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Research Article

Kinetics Investigation on Mushroom Tyrosinase Inhibition of Proso Millet

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Proso millet *(Panicum miliaceum)* is rich in nutritive components and is widely used as a human food, feed and forage for animals, and fuel. This study investigated the effect of a proso millet extract on the inhibition of tyrosinase, a key enzyme in melanogenesis. High performance liquid chromatography analysis indicated that the proso millet extract contained phenolic tyrosinase inhibitors, such as syringic acid, *p*-coumaric acid, and ferulic acid. The extract had an IC_{50} for inhibition of tyrosinase activity of 14.02 mg/mL. A Lineweaver-Burk double reciprocal plot showed that the proso millet extract functioned as a mixed competitive and noncompetitive inhibitor. Proso millet has potential as a tyrosinase inhibitor that may have applications in the cosmetics industry.

1. Introduction

Proso millet (*Panicum miliaceum* L.) was first domesticated in East Asia more than 10,000 years ago and is now one of the world's most important and ancient domesticated crops [1]. It has very short growing season (10 weeks) and, aside from wheat and barley, is the longest-used summer cereal crop [2]. Proso millet is used as a human food, feed and forage for livestock, and fuel [3].

There have been many studies of proso millet due to its economic importance. For example, some studies have proposed morphological and molecular analysis to identify proso millet [4–6]. Other studies have examined germination [7] and growth [8, 9]. Two recent publications reviewed the agronomic characteristics of proso millet [10, 11].

Proso millet is a rich source of protein, minerals, and vitamins, and many studies have characterized its biomolecular components. The protein content of proso millet is about 12% by dry weight, and the protein quality (essential amino acid index) is about 51% [2]. The major biomolecular components of extracted aromatic hydrocarbons and ethers are miliacin, α-amyrin methyl ether, and pentacyclic triterpene methyl ethers, and the relative abundance is miliacin nearly 90% [1].

Some previous studies have proposed biomedical applications for proso millet. In particular, the protein of proso millet may prevent liver injury induced by D-galactosamine [12] and reduce the plasma concentration of high-density lipoprotein [13]. Proso millet is also a gluten-free grain and is an acceptable food for individuals with coeliac disease or gluten sensitive enthesopathy [14]. Proso millet may also prevent hair loss induced by cisplatin-based chemotherapies [15]. Proso millet affects adipocyte differentiation and downregulates adipogenic genes and fatty acid accumulation in adipocytes [16].

There is increasing interest in the use of plant natural products for inhibition of melanogenesis [17, 18]. However, little is known about the effect of proso millet on tyrosinase activity. The objective of this study was to determine effect of a proso millet extract on the kinetics of tyrosinase activity as a preliminary assessment for its use in cosmetic applications.

2. Materials and Methods

2.1. Materials. Acetic acid was from Panreac (Barcelona, Spain), acetonitrile and disodium hydrogen phosphate were from J. T. Baker (Phillipsburg, NJ, USA), ferulic acid was from ChromaDEX (Irvine, California), gallic acid was from Alfa Aesar (Ward Hill, MA, USA), kojic acid and (s)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid (L-dopa) were from Acros (New Jersey, USA), and methyl paraben was from Supelco (Bellefonte, USA). Mushroom tyrosinase, caffeic acid, *p*-coumaric acid, and sodium dihydrogen phosphate were from Sigma-Aldrich (Saint Louis, MO, USA). Syringic acid was from MP Biomedicals (Santa Ana, California, USA), and proso millet (milletGen®) was from Healthmate Co. Ltd. (Changhua, Taiwan).

2.2. Preparation of Proso Millet Extract. One gram of proso millet and 9 mL of deionized water were mixed and sonicated in an ultrasonic bath for 30 min. The supernatant was collected after centrifugation and then passed through a filter with a 0.45 μm pore size. A 0.1 mg/mL methyl paraben solution was dissolved in 70% methanol and passed through a 0.45 μm filter, and the filtrate was utilized as an internal standard in high performance liquid chromatography (HPLC) analysis. For HPLC analysis, the proso millet solution was prepared by mixing 180 μL of the proso millet supernatant with 10 μL of the internal standard solution. For tyrosinase inhibition, the proso millet solution was diluted with deionized water into a series of solutions (6.25, 12.5, 17.5, 20, and 25 mg/mL).

2.3. Calibration and Validation. Standard solutions of syringic acid, *p*-coumaric acid, and ferulic acid were prepared at 0.5 mg/mL in methanol, and series of diluted standards were prepared for HPLC calibration curves (syringic acid: 0.45, 0.563, 0.9, 1.125, and 2.25 μg/mL; *p*-coumaric acid: 1.5, 1.8, 2.25, 3, and 4.5 μg/mL; ferulic acid: 0.75, 0.9, 1.125, 1.5, 2.25, and 4.5 μg/mL) with internal standard solution (18 : 1). The criterion of a signal to noise (S/N) ratio of at least 3 : 1 was used as the detection limit. Recovery of a standard marker was defined as the ratio of the detected amount to the added amount.

2.4. HPLC Analysis. The HPLC system (Agilent 1200 Infinity Series, Agilent, USA) had a reverse-phase column (Cosmosil 5C18-AR II, 5 μm, 25 cm × 4.6 mm ID, Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of a mixture of 0.5% acetic acid and 80% acetonitrile. The percentage of 0.5% acetic acid in the mobile phase was 100% at 0 min, 80% at 0–10 min, 70% at 10–20 min, 40% at 20–30 min, and 0% at 30–40 min. The flow rate was 0.8 mL/min and absorbance was measured at 280 nm.

2.5. Tyrosinase Inhibition. Based on a previous report [19], 40 μL of proso millet solution (6.25 to 25 mg/mL), 40 μL of tyrosinase solution (5.544 μg/mL or 20 U/mL), and 120 μL of 5 mM L-dopa solution were loaded into the wells of a 96-well plate at 37°C. After 30 min, the absorbance was measured at 475 nm. Kojic acid (0.035 to 0.2 mg/mL) was the positive control, and deionized water was the blank control. The percent tyrosinase inhibition was defined as $100\% \times \Delta OD_{sample}/\Delta OD_{control}$, where ΔOD is the absorbance change at 475 nm. The half inhibitory concentration (IC_{50}) was obtained by regression analysis.

2.6. Kinetic Properties. The Lineweaver-Burk equation can be derived from the Michaelis-Menten equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}, \quad (1)$$

where V, V_{max}, K_m, and $[S]$ are reaction rate, maximum reaction rate, Michaelis-Menten constant, and substrate concentration, respectively. The V_{max} and K_m can be obtained by the y-intercept ($1/V_{max}$) and x-intercept ($-1/K_m$) from a plot of $1/V$ versus $1/[S]$ (Lineweaver-Burk plot).

Before constructing the Lineweaver-Burk plot, the effect of the proso millet extract concentration on tyrosinase activity was determined. The concentration of L-dopa (substrate) was set 1.25 mM, and 120 μL in a sodium phosphate buffer (pH 6.8) was used for testing in a 96-well plate. A 40 μL solution of proso millet extract (0 to 7 mg/mL) and a 40 μL solution of tyrosinase (2.772 to 11.088 μg/mL) were mixed with L-dopa.

For measurement of tyrosinase inhibition, a 40 μL solution of solution (0 to 7 mg/mL) and a 40 μL solution of tyrosinase (5.544 μg/mL) were added to each well of a 96-well plate. Then a 120 μL of an L-dopa solution (0.0625 to 0.25 mM) in a sodium phosphate buffer (pH 6.8) was added to initiate the reaction. A Lineweaver-Burk plot was used to assess the effect of proso millet extract on tyrosinase kinetics.

3. Results and Discussion

3.1. HPLC Analysis. Figure 1 shows a representative HPLC chromatogram of the proso millet extract and the internal standard, methyl paraben, which is a stable compound and separated from other peaks. The identified bioactive constituents are syringic acid, *p*-coumaric acid, and ferulic acid. The detection limit was 0.045 μg/mL for syringic acid, 0.078 μg/mL for *p*-coumaric acid, and 0.075 μg/mL for ferulic acid. The recoveries were 97.55% for syringic acid, 97.01% for *p*-coumaric acid, and 96.77% for ferulic acid. The calibration curves of standards were as follows: $y = 264.31x - 0.0153$, $R^2 = 0.9962$ for syringic acid; $y = 255.56x + 0.0562$, $R^2 = 0.9991$ for *p*-coumaric acid; $y = 188.42x - 0.0123$, $R^2 = 0.9922$ for ferulic acid. The proso millet extract had 2.81 μg/g syringic acid, 31.88 μg/g *p*-coumaric acid, and 12.52 μg/g ferulic acid.

3.2. Effect of Proso Millet on Tyrosinase Activity. We used kojic acid as a positive control (Figure 2). Figure 3 shows the inhibitory effect of the proso millet extract. These results indicate that kojic acid and proso millet reduced tyrosinase activity in a dose-dependent manner. The IC_{50} of kojic acid was 0.05 mg/mL and the IC_{50} of the proso millet extract was 14.02 mg/mL.

3.3. Kinetics of Tyrosinase Inhibition. Figure 4 shows the relationship between the activity and concentration of tyrosinase

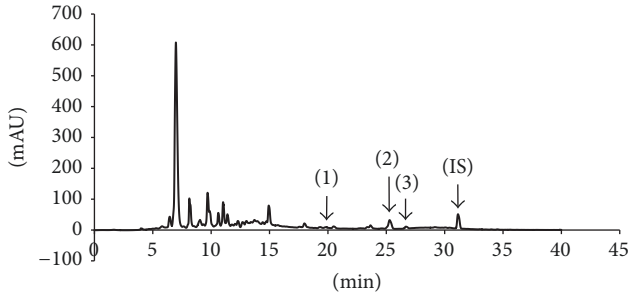

Figure 1: Representative HPLC chromatogram of a proso millet extract ((1) syringic acid; (2) *p*-coumaric acid; (3) ferulic acid) and the internal standard (IS), methyl paraben.

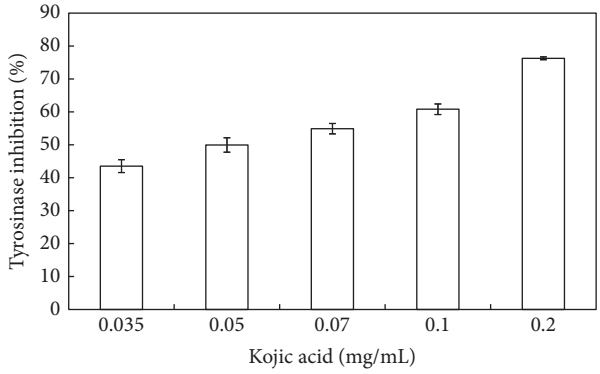

Figure 2: Effect of the kojic acid concentration on inhibition of tyrosinase.

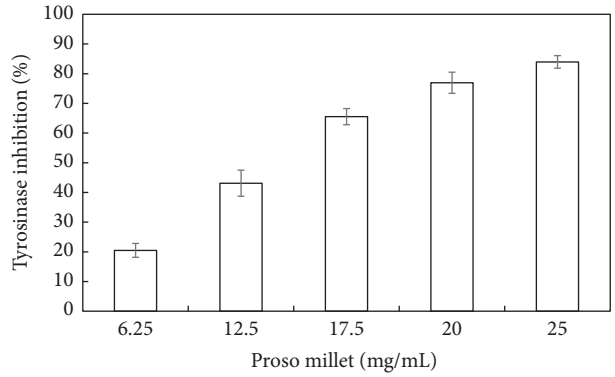

Figure 3: Effect of the proso millet extract concentration on the inhibition of tyrosinase.

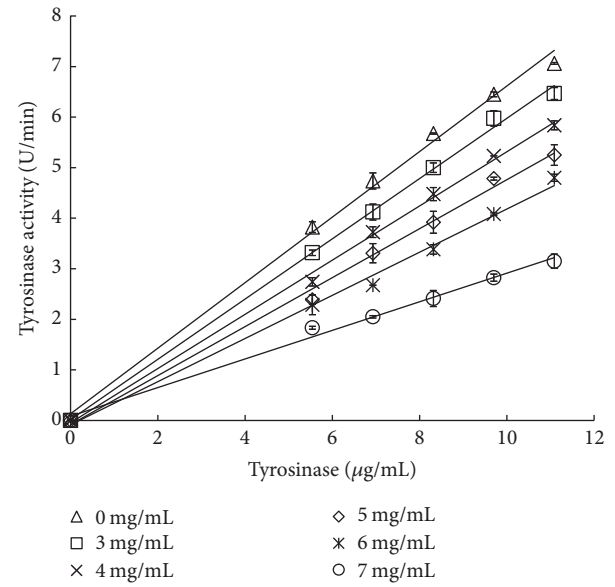

Figure 4: Effect of proso millet extract concentration (0~7 mg/mL) on the relationship between tyrosinase concentration and activity.

in the presence of different concentrations of the proso millet extract when using L-dopa (1.25 mM) as a substrate. These results indicate almost linear relationships between the tyrosinase concentration and activity for each concentration of proso millet extract and that tyrosinase activity decreased as the concentration of the proso millet extract increased, in agreement with the results in Figure 3. This effect may be attributed to the presence of phenolic tyrosinase inhibitors [20–22], such as syringic acid [23], *p*-coumaric acid [24, 25], and ferulic acid [25], which can act as alternative substrates (competitive inhibitors) of tyrosinase [26].

We examined the mechanism of this inhibitory effect using a Lineweaver-Burk double reciprocal plot of reaction rate versus L-dopa concentration with different concentrations of the proso millet extract (Figure 5(a)). The results indicate that the y-intercept and x-intercept depend on the concentration of the proso millet extract. These changes in the apparent $V_{\max}$ and K_m indicate that the proso millet extract inhibited tyrosinase by competitive and noncompetitive mechanisms. Previous research reported that kojic acid and n-acetyl-pentapeptides have similar effects [27], but *Vitis vinifera* leaf extracts exhibit competitive inhibition [19].

The equilibrium constant (KI) for inhibitor binding with free tyrosinase, determined by linear regression of the apparent $K_m/V_{\max}$ versus proso millet extract concentration, was 3.17 mg/mL (Figure 5(b)). The KI for inhibitor binding with the enzyme-substrate complex [28], determined by linear regression of the apparent $1/V_{\max}$ versus proso millet extract concentration, was 24.28 mg/mL (Figure 5(c)). The lower the KI is, the stronger the affinity is. Therefore, proso millet extract has a stronger affinity with free tyrosinase than tyrosinase-L-dopa complex.

4. Conclusion

The cosmetics industry is interested in plant natural tyrosinase inhibitors for potential use as inhibitors of melanogenesis. This study investigated the kinetics of tyrosinase inhibition by a proso millet extract. The results indicate that this extract reduced the tyrosinase activity in a dose-dependent manner, with an IC_{50} of 14.02 mg/mL. A Lineweaver-Burk double reciprocal plot indicated that the proso millet extract functioned as a mixed competitive and noncompetitive inhibitor of tyrosinase. Based on HPLC analysis, the bioactive constituents of the proso millet extract may be phenolics,

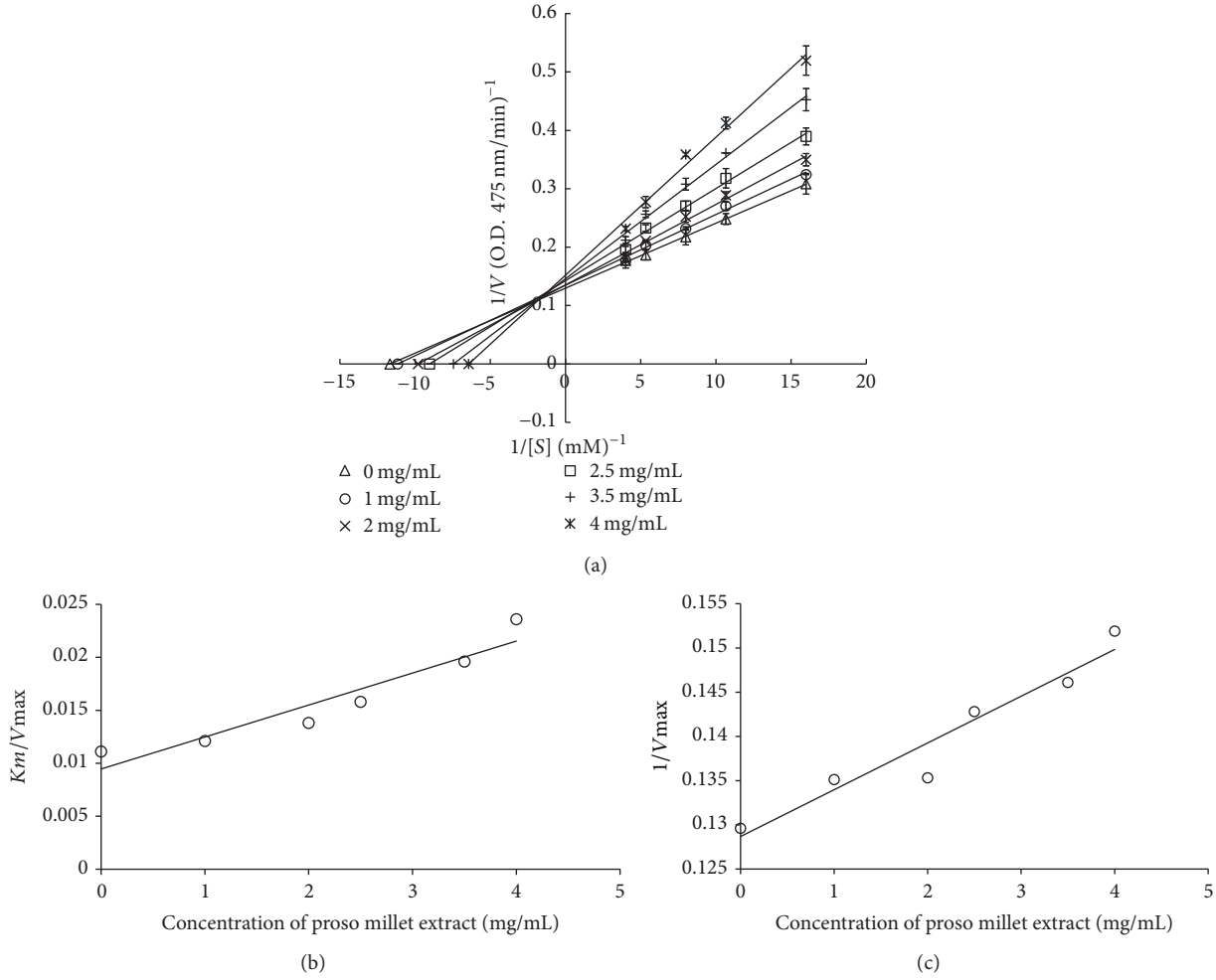

Figure 5: (a) Lineweaver-Burk double reciprocal plot of the effect of proso millet extract concentration (0~4 mg/mL) on tyrosinase activity. (b) Apparent K_m/V_{max} as a function of proso millet extract concentration. (c) Apparent $1/V_{max}$ as a function of proso millet extract concentration.

including syringic acid, *p*-coumaric acid, or ferulic acid. These results provide a foundation for the potential use of proso millet as an inhibitor of melanogenesis.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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