Enzymatic Sulfation of Isoamyl Gallate and (−)-Epigallocatechin Gallate by Bacterial Arylsulfotransferase

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A novel type of arylsulfotransferase which was obtained from a human intestinal bacterium catalyzes the sulfation of polyphenols related to tannins. Isoamyl gallate and (−)-epigallocatechin gallate were found to be sulfated rapidly using *p*-nitrophenylsulfate as a donor substrate. In the case of isoamyl gallate, two sulfated products, 3-monosulfate and 4-monosulfate, were isolated when an equimolar amount of *p*-nitrophenylsulfate was incubated with isoamyl gallate. In the case of (−)-epigallocatechin gallate, 4′-monosulfate was isolated at an equimolar incubation of donor and acceptor. Thus, arylsulfotransferase was useful for the convenient preparation of sulfate esters of these polyphenols.

Keywords arylsulfotransferase; gallate ester; tannin; intestinal bacteria

Introduction

Arylsulfotransferase (EC 2.8.2.1), which is widely distributed in human organs, such as the liver, kidney, brain, lung and intestinal epitherial cells,[1−3] plays an important role in sulfate conjugation. This enzyme catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to aliphatic hydroxyl and phenolic compounds. Several studies have been reported on the sulfation of flavones and tannins. For instance, quercetin was well sulfated by plant flavonoid sulfotransferase,[4,5] and various sulfated flavones were found in the plant kingdom.[6]

We discovered a novel type of arylsulfotransferase from *Eubacterium* A-44, which is a dominant bacterium in the human intestine.[7] The bacterial enzyme catalyzes the transfer of sulfate groups from phenol sulfate esters, but not from PAPS, to other phenolic compounds with strict specificity. Therefore, human intestinal bacteria may play an important role in the sulfate conjugation of flavones and tannins ingested as components of vegetable food. We had already reported on the enzymatic sulfation of flavones[8] and tannins[9] by bacterial sulfotransferase, and showed that (+)-catechin-4′-monosulfate and -5,4′-disulfate were produced by sulfation of (+)-catechin. Alkyl gallate and (−)-epigallocatechin gallate (EGCG) were well sulfated, but the positions of the sulfated hydroxyl groups in their molecules were not determined. Thus, it is indispensable to the investigation of the substrate specificity of the enzyme that the relationship between the position of the sulfated hydroxyl groups and the activity of the acceptors is clarified. Especially, EGCG is a main constituent of Japanese green tea, and therefore it would be interesting to investigate the effect of intestinal bacteria on the metabolism of EGCG.

The present paper describes both the isolation of enzymatically sulfated isoamyl gallate (IG) and EGCG by bacterial sulfotransferase and the elucidation of their chemical structures. It also discusses the substrate specificity of the enzyme through the position of sulfated hydroxyl groups in their molecules.

Materials and Methods

Chemicals *p*-Nitrophenyl sulfate (PNS) was purchased from Sigma Chemical Co. (U.S.A.). Diethylaminoethyl (DEAE)-cellulose was from Brown Co. (U.S.A.) and hydroxyapatite was from Seikagaku Corporation (Japan). General anaerobic medium (GAM) broth was from Nissui Seiyaku Co., Ltd. (Japan). All other chemicals were of analytical reagent grade.

Partial Purification of Arylsulfotransferase Arylsulfotransferase was partially purified under the same procedure as described in our previous report.[9] After DEAE-cellulose column chromatography, the active fractions were collected and treated with ammonium sulfate (70% saturation). The resulting precipitate was dissolved in 10 mM of a sodium phosphate buffer (pH 6.0) and was dialyzed against the same buffer. The inner dialysate was applied to a column of hydroxyapatite, and the column was washed with 10 mM of a sodium phosphate buffer (pH 6.0) and then eluted with a linear gradient between 10—300 mM sodium phosphate buffer (pH 6.0). The resulting enzyme solution (11.2 units/mg protein) was used for the sulfation of tannins. The assay method and the definition of the enzyme activity were described in our previous reports.[7,10]

Enzymatic Sulfation of EGCG and IG Tannins were dissolved in 0.1 M Tris–HCl buffer (pH 8.0). In the case of the sulfation of EGCG, a reaction mixture contained 17 ml of 4 mM EGCG, 1.4 ml of 50 mM PNS, 4.8 ml of 25 mM ascorbic acid, 4.8 ml of 1 mM ethylenediaminetetraacetic acid (EDTA), 70 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 5 ml of arylsulfotransferase (10.0 units/ml). The mixture was incubated for 5 h at 37 °C. Enzymatic sulfation of IG was carried out under the same components and conditions as described above, except that 20 mM IG and 6.0 ml of 50 mM PNS were added into the reaction mixture and the incubation period of time was 8 h. The enzyme solution was added to the reaction mixture every two hours.

Isolation of Sulfated Tannins The reaction mixture was lyophilized and the resulting residue was applied to a reverse-phase high performance liquid chromatography (HPLC, CHEMCOSORB 7-ODS-H, column size 4.6 × 150 mm). The column was eluted with 10% MeOH and 30% MeOH for the products of EGCG and IG, respectively. Each product was collected by monitoring absorbance at 280 nm.

Structural Determination of Sulfated Products of EGCG and IG Nuclear magnetic resonance (NMR) spectra were measured on a Varian VXR 500 spectrometer (500 MHz for ^{1}H and 126 MHz for ^{13}C) in MeOH-d_4 + D_2O solution. The chemical shifts are given in δ (ppm) values relative to the solvent (MeOH-d_4, δ_H 3.34 ppm for ^{1}H and δ_C 48.0 ppm for ^{13}C) in tetramethylsilane scale. The assignments of IG sulfates and EGCG sulfate were based on the comparisons with those of IG and EGCG, respectively, which were confirmed by ^{1}H- and ^{13}C-NMR spectra. Fast atom bombardment mass spectrum (FAB-MS) was recorded on a VG-70 SE mass spectrometer using glycerol as a matrix agent.

Analysis of the Sulfated Products by Cellulose Thin Layer Chromatography (TLC) Electrophoresis Electrophoresis was carried out under the same condition as described in our previous report.[9] The sulfated products were spotted on a cellulose TLC plate and subjected to high voltage electrophoresis for 60 min in a HCOOH : AcOH : H_2O buffer (33 : 147 : 1820, v/v/v), pH 1.9 at 1000 V, and detected under an ultraviolet (UV) lamp (365 nm).

Results

Our previous study showed that (+)-catechin-4′-monosulfate and -5,4′-disulfate were produced in the

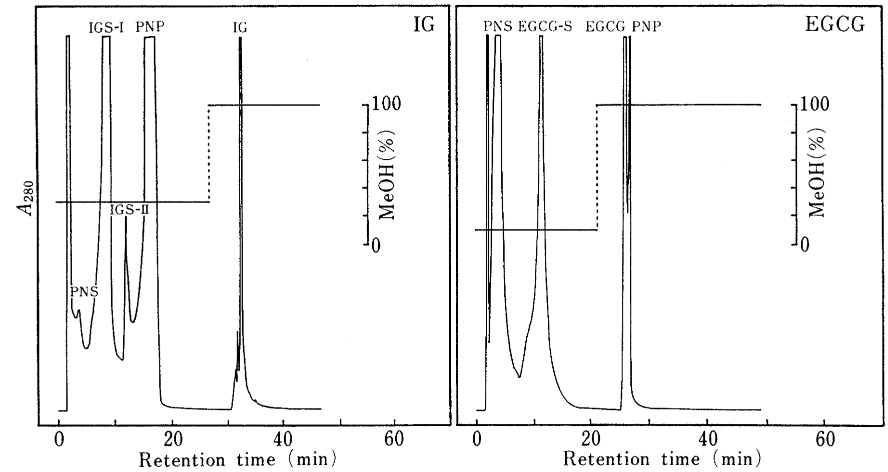


Fig. 1. Isolation of IG-O-Sulfate and EGCG-O-Sulfate by Reverse-Phase HPLC

Conditions of analysis and elution of the reaction products were described in Materials and Methods.

enzymatic sulfation of (+)-catechin by bacterial sulfotransferase.[9] We also reported that while gallic acid was not sulfated, alkyl gallates were effectively sulfated, and the degree of sulfation activity increased in proportion to the length of alkyl group. EGCG was a slightly poor substrate as compared with (+)-catechin, (−)-epicatechin and (−)-epicatechin gallate. Thus, the sulfation activity varied depending on each substrate's structure and the position of hydroxyl groups. Using IG and EGCG as examples of polyphenolic compounds related to tannin, we attempted to isolate sulfated products and to identify their chemical structures.

The enzymatic sulfation of IG reached a plateau in 8 h by monitoring the absorbance at 405 nm when equivalent molar amounts of PNS and IG were incubated. Two products were detected in the reaction mixture by reverse-phase HPLC. The products were designated as IGS-I, with a retention time of 8.5 min, and IGS-II, with a retention time of 12.1 min (Fig. 1). Even if excess amounts of PNS over IG were incubated, only IGS-I and IGS-II were produced, but no other products were formed. When EGCG was enzymatically sulfated with an equivalent molar of PNS to EGCG, the reaction reached a plateau after 5 h of incubation, and only one product was detected by monitoring the absorbance at 280 nm. The product was designated as EGCG-S, and its retention time was 11.3 min (Fig. 1).

Electrophoretic experiments would be important to structural elucidation because sulfation causes a change in the polarity of the compound. The sulfated tannins have higher mobility to anode than the corresponding tannins. IGS-I and IGS-II were more mobile to anode than EGCG-S, though IG and EGCG were not mobile. The mobilities of IGS-I, IGS-II and EGCG-S were 5.0, 5.5 and 2.0 cm to anode, respectively, under the same conditions. Therefore, it was suggested that IGS-I and IGS-II contained the same number of sulfate groups.

NMR The IG sulfates showed an $(M-H)^-$ ion peak at m/z 319, corresponding to the molecular formula $C_{12}H_{16}O_8S$, in negative ion FAB-MS. The structures of these sulfates were determined based on the ^{1}H- and ^{13}C-NMR analyses as follows. The ^{1}H-NMR spectrum of IGS-I showed two unequivalent aromatic proton signals

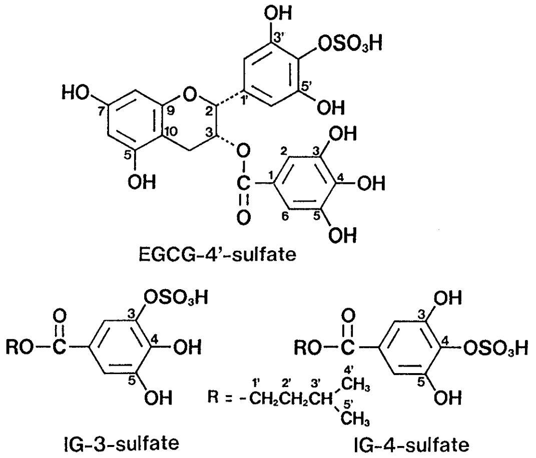


Fig. 2. Structures of Sulfated IG and EGCG

with *meta*-coupling (δ: 7.33 and 7.66, each 1H, d, $J=2$ Hz), in contrast to a singlet (δ: 7.08, s, 2H) due to the galloyl protons of IG, clearly indicating that the sulfation took place at the O-3 hydroxyl group. The nonequivalent nature of the aromatic carbons in IGS-I was also disclosed by the ^{13}C-NMR spectrum, which exhibits six sp^2 carbon resonances at δ: 113.6, 116.1, 120.9, 140.4, 143.0 and 146.6, along with the signal of an ester carbonyl carbon (δ: 167.0) and those due to the isoamyl residue [21.9 (C-4′, C-5′), 25.4 (C-3′), 37.6 (C-2′) and 63.5 (C-1′)]. The signals at 113.6 and 116.1, attributable to C-2 and C-6, shifted lower than the signal (2C, δ: 108.9) of the equivalent C-2 and C-6 in IG. The signal at 143.0, due to the C-4 signal in IGS-I, also showed a significant downfield shift from that (δ: 138.7) of IG, while the C-3 signal was shifted to an upper field (δ 145.4 in IG→140.4 in IGS-I). These shifts are consistent with our previous findings[9] that upon sulfation of a phenolic hydroxyl group, the sulfated carbon is shifted by *ca.* 5 ppm toward the upper field, and the *ortho* and *para* carbons show a marked downfield shift ($\Delta\delta$: 4—7 ppm), while the *meta* carbon is almost unaffected. This data showed that IGS-I was characterized as a 3-O-sulfate of IG (Fig. 2).

The ^{1}H-NMR spectrum of IGS-II [δ: 1.02 (6H, d, $J=7$ Hz), 1.68 (2H, q, $J=7$ Hz), 1.82 (1H, tq, $J=7$ Hz), 4.33 (2H, t, $J=7$ Hz), 7.08 (2H, s)] was almost superimposable on that of IG. On the other hand, comparison of the ^{13}C-NMR spectrum of IGS-II with that of IG revealed a remarkable upfield shift of the signal attributable to C-4 (δ: 138.7→128.1), and downfield shifts of C-3, C-5 and C-1 [δ: 145.4 (2C)→151.2 (2C) and 120.7→126.1]. The other signals were essentially the same as those of IG. Based on these data, IGS-II was characterized as the 4-O-sulfate of IG (Fig. 2).

TABLE I. ^{1}H- and ^{13}C-NMR Data for EGCG and EGCG-S [J (Hz) in Parentheses]

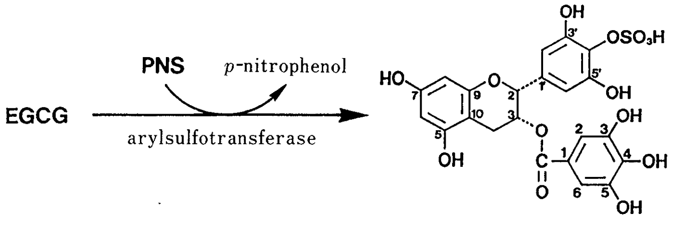
Position	EGCG[a]		EGCG-S	
	δ_H	δ_C	δ_H	δ_C
EGC-part				
2	5.00 br s	77.5	5.07 br s	77.3
3	5.56 m	68.9	5.57 m	68.8
4	2.88 dd (2, 17) 3.02 dd (4.5, 17)	25.7	2.90 dd (2.5, 17) 3.02 dd (4.5, 17)	25.8
5		156.7		156.8
6	6.00 s	95.4[b]	5.99[b] d (2)	95.6[b]
7		156.7		156.8
8	6.00 s	94.8[b]	6.02[b] d (2)	94.9[b]
9		156.1		156.0
10		98.4		98.4
1′		129.7		137.0
2′	6.55 s	105.8	6.65 s	107.0
3′		145.6		150.7
4′		132.7		128.4
5′		145.6		150.7
6′	6.55 s	105.8	6.65 s	107.0
Galloyl-part				
1		120.4		120.4
2	6.99 s	109.2	6.99 s	109.3
3		145.2		145.3
4		138.7		138.9
5		145.2		145.3
6	6.99 s	109.2	6.99 s	109.3
CO		166.6		166.7

a) Measured in MeOH-d_4. Signal assignments were confirmed by ^{1}H–^{13}C long-range shift correlation spectroscopy. b) The assignments in each column may be interchangeable.

The ^{1}H-NMR spectrum of EGCG-S was closely similar to that of EGCG (Table I). Unlike the 2H singlet (δ: 6.00) of EGCG, the H-6 and H-8 signals of EGCG-S appeared at δ: 5.99 and 6.02 as doublets ($J=2$ Hz). However, the chemical shift difference of these signals between EGCG-S and EGCG was negligible, suggesting that the A-ring is unaffected on sulfation.[9] The signal patterns of the B-ring protons (2H, s) and the galloyl protons (2H, s) in EGCG-S are analogous to those of IGS-I, but not to those of IGS-II. The sulfation in EGCG is therefore considered to have taken place at O-4′ of the B-ring or O-4 of the galloyl group. The ^{13}C-NMR signals of EGCG-S are almost superimposable on those of EGCG, except for three signals at δ: 128.4, 137.0 and 150.7 (2C), attributable to the B-ring carbons (Fig. 2 and Table I). This analogy indicates that the sulfation in EGCG-S took place at the 4′-hydroxyl group of B-ring, but not at the galloyl moiety. Taking the shifts of the sulfated carbon, and *ortho* and *para* carbon signals upon sulfation into consideration,[9] the signal at δ: 128.4 is reasonably assigned to C-4′, which is shifted upfield by 4.3 ppm compared to that of EGCG. Similarly, the other two signals (δ: 137.0 and 150.7) were assigned to C-1′, and C-3′ (C-5′), which are shifted downfield by 5.1 and 7.3 ppm from those of EGCG.

Discussion

Human intestinal flora play an important role in the metabolism of polyphenols related to tannins because there is every possibility that tannins are ingested as components of vegetable food. Especially, it is known that green tea contains several tannins, among which EGCG, accompa-



EGCG-4′-sulfate

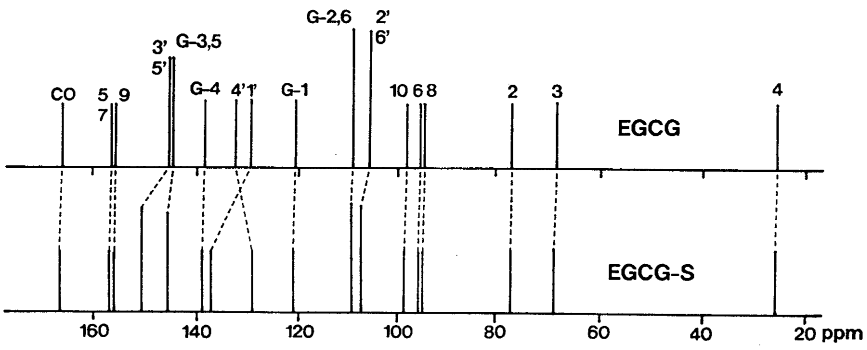


Fig. 3. ^{13}C-NMR Spectral Comparison of EGCG and EGCG-S
G: galloyl.

nied by (−)-epicatechin and (−)-epicatechin gallate, is the main phenolic compound in the infusion.[11)] Furthermore, Fujita *et al.*[12)] reported that EGCG involves an inhibitory effect on carcinogenesis.

Intestinal bacterial sulfotransferase effectively sulfated polyphenols, such as (+)-catechin, (−)-epicatechin, EGCG, and so on, but gallic acid was barely sulfated because the bacterial sulfotransferase was negatively influenced by free carboxyl groups. Previous studies[9,10)] suggested that the sulfation was associated with the position of the hydroxyl groups of polyphenols. In the present study, IG, in which the carboxyl group is masked with the alkyl group, was used for determining the position of the sulfation of galloyl groups. The sulfation took place at the 3- or 4-hydroxyl group of IG. However, IG-disulfate was not produced under the present conditions. This phenomenon is similar to the enzymatic sulfation of dopamine, which is one of the neurotransmitters and contains a catechol moiety, since one of the hydroxyl groups at the 3- and 4-positions in dopamine was sulfated.[13)] It was observed that the sulfate group of dopamine-3-sulfate was transferred to the 4-hydroxyl group of dopamine by the enzyme, but the transfer of the 3-sulfate group in IGS-I to the 4-hydroxyl group of IG was not detected under the present conditions. Based on the results from HPLC, a twelve-fold production of IG-3-sulfate over IG-4-sulfate was confirmed, so that the 3-hydroxyl group of IG is regarded as more susceptible to enzymatic sulfation than its 4-hydroxyl group. The 4′-hydroxyl group in EGCG alone was sulfated, and di- and trisulfate were not produced. Although EGCG contains two trihydroxyphenyl moieties, the trihydroxyphenyl moiety in the galloyl group at the 3-position of EGCG was not sulfated. The hydroxyl group at the 5-position of EGCG was not sulfated, either, despite the fact that the 5-hydroxyl group of (+)-catechin was sulfated and the sulfation took place after the sulfation at the 4′-hydroxyl group.[10)] These results suggested that ring-B is more susceptible to sulfation than ring-A.

Bacterial arylsulfotransferase is useful for the specific sulfation of tannins and would play an important role in terms of the metabolism of phenolic compounds in food.

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