# Increase of Melanogenesis in the Presence of Fatty Acids

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## Summary

Tyrosinase catalyzes the oxidation of phenolic compounds to the corresponding quinines which are subsequently converted to melanin pigments. Loss of the hair shaft melanin is associated with decrease of tyrosinase activity in the bulb of melanocytes. Activators of tyrosinase with stimulatory effects on melanogenesis are beneficial for the preventation of hair whitening and treatment of hypopigmentation disease such as oculocutaneous albinism 1B. This paper reports activation of polyphenol oxidase activity by fatty acids using dopamine hydrochloride as substrate. The results showed that saturated fatty acids are more potent tyrosinase activators than unsaturated ones. Measuring important kinetic constants, $V_{max}$ and $K_m$ revealed that tyrosinase activation followed a mixed type mechanism depending on fatty acid concentration, chain length and double bonds.

**Key words:** Tyrosinase activator, fatty acids, saturated, unsaturated.

## Introduction

Tyrosinase or polyphenoloxidase, PPO (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) uses molecular oxygen to catalyse two different reactions: the oxidation of monophenols, e.g., tyrosine, to their corresponding *o*-diphenols (monophenolase or cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity) [1]. Figure 1 shows that *o*-quinones, thus generated, polymerize to melanin or melanin-like pigments in fungi, plants, unicellular bacteria and mammalian cells [2].

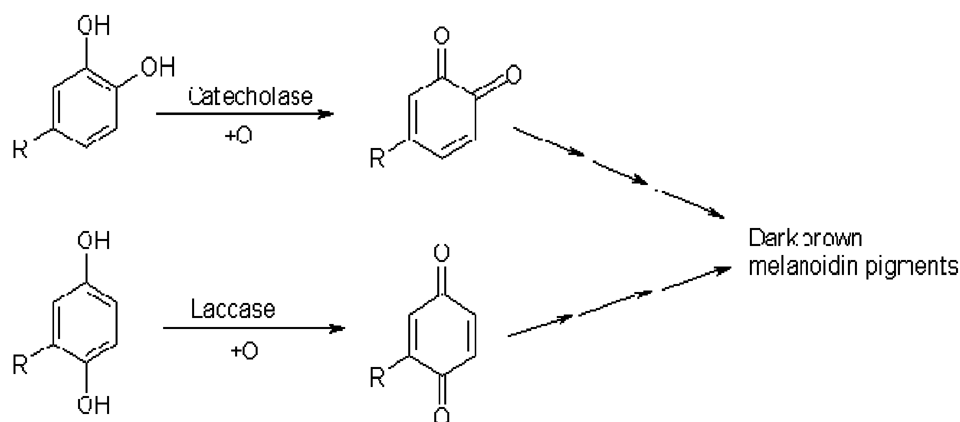



Figure 1. Formation of melanin pigments by action of tyrosinase.

Polyphenol oxidases are copper-containing monooxygenase widely distributed in the living world. They are also known as phenolases, tyrosinase and phenol oxidases. These terms are often used without any rule, even if tyrosinase is the term usually adopted for animal including human enzymes, and refers to the ‘‘typical’’ substrate, tyrosine [3]. Melanin is involved in the defense mechanism of human skin against the harmful effects of UV radiation due to its ability to absorb and reflect UV energy and its ability to scavenge oxidative free radicals [4, 5]. Tyrosinase is composed of three domains, of which the central domain contains active site [6]. The active site of tyrosinase consists of two copper atoms (Fig. 2). Six histidine residues bind a pair of copper ions in the active site of tyrosinase, which interact with both molecular oxygen and its phenolic substrate [7, 8]. The widely varying Cu–Cu distances have been calculated for the met polyphenoloxidase, ranging from 2.8 to 3.6Å [9- 11].

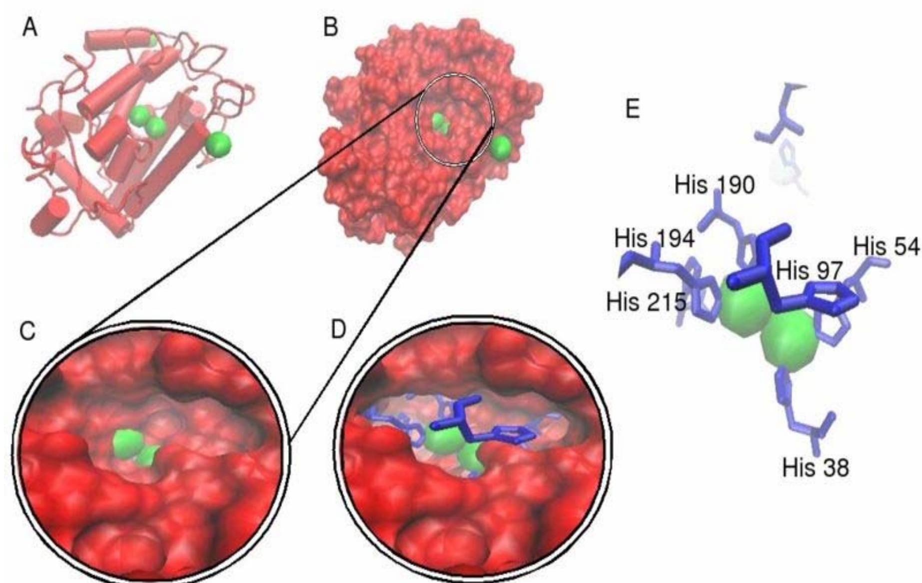



Figure 2. Positions of two copper atoms (green) in the active site of tyrosinase.

The most commercial importance of tyrosinase is evident in food, cosmetic and pharmaceutical industries. In the food industry, it is the main enzyme involved in the undesirable browning of fruits and vegetables during processing and storage. In pharmaceutical preparations tyrosinase is an important enzyme due to its role in the synthesis or modification of high-value compounds like the phytoestrogen coumestrol, known for its estrogenic activity, and L-DOPA, which is used for the treatment of Parkinson's disease. A case study has reported a patient with long-standing Parkinson's disease who noted that his white hair turned grey and darkened 8 months after the addition of carbidopa to his established levodopa (L-dopa) therapy and 4 months after the introduction of bromocriptine [12]. This is the only report authors found in literature about tyrosinase activation by a medicine. Tyrosinase activators are rarely studied as most industries are more interested on inhibitors of the enzyme in order to reduce the adverse effects of melanin formation in, for example, processed food and human skin.
Fatty acids are major components of biological cell membranes that play important roles in intracellular signaling and as precursors for ligands that bind to nuclear receptors [13, 14]. Recently it has been demonstrated that fatty acids regulate the degradation of tyrosinase by modulating the ubiquitination of tyrosinase, which leads to an increase or a decrease in its degradation by proteasomes. In fact, fatty acids are able to regulate the proteasomal degradation of tyrosinase [15, 16]. The aim of this research was evaluation of fatty acid efficiency as tyrosinase activators.

## Materials and Methods

### Chemicals

Mushroom tyrosinase (5units/mg) was purchased from Fluka, dopamine hydrochloride and MBTH (3-methyl-2-benzothiazolinone hydrazone) from Sigma (St. Louis, MO. USA), and all other chemicals including fatty acids, emulsifiers and various solvents from Merck (Darmstadt, Germany).

### Preparation of the enzyme and substrate solution

a) Enzyme solution: Pure mushroom tyrosinase (1mg/ml) was diluted to 1/160 of its original concentration without further purification.
b) Substrate solution: Dopamine hydrochloride, L-DOPA (55 mM) was freshly prepared in phosphate buffer (pH 6.8) containing 2% (v:v) dimethyl foramide (DMF) in 0.08% phosphoric acid and 5 mM 3-Methylbenzthiazolinone-2-Hydrazone (MBTH). The solution was stored in dark until use in order to prevent its color change by the action of direct light.
c) Fatty acids were dissolved in phosphate buffer (pH 6.8) using surfactants. The primary stock was made in millimolar concentration and then the macromolar concentrations were then prepared from the primary stock solutions (5.0-1000 μM).

### Enzymatic assay

The diphenolase activity was determined by a spectrophotometric method, as described by Chan [17]. The enzymatic reaction was initiated by addition of a known amount of the enzyme to a solution of substrate containing L-DOPA, DMF and MBTH in phosphate buffer (pH 6.8). L-DOPA is the main substrate that

converts to quinone by enzyme activity. MBTH is a strong nuocleophile that is attached to produce a pink complex. DMF is added to the reaction mixture in order to keep the resulting complex in solution state during the course of investigations. The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1.0 ml contained 10 μl enzyme solution (a), 950 μl substrate solution (b) and 40 μl phosphate buffer (pH 6.8). To estimate and compare the activities measured in this study, the molar extinction coefficient of the product was 3700 per mole per centimeter.

**Effect of activators on the enzymatic activity**

To investigate the effect of fatty acids on the activity of enzyme, different concentrations (5.0-1000 μM) of each activator were pre-incubated with enzymes at room temperature. Tyrosinase activity was measured by replacing the phosphate buffer with each activator.

**Kinetic analysis**

Kinetic constants of various substrates concentrations were determined by measuring the initial velocity as a function of substrate concentrations.

Enzymatic activity of tyrosinase was then measured in the presence of known amount of each activator using a constant substrate concentration under assay conditions. Michaels–Menten constant ($K_m$) and maximum velocity ($V_{max}$) of the tyrosinase were determined from the Lineweaver–Burk plots.

## Results and Discussions

The activatory effect of 5 fatty acids on the oxidation of dopamine hydrochloride by tyrosinase was investigated. The study was designed so that the result would be able to show the effect of alkyl chain length (12 to 18 carbon atoms), type of the fatty acids and presence of double bond. Table 1 shows the chemical structures of the fatty acids used as activators. The rate of tyrosinase reaction on dopamine hydrochloride was studied at room temperature (20ºC) in the absence and presence of activators.

Table 1. The chemical structure of tyrosinase activators used.

| Common name | Systematic name | Chemical formula |
|---|---|---|
| Lauric acid | *n*- Dodecanoate | $CH_3(CH_2)_{10}COOH$ |
| Myristic acid | *n*- Tetradecanoate | $CH_3(CH_2)_{12}COOH$ |
| Palmitic acid | *n*- Hexadecanoate | $CH_3(CH_2)_{14}COOH$ |
| Stearic acid | *n*- Octadecanoate | $CH_3(CH_2)_{16}COOH$ |
| Oleic acid | *cis*-$\Delta^9$- Octadecanoate | $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ |

**All fatty acids enhanced tyrosinase activity**

The result showed that all four saturated and the only unsaturated fatty acid as oleic acids studied act as activators of tyrosinase and the order of activation capability depended primary on the concentration of fatty acids. It was also found that the longer the chain length, the more potent activator of tyrosinase was the fatty acid. However, there was an exception, i.e. stearic acid with 18 carbon atoms did not follow the order in chain length and appeared after $C_{12}$. Therefore, among saturated fatty acids, palmitic ($C_{16}$) was the strongest activator and lauric ($C_{12}$) the weakest (Table 2). The strength of activators also depended on the nature of fatty acid in terms of saturation. It was found that the only unsaturated (18: $1^9$) was even weaker than shortest saturated fatty acid.

**Table 2**. The effect of fatty acids on biological activity of tyrosinase.

| Activator | No. of C: double bond | Activation (%) |
|---|---|---|
| Lauric acid | 12: 0 | 139.1 |
| Myristic acid | 14: 0 | 178 |
| Palmitic acid | 16: 0 | 276.2 |
| Stearic acid | 18: 0 | 157.8 |
| Oleic acid | 18: $1^9$ | 125.5 |

As mentioned above, tyrosinase activation has not been studied in detail. However, sodium dodecyl sulfate (SDS) has been reported to act as positive effectors of the enzyme [18-20]. It has been shown that activation process of tyrosinase is related to a limited conformational change due to the binding of small amounts of SDS to the latent enzyme. In the case of fatty acids as tyrosinase effectors, it can be predicted that due to high hydrophobicity present in enzyme structure [6] the long hydrophobic tail of fatty acids, can bind to effector site of tyrosinase with relatively strong hydrophibic attractions, leading to conformational change of enzyme at its active site. This conformational change enhances the affinity of active site towards its substrate which results in a more kinetically favorable reaction. On the other hand, conformational studies have revealed that, in addition to its active site, tyrosinase posses an effector site to which the effector can bind [21]. Partial biding of the substrate to the active site may change the conformation of the enzyme so that the hydrophobic pocket (effector site) expands. Therefore, longer alkyl chains would accommodate and bind more tightly. The double bond in oleic acid cause to curvature in its tail. Therefore, the hydrophibicity of oleic acid's tail was decreased than linear state and the joining of oleic acid to tyrosinase surface leads to much weaker hydrophobic interaction than saturated fatty acids. It was also observed in high concentration of each fatty acid, up of 100 µM, the order of activator potency was increased. It could, then, be stated that high hydrophibicity in tyrosinase environment induces enzyme biological activity.

Another interesting point that may explain the activator role of fatty acids is that the multiple alignment of tyrosinase in some species has shown that most of conserved amino acids possess positively charged basic groups as their side chains (Fig. 3).

Therefore, some positive charges are present and conserved naturally in tyrosinase. Negative charge of fatty acids may make them more favorable for binding to these positive charges leading to ionic bond on enzyme structure.

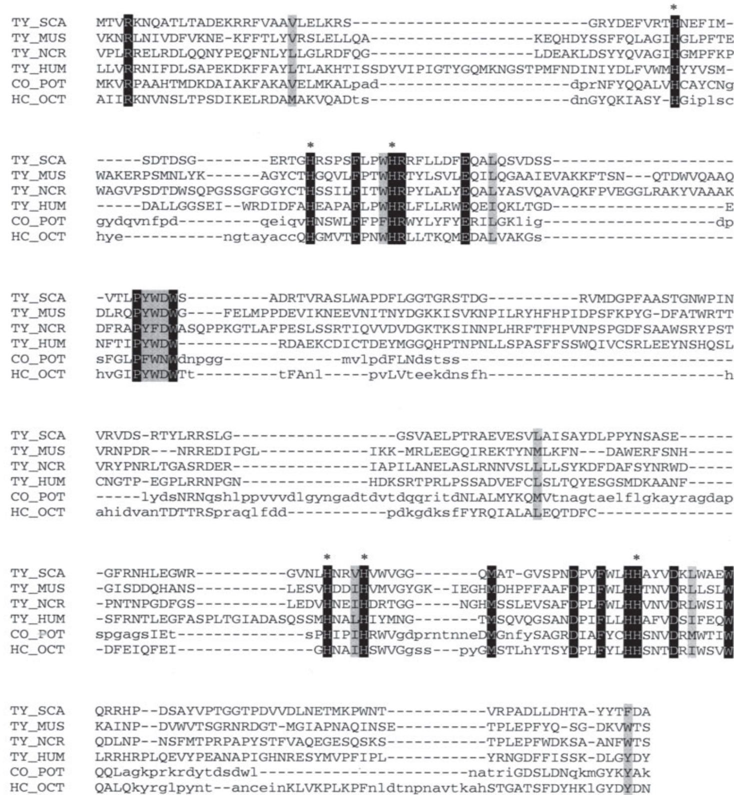



Figure 3. Multiple sequence alignment of the type 3 copper proteins**.** *TY_SCA* indicates the amino acid sequence of the *S. castaneoglobisporus* tyrosinase. The other sequences are taken from the GenBankTM data base: *TY_HUM*, human tyrosinase; *TY_MUS*, *A. bisporus* (mushroom) tyrosinase; *TY_NCR*, *N. crassa* tyrosinase; *CO_POT*, potato catechol oxidase; *HC_OCT*, octopus hemocyanin. Structurally superimposable residues in *CO_POT* and *HC_OCT* are shown by *capital letters*. The identical and conserved residues are displayed in *black* and *gray shading*, respectively. The *asterisks* indicate the His residues participating in copper binding [22].

**Kinetic studies**

The activatory mechanisms of fatty acids on mushroom tyrosinase, during the oxidation of dopamine hydrochloride, were determined from Linweaver-Burk double reciprocal plots. The kinetic constants ($K_m$ and $V_{max}$) were calculated from the double reciprocal plots in the case of each activator. The double reciprocal Linweaver-Burk plots of the diphenolase activity in presence of fatty acids have been drawn. The plots of $1/V_0$ versus $1/[S]$ gave a family of straight lines with different slopes that intersects together in a fixed point. Figure 4 shows the double-reciprocal plots of the enzyme activated by fatty acids.

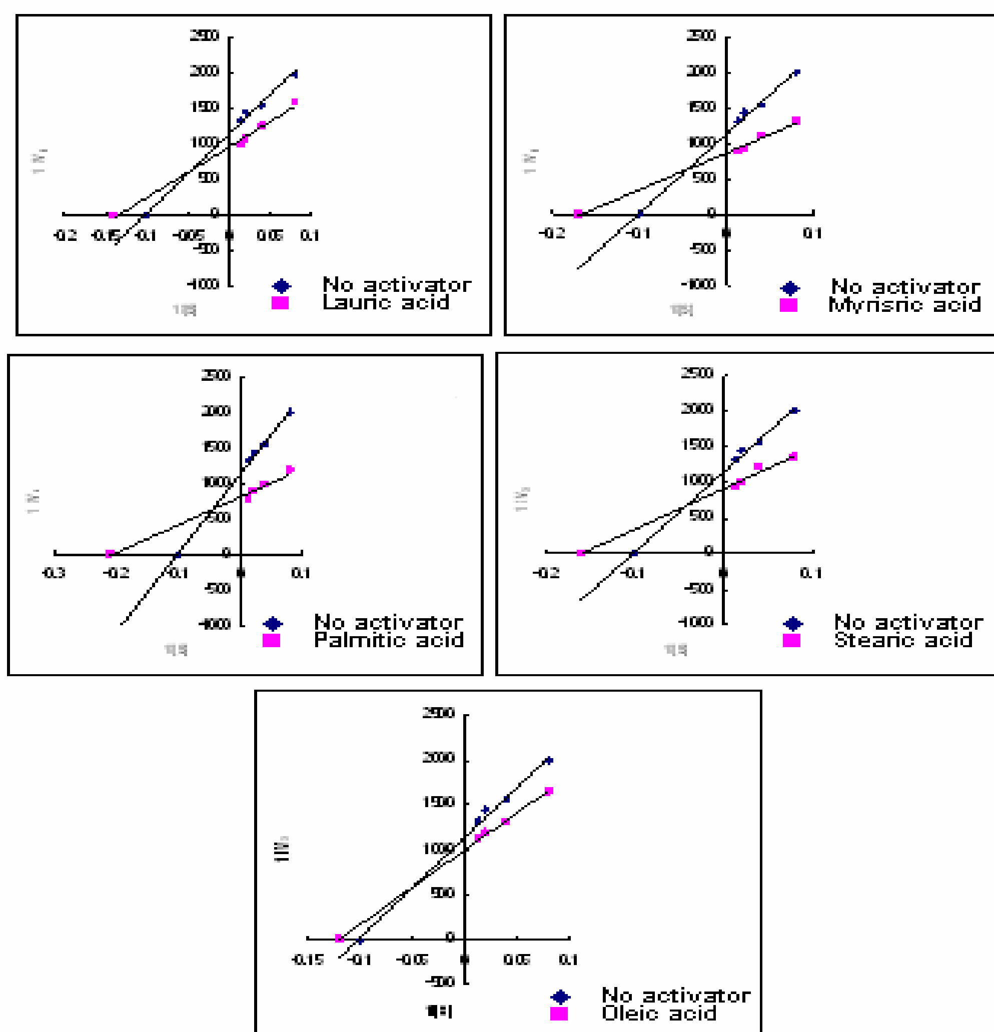
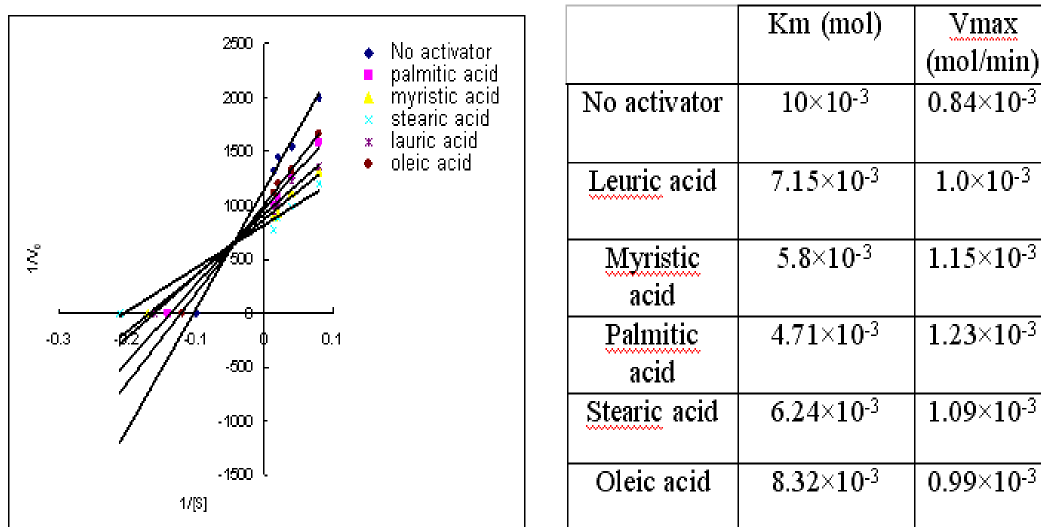



Figure 4. Lineweaver-Burk plots, for activation of tyrosinase action on dopamine hydrochloride by fatty acids.

Kinetic studies showed that both Michaelis–Menten constant, $K_m$ and Maximum velocity, $V_{max}$ were affected by activators. In the presence of all types of fatty acids a decrease in $K_m$ and an increase in $V_{max}$ were observed (Fig. 5). These findings show that all tested fatty acids enhanced the affinity of tyrosinase towards its substrates, i.e. they all are mixed-type activators. Therefore, fatty acids could bind to both free enzyme and enzyme-substrate complex. Palmitic acid caused the highest increase in $V_{max}$ and decrease in $K_m$ as compared with other fatty acids and oleic acid induced less change in both kinetic parameters, i.e. it is the weakest positive effectors among fatty acid used in the present investigation.




| | Km (mol) | Vmax (mol/min) |
|---|---|---|
| No activator | $10\times10^{-3}$ | $0.84\times10^{-3}$ |
| Leuric acid | $7.15\times10^{-3}$ | $1.0\times10^{-3}$ |
| Myristic acid | $5.8\times10^{-3}$ | $1.15\times10^{-3}$ |
| Palmitic acid | $4.71\times10^{-3}$ | $1.23\times10^{-3}$ |
| Stearic acid | $6.24\times10^{-3}$ | $1.09\times10^{-3}$ |
| Oleic acid | $8.32\times10^{-3}$ | $0.99\times10^{-3}$ |

Figure 5. Alternations in kinetic parameters ($K_m$ and $V_{max}$) of tyrosinase in the presence of fatty acids.

## Conclusions

The results obtained in the present study indicate that fatty acids are moderate activators of tyrosinase action on dopamine hydrochloride. It was shown that all saturated and unsaturated fatty acids enhanced tyrosinase activity and the order of activation potency depended on their concentration, chain length and presence or absence of saturation. It was also shown that both of kinetic parameters ($K_m$ and $V_{max}$) were affected by fatty acids. This type of kinetic behavior is typical of a mixed-type activator meaning that fatty acids could bind to both the free enzyme and enzyme-substrate complex. However, to comment this type of activation, more detailed investigations are needed. On the other hand, the degree of un-saturation that was not studied in this research should be investigated using 1-4 double bonded fatty acids. In conclusion, fatty acids are potent tyrosinase activator and stimulator of melanogenesis with potential for the treatment of hypopigmentation disease and preventation or reversing the reactions that result in hair graying. Preventation of hair greying is one of the main cosmetic goals of drug design. Natural fatty acids could successfully offer a step towards this aim and further studies are undertaking within our research group.

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