Equol Inhibits Mushroom Tyrosinase in Vitro through Tight Binding

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Equol, an intestinal metabolite of daidzein, inhibited more potently mushroom tyrosinase in vitro than other inhibitors, genistein and kojic acid. We investigated the mechanism underlying tyrosinase inhibition by equol. Treating racemic equol with tyrosinase produced 3-hydroxyequol. Because the optical activity of the product showed <25% enantiomeric excess, the reaction was not highly stereospecific. Using enzyme-linked immunosorbent assays with an anti-equol monoclonal antibody, we observed that equol bound to pre-coated tyrosinase in a dose-dependent manner. Our results suggested the formation of a stable equol–tyrosinase complex.

Key words equol; tyrosinase; genistein; enzyme-linked immunosorbent assay (ELISA)

INTRODUCTION

Soy isoflavones have been of interest to researchers because of their potential use in maintaining human health. Recent studies have suggested that isoflavones are metabolized by the intestinal microflora, with the resulting products differing between individuals. An “equol producer” can convert daidzein to equol (1, Fig. 1A) in the intestine. Recent studies have reported the biological activity of equol, which is often stronger than that of its substrate daidzein. However, the activity of equol does not differ critically from that of genistein.

Because we isolated equol-metabolizing bacteria from human feces, we tried to find distinct activity of equol than genistein.

MATERIALS AND METHODS

Chemicals and Reagents Genistein and racemic equol were obtained from LC Laboratories (Woburn, MA, U.S.A.). (S)-Equol was isolated as bacterial metabolites. L-Tyrosine was purchased from Wako Pure Chemical Corporation (Osaka, Japan), and mushroom tyrosinase from Sigma-Aldrich (St. Louis, MO, U.S.A.). The bovine serum albumin (BSA) used was a product of Bio-rad (Hercules, CA, U.S.A.). Tween-PBS (TPBS) was prepared by mixing PBS with 0.05% Tween 20®.

Mushroom Tyrosinase Inhibition The reactions were conducted in 96-well plates using the method described by Chen et al., with minor modifications. In brief, solvent mixtures containing 1.0 mM L-tyrosine (170 µL) dissolved in phosphate buffer (50 mM, pH 6.8) were placed in wells with ethanolic samples (10 µL) and mushroom tyrosinase (1000 U/mL, 20 µL). After brief, gentle shaking, the reaction was allowed to proceed for 30 min at ambient temperature in the air-conditioned room set at 23 ± 5°C. The amount of dopaquinone produced was measured by determining the absorbance at 490 nm using a Sunrise™ microplate reader (Tecan, Mönndorf, Switzerland). Because temperature affects enzymatic browning, the enzyme activities were compared by analyzing the results obtained from a single 96-well plate for each examination.

LC-MS/MS Analysis A Wakosil-II 3C18HG column (i.d. 3.0 × 150 mm, Wako Pure Chemical Corporation) was used for sample separation. The solvent mixture, H2O:MeOH:HCOOH (550:450:1), was used at a flow rate of 0.3 mL/min at 40°C. Mass spectrometry was performed using the same HPLC column connected to a 3200 QTRAP (AB Sciex, Framingham, MA, U.S.A.) with a negative electrospray interface.

Isolation of the Reaction Product from Tyrosinase-Treated Equol Racemic equol in EtOH (10 mM, 30 mL) was dissolved in phosphate buffer (50 mM, pH 6.8) and pre-coated to a 96-well plate. The coated plate was incubated at 4°C for 1 h. The plates were washed with PBS-Tween and the wells were filled with mushroom tyrosinase (1000 U/mL, 20 µL) and equol (3-hydroxyequol, 25 µM). After brief, gentle shaking, the plate was incubated for 1 h at 23°C. The mixture was then diluted 700-fold with TPBS and the absorbance at 490 nm was measured. The reaction mixture was precipitated with 10% trichloroacetic acid (TCA). The precipitate was collected and dissolved in a solution containing 0.5% formic acid and 0.1% TFA. The samples were analyzed by LC-MS/MS. The results were compared with those of the control mixture without mushroom tyrosinase.

Fig. 1. Inhibitory Effect of Equol on Mushroom Tyrosinase in Vitro
(A) Structures of equol and 3-hydroxyequol. (B) Dose-dependent activity of equol, genistein, and kojic acid. Data represent the mean ± standard deviation (S.D.) of three experiments.

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added to 50mM phosphate buffer (pH 6.8) containing tyrosinase (110 U/mL, 270 mL). The reaction mixture was stirred for 2d at room temperature and the product was extracted with EtOAc (300 mL × 2). The extract was washed with water and saturated brine. After drying over anhydrous Na2SO4, the extract was concentrated in vacuo. The solvent was removed, and nitrogen gas was used to break the reduced pressure of the flask to avoid oxidation of the product. The concentrate was then separated by elution with a solvent mixture of hexane/EtOAc (1:1) using a 1-mm-thick Merck silica gel plate. Finally, 3ʹ-hydroxyequol (11.0 mg) and unreacted equol (28.0 mg) were obtained.

3ʹ-Hydroxyequol (2): LC-MS/MS(ESI−) m/z: 257 [M−H]−; 1H-NMR (400 MHz, acetone-d6) δH 7.98 (3H, brs, OH), 6.88 (1H, d, J = 8.2 Hz, H-5), 6.79 (1H, d, J = 8.1 Hz, H-5ʹ), 6.79 (1H, d, J = 2.1 Hz, H-2), 6.65 (1H, dd, J = 2.1, 8.1 Hz, H-6ʹ), 6.36 (1H, dd, J = 2.4, 8.2 Hz, H-6), 6.28 (1H, d, J = 2.4 Hz, H-8), 4.18 (1H, dd, J = 3.5, 10.5 Hz, H-2a), 3.90 (1H, dd, J = 10.5, 10.5 Hz, H-2b), 2.99 (1H, m, H-3), 2.87–2.78 (2H, m, H-4).; 13C-NMR (100 MHz, acetone-d6) δC 157.5 (C-7), 155.9 (C-8a), 146.0 (C-3), 144.7 (C-4), 134.3 (C-1), 131.0 (C-5), 119.4 (C-6), 116.2 (C-5ʹ), 115.3 (C-2), 114.0 (C-4a), 108.8 (C-6ʹ), 103.6 (C-8), 71.6 (C-3), 32.6 (C-4). 

Characterization of the Anti-equol Monoclonal Antibody
The reactions were conducted in a similar manner to a previously described method for preparing anti-equol monoclonal antibodies. A 96-well enzyme-linked immunosorbent assay (ELISA) plate coated with 4ʹ-carboxymethylequol-BSA (0.2 µg/well) was washed with 200 µL TPBS (×3) and then blocked with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan). After washing the plate, TPBS (100 µL) was added to the wells along with the competitors in EtOH (50 µL) and anti-equol monoclonal antibody solution (4 µg/mL in TPBS, 50 µL). After incubation for 2h at room temperature, the plate was washed and developed following the general procedure used by our group, using horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) and a commercially available ELISA TMB kit (Nacalai Tesque). After quenching the reaction with 2M phosphoric acid (100 µL), the absorbance was measured at 450/650 nm using a Tecan Sunrise™ microplate reader.

Identification of the Tyrosinase–Equol Complex Using an Anti-equol Monoclonal Antibody
The method used was similar to the ELISA procedure described above. In brief, the proteins (1.0 mg/mL) were placed in 96-well ELISA plates (100 µL/well) and stored at 4°C overnight. The plate was washed with 200 µL TPBS (×3) and then blocked. After washing with TPBS, 100 µL of racemic equol solution (6.25–100 µM in TPBS) was added and incubated for 2h at room temperature. After washing with TPBS, the resulting protein complex was conjugated with 100 µL of anti-equol monoclonal antibody (5 µg/mL) for 2h at room temperature. After washing with TPBS, the anti-equol antibody in the well was treated with HRP-conjugated anti-mouse IgG and was evaluated using the ELISA TMB kit.

RESULTS AND DISCUSSION
In this study, we first analyzed tyrosinase inhibition by soy isoflavones and metabolites. Genistein showed stronger activity than daidzein (data not shown), which is in accordance with the results of a previous study. We then examined the activity of equol in a dose-dependent manner, comparing it to genistein and kojic acid (a potent tyrosinase inhibitor). Equol exhibited a stronger inhibitory activity than kojic acid (Fig. 1B). Also, the activity was much stronger than genistein. The results showed that the microbial product was a more potent inhibitor of tyrosinase than was the “natural” isoflavone.

We next attempted to elucidate the mechanism underlying the inhibition of tyrosinase by equol. We treated commercially available racemic equol with tyrosinase, and found a new spot on TLC. The product reacted with FeCl3 more effectively than did the equol in the plate. Because tyrosinase generally produces catechol such as dihydroxyphenylalanine from l-tyrosine, this product on the TLC might have catechol moiety. Indeed, the LC-MS/MS analysis also indicated the incorporation of one oxygen atom to equol. From a structural perspective, three catechols, 5-, 7-, and 3ʹ-hydroxyequol, can be produced from one equol molecule. On the 1H-NMR spectrum, no 2H proton signal was found in aromatic region, whereas equol has two 2H proton signals for the B-ring. The spectrum also suggested two 1,2,4-trisubstituted benzene rings. From these results, we determined that this product was 3ʹ-hydroxyequol (2, Fig. 1A). The structure was also supported by heteronuclear multiple bond connectivity (HMBC) spectrum that showed a correlation between the 6ʹ-proton (δH 6.65) and a methine carbon (δC 38.9) of the C-ring, in addition to a 4ʹ-carbon (δC 144.7).

The naturally occurring enantiomers of equol is (S)-equol, and enzymatic reactions often have different reaction rates for enantiomers. We therefore investigated the stereochemistry of 3ʹ-hydroxyequol and unreacted equol. The recovered equol retained no optical activity (Fig. 2A), as ascertained with HPLC analysis using a Sumichiral OA-7000. 3ʹ-Hydroxyequol showed two peaks under the same HPLC conditions (Fig. 2A), although these were not entirely distinct. The absolute stereochemistry of 3ʹ-hydroxyequol in Fig. 2A was confirmed from the tyrosinase reaction using optically active (S)-equol obtained from intestinal bacteria. There was no HPLC peak corresponding to (R)-3ʹ-hydroxyequol from the experiment. As can be seen in Fig. 2A, the peak for (S)-3ʹ-hydroxyequol was slightly higher than that for the (R)-form. However, the selectivity of the enzymatic reaction was not constrained (e.e. < 25%). Under these experimental conditions described in Materials and Methods, 3ʹ-hydroxyequol production persisted with a lower yield than that of the unreacted equol. As a result, unreacted equol still contained a similar amount of each enantiomer (Fig. 2A). Our results indicated that mushroom tyrosinase oxidized equol without exhibiting strict stereochemical selectivity. Indeed, both enantiomers, which were obtained from the same supplier (Cayman Chemical), inhibited tyrosinase in a similar manner (Fig. 2B). Thus, our findings indicated that both equol enantiomers were good substrates of tyrosinase and were metabolized to 3ʹ-hydroxyequol.
inhibitory mechanism of equol results from its strong binding to the active site of the enzyme. We used the anti-equol monoclonal antibody\(^7\) to confirm this. We first examined the reactivity of the antibody to 3ʹ-hydroxyequol and optically active equols. Competitive ELISA showed that the antibody reacted with both equol enantiomers in a similar manner (Fig. 4A), as we had previously predicted using racemic equol and bacterial \((S)\)-equol.\(^7\) In addition, the anti-equol antibody bound to 3ʹ-hydroxyequol (Fig. 4A), although its capacity for doing so was slightly lower than that for binding to equol.

From the profile illustrated in Fig. 4A, we deduced that the anti-equol antibody would detect the presence of protein that binds to equol or 3ʹ-hydroxyequol. We then used the antibody to demonstrate the existence of a tyrosinase–equol complex. Tyrosinase coated on an ELISA plate was incubated with racemic equol and the plate was developed using anti-equol monoclonal antibody and HRP-conjugated anti-mouse IgG. The tyrosinase formed a complex that was recognized by the anti-equol monoclonal antibody (Fig. 4B). Another protein, BSA, did not capture equol. The complex formation depended on the amount of tyrosinase (data not shown) when treated with the same equol solution (100 \(\mu\text{M}\)). We therefore concluded that the reaction mixture containing equol and tyrosinase produced a complex that was stable during ELISA and could endure multiple TPBS washes. However, because of the profile of the anti-equol antibody (Fig. 4A), we were unable to determine whether it was equol or 3ʹ-hydroxyequol that formed the complex with the enzyme.

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Conflict of Interest The authors declare no conflict of interest.

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