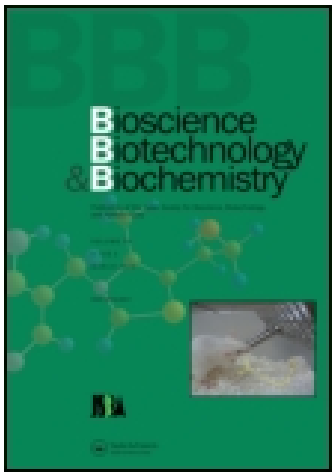

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Enzymatic Synthesis of (+)Catechin-α-glucoside and Its Effect on Tyrosinase Activity

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The glycosidation of (+)catechin, which has five hydroxyl groups with cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) and soluble starch has been studied. One of the transfer products was purified and its structure was determined to be (+)catechin 3′-*O*-α-D-glucopyranoside. This glucoside noncompetitively inhibited the activity of tyrosinase from mushroom, IC_{50} being 5.8 mM, but didn't inhibit that from mouse melanoma. In contrast, arbutin (hydroquinone-*O*-β-D-glucopyranoside) inhibited both tyrosinases.

In general, a substrate analogue of an enzyme can be expected to inhibit the corresponding enzyme activity. In the case of tyrosinase, some polyphenols and their derivatives are known as inhibitors. A tyrosinase inhibitor can be applied to prevent discoloring of perishable foods or to suppress melanogenesis in animal cells. For example, arbutin, which has been extracted from a plant and has been applied to cosmetics, was found to inhibit tyrosinase activity.[1)]

We have synthesized various polyphenol glycosides with glycosyl transfer enzymes to examine their effect on tyrosinase activity. In this paper, we report the enzymatic synthesis of (+)catechin glucoside, and its effect on the activity of tyrosinases.

Materials and Methods

Enzymes. Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from *Bacillus macerans* was purchased from Amano Pharmaceutical Co. (Nagoya, Japan), and Tyrosinase (EC 1.14.18.1) from *Agricus bisporus* (mushroom) was purchased from Sigma Chemicals Co. (St. Louis, MO). Glucoamylase (EC 3.2.1.3) was prepared from a culture broth of *Aspergillus niger* according to the method of Tsujisaka *et al.*[2,3)]

Tyrosinase from mouse melanoma. Tyrosinase from mouse melanoma was prepared according to the modified method of Hashimoto *et al.*[4)] Mouse melanoma B16-FO (ATCC CRL 6322) was cultured in Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum) at 37°C for 5 or 6 days. The cells were harvested by trypsinization when the culture was between 50 to 80% confluent. The cells collected by centrifugation at 180 × *g* for 5 min were suspended in an ice-cooled 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% Triton X-100. After stirring for 20 min, the suspension was centrifuged at 30,000 × *g* for 30 min. This extract was used as a crude tyrosinase solution.

Substrates. Soluble starch, (+)catechin and 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) were obtained from Nacalai Tesque Co. (Kyoto, Japan).

Enzyme activity. The activity of CGTase was assayed by using soluble starch as a substrate. Fifty μl of the enzyme solution was added to 450 μl of a 50 mM sodium phosphate buffer (pH 7.0) containing 0.5% soluble starch, and the mixture was incubated at 40°C for 10 min. Then, 1.0 ml of 0.5 N HCl was added to the mixture to stop the enzyme reaction, before 2.5 ml of a 0.05% KI solution containing 0.005% I_2 was added. After keeping at room temperature for 20 min, the absorbance of 660 nm of the solution was measured. One unit of enzyme activity is defined as the amount of enzyme which reduced by 0.5 the unit absorbance at 660 nm per min under the conditions just described.

Thin-layer chromatography (TLC). TLC was carried out with the ascending method, using silica gel 60 plates (Merck Co., Frankfurt, Germany) and ethyl acetate–acetic acid–water (3 : 1 : 1, v/v) as the solvent. Spots were detected by spraying with H_2SO_4–methanol (1 : 2, v/v) and subsequent heating.

High-performance liquid chromatography (HPLC). HPLC was carried out under the following conditions: column, TSK-GEL ODS-120T (4.6 × 250 mm, Tosoh Co., Tokyo, Japan); solvent, methanol–10 mM phosphate (15 : 85, v/v); flow rate, 1.0 ml/min; pump, Tosoh CCPM; detector, Tosoh UV-8000 (at 279 nm).

Preparation of the glucoside. CGTase (6000 units) was added to 100 ml of a 10 mM sodium phosphate buffer (pH 6.5) containing 5% soluble starch and 2% (+)catechin. After incubating at 40°C for 40 h, 5 mg of glucoamylase was added to the mixture, and the mixture was incubated at 40°C for 1 h more to convert (+)catechin oligoglucoside to (+)catechin glucoside. The mixture was then concentrated *in vacuo* to 20 ml. The concentrate was applied to a column of silica gel C-200 (Wako Pure Chemical Industries, Osaka, Japan) that had been equilibrated with ethyl acetate–methanol (5 : 95, v/v), and the products were eluted with ethyl acetate–methanol (10 : 90, v/v), the fractions containing (+)catechin glucosides being collected. After being concentrated, in glucoside was separated by preparative high-performance liquid chromatography under the conditions described next.

Preparative high-performance liquid chromatography (PrepHPLC). PrepHPLC was carried out under the following conditions: column, μBondasphere 5 μ C_{18}-100 Å (19 × 150 mm, Nihon Waters, Tokyo, Japan); solvent, methanol–10 mM phosphate (18 : 82, v/v); flow rate, 5.0 ml/min; pump. Tosoh CCPM; detector, Tosoh UV-8000 (at 279 nm).

Measurement of the inhibition of tyrosinase activity. A sodium phosphate buffer (0.1 M, pH 6.8) was used throughout the experiments. The buffer solution (0.7 ml), 1.0 ml of the glucoside solution and 0.3 ml of 2000 units/ml

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Abbreviations. CGTase, cyclodextrin glucanotransferase; L-DOPA, 3-(3,4-dihydroxyphenyl)-L-alanine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PrepHPLC, preparative high-performance liquid chromatography.

of tyrosinase were mixed and preincubated at 25°C for 5 min in a sample cuvette. In a reference cuvette, the buffer solution was used in place of tyrosinase. After preincubation, 1.0 ml of 2.5 mM L-DOPA in the buffer was added to each cuvette and mixed. The increase in absorbance at 475 nm was recorded for 1 min. A control test was carried out by using the buffer without the glucoside. The residual activity was calculated according to the following formula:

$$\text{Residual activity } (\%) = A/Ac \times 100$$

(Ac, increase in absorbance at 475 nm per min in the control test; A, increase in absorbance at 475 nm per min.)

When using tyrosinase from mouse melanoma, the reaction mixture was incubated at 37°C, and the concentration of L-DOPA was 10 mM, the other conditions being the same as those just described.

Results

Preparation and isolation of the enzymatic reaction products

Typical chromatograms of the reaction products by TLC and HPLC are shown in Figs. 1 and 2. By TLC, two main spots a and b, and two minor spots c and d appeared (Fig. 1). (+)Catechin was detected near the solvent front (R_f 0.9). By HPLC, at least four products (peaks A, B, C, and E in Fig. 2) were detected around peak D of (+)catechin. Peak E corresponded to spot a, and peaks A, B, and C corresponded to spot a or b. Fractions corresponding to peak E were pooled and further purified as described in the Materials and Methods section. The other reaction products could not be purified homogenously, because their retention times were too close to each other for PrepHPLC. Deionization was carried out by PrepHPLC under the same conditions as those given in the materials and methods section, except for using methanol–water (18:82, v/v) as the mobile phase. The yield of compound E from 2.0 g of (+)catechin was 38 mg. Compound E was homogeneous by TLC and HPLC, and we used this fraction for further studies.

Structural analysis of compound E

The molecular weight of compound E was estimated to be 452 daltons by FAB mass spectrometry; that gave $[M+H]^+$ at m/z 453 and $[M+Na]^+$ at m/z 475. The ^{13}C-NMR spectrum revealed the presence of (+)catechin and a glucose moiety, while the ^{1}H-NMR spectrum revealed the presence of (+)catechin and glucose in a molar ratio of 1:1 (Table I). The binding position of glucose was concluded to be C-3′ from the result that the chemical shift of H-2′ (δ 7.29) was lower than that of free (+)catechin. The α-configuration of the anomeric carbon in glucose could be assigned on the basis of the coupling constant (J = 3.7 Hz) in the ^{1}H-NMR spectrum. From these results, we conclude that the structure of compound E was (+)catechin 3′-*O*-α-D-glucopyranoside (Fig. 3).

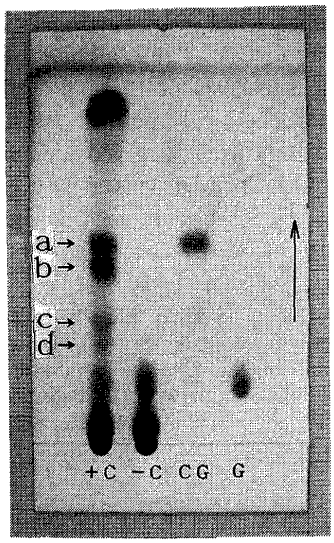

Fig. 1. Thin-layer Chromatogram of the Reaction products from (+)Catechin and Soluble Starch with CGTase.

+C, reaction products incubated with (+)catechin; −C, reaction products incubated without (+)catechin; CG, isolated (+)catechin glucoside; G, glucose. The products are shown by small arrows, and details are given in the Materials and Methods section.

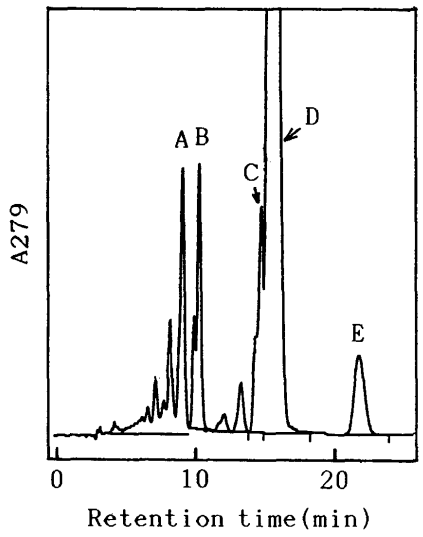

Fig. 2. High-performance Liquid Chromatogram of the Reaction Products from (+)Catechin and Soluble Starch with CGTase.

Peaks A, B, C, and E are products from (+)catechin and soluble starch incubated with CGTase. Peak D is (+)catechin. 3μl of the sample solution was applied to HPLC, details being described in Materials and Methods.

Table I. ^{13}C- and ^{1}H-NMR Data for (+)Catechin Glucoside

Position	δ_C	δ_H
2	83.6	4.57 (1H, d)
3	69.6	3.99 (1H, dd)
4	29.8	2.49–2.83 (2H, m)
4a	102.3	—
5	158.4	—
6	97.2	5.84 (1H, d)
7	157.7	—
8	96.3	5.93 (1H, d)
8a	158.6	—
1′	132.5	—
2′	118.0	7.29 (1H, d)
3′	147.3	—
4′	150.1	—
5′	119.2	6.84 (1H, d)
6′	124.8	6.97 (1H, d)
Glucose		
1	101.7	5.34 (1H, d, J = 3.7 Hz)
2	74.3	3.57 (1H, dd, J = 3.7, 9.8 Hz)
3	75.2[a]	3.45 (1H, t)[b]
4	72.0[a]	3.88 (1H, t)[b]
5	75.7[a]	3.31 (1H, dd)[b]
6	63.0	3.76 (2H, dd)

Chemical shifts are indicated in ppm downfield from internal TMS, as measured in CD_3OD.

[a,b] These assignments might be interchangeable.

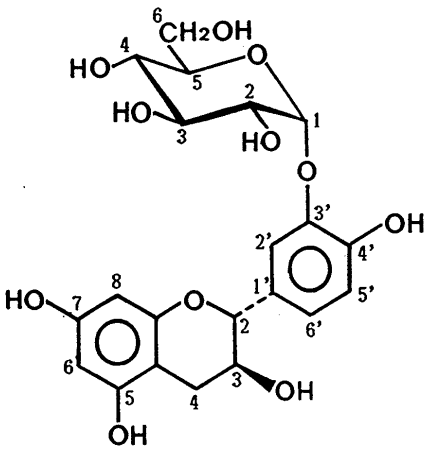

Fig. 3. Structure of (+)Catechin 3'-*O*-α-D-glucopyranoside.

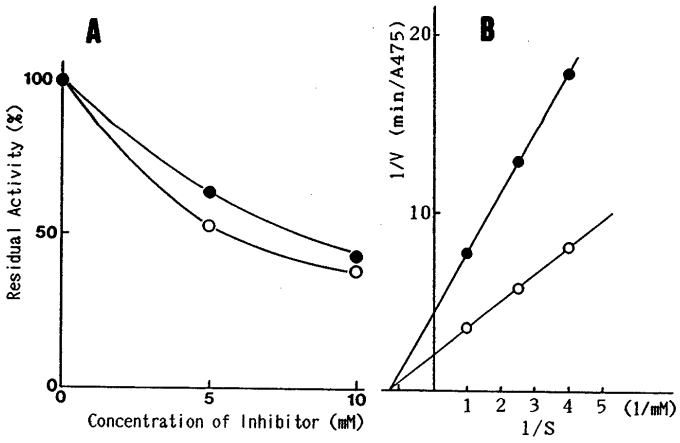

Fig. 4. Effects of (+)Catechin Glucoside and Arbutin on the Activity of Tyrosinase.

A. Tyrosinase from mushroom was used; ○, inhibition by (+)catechin glucoside; ●, inhibition by arbutin. B. Lineweaver–Burk plots of tyrosinase activity with (●) or without (○) (+)catechin glucoside (10 mM). The conditions were the same as those used in the standard enzyme assay described in Materials and Methods.

Inhibition by (+)catechin glucoside of tyrosinase activity

The (+)catechin glucoside inhibited the tyrosinase from mushroom almost as strongly as arbution (hydroquinone-*O*-β-D-glucopyranoside) did as shown in Fig. 4A. The IC_{50} value of (+)catechin glucoside for tyrosinase from mushroom was 5.8 mM, that of arbutin being 8.4 mM. The inhibitory mechanism of the (+)catechin glucoside was noncompetitive as shown in Fig. 4B, which is different from the competitive inhibition by arbutin.[5] However, the (+)catechin glucoside didn't inhibit the tyrosinase activity in a cell-free extract from mouse melanoma, while arbutin did inhibit this activity (Table II).

Table II. Comparison of the Inhibition by (+) Catechin Glucoside and Arbutin of the Activity of Tyrosinases.

Procedures for measuring the inhibition of tyrosinase activity are described in Materials and Methods.

The data shows the concentration which inhibited 50% of tyrosinase activity.

Tyrosinase	(+) Catechin glucoside (mM)	Arbutin (mM)
Mushroom	5.8	8.4
B16 mouse melanoma	>10	4.8

Discussion

Various kinds of oligoglucosides have been synthesized with glycosidases or glycosyl transfer enzymes.[6] However, there are few examples of polyphenols working as the acceptor of such enzymic reactions. An example of galactose being transferred to hydroxybenzen derivatives with galactanase (EC 3.2.1.89) has been reported.[7] We have revealed that glucose or oligoglucan was transferred to (+)catechin with CGTase to produce (+)catechin-α-glucoside. The yield of the purified glucoside (compound E) from 2.0 g of (+)catechin was 38 mg (1.2 mol/mol%). HPLC analysis (peak area) of the enzymatic reaction mixture suggested that 4.3% of (+)catechin was converted to compound E in the reaction mixture.

In this study, only (+)catechin 3'-glucoside was isolated, but a series of (+)catechin glucosides were synthesized with CGTase in the reaction mixture. These other reaction products, which were detected by TLC and HPLC, seem to have been (+)catechin glucosides including various oligoglucosides, because they showed UV-absorption to indicate the presence of (+)catechin in the molecular structure and were also produced in the presence of starch. Some of these other products were converted to (+)catechin glucoside (compound E) by the action of glucoamylase (data not shown). Therefore, the overall transfer ratio from (+)catechin to its glycosides was 18.3% on the basis of the HPLC analysis.

(+)Catechin glucoside inhibited the activity of the tyrosinase from mushroom, but no inhibitory effect on the tyrosinase activity in a cell-free extract from mouse melanoma was observed. On the other hand, arbutin inhibited the tyrosinase activity from both origins (Table II). These inhibitors had different characteristics, the inhibitory mechanism of (+)catechin glucoside against the activity of tyrosinase from mushroom being noncompetitive, while that of arbutin was competitive.[5] These results show that the tyrosinase from mushroom can not always be used as a model enzyme to obtain the inhibitor of tyrosinase from mammalian origins.

Although some naturally occurring (+)catechin glucosides have been reported,[8,9] they all had the β-configuration of glucose. The synthesis of these polyphenol glycosides with an organic chemical method is not easy because it results in a mixture of glycosides with α- and β-configuration. Our present investigation suggests that various kinds of polyphenol-α-glucoside could be enzymatically synthesized with comparative ease.

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