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Inhibitory activities of hydroxyphenolic acid–amino acid conjugates on tyrosinase

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ABSTRACT

The inhibitory effects of hydroxyphenolic acid–aromatic amino acid conjugates on the activity of mushroom tyrosinase were investigated. Amongst the hydroxyphenolic acid–amino acid derivatives, protocatechuic acid–amino acid amide (PA-AA-NH_2) showed highly increased tyrosinase inhibitory activity. The results show that it could strongly inhibit both the monophenolase and diphenolase activities of tyrosinase. The IC_{50} values, inhibition type, and K_I values of these hydroxyphenolic acid derivatives (PA-F-NH_2, PA-W-NH_2, PA-Y-NH_2) were evaluated and compared. Kinetic analyses of PA-AA-NH_2 as a tyrosinase inhibitor revealed that it acted as a reversible mixed-I type inhibitor, possibly by chelating copper at the active site of tyrosinase. These results suggest that aromatic amino acid conjugation assisted PA in binding to the active site of mushroom tyrosinase where it interrupted access to the substrate.

1. Introduction

Melanin is a dark pigment produced by skin cells in the innermost layer of the epidermis. When the skin is exposed to UV radiation, the formation of abnormal melanin pigment occurs, constituting a serious aesthetic problem that is particularly prevalent in middle-aged and elderly individuals (Parvez et al., 2006). Tyrosinase plays an important role in the biosynthesis pathway of melanin from tyrosine. Tyrosinase catalyses two distinct reactions involving molecular oxygen, the hydroxylation of tyrosine to L-DOPA as a monophenolase and the oxidation of L-DOPA to dopaquinone as a diphenolase. Dopaquinone is non-enzymatically converted to dopachrome and later to dihydroxyindols, which induce the production of melanin pigments (Parvez, Kang, Chung, & Bae, 2007). Therefore, the development of tyrosinase inhibitors is of great concern in the medical, agricultural, and cosmetic industries (Fenoll et al., 2001; Friedman, 1996).

Hydroxyphenolic acids and its esters, one group of dietary polyphenols, are known for their tyrosinase inhibitory activities and antioxidant activities (Rice-Evans, Miller, & Paganga, 1996). Amongst them, protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid) are known to have potent antioxidant activity (Kawabata, Okamoto, Kodama, Makimoto, & Kasai, 2002; Kubo, Chen, & Nihei, 2003; Kubo, Kinst-Hori, Chaudhuri, et al., 2000; Kubo, Kinst-Hori, Kubo, et al., 2000; Nakamura, Torikai, & Ohigashi, 2001; Saito, Okamoto, & Kawabata, 2004a, 2004b; Saito, Okamoto, Kawabata, & Kasai, 2003). In the case of gentisic acid (2,5-dihydroxybenzoic acid), its alkyl esters seem to be a good inhibitor of melanogenesis (Curto et al., 1999). Previously, our group found that the addition of aromatic amino acids to kojic acid strongly increased tyrosinase inhibitory activity and storage stability (Noh, Kwak, Kim, & Lee, 2006; Noh, Kwak, Seo, & Lee, 2009). In the present investigation, we conjugated aromatic amino acids to hydroxyphenolic acids, (protocatechuic acid (PA), α-resocylic acid (RA), gentisic acid (GT), and gallic acid (GA)) and then examined their tyrosinase inhibitory activities. Despite their close structural similarities, these compounds showed quite different levels of tyrosinase inhibitory activity. After choosing the most potent tyrosinase inhibitor, we carried out a kinetic study of the diphenolase inhibitory activity of mushroom tyrosinase.

2. Materials and methods

2.1. Reagents

Aminomethyl surface-layered polystyrene (AM SURE®) (100–200 mesh, 0.76 mmol/g) resin, Libra tubes® (15 ml, 5 ml) and Fmoc-L-amino acids were purchased from BeadTech Inc. (Seoul, Korea). Mushroom tyrosinase (EC 1.14.18.1), tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), protocatechuic acid (PA), α-resocylic acid (RA), gentisic acid (GT), and gallic acid (GA) were obtained from Aldrich (St. Louis, MO, USA). Fmoc-Rink amide linker, benzotriazol-1-yl-oxytris(dimethylamino)-phosphoniumhexafluorophosphate (BOP), *o*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) were purchased from GL Bio-Chem (Shanghai, China). *N*-Methyl-2-pyrrolidone (NMP) was purchased from Junsei Chemicals

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(Tokyo, Japan). Piperidine and dichloromethane (DCM) were from Dae-Jung Chemicals (Siheung, Korea), Tetrahydrofuran (THF) was from Sam Chun Chemicals (Pyongtack, Korea), *N*,*N*-Dimethyl-formamide (DMF) from Mallinckrodt Backer (Kentucky, USA), Trifluoroacetic acid (TFA) from Acros Organics (New Jersey, USA), and phenol from DC Chemical (Seoul, Korea). All solvents were of reagent grade and used without further purification.

2.2. Synthesis of hydroxyphenolic acid–amino acid amides

The Fmoc-Rink amide linker (2 equiv.) was anchored onto AM SURE® resin (0.76 mmol/g) containing HBTU (2 equiv.), HOBt (2 equiv.), and DIPEA (4 equiv.) in NMP at 30 °C for 3 h. The resins were filtered, washed with NMP and DCM, and then dried *in vacuo*. The loading level of the linker onto the resins was between 0.7 and 0.83 mmol/g resin, which was determined by Fmoc titration. After deprotection of the Fmoc groups with 20% piperidine in NMP for 30 min, *N*-Fmoc-amino acid (2 equiv.) in DMF was coupled to the resin using the general protocol of the BOP-mediated solid phase Fmoc/tBu strategy. After removing the Fmoc group, hydroxyphenolic acid (2 equiv.) was added to the resin containing HBTU (2 equiv.), HOBt (2 equiv.), DIPEA (4 equiv.), followed by shaking for 2 h. The reaction was monitored by the Kaiser's Ninhydrin Test. Finally, the resin was treated with reagent K [TFA/thioanisole/phenol/water/EDT (82.5/5/5/5/2.5 v/v)] for 30 min at room temperature and filtered. The crude peptide in the filtrate was concentrated in a light vacuum and precipitated with cold diethyl ether, yielding a white powder. The powder was further washed with diethyl ether and dried *in vacuo* and recrystallised from ethyl acetate–benzene to give the desired hydroxyphenolic acid–amino acid amide conjugates (O'bien et al., 1969). Product yields were 70–90%, based on the amount of the first amino acid on the resin. The products were identified by NMR and electrospray ionisation mass spectroscopy (ESI-MS). The purities of the products were determined by HPLC (YoungLin Autochro 2000), using the following conditions: Waters μBondapak C18 reverse phase column (125 Å, 10 μm, 3.9 × 150 mm); gradient elution with *A*: 0.1% TFA/water, *B*: 0.1% TFA/acetonitrile; from 10% to 90% B over 50 min; flow: 1 ml/min; detection: UV, 220 and 260 nm.

2.3. Enzyme assay

Tyrosinase catalyses the oxidation of two substrates, tyrosine and L-DOPA. Tyrosine was used as the substrate for the tyrosinase monophenolase activity assay, and L-DOPA was used as the substrate for the tyrosinase diphenolase activity assay. Enzyme activities were monitored by dopachrome formation at 475 nm. The reaction media (3 ml) for tyrosinase activity contained 0.5 mM tyrosine or L-DOPA in 50 mM Phosphate buffer (pH 6.8) along with the indicated concentration of inhibitor dissolved in ethanol. The final concentration of mushroom tyrosinase was 33.3 μg/ml for monophenolase activity and 6.67 μg/ml for *o*-diphenolic activity. The reaction mixture was stirred at 37 °C for 10 min, then immediately cooled in an ice bath. After standing in a ice bath for 10 min, the UV absorbance of the solution was measured at 475 nm. The same solution but without the test substance was also prepared, and its UV absorbance was measured at 475 nm. The % inhibition was calculated using the following formula: $[(A–B)/A] \times 100$ (*A*: absorbance of control solution; *B*: absorbance of test substance solution). The extent of inhibition was expressed as the percentage necessary for 50% inhibition (IC_{50}). The effects of inhibition on melanin formation was performed in B16 cells. The melanin contents of B16 cells were measured spectrophotometrically in the experimental period. Each experiment was performed in triplicate and the data were averaged.

2.4. Determination of inhibition type and inhibition constant

The inhibition type and inhibition constant were determined by the Lineweaver–Burk plot from the second plot of the apparent K_m/V_m versus the concentration of inhibitor.

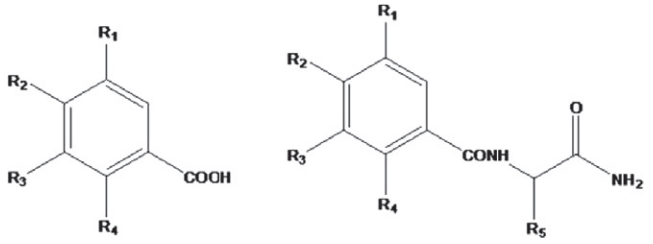
2.5. Determination of copper ion chelating ability

Stock solution of inhibitor (2 mM) was prepared in ethanol. Then 50 μM of the test solution was prepared in a cuvette containing PBS (50 mM, pH 6.8) and the absorbance was recorded in the 200–600 nm range. A repeated scan was recorded after incubating with 100 μM $CuSO_4$ or mushroom tyrosinase (138 units) at 25 °C for 30 min.

3. Result

3.1. Tyrosinase inhibitory activity and melanogenesis inhibitory activity of hydroxyphenolic acid–amino acid amides

The phenylalanine (F), tryptophan (W), and tyrosine (Y) were conjugated to PA, RA, GT, and GA. Hydroxyphenolic acids and their amino acid conjugates (20 μM); PA (A), PA-F-NH_2 (A-1), PA-W-NH_2 (A-2), PA-Y-NH_2 (A-3), RA (B), RA-F-NH_2 (B-1), RA-W-NH_2 (B-2), RA-Y-NH_2 (B-3), GT (C), GT-F-NH_2 (C-1), GT-W-NH_2 (C-2), GT-Y-NH_2 (C-3), GA (D), GA-F-NH_2 (D-1), GA-W-NH_2 (D-2), and GA-Y-NH_2 (D-3) (see Fig. 1 for structures) were tested for their inhibitory activity on tyrosinase using tyrosine as a substrate. The results (Fig. 2) show that compounds of A-1, A-2, and A-3 possess prominent inhibitory activity, whereas RA-AA-NH_2, GT-AA-NH_2 and GA-AA-NH_2 hardly have any inhibitory activity on the enzyme. The melanogenesis inhibitory activity of hydroxyphenolic acids and their amino acid conjugates were determined by measuring melanin contents after treating 100 μM of each samples in B16 cells. In the case of A-1, A-2, and A-3, they also showed high melanogenesis inhibitory activity even though PA itself increased the melanin contents. Other hydroxyphenolic acid–amino acid conjugates have little or no effect on melanogenesis inhibitory activity or increased melanin contents. Amongst these hydroxyphenolic acid–amino acid conjugates, PA–aromatic amino acid conjugates afforded higher tyrosinase inhibitory activity and melanogenesis inhibitory activity than PA alone.



PA (A): R_1=R_4=H; R_2=R_3=OH

RA (B): R_2=R_4=H; R_1=R_3=OH

GT (C): R_2=R_3=H; R_1=R_4=OH

GA (D): R_4=H; R_1=R_2=R_3=OH

A-1: R_1=R_4=H; R_2=R_3=OH; R_5=CH_2-C_6H_5

A-2: R_1=R_4=H; R_2=R_3=OH; R_5=CH_2-C_8H_8N

A-3: R_1=R_4=H; R_2=R_3=OH; R_5=CH_2-C_6H_4-OH

Fig. 1. Chemical structure of hydroxyphenolic acid and hydroxyphenolic acid–amino acid amides: PA (A), PA-F-NH_2 (A-1), PA-W-NH_2 (A-2), PA-Y-NH_2 (A-3), RA (B), RA-F-NH_2 (B-1), RA-W-NH_2 (B-2), RA-Y-NH_2 (B-3), GT (C), GT-F-NH_2 (C-1), GT-W-NH_2 (C-2), GT-Y-NH_2 (C-3), GA (D), GA-F-NH_2 (D-1), GA-W-NH_2 (D-2), and GA-Y-NH_2 (D-3).