Isolation and Identification of Acetyl-CoA Carboxylase Inhibitors from Green Tea (*Camellia sinensis*)

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An aqueous methanol extract from green tea showed potent acetyl-CoA carboxylase inhibitory activity. An active compound was isolated from the extract and identified as (–)-epigallocatechin gallate by instrumental analyses. The IC_{50} value of (–)-epigallocatechin gallate was 3.1×10^{-4} M. Among tea catechins and related compounds, nearly equal activity was found in (–)-epigallocatechin gallate and (–)-epicatechin gallate, whereas (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, gallic acid and methyl gallate each had no inhibitory activity. These results indicate that the 3-*O*-gallate group of the catechin structure was necessary for this activity. (–)-Epigallocatechin gallate inhibited triglyceride accumulation in 3T3-L1 cells at a concentration of 1.0×10^{-7} M or higher.

Key words: acetyl-CoA carboxylase inhibitor; green tea; catechins

Acetyl-CoA carboxylase (ACC, EC 6.4.1.2) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, this reaction being the rate-limiting step of fatty acid biosynthesis. Its activity is regulated by the phosphorylated/dephosphorylated form,¹⁾ as well as by changes in enzyme quantity.²⁾ The inhibitors of ACC would thus lead to avoiding obesity as a risk factor of many chronic diseases. In addition, it has recently been reported that malonyl-CoA played an important role in glucose-stimulated insulin secretion from pancreatic β -cells.³⁾ Hence, ACC inhibitors have the possibility for clinical use with hyperlipidemic or diabetic patients. We have started to search for ACC inhibitors in foodstuffs, since daily consumption of such inhibitors from foods might result in a healthy effect without using medicines. Although 5-(tetradecyloxy)-2-furoic acid (TOFA),⁴⁾ 2-tetradecanylethylglutarate⁵⁾ and tetramethylhexadecanedioic acid⁶⁾ are known as ACC inhibitors, these compounds do not originate from foodstuffs. This paper reports the isolation, identification, and structure-activity relationship for ACC inhibitors from plant foodstuffs and their biological effects on lipid metabolism.

Materials and Methods

Materials. All plant foodstuffs were obtained from market sources. Authentic samples of (–)-epigallocatechin gallate (EGCg) and 4-hydroxy-3-nitrophenylacetic acid were purchased from Wako Pure Chemical Industries and Sigma Chemical Co., respectively. All other

chemicals were of reagent grade.

Instrumental analyses. ¹H- and ¹³C-NMR spectra were measured with a Bruker AM500 spectrometer operating at 500 MHz for ¹H and at 125 MHz for ¹³C. FD-MS data were measured with a JEOL JMS-SX102A mass spectrometer, and optical rotation was measured with a JASCO DIP-370 spectrometer.

Measurement of ACC inhibitory activity. Rat liver ACC was successively purified by ammonium sulfate precipitation, calcium phosphate gel chromatography and DEAE-cellulose ion-exchange chromatography, according to the method of Tanabe *et al.*⁸⁾ The enzyme fraction obtained by DEAE-cellulose chromatography was dissolved in a 100 mM potassium phosphate buffer (pH 7.5, 5.6 mU/ml) containing 250 mM sucrose, 5 mM 2-mercaptoethanol and 1 mM EDTA, and was used as the enzyme solution. A 50 mM Tris-HCl buffer (pH 7.5, 160 μ l) containing 1.3 mM potassium citrate, 13 mM MgCl₂, 4.7 mM glutathione, 0.16 mM acetyl-CoA and 0.13 mM 4-hydroxy-3-nitrophenylacetic acid (internal standard) were mixed with the enzyme solution (20 μ l) and the test solution (10 μ l) as described next. After preincubating for 5 min, a 50 mM tris-HCl buffer (pH 7.5, 10 μ l) containing 75 mM ATP and 500 mM KHCO₃ was added, and the reaction was conducted for 15 min at 37°C. Malonyl-CoA produced during the reaction was measured by HPLC with a reverse-phase column (Finepak SIL C18-10, 4.6 $\times$ 250 mm, JASCO). Gradient elution (5–15% MeCN containing 200 mM KHPO₄) was performed at a flow rate of 1 ml/min and at 40°C (Fig. 1).

Screening for ACC inhibitors in foodstuffs. The edible parts of each foodstuff were extracted with 50% aqueous MeOH (10 ml/g fr. wt.). The extract was partitioned between EtOAc and water, and the EtOAc layer was washed with 5% NaHCO₃ to remove acidic substances such as citrate and fatty acid which would affect ACC activity. The resulting extract was evaporated, redissolved in DMSO (10 ml/g fr. wt.), and submitted to ACC inhibitory activity measurements.

Isolation of the ACC inhibitor from green tea. Green tea (5 g) was extracted with 50% aqueous MeOH (50 ml). The MeOH extract was partitioned between EtOAc and

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Fig. 1. HPLC Profile in the Measurement of Acetyl-CoA Carboxylase Inhibitory Activity.

water, the resulting EtOAc solution being submitted to preparative TLC (Merck) developed with CHCl_3 -MeOH-formic acid (16:4:1). The active fraction was dissolved in MeOH, and put in a column of Cosmosil 75C18-OPN (Nacalai Tesque, 20×95 mm). Stepwise elution was performed from water to MeOH. The active fraction obtained from 30% MeOH eluate was further purified by preparative HPLC in an Inertsil PREP-ODS column (GL Science, 20×250 mm), eluting with MeCN-water (1:3) at a flow rate of 6.0 ml/min and detecting at UV251 nm. The pure active principle (55.8 mg) was obtained as purple amorphous powder and identified as **EGCg** by comparing its physicochemical properties with those of an authentic specimen.

Preparation of (-)-epicatechin gallate (ECg) and (-)-epigallocatechin (EGC). Green tea (20 g) was extracted with 50% aqueous MeOH, and the extract was partitioned between EtOAc and water. The resulting EtOAc solution was successively submitted to silica gel (Wako Pure Chem. Ind., 27×180 mm, CHCl_3 -MeOH stepwise elution) and Sephadex LH-20 (Pharmacia, 20×400 mm, CHCl_3 -MeOH (1:1)) columns, and to preparative TLC (Merck, CHCl_3 -MeOH (7:3)) to obtain **ECg** (195.9 mg). The $^1\text{H-NMR}$ spectrum of the isolate was in good accordance with the spectral data in the literature.⁷ **EGCg** (50 mg) and tannase (*Aspergillus ory-*

zae origin, 415U, Wako Pure Chem. Ind.) were suspended in a 50 mM citrate buffer (pH 5.5, 10 ml), and the enzymatic reaction was conducted at 37°C for 90 min. The reaction mixture was partitioned between EtOAc and water, and the EtOAc layer was washed with 5% NaHCO_3 to obtain **EGC** (4.0 mg). The $^1\text{H-NMR}$ spectrum of the isolate was in good accordance with the spectral data in the literature.⁷ **ECg** and **EGC** were dissolved in DMSO at a concentration of 5 mM and then submitted to ACC inhibitory activity measurement.

Triglyceride accumulation in 3T3-L1. 3T3-L1 cells were kindly provided by Prof. Y. Kitagawa of Nagoya University. Cultivation of 3T3-L1 cells and their conversion to adipocytes were carried out according to the method of Kharroubi *et al.*⁹ After converting to adipocytes, the cells were treated for 6 days with Dulbecco's modified Eagle's medium (DMEM, Gibco) containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (5 mg/ml), 10% controlled process serum replacement type-1 (CPSR-1, Sigma Chem. Co.) and **EGCg** at the concentration indicated in the figure legend. After intracellular lipid extraction by the Folch method,¹⁰ Triglyceride (TG) concentration was enzymatically measured by a commercial kit (Wako Pure Chem. Ind.).

Results and Discussion

Primary screening was carried out to select a foodstuff with high ACC inhibitory activity. The tested foodstuffs and results (% inhibition) were as follows: green tea (57), poaal tea (32), Asiatic ginseng (31), black tea (30), lily flower (29), gymnema-cha (25), rakanka (24), Welsh onion (22), garland chrysanthemum (21), green soybean (19), Chinese gutta percha (15), green gram sprouts (12), enokitake (11), dokudami-cha (3), woody ear (0). The green tea showed the highest activity among the 15 foodstuffs tested. Although black tea and poaal tea also showed inhibitory activity, their activity were lower than that of green tea. This result indicates that the fermentation of tea lowered the inhibitory activity. We thus selected green tea as the starting material.

Green tea (5 g) was extracted with 50% aqueous methanol. The extract was successively fractionated by solvent partition, preparative TLC, Cosmosil column chromatography and reverse-phase HPLC. The final preparative HPLC run gave an active compound (55.8 mg) as purple amorphous powder. The physicochemical properties of the active compound were as follows: mp 215–216°C; $[\alpha]_D^{25} -179^\circ$ (c 0.12, ethanol); FD-MS m/z : 459 ($[\text{M}+\text{H}]^+$), 290, 170; $^1\text{H-NMR}$ δ_{H} (acetone- d_6): 2.91 (1H, dd, $J=4.2, 16.5$), 3.01 (1H, dd, $J=2.3, 16.5$), 5.06 (1H, s), 5.55 (1H, s), 6.02 (1H, d, $J=1.9$), 6.05 (1H, d, $J=1.9$), 6.61 (2H, s), 7.01 (2H, s); $^{13}\text{C-NMR}$ δ_{C} (acetone- d_6): 26.6, 69.2, 78.0, 95.8, 96.4, 99.0, 106.7, 109.9, 121.8, 130.7, 133.1, 138.8, 145.9, 146.2, 157.1, 157.4, 157.7, 166.0.

Since its mass and NMR spectra were consistent with those of authentic **EGCg**, the active compound was identified as **EGCg** with an IC_{50} value of 3.1×10^{-4} M.

Green tea contains a number of catechin derivatives other than **EGCg**. In order to examine the structure-ac-

Fig. 2. Acetyl-CoA Carboxylase Inhibitory Activity of EGCg and Related Compounds.

Each compound was dissolved in DMSO at a concentration of 5 mM, and an aliquot of this solution (10 μ l) was used for the inhibitory activity measurements. Inhibitory activity is shown as the activity relative to that of EGCg as 100%.

Fig. 3. Effect of EGCg on 3T3-L1 Triglyceride Accumulation at Various Concentrations.

Avidin (1.2 mg/l) was used as a positive control. See the Materials and Methods section for details of the procedure.

tivity relationship of catechins, (–)-epigallocatechin (EGC) was prepared from EGCg by enzymatic hydrolysis, and (–)-epicatechin gallate (ECg) was isolated from green tea. The ACC inhibitory activities of these catechins and of commercially available (+)-catechin (C), (–)-epicatechin (EC), gallic acid and methyl gallate are summarized in Fig. 2. Since C and EC showed no inhibitory activity, the catechin skeleton itself was insufficient to develop activity. ECg showed almost the same activity as EGCg, although EGC showed no inhibitory activity. Neither free gallic acid nor methyl gallate showed any inhibitory activity. These results indicate that both the catechin skeleton and 3-O-gallate appendix were necessary to demonstrate inhibitory activity.

3T3-L1 cells, a subclone of mouse fibroblast 3T3 cells, differentiate into cells with morphological and biochemi-

cal characteristics of adipocytes, and can accumulate a large amount of TG. The effect of EGCg on intracellular TG accumulation was thus measured (Fig. 3). The intracellular TG concentration was reduced to about one-half by the avidin (1.2 mg/l) treatment after the conversion of adipocytes. EGCg reduced the intracellular TG concentration in a dose-dependent manner and had comparable activity to avidin at a concentration of 1.0×10^{-5} M. A microscopic examination showed EGCg to have no effect on the cell viability at a concentration of 1.0×10^{-5} M or lower, although cell toxicity was apparent at a concentration of 1.0×10^{-4} M.

It has been reported that green tea and tea catechins have such biological and biochemical effects as anti-mutagenic,¹¹⁾ anti-carcinogenic,¹²⁾ anti-retro viral¹³⁾ and anti-hypercholesterolemic.¹⁴⁾ Moreover, orally administered EGCg was absorbed into the circulation system by rats.¹⁵⁾ The present results suggest a possible new function of green tea which might reduce fatty acid biosynthesis.

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