

# Exploring the inhibitory effects of glutamic acid on melanin production: Mechanistic insights and molecular docking analysis

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**Abstract.** Glutamic acid is widely recognized as safe and has various applications in the medical and food industries. This study demonstrated its significant inhibition of tyrosinase, acting as a mixed-type inhibitor according to enzymatic kinetic analysis. Fluorescence spectroscopy analysis and investigation of tyrosinase activity under different pH confirmed that glutamic acid induced changes in the protein structure of tyrosinase, leading to its reduced activity through acidification and binding effects. Additionally, glutamic acid was found to inhibit L-DOPA auto-oxidation, thereby preventing further formation of dopachrome. The IC<sub>50</sub> values for glutamic acid inhibiting tyrosinase activity and L-DOPA auto-oxidation were detected to be 4.69 mM and 0.72 mM, respectively. Glutamic acid had a better inhibitory effect on L-DOPA auto-oxidation than tyrosinase activity. The L-DOPA auto-oxidation process can also lead to the formation of melanin, and its inhibition by glutamic acid further supported its potential in controlling melanin synthesis. Moreover, glutamic acid demonstrated a dose-dependent decrease in melanin production in B16 cells while maintaining cell viability. Western blot analysis revealed decreased protein expression of TYR and TRP-1, both of which are involved in melanin production, with increasing concentrations of glutamic acid. Molecular docking analysis suggested a potential mechanism involving the disruption of copper binding sites on tyrosinase. These findings underscore the potential of glutamic acid as a promising agent for controlling melanin production and associated disorders.

## 1. Introduction

Extended exposure to ultraviolet rays can result in skin pigmentation, serving as a protective mechanism to prevent skin cancer [1]. However, evidence suggests a complex relationship between melanin deposition and photoprotection, as excessive melanin production can result in the formation of dark spots, adversely affecting facial aesthetics. Thus, based on World Health Organization (WHO), skin-lightening treatments are used by half of the populations in South Korea, Malaysia, and the Philippines, with 40% of Chinese women regularly using skin-lightening products [2]. Skin pigmentation-related dark spots and hyperpigmentation are crucial research topics in medical aesthetics.

Melanin consists of eumelanin and pheomelanin, which are catalyzed to generate by tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and -2 (TRP-2) found in mammals, plants, and microorganisms [3]. Tyrosinase facilitates the primary rate-limiting step in the oxidation of tyrosine, transforming it into dopaquinone, and subsequently undergoing conversion to dopachrome. [4]. Besides, dopaminequinone forms pheomelanin when glutathione or cysteine exists.

Additionally, tyrosinase, TRP-1, and TRP-2 catalyze the sequential generation of indole-5,6-quinone, 5,6-dihydroxyindole-2-carboxylic acid and indole-2-carboxylic acid-5,6-quinone, ultimately leading to the formation of eumelanin. Tyrosinase belongs to type-III copper enzymes, along with catechol oxidases and hemocyanins [5]. The type-III copper enzymes feature a distinctive binuclear copper center, with each copper ion being coordinated by three highly conserved histidine residues. Tyrosinase exhibits broad substrate specificity, catalyzing two distinct oxidative reactions: monophenols *o*-hydroxylation and *o*-diphenols oxidation for monophenolase and diphenolase activity, respectively [6]. In addition, several protein structures of tyrosinases have been identified and submitted to the protein data bank including accession numbers 2Y9X from *Agaricus bisporus*, 6JU9 from *Aspergillus oryzae*, and 5ZRE from *Burkholderia thailandensis* [7-9].

Glutamic acid, an acidic amino acid, holds significant importance in protein metabolism within organisms, participating in numerous vital chemical reactions across animals, plants, and microorganisms. With its FEMA (Flavour Extract Manufacturers Association) number 3285, it is considered Generally Recognized as Safe

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(GRAS). Glutamic acid, typically present as monosodium glutamate (MSG), functions as a flavor enhancer in human and animal diets. It collaborates with  $\gamma$ -aminobutyric acid (GABA) to promote feed intake by stimulating orexigenic neurons in the hypothalamus [10]. Medically, glutamate plays a vital role in detoxifying ammonia in the brain, particularly during impaired liver function and hyperammonemia. This detoxification process requires continuous glutamate synthesis, likely involving pyruvate carboxylase (PC) and glutamate dehydrogenase (GDH) [11]. Several tyrosinase inhibitors have been identified such as phenols, flavonols, anthocyanidins, curcuminoids, coumarins, chalcones, stilbenes, lignans, terpenoid derivatives, quinone derivatives, pyridine, piperidine, thiosemicarbazones, azole, benzaldehyde derivatives, kojic acid analogs, and carboxylic acids [12]. In this study, glutamic acid belonging to carboxylic acids was used to explore its anti-melanin capacity. Glutamic acid not only can inhibit tyrosinase activity but also has the effect of inhibiting L-DOPA auto-oxidation.

## 2. Materials and methods

### 2.1. Anti-tyrosinase activity of glutamic acid

Mushroom tyrosinase purchased from Sigma-Aldrich (MO, USA) was performed to assess the anti-tyrosinase activity of glutamic acid (1, 2, 4, 8, and 16 mM). A mixture containing 100  $\mu$ L of tyrosinase (360 U/mL), 300  $\mu$ L of L-DOPA (5 mM), and 200  $\mu$ L of glutamic acid was incubated for 5 min at room temperature. The inhibition rate of tyrosinase activity was measured at 475 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Deionized water served as a control for assessing the anti-tyrosinase activity of glutamic acid.

### 2.2. Enzymatic kinetic analysis

Varying concentrations of glutamic acid (ranging from 0 to 8 mM) and L-DOPA (ranging from 0 to 4 mM) were utilized for the assessment of anti-tyrosinase activity. Mushroom tyrosinase, purchased from Sigma-Aldrich at a final activity concentration of 60 U/mL, was used for the enzymatic kinetic studies. The activity of tyrosinase was measured at room temperature for 5 min using a microplate spectrophotometer set at 475 nm. The enzymatic velocity was determined using the formula  $[v = \frac{[OD_{475}(\text{glutamic acid}) - OD_{475}(\text{control})]}{3700L \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \cdot 0.6 \text{ cm} \cdot \text{time (min)}}$ . Michaelis-Menten analysis, encompassing calculating  $V_{\text{max}}$  and  $K_m$ , was analyzed using GraphPad Prism (GraphPad Software, MA, USA). To evaluate the  $K_i$  value, a mixed model inhibition approach was employed.

### 2.3. Fluorescence spectra analysis

A fluorescence assay was conducted using a microplate reader (Infinite 200Pro, Tecan, Switzerland) with mushroom tyrosinase (Sigma-Aldrich) at a final concentration of 60 U/mL. Various concentrations of glutamic acid (0, 2, 4, 8, and 16 mM) were added to the mushroom tyrosinase.

### 2.4. Inhibition of L-DOPA auto-oxidation

Different concentrations of glutamic acid were added to 2.5 mM L-DOPA and reacted at 37°C for 12 h. The auto-oxidation of L-DOPA was determined at 475 nm using a microplate spectrophotometer (Molecular Devices). Deionized water served as a control for assessing the ability of glutamic acid to prevent the auto-oxidation of L-DOPA.

### 2.5. Effect of glutamic acid on cell viability

The impact of glutamic acid on B16 cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. B16 cells were added to a 96-well plate and cultivated at 37°C with 5% CO<sub>2</sub> for 24 h. Various concentrations of glutamic acid (0, 1, 2, 4, 8, 16 mM) were added to the cells for 24 h. After incubation, the supernatant was removed, and the cells were treated with a medium containing 6  $\mu$ L of MTT solution (5 mg/mL). After a 4-h incubation at 37°C with 5% CO<sub>2</sub>, the formazan crystals produced by the cells were dissolved using dimethyl sulfoxide. The absorbance of the formed formazan was analyzed at 570 nm using a microplate spectrophotometer (Molecular Devices).

### 2.6. Melanin content of B16 cell

The cells were subjected to various concentrations of glutamic acid (0, 1, 2, 4, 8, 16 mM) for 1 h. Subsequently, to induce melanin production,  $\alpha$ -melanocyte-stimulating hormone (1  $\mu$ M) was introduced to the cells, followed by a 48-h incubation period. After incubation, the cells were harvested, and 200  $\mu$ L of 1 M NaOH was added to dissolve the melanin particles. The mixture was then incubated at 60°C until complete dissolution of melanin. The melanin content was quantified at 405 nm using a microplate spectrophotometer (Molecular Devices).

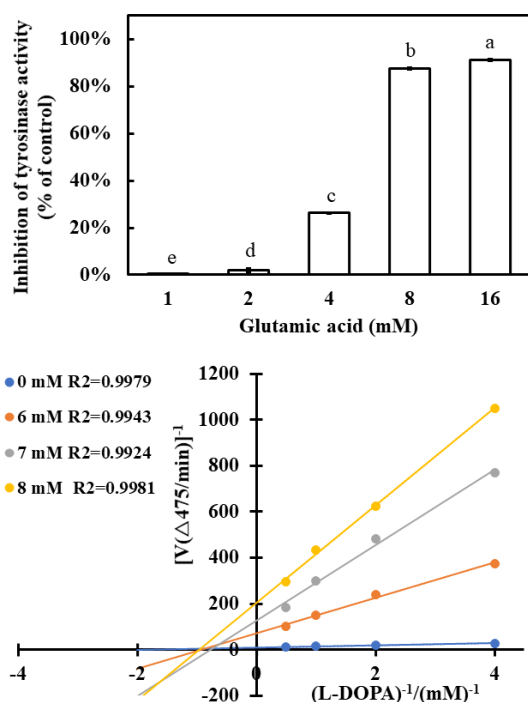
### 2.7. Autodocking analysis

The protein structure of mushroom tyrosinase from *Agaricus bisporus* (PDB no. 2y9x) was utilized for molecular docking analysis. Autodocking analysis was conducted to predict the binding affinity of glutamic acid to mushroom tyrosinase via AutoDock Vina (<https://autodock.scripps.edu>) [13].

### 3. Result and discussion

#### 3.1. Inhibition of tyrosinase by glutamic acid and its enzymatic kinetic assay

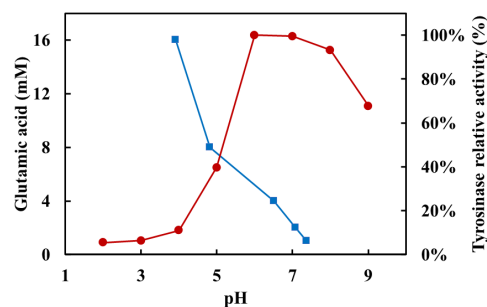
Many carboxylic acids have been demonstrated to possess the capability of inhibiting tyrosinase activity such as acrylic acid, propanoic acid, pyruvic acid, 2-oxo-octanoic acid, and 2-oxo-butanoic acid [12, 14]. Glutamic acid, a type of carboxylic acid, also exhibited anti-tyrosinase activity. An 87.6% suppression of tyrosinase activity was achieved with 8 mM glutamic acid (Fig. 1). The IC<sub>50</sub> value for glutamic acid, calculated using GraphPad Prism, was determined to be 4.69 mM. Varying concentrations of glutamic acid and L-DOPA were used for enzymatic kinetic assay. Calculations performed using GraphPad Prism yielded the following V<sub>max</sub> and K<sub>m</sub> values for glutamic acid across concentrations of 0, 6, 7, and 8 mM: V<sub>max</sub> - 0.423, 0.069, 0.046, 0.022, and K<sub>m</sub> - 1.028, 2.982, 4.551, 2.499. The inhibition constant (K<sub>i</sub>) for glutamic acid was determined to be 0.251 mM. These findings suggest that glutamic acid functioned as a mixed-type inhibitor against mushroom tyrosinase. Glutamic acid showed a low inhibitory effect on tyrosinase, compared with some competitive and non-competitive tyrosinase inhibitors [12].



**Fig. 1.** Anti-tyrosinase activity of glutamic acid and Lineweaver-Burk graph on tyrosinase of glutamic acid.

The nearly 100% of mushroom tyrosinase activity (Sigma-Aldrich) could be sustained within the pH range of 6 to 7 (Fig. 2). As the pH decreased, the activity of tyrosinase declined, implying the acidification of glutamic acid for tyrosinase inhibition [15]. This acidification process leads to the unfolding of tyrosinase, resulting in a reduction in its catalytic activity. Moreover, at a pH range of 6 to 7, 4 mM glutamic acid still

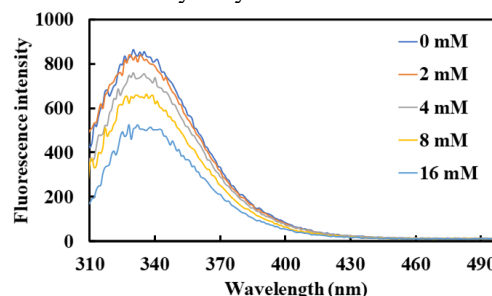
exhibited anti-tyrosinase activity of 26.3%. This indicated that the binding effect of glutamic acid on tyrosinase was still observed.



**Fig. 2.** Tyrosinase relative activity under different pH (100 mM glycine/HCl buffer at pH 2-3; 100 mM sodium acetate/acetic acid buffer at pH 4-5; 100 mM sodium phosphate buffer at pH 6-8 and 100 mM glycine/NaOH buffer at pH 9), and the pH values at different concentrations of glutamic acid. Circle refers to tyrosinase relative activity, and square refers to glutamic acid concentration.

#### 3.2. Effect of glutamic acid on tyrosinase

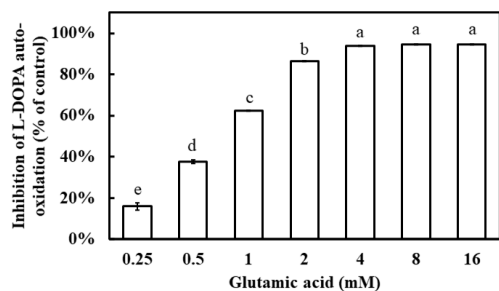
Tryptophan, tyrosine, and phenylalanine were utilized as effective fluorescent indicators to monitor the conformational changes of tyrosinase [16]. Tyrosinase showed intense emission at around 330-340 nm when excited at 280 nm (Fig. 3). As the concentration of glutamic acid increased, the fluorescence intensity of tyrosinase declined. Specifically, the addition of 16 mM glutamic acid resulted in a 41.3% reduction in the fluorescence intensity of tyrosinase at 335 nm.



**Fig. 3.** Fluorescence spectra of tyrosinase treated with various concentrations of glutamic acid.

#### 3.3. Inhibition of L-DOPA auto-oxidation by glutamic acid

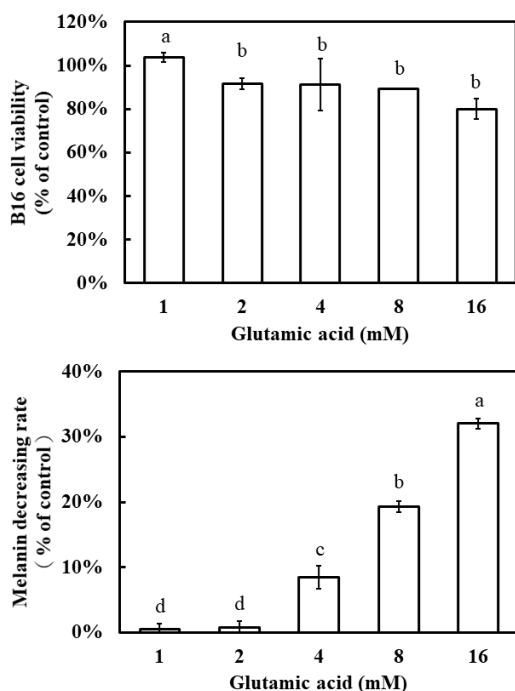
L-DOPA can undertake auto-oxidation to produce quinones and reactive oxygen species, exacerbating the existing oxidative stress condition in the nigro-striatal regions [17]. As glutamic acid increased, the rate of L-DOPA auto-oxidation inhibition also increased (Fig. 4). When glutamic acid was 1 mM, the rate of L-DOPA auto-oxidation inhibition reached 62.4%. The IC<sub>50</sub> value for glutamic acid, calculated using GraphPad Prism, was measured to be 0.72 mM. Glutamic acid had a better inhibitory effect on L-DOPA auto-oxidation than tyrosinase activity. This suggested that glutamic acid could be combined with a better tyrosinase inhibitor as a whitening agent.



**Fig. 4.** Inhibition of L-DOPA auto-oxidation treated with various concentrations of glutamic acid.

### 3.4. Anti-melanin assay of glutamic acid

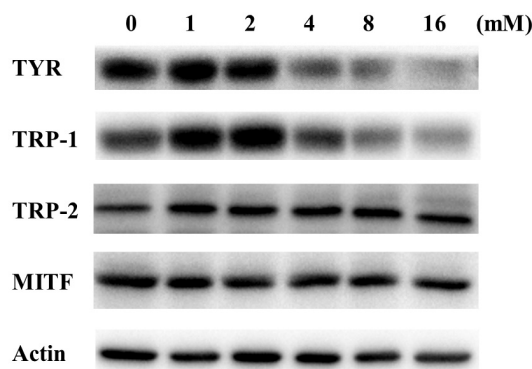
To further investigate the impact of glutamic acid on melanin production, B16 cells were utilized for viability assay. The result indicated that even if 16 mM glutamic acid was added to B16 cells, the cell viability rate could still reach 80% (Fig. 5). The production of melanin exhibited a dose-dependent manner. When glutamic acid was 16 mM, the melanin decreasing rate can achieve 32%. Glutamic acid indeed had a significant inhibitory effect on melanin.



**Fig. 5.** Effect of glutamic acid on the viability and melanin content of B16 cells.

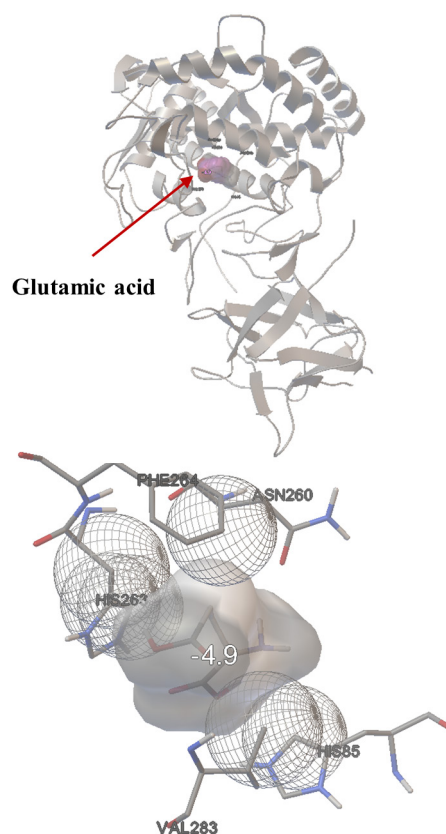
To confirm the protein expression of TYR, TRP-1, TRP-2, and MITF involved in melanin production [18], western blot analysis was performed on B16 cells treated with different concentrations of glutamic acid.  $\beta$ -Actin was utilized as a positive control for sample normalization. The results showed a significant reduction in the protein expression of TYR, and TRP-1 with high concentrations of glutamic acid (Fig. 6). This result revealed that glutamic acid not only inhibited tyrosinase but also attacked TRP-1. The reduction of TRP-1 protein can reduce the catalytic conversion of

5,6-dihydroxyindole-2-carboxylic acid to indole-2-carboxylic acid-5,6-quinone and indirectly reduce the generation of eumelanin [18].



**Fig. 6.** Protein expression of TYR, TRP-1, TRP-2, MITF, and  $\beta$ -actin of B16 cells treated with various concentrations of glutamic acid.

### 3.5. Molecular docking of glutamic acid with tyrosinase



**Fig. 7.** The molecular docking of glutamic acid with tyrosinase (2Y9X) was conducted. The protein-ligand complexes were analyzed using AutoDock Vina (<https://autodock.scripps.edu>).

Because acidification and binding effects of glutamic acid on tyrosinase were observed, molecular docking analysis was analyzed to predict the binding affinity between glutamic acid and mushroom tyrosinase (Fig. 7). The affinity of glutamic acid to tyrosinase was  $-4.9$  kcal/mol according to the AutoDock Vina [13]. One hydrogen bond was identified between glutamic acid and

Asn260 of tyrosinase. Additionally, His85, His263, Phe264, and Val283 on tyrosinase exhibited close contact with glutamic acid. His85 and His263 were involved in binuclear copper-binding sites [8]. This implies that glutamic acid may bind to tyrosinase, disrupting the copper binding and consequently reducing its activity. Furthermore, this result could lead to the design of new compounds that target these sites for the treatment of hyperpigmentation and other melanin-related disorders.

#### 4. Conclusion

In conclusion, this study demonstrates the inhibitory potential of glutamic acid against tyrosinase activity. Glutamic acid showed a significant anti-tyrosinase effect, functioning as a mixed-type inhibitor with an IC<sub>50</sub> value of 4.69 mM. It induced acidification and binding effects, resulting in the inactivation of tyrosinase, a finding further verified by the fluorescent indicator assays. Furthermore, the inhibition of L-DOPA auto-oxidation by glutamic acid, with an IC<sub>50</sub> value of 0.72 mM, supported its additional inhibitory effect. The enzymatic kinetic analysis and the determination of L-DOPA auto-oxidation can guide the development of new formulations with precise concentrations of glutamic acid for effective melanin inhibition. Moreover, glutamic acid displayed a dose-dependent reduction in melanin production in B16 cells, alongside decreased protein expression of TYR and TRP-1. Molecular docking analysis suggested a potential binding affinity of glutamic acid to tyrosinase, disrupting copper binding. These findings collectively highlight glutamic acid's potential as a therapeutic agent for regulating melanin production and addressing related disorders. Because glutamic acid was recognized as safe and had been widely used in the food industry for a long time, it also could be applied in the cosmetic industry, where controlled melanin synthesis is essential for products aimed at skin lightening. Further research could explore the long-term effects and safety of glutamic acid when used as a skin-lightening agent, as well as its potential synergistic interactions with other compounds in cosmetic formulations.

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