Isolation and Identification of \( \alpha \)-Glucosidase Inhibitors from Tochu-cha (Eucommia ulmoides)

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\( \alpha \)-Glucosidase inhibitory activity was found in aqueous methanol extracts of tochu-cha, dried leaves of Eucommia ulmoides (Eucommiaceae). Five active principles against yeast enzyme were isolated and characterized. Among them, quercetin (1; \( K_i; 8.5 \times 10^{-6} \) M) was considered to contribute mostly to the activity of the tochu leaves. In regard to an animal \( \alpha \)-glucosidase, rat intestinal sucrase activity was also inhibited by 1.

Key words: \( \alpha \)-glucosidase inhibitor; quercetin; tochu-cha; Eucommia ulmoides

\( \alpha \)-Glucosidase is an enzyme that catalyzes the final step in the digestive process of carbohydrates, and hence \( \alpha \)-glucosidase inhibitors could retard the use of dietary carbohydrates to suppress postprandial hyperglycemia. The possibility of clinical use of such inhibitors for diabetic or obese patients has been attempted by acarbose, which has been shown to effectively reduce the intestinal absorption of sugars in humans. On the other hand, \( \alpha \)-glucosidase has an important function for post-translational processing of sugar chains expressed on the cell surface which is critical to the cell–cell recognition. It has been recently reported that nojirimycin, a potent \( \alpha \)-glucosidase inhibitor, inhibits the HIV infection of T-cells. In the course of our search for physiologically active food ingredients, we tried to isolate and identify \( \alpha \)-glucosidase inhibitors from plant foodstuffs.

Primary screening experiments were done using a readily available yeast enzyme. Among the twenty-eight plant foodstuffs tested (see Experimental), aqueous methanol extracts of tochu-cha, dried leaves of Eucommia ulmoides (Eucommiaceae) showed high inhibitory activity against yeast \( \alpha \)-glucosidase. Column chromatographic fractionation of the extracts followed by HPLC separation yielded quercetin (1), catechin-(7,8-b,c)-4x-(3,4-dihydroxyphenyl)-2(3H)-pyranone (2), catechin-(7,8-b,c)-4β-(3,4-dihydroxyphenyl)-2(3H)-pyranone (3), kaempferol-3-O-β-glucoside (4), and eucommiol (5) as the inhibitors (Fig. 1).

The chemical structures including their stereochemistry were deduced from the results of spectroscopic analyses and confirmed by comparison their physicochemical data with those of an authentic specimen or data from the literature. The compounds except 5 were first isolated from tochu leaves. The inhibitory activities of the isolated compounds 1–5 (24 \( \mu \)M) are depicted in Fig. 2. The \( K_i \) values of 1, 2, and 3 were 8.5 \( \times \) 10\(^{-6} \) M, 2.5 \( \times \) 10\(^{-5} \) M, and 2.5 \( \times \) 10\(^{-5} \) M, respectively, indicating that 1 was the most effective inhibitor of the isolates. The 4 and 5 were not measured because of their low yields. This is the first report on the inhibitory activity of 2-5 against \( \alpha \)-glucosidase, while 1 was already known to inhibit yeast \( \alpha \)-glucosidase. It is interesting that among the isolates, 5 has a polyhydroxylated cyclopentane structure, which has the possibility to mimic the glucose molecule although its inhibitory activity against \( \alpha \)-glucosidase is not very high.

There have been no reports on the inhibitory activity of the isolated compounds against animal \( \alpha \)-glucosidase. To examine the efficiency of the tochu constituents for inhibition of mammalian enzymes in the digestive tract, the inhibitory activity of the isolates was measured using a rat intestinal sucrase that is a key enzyme for use of dietary sucrose. Quercetin (1) showed a moderate inhibitory activity at the IC\(_{50}\) of 2.9 \( \times \) 10\(^{-4} \) M, but no other compounds showed inhibition even at the 10-fold higher concentration compared to 1. The fact that quercetin, a common plant flavonol, inhibits mammalian intestinal \( \alpha \)-glucosidase was thus disclosed. Since 1 is the major inhibitory constituent in tochu leaves despite its lower activity than a typical inhibitor such as nojirimycin, it may be expected to suppress use of carbohydrates in intestine when tochu-cha is used as a beverage.

Experimental

Screening of \( \alpha \)-glucosidase inhibitors. Edible parts of each foodstuff were extracted with 50% MeOH (10 ml/g fr. wt.) and the extracts were measured for \( \alpha \)-glucosidase inhibitory activity. The inhibitory assay was
done by the chromogenic method with a slight modification. Yeast \(\alpha\)-glucosidase (10 \(\mu\)g/ml, 70 U/mg, Wakun Pure Chem. Ind.) was dissolved in 100 mM phosphate buffer (PB, pH 7.0) containing 0.2% BSA and 0.02% NaN\(_3\), and used as an enzyme solution. \(p\)-Nitrophenyl-\(\alpha\)-D-glucopyranoside (NPG, Kanto Chem. Co., 5 mm) in the same buffer (pH 7.0) was used as a substrate solution. The enzyme solution (50 \(\mu\)l) and the methanol extracts (10 \(\mu\)l) of the tested materials were mixed in a well of a microtiter plate and measured for \(\Delta A_{405}\) at zero min with a microplate reader. After incubation for 5 min, the substrate solution (50 \(\mu\)l) was added and the reaction was done for 5 min at room temperature. The increase in absorbance from zero time was measured. Tested foodstuffs and the results (\% inhibition) were as follows: spinach (0), mustard (22), radish (1), Japanese horseradish (35), corn (16), cucumber (29), garlic chrysanthenum (6), burdock (12), dandelion (3), pepper (3), perilla (0), ginger (26), muoga ginger (6), carrot (40), tochu (92), eggplant (46), red pepper (8), potato (3), bell pepper (31), apple (43), sweet potato (0), kiwi fruit (74), adzuki bean (5), broad bean (0), lemon (66), yamanoimo (3), Welsh onion (86), onion (43).

**Isolation of active principles from tochu-cha.** Dried leaves (200 g) of *E. ulmoides*, tochu-cha, were pulverized and extracted twice with 50% MeOH (2 liters each) at room temperature. The extracts were partitioned between EtOAc-H\(_2\)O and the EtOAc soluble fraction was further separated into the neutral-philic (1.02 g), and acidic (1.24 g) fractions with 5% NaHCO\(_3\). The neutral-philic fraction was chromatographed on silica gel using a CHCl\(_3\)-MeOH gradient to obtain two active fractions. The less polar fraction (CHCl\(_3\)-MeOH = 9:1) was subjected to silica gel prep. TLC (hexane-acetone = 45:55) followed by HPLC (Inertisil PREP-ODS, GL Science, 20 \(\times\) 250 mm, MeCN-H\(_2\)O = 4:6), to give queretin (1, 22.2 g) as a yellow amorphous powder. The physicochemical properties of 1 coincided well with those of an authentic specimen. The more polar fraction (CHCl\(_3\)-MeOH = 8:2) was also subjected to the prep. TLC and HPLC under the same conditions as above except for using MeOH-H\(_2\)O = 43:57 as the mobile phase in HPLC to give catechin-(7,8-b-c)-4x-(3,4-dihydroxy-phenyl)-(2(3H)pyranone (2, 3.2 mg) and catechin-(7,8-b-c)-4b-(3,4-di-hydroxyphenyl)-2(3H)pyranone (3, 2.0 mg) both as a red amorphous powder. The physicochemical properties of 2 and 3 coincided well with those reported in the literature.\(^9\)

The acidic fraction, which also showed moderate inhibitory activity against \(\alpha\)-glucosidase, was chromatographed successively on Sephadex LH-20 (MeOH) and on Cosmosil 75C\(_18\)-OPN (MeOH-H\(_2\)O). Active fractions were subjected to HPLC (MeCN-H\(_2\)O = 1:3) with the same column as 1 to give kaempferol-3-\(\beta\)-glucoside (4, 2.3 mg) as a yellow amorphous powder. The mass and NMR spectral data of 4 well supported its structure and the \({}^{13}\)C NMR data was identical to those reported in the literature.\(^9\) The isolation of 4 from the acidic fraction seems to arise from an insufficient solvent-partitioning due to its partial solubility in water. For the isolation of water-soluble active principles, pulverized leaves (200 g) of *E. ulmoides* were mixed with activated charcoal (100 g), and then extracted with 50% MeOH (2 liters) at room temperature. The extracts were centrifuged and the supernatant was filtered through cellulose acetate membrane (0.45 \(\mu\)m pore size). The filtrate was chromatographed on Biogel P-2 (10% EtOH). The active fractions were subjected to HPLC (Finepak SIL C\(_18\), JASCO, 4.6 \(\times\) 150 mm, MeOH-H\(_2\)O, linear gradient, and Finepak SIL NH\(_2\)-5, JASCO, 4.6 \(\times\) 250 mm, MeCN-H\(_2\)O = 4:1) to give eucommiol (5, 2.8 mg) as a colorless syrup. The physicochemical properties of 5 was identical to those reported in the literature.\(^5\)

**Evaluation of inhibitory activity of isolates.** Each isolated compound was dissolved in MeOH (24 \(\mu\)l, final concentration). Yeast \(\alpha\)-glucosidase (0.1 \(\mu\)g/ml) was dissolved in 10 mM PB (pH 7.0) containing 0.2% BSA and used as an enzyme solution. NPG (5 mm) in the same buffer (pH 7.0) was used as a substrate solution. PB (10 mM, pH 7.0, 2 ml, substrate (200 \(\mu\)l) and inhibitor (100 \(\mu\)l) were mixed in a test tube and incubated for 5 min at 37°C. Enzyme solution (200 \(\mu\)l) was then added and the reaction was done for 5 min at 37°C. The reaction was stopped by adding 0.25 \(\mu\)l Na\(_2\)CO\(_3\) (1.5 ml). The increase in absorbance at 405 nm was measured.

**Rat intestinal sucrase inhibitory activity.** Rat small intestinal sucrase-isomaltase complex was solubilized with Triton X-100.\(^8\) Sucrase activity was measured as described by Dahlqvist\(^9\) with 20 \(\mu\)m sucrose as a substrate. Compounds 1-5 were dissolved in 50% DMSO and used for the assay.

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**References**