that octanoic acid and hexanoic acid were mixed-type inhibitors and (2*E*,4*E*)-hexa-2,4-dienoic acid and (2*E*)-but-2-enoic acid were noncompetitive inhibitors. The inhibition constants have been determined and compared.

Keywords Tyrosinase · Fatty acid · Monophenolase activity · Diphenolase activity · Inhibition kinetics

Introduction

Tyrosinase (EC 1.14.18.1), a multifunctional copper-containing enzyme, is widely distributed in the fungi, plants, and animals [1]. It has two Cu (II) ions (Cu_A and Cu_B) in

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the active site of tyrosinase bind to six histidine residues, Cu_A binds to the nitrogen atoms of His_{38}, His_{54}, and His_{63}, and Cu_B binds to those of His_{190}, His_{194}, and His_{216} [2]. The active site of tyrosinase has three states: oxidized (E_{oxy}), reduced (E_{met}), and deoxidized states (E_{deoxy}) [3]. Tyrosinase is a key enzyme in the melanin biosynthesis; it plays an important role in catalyzing hydroxylation of monophenol to *o*-diphenol and the oxidation of diphenol to *o*-quinones [4]. Its abnormal expression is responsible for the various dermatological disorders, such as melasma, freckles, senile lentigines, age spots, and sites of actinic damage [1, 5] and it also contributes to the browning of some fruits and vegetables during handling and storage which will debase the taste and nutrition value of them [6]. For the import role of tyrosinase in the melanin biosynthesis, the control of the tyrosinase activity is of great importance in preventing formation of the unfavorable darkening.

It is well-known that tyrosinase can be inhibited by aromatic aldehydes [7], aromatic acids [8], tropolone [9], and kojic acid [10], α-cyano-4-hydroxycinnamic acid [11], 4-chlorosalicylic acid[12], phloridzin dihydrate [13], cefazolin and cefodizime [14], azelaic acid [15], and so on. Some inhibitors of tyrosinase were also found in the antimicrobial activities [16, 17]. Some inhibitors have been applied in cosmetic and medicine, but the effects of fatty acids as inhibitors of tyrosinase were rarely investigated. The purpose of the present work is, therefore, to carry out a kinetic study of the inhibition of the diphenolase activity of mushroom tyrosinase and to evaluate the kinetic parameters and the inhibition mechanisms. In addition, these dates can provide the basis for development of novel effective tyrosinase inhibitors.

Materials and Methods

Materials

Octanoic acid (a), (2*E*,4*E*)-hexa-2,4-dienoic acid (b), hexanoic acid (c), (2*E*)-but-2-enoic acid (d), and butyric acid (e) were purchased from Sinopharm Chemical Reagent Co (China). L-3, 4-dihydroxyphenylalanine (L-dopa) and L-Tyrosine (L-Tyr), dimethylsulfoxide (DMSO) were obtained from Aldrich (USA). Tyrosinase (EC 1.14.18.1) from mushroom was also from the Sigma Chemical Co. The specific activity of the enzyme was 6,680 U/mg. All other reagents were homemade and of analytical grade. The water used was re-distilled and ion free.

Assay of the Monophenolase Activity and Diphenolase Activities

The monophenolase activity and diphenolase activity assays were performed as previously reported [4]. In this investigation, L-Tyr was used as the substrate for the monophenolase activity assay and L-dopa was used as the substrate for the diphenolase activity assay. The reaction media (3 ml) for activity assay contained 0.5 mM L-Tyr or 0.5 mM L-dopa in 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH6.8) and 0.1 ml of different concentrations of inhibitor (dissolved in DMSO as previously). Of the aqueous solution of mushroom tyrosinase, 0.1 ml was added to the mixture. The final concentrations of mushroom tyrosinase were 33.33 μg/ml for the mensuration of the monophenolase activity and 6.67 μg/ml for the mensuration of the diphenolase activity [18, 19]. The enzyme activity was determined by following the increasing absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3,700 ($M^{-1}cm^{-1}$) [7] by using a Beckman UV-650 spectrophotometer. The

temperature was controlled at 30°C. The inhibitor was first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution was 3.3%. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. The inhibition type was assayed by the Lineweaver-Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

Results

Effects of Octanoic Acid, (2E,4E)-hexa-2,4-Dienoic Acid, Hexanoic Acid, (2E)-but-2-Enoic Acid, and Butyric Acid On Monophenolase Activity Of Mushroom Tyrosinase

The inhibitory effects of compounds (a), (b), (c), (d), and (e) (see Fig. 1 for the structures) at different concentrations on the oxidation of L-Tyr by the tyrosinase were assayed. The kinetic course of the oxidation of the substrate in the presence of compounds (a)-(e) was shown in Fig. 2. The monophenolase activity of mushroom tyrosinase was assayed using L-Tyr as substrate, the lag period and characteristic of monophenolase activity were observed simultaneously with the appearance of the first stable product—dopachrome. The system reached a constant rate after the lag period, which was estimated by extrapolation of

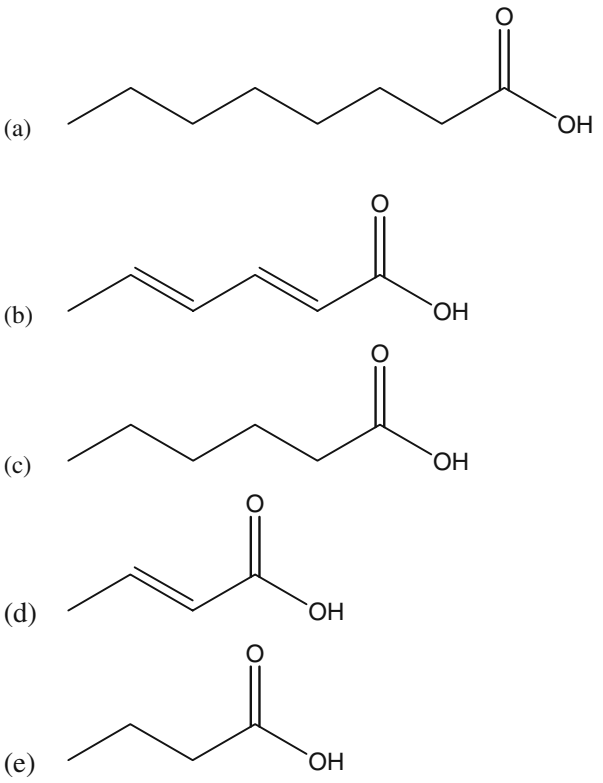

Fig. 1 Chemical Structures of octanoic acid (**a**), (2*E*,4*E*)-hexa-2,4-dienoic acid (**b**), hexanoic acid (**c**), (2*E*)-but-2-enoic acid (**d**), and butyric acid (**e**)

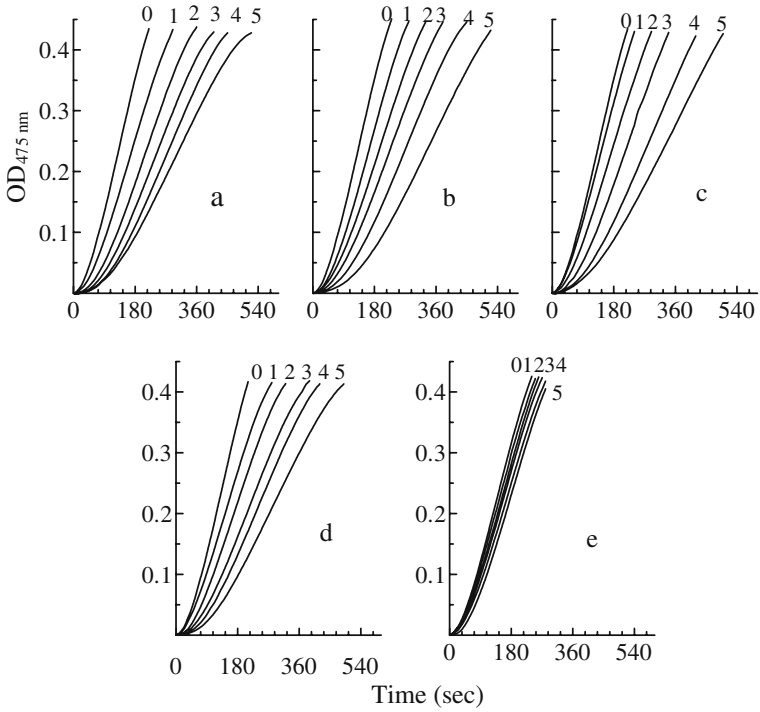

Fig. 2 Progress curves for the inhibition of monophenolase activity of mushroom tyrosinase by tested fatty acids: octanoic acid (**a**), (2*E*,4*E*)-hexa-2,4-dienoic acid (**b**), hexanoic acid (**c**), (2*E*)-but-2-enoic acid (**d**), and butyric acid (**e**). The concentrations of (**a**) and (**b**) for curve 0-5 were 0, 1, 2, 3, 4 and 5 mM, respectively. The concentrations of (**c**), (**d**), and (**e**) for curve 0-5 were 0, 4, 8, 12, 16, and 20 mM, respectively

the linear portion of the product accumulation curve to the abscissa [8]. After the reaction system reached the steady state, the curve of product increased linearly with increasing reaction time; the slope of the line denoted the steady-state rate. With the increasing of the compounds' concentrations, the steady-state rates decreased. The inhibitory effects of fatty acids on the monophenolase activity showed dose-dependent as Fig. 3(I). The inhibition strength followed the order: (a)>(b)>(c)>(d)>(e). The IC_{50} values of these compounds were determined and listed in the Table 1 for comparison. The octanoic acid was the most potent inhibitor of the monophenolase activity of mushroom tyrosinase among these compounds. On the other hand, the lag period of the enzyme was lengthened by fatty acids tested. The relationship of the lag period against the inhibitor concentrations was showed as Fig. 3(II). It was indicated that the (a), (b), (c), and (d) could prominently extend the lag period, while the compound (e) had a weak effect. When the concentration of compound (a), (b), (c), (d), and (e) reached to 5 mM, they lengthened the lag period from 29.5 to 118.5 s, 99.2 s, 46.5 s, 36.5 s, and 31.5 s, respectively. They extended the lag period of monophenolase by 302%, 237%, 57.6%, 23.7%, and 6.8%, respectively.

Effects of Fatty Acids on Diphenolase Activity of Mushroom Tyrosinase

The progress curve of the oxidation reaction of L-dopa by mushroom tyrosinase was a line passing through the origin, which indicated that the formation of product was in proportion to

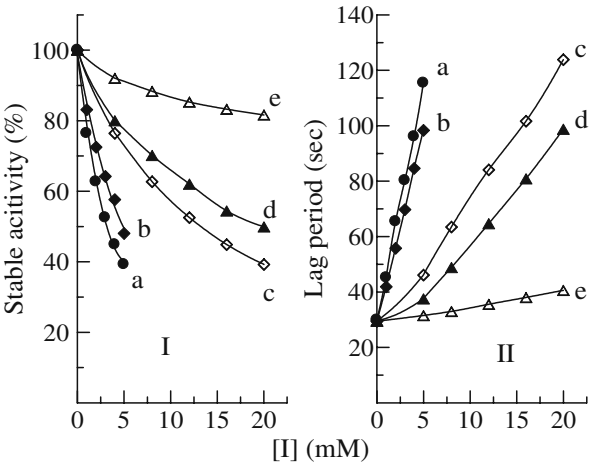

Fig. 3 Effects of fatty acids on the steady-state rate (*I*) or the lag period (*II*) of monophenolase activity for the oxidation of L-tyrisine. Curves of **a**, **b**, **c**, **d**, and **e** denote the compounds of octanoic acid, (2*E*,4*E*)-hexa-2,4-dienoic acid, hexanoic acid, (2*E*)-but-2-enoic acid and butyric acid, respectively

the reaction time. The value of the slope of the line indicated the diphenolase activity. The inhibitory concentration effect of the fatty acids tested on the diphenolase activity of tyrosinase was assayed. The diphenolase activity decreased with increasing inhibitor concentrations (Fig. 4). The IC_{50} values of compounds (a), (b), (c), and (d) were determined to be 2.15, 0.90, 3.35, and 12.05 mM, respectively. While the compound (e) only inhibited the diphenolase activity by 34.0% when its concentration reached to 16 mM. The data were summarized in Table 1 for comparison with the arbutin as positive control. Song et al reported that arbutin was a competitive tyrosinase inhibitor with IC_{50} of 5.30 mM [20]. The inhibition strength follows the order: (b) >(a)>(c)>(d)>(e). The results showed that the unsaturated fatty acids exhibited more potent inhibitory effects than saturated fatty acids, as (b)>(c)and (d)>(e); and that with the carbon chains prolongation, the inhibitory effects of fatty acids on the enzyme activity become more potent, as (a) >(c)>(e) and (b)>(d).

The Inhibition Mechanisms of Fatty Acids on Diphenolase Activity of Mushroom Tyrosinase

The inhibition mechanisms by fatty acids against the tyrosinase for the oxidation of L-dopa were studied. The relationship of enzyme activity with the enzyme concentration in the presence of different concentrations of inhibitors gave a family of straight lines, which all passed through

Table 1 Inhibition constants of octanoic acid (a), (2*E*,4*E*)-hexa-2,4-dienoic acid (b), hexanoic acid (c), (2*E*)-but-2-enoic acid (d), and butyric acid (e) with mushroom tyrosinase.

Compounds	IC_{50} (mM)		Inhibition type	Inhibition constants	
	Monophenolase activity	Diphenolase activity		K_I (mM)	K_{IS} (mM)
(a)	3.06	2.15	Mixed	1.54	3.08
(b)	4.95	0.90	Non-competitive	0.91	0.91
(c)	13.20	3.35	Mixed type	2.51	5.10
(d)	20.00	12.05	Non-competitive	12.00	12.00
(e)	>20.00	>16.00	Not detected		

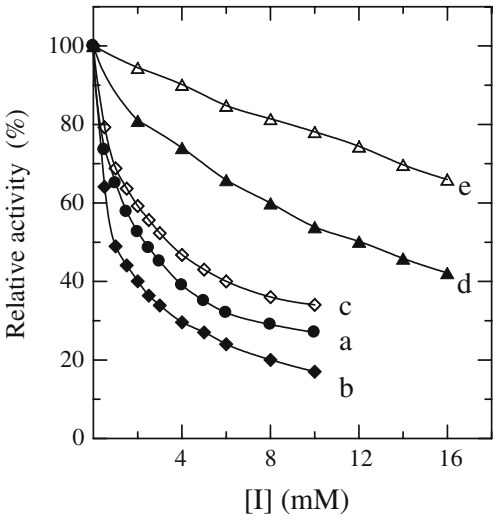

Fig. 4 Inhibitory effects of fatty acids on the diphenolase activity for the oxidation of L-dopa. Curves of **a**, **b**, **c**, **d,** and **e** denote the compounds of octanoic acid, (2*E*,4*E*)-hexa- 2,4-dienoic acid, hexanoic acid, (2*E*)-but-2-enoic acid and butyric acid, respectively

the origin, indicating that the inhibition mechanism of fatty acids on diphenolase activity of mushroom tyrosinase belonged to be reversible. Increasing the inhibitor concentration resulted in a descending of the slope of the line. The presence of compound (a) did not bring down the amount of the efficient enzyme, but just resulted in the inhibition of enzyme activity.

The Inhibition Type of Fatty Acids on Diphenolase Activity of Mushroom Tyrosinase

The inhibitory kinetics of mushroom tyrosinase by saturated fatty acids (a) and (c) had been studied. When using (a) as inhibitor, the double-reciprocal plots of the enzyme inhibited were showed as Fig. 5. Under the experimental conditions employed, the oxidation reaction of L-dopa by mushroom tyrosinase followed Michaelis-Menten kinetics. Double-reciprocal plots yielded a family of straight lines intersected at the second quadrant. Thus, it was a mixed type inhibitor, indicating that compound (a) inhibited the enzyme activity not only by binding with the free enzyme but also with the enzyme-substrate complex. The equilibrium constants for inhibitor binding with the free enzyme (K_I) and with enzyme-substrate complex (K_{IS}) were obtained from the slope or the vertical intercept versus the inhibitor concentration, respectively. From the Fig. 5(II) and Fig. 5(III), the values of K_I and K_{IS} of compound (a) were determined to be 1.54 and 3.08 mM, respectively. The results were summarized in Table 1. For the compound (c), the inhibition behavior is as same as the compound (a). The values of K_I and K_{IS} of compound (c) were determined to be 2.51 and 5.10 mM, respectively. The results were also listed in Table 1 for comparison.

The inhibitory kinetics of mushroom tyrosinase by unsaturated fatty acids: (b) and (d) had been studied. Figure 6 showed the double-reciprocal plots of the enzyme inhibited by (b) at different concentrations. The double-reciprocal plots yielded a family of straight lines intersected at the *x*-abscissa. The inhibition of (b) on the enzyme belonged to non-competitive type, indicating that the equilibrium constants for inhibitor binding with the free enzyme (K_I) and with enzyme-substrate complex (K_{IS}) were the same. Plot of the vertical intercept versus the inhibitor concentration (in Fig. 6 II) gave a line and the values of K_I and K_{IS} of compound (b) were determined to be 0.91 mM. The results were summarized in Table 1. For the compound (d), the inhibition behavior is as

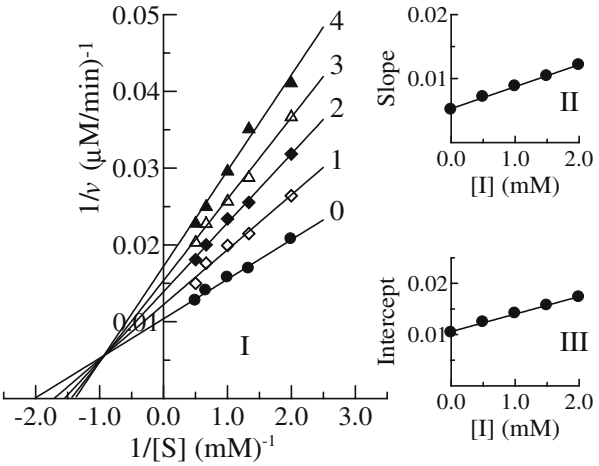

Fig. 5 Determination of the inhibitory type and inhibition constants of octanoic acid. (*I*) Lineweaver–Burk plots for diphenolase activity inhibited by octanoic acid. The concentrations of octanoic acid for line 0-5 were 0, 0.5, 1.0, 1.5 and 2.0 mM, respectively. (*II*) represents the plot of slope versus the concentration of octanoic acid for determining the inhibition constants K_I , (*III*) represents the plot of intercept versus the concentration of octanoic acid for determining the inhibition constants K_{IS}

same as the compound (b). The values of K_I and K_{IS} of compound (d) were determined to be 12.00 mM. The results were also listed in Table 1 for comparison.

Discussion

Tyrosinase exhibits both monophenolase and diphenolase activities. This paper reported the effects on the activities of tyrosinase by (a), (b), (c), (d), and (e). We used L-dopa as substrate to determine the diphenolase activity of the enzyme, and L-tyrosine to the monophenolase

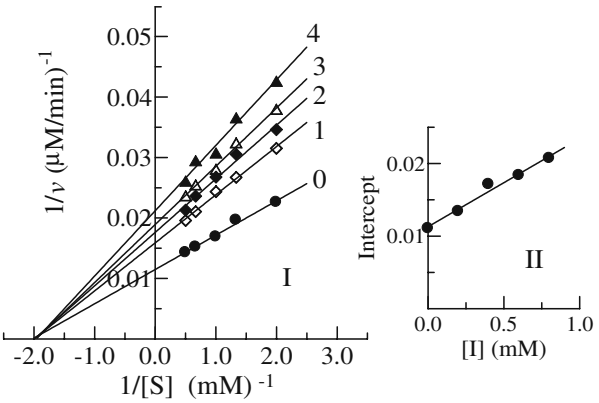

Fig. 6 Determination of the inhibitory type and inhibition constants of (2*E*,4*E*)-hexa-2,4-dienoic acid. (*I*) Lineweaver–Burk plots for diphenolase activity inhibited by (2*E*,4*E*)-hexa-2,4-dienoic acid. The concentrations of (2*E*,4*E*)-hexa-2,4-dienoic acid for line 0-5 were 0, 0.25, 0.50, 0.75, and 1.0 mM, respectively. (*II*) represents the plot of intercept versus the concentration of (2*E*,4*E*)-hexa-2,4-dienoic acid for determining the inhibition constants K_I

activity. The results showed that all of the fatty acids could inhibit both the monophenolase activity and diphenolase activity of tyrosinase. For the inhibitory effects on diphenolase activity, (b)>(a)>(c)>(d)>(e), indicated that the unsaturated fatty acids had stronger inhibition than saturated fatty acids and the longer the carbon chains were, the stronger the inhibition was. The inhibition mechanisms of (a), (b), (c), and (d) were reversible, (e) had a weak effect on diphenolase activity. Saturated fatty acids (a) and (c) exhibited mixed inhibition type, while unsaturated fatty acids (b) and (d) exhibited noncompetitive inhibition type. For the inhibitory effects on monophenolase activity, the unsaturated fatty acids exhibited stronger inhibitory effects than saturated fatty acids, as (b)>(c) and (d)>(e); and the longer the carbon chains were, the stronger the inhibition was, as (a)>(c)>(e) and (b)>(d). All of compounds could extend the lag period of monophenolase activity.

Mushroom tyrosinase can host substrates of various sizes; it seems that the interaction of the substrate residue with the pocket of the active site seriously influences the impact of the docking head of the substrate which coordinates to the copper ion in the active site. As a matter of fact, the docking style of different phenols of various sizes is not expected to be too different in the mushroom tyrosinase active site. But the way the residues of these substrates lay in the pocket of the active site would be predictably different. Thus, the overall conformational changes of mushroom tyrosinase results from the sum of the interactions of both head and body of the substrate with the active site and the pocket of the active site [21]. In the process of catalysis, tyrosinase has three existing forms, E_{met}, E_{oxy}, and E_{deoxy}. The E_{deoxy} form [Cu(I)-Cu(I)] is a reduced species, which binds oxygen to give the E_{oxy} [Cu(II)-O22–Cu(II)]. In the E_{oxy} form, molecular oxygen is bound as peroxide in μ-η2: η2 side-on bridging mode, which destabilizes the O–O bond and activates it. E_{met} form [Cu(II)-Cu(II)] is assumed as a resting enzymatic form, where Cu(II) ions are normally bridged to a small ligand, such as a water molecule, or hydroxide ion[2]. Both E_{met} form and E_{oxy} form can catalyze the diphenol substrate; the E_{oxy} form can also catalyze the monophenol substrate but the E_{met} form cannot. The E_{deoxy} form can combine with oxygen [18]. The fatty acids may disrupt the tertiary structure of the tyrosinase through intermolecular hydrogen bonding [22]. The unsaturation of the molecule probably does not alter the tyrosinase inhibitory activity of the fatty acids, so the unsaturated fatty acids can bring more conformational instability to the tyrosinase on interaction [23]. But the saturated fatty acids would pack better therefore not much conformational change when they interact with the tyrosinase residue. Therefore fatty acids with unsaturation (b and d) are stronger inhibitors as compared to their saturated analogs (c and e) for diphenolase activity. Nazzaro-porro Marcella et al. [24] also proved that fatty acids with unsaturation are stronger inhibitors as compared to the saturated ones. Interestingly, this conclusion is the same with the research by Ando et al. [25], which investigated that fatty acids regulate pigmentation via proteasomal degradation of tyrosinase. It provides a novel view to the research of tyrosinase.

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