Enzymatic Sulfation of Quercetin by Arylsulfotransferase from a Human Intestinal Bacterium

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A novel type of arylsulfotransferase was partially purified from human intestinal bacteria and its enzymatic properties were examined. Polyphenols such as chalcone, xanthone and flavonoid were found to be sulfated by the bacterial arylsulfotransferase though the sulfation activity varied depending upon the positions of the hydroxyl groups. Quercetin, as an example of a flavonol, was rapidly sulfated when *p*-nitrophenyl sulfate (PNS) was taken as a donor substrate. At a ten-fold molar excess of PNS over quercetin, two products, the 3,3′-disulfate and 3,3′,7-trisulfate derivatives, were formed, but the 4′- and 5-hydroxyl groups were not sulfated. In the case of equimolar or two-fold molar excess of PNS to quercetin, only the 3,3′-disulfate was produced and no monosulfate was formed. The enzymatic procedure is useful as a specific and convenient method for the preparation of polyphenol sulfate esters.

Keywords arylsulfotransferase; quercetin; enzymatic sulfation; sulfate conjugation; flavonoid; intestinal bacteria

Sulfate conjugation and glucuronide conjugation are widely known as major metabolic pathways for the detoxification of endogenous and exogenous phenolic compounds. Such conjugations make phenolic compounds water-soluble and generally result in loss of their biological activity and toxicity. Arylsulfotransferase (EC 2.8.2.1) was first discovered in guinea pig liver,[1] and thereafter the enzyme was found widely in brain, kidney, intestinal epithelial cells and so on.[2-4] The enzyme catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to various alcohol groups.

A similar type of arylsulfotransferase was also found in the plant kingdom, such as in *Flaveria chloraefolia* and *Flaveria bidentis*.[5] The enzyme also transfers the sulfate of PAPS to flavonoids, forming various flavonoid sulfates. Flavonoid sulfates have been reported to be widely distributed in a number of plant families, especially in the Compositae and in such genera as *Brickellia* and *Flaveria*.

We discovered a novel type of sulfotransferase from a human intestinal bacterium, *Eubacterium* A-44.[6] This enzyme catalyzes transfer of the sulfate group from phenol sulfate esters, but not from PAPS, to other phenols with strict specificity. The bacterial enzyme is several thousand-fold more active than the enzymes of mammalian and plant origins, and is expected to be useful for the preparation of sulfated phenols.

This enzyme sulfated polyphenols, such as chalcones, xanthones and flavones,[7] but the enzyme activity varied depending upon the positions of their hydroxyl groups. The present paper describes the positions of sulfated hydroxyl groups using quercetin as a typical example.

Materials and Methods

Chemicals *p*-Nitrophenyl sulfate (PNS) and 4-methylumbelliferyl sulfate (MUS) were purchased from Sigma Chemicals, Co. (U.S.A.). Tyramine was purchased from Nacalai Tesque and diethylaminoethyl-cellulose (DEAE-cellulose) was from Brown Co. (U.S.A.). General anaerobic medium (GAM) broth was from Nissui Seiyaku Co., Ltd. (Japan). All other chemicals were of analytical reagent grade.

Partial Purification of Arylsulfotransferase *Eubacterium* A-44, isolated from human feces, was cultured under the same procedure as described in our previous report,[6] except that 1 mM PNS was contained in the culture medium as an inducer in place of phenolphthalein disulfate.[7] The crude extract of the enzyme was prepared according to the method described in our previous report[8] and did not contain any arylsulfatase activity using PNS or MUS as a substrate.

The extract was applied to a column of DEAE-cellulose (3.5 × 20 cm) previously equilibrated with 0.1 M acetate buffer (pH 5.5). The column was washed with 500 ml of the same buffer containing 0.14 M KCl and eluted with 500 ml of the buffer containing 0.25 M KCl. The active fractions were collected, pooled and then dialyzed against the same buffer for 15 h with one change of the outer dialysate. The resulting enzyme solution (3.5 unit/mg protein) was used for the sulfation of quercetin. The assay method and the definition of the enzyme activity were described in our previous reports.[6,8]

Enzymatic Preparation of Sulfated Quercetin Quercetin, as an acceptor, was dissolved in 70% dimethylsulfoxide (DMSO). A reaction mixture contained 20 ml of 5 mM quercetin, 20 ml of 50 mM PNS, 10 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 6 ml of the enzyme (21 unit). The mixture was incubated with shaking for 20 h at 37 °C. DMSO at the final concentration (11.6%) decreased the enzyme activity by 20—30%.

The reaction mixture was lyophilized and the resulting residue was dissolved in an appropriate volume of water, and filtered through glass wool (GRW-03 Nippon Rikagaku Kikai). The filtrate was subjected to preparative paper partition chromatography (PPC) (Toyo No. 51B) and developed with *n*-BuOH–AcOH–H_2O (4:1:2 v/v/v). After drying the paper, each spot was detected under a UV lamp (365 nm) in a fluorescence analysis cabinet (Model C-4, Spectronics Corporation). The bands corresponding to quercetin-*O*-sulfate were cut out and extracted separately with water overnight. Each extract was filtered through the glass wool and lyophilized to dryness.

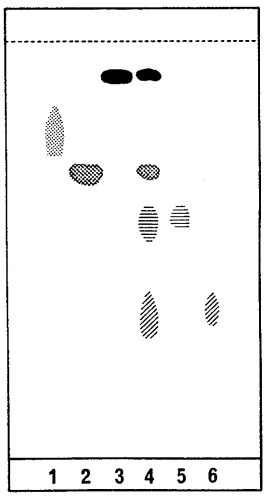
Structural Determination of Quercetin-*O*-sulfate The ultraviolet (UV) spectra of flavonoids, in general, have two major absorption peaks which are commonly referred to as band I (usually 300—380 nm) and band II (usually 240—280 nm). It is possible to correlate the shifts of each band obtained by the addition of spectral shift reagents[9,10] in the UV spectra with the flavonoid sulfation pattern.

Analysis of the Reaction Products by Paper Electrophoresis The reaction products were spotted on the center of a filter paper (51B, Advantec Toyo, 60 × 60 cm) and electrophoresis was carried out for 1 h in HCOOH–AcOH–H_2O buffer (33:147:1820), pH 1.9 at 1.5 kV (Toyo HPE-406, Toyo solid-state power supply model PS-2510). After drying the paper, spots were detected under a UV lamp (365 nm) and by spraying with 1 N NaOH, and then their mobilities were determined.

Results

Bacterial arylsulfotransferase activity was measured using chalcones, xanthones and flavones as acceptors and PNS as a donor of sulfate.[7] The sulfation activity was markedly variable, depending upon the positions of hydroxyl groups of these polyphenols, and the sulfated positions were not identified. Therefore, quercetin was used as an example and enzymatically sulfated to identify the reaction products.

It was difficult to determine spectrometrically *p*-nitrophenol (PNP) produced on the sulfation of quercetin in the case of PNS as a donor substrate, because quercetin is yellow-colored in the solution, as well as PNP, one of the



lane	*Rf* value
1. quercetin	0.83
2. *p*-nitrophenyl sulfate	0.68
3. *p*-nitrophenol	0.92
4. reaction mixture	—
5. Y_1	0.60
6. Y_2	0.39

Fig. 1. Paper Partition Chromatogram of Sulfated Quercetin

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

TABLE I. UV Absorption Maxima of Sulfated Quercetins

Shift reagents	λ_{max}			
	Y_1		Y_2	
	I	II (sh)	I	II (sh)
None	372	251 (261)	370	250 (270)
NaOMe	435↑	278	450↑	270
$AlCl_3$	443	273	443	272
$AlCl_3$/HCl	427	263	422	262
AcONa	382	275	383	250 (270)
AcONa/H_3BO_3	370	265	366	250 (265)

I, band I; II, band II; sh, shoulder. ↑: remarkable increase in intensity. Each compound was dissolved in MeOH.

products. Therefore, MUS was used as a donor for the sulfation of quercetin and 4-methylumbelliferone produced by the sulfate transfer reaction was fluorometrically (ex. 330 nm, em. 450 nm) determined. The reaction reached a plateau after 2 h incubation, when two or three hydroxyl groups were sulfated among the five hydroxyl groups of quercetin (data not shown).

A ten-fold molar excess of PNS over quercetin was incubated in the reaction mixture for 20 h at 37 °C for the preparation of sulfated quercetin. The reaction products were sufficiently concentrated to dryness *in vacuo* and analyzed by PPC. Two yellowish and UV-absorbing spots were detected as products with low mobility in *n*-BuOH–AcOH–H_2O compared with other known compounds (lane 4, in Fig. 1). They were designated as Y_1 (yellow spot 1) of higher *Rf* value and Y_2 (yellow spot 2) of lower *Rf* value. Donor PNS decreased, acceptor quercetin disappeared and PNP from PNS was clearly formed. Based on these results, it seems that Y_1 and Y_2 are sulfated quercetins. A mixture of Y_1 and Y_2 was prepared on a larger scale and subjected to preparative PPC in the same solvent system. The two bands were cut out and extracted with water. Each extract was concentrated to dryness. The products were analyzed by PPC and it was confirmed that they were well separated (lanes 5 and 6 in Fig. 1). Furthermore, Y_1 and Y_2 were positive in the Lassaigne test, which is a qualitative test of

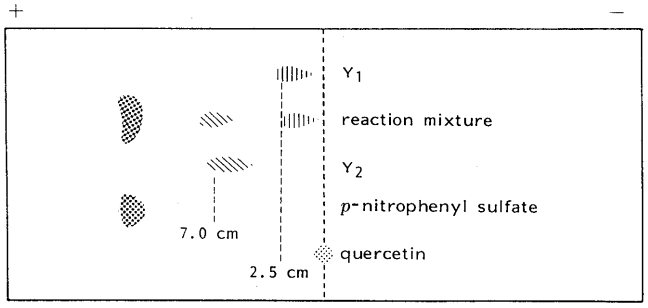


Fig. 2. Electrophoregram of Sulfated Quercetins

1.5 kV, 1 h, pH 1.9. Solvent system, HCOOH–AcOH–H_2O (33 : 147 : 1820 v/v/v).

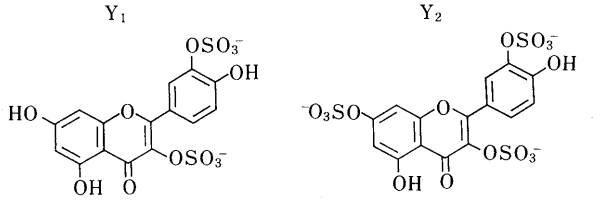


Fig. 3. Structures of Sulfated Quercetin

sulfur, suggesting that Y_1 and Y_2 were sulfated quercetins. Both compounds had two absorption maxima characteristic of flavonoids in their UV spectra, and were positive to the Mg–HCl reaction. Therefore, it was proved that Y_1 and Y_2 retained the flavonoid skeleton. The positions of the sulfate groups of quercetin were further investigated in terms of the shifts of UV absorption maxima with several shift reagents (Table I).

The addition of NaOMe caused a bathochromic shift of band I in the UV spectra of Y_1 and Y_2 with increased intensity, respectively, indicating that the hydroxyl group at the 4′-position in Y_1 and Y_2 is free, and the 3-hydroxyl group is not free. $AlCl_3$ also caused a bathochromic shift of band I in both compounds, indicating that the 5-hydroxyl group is free. Further addition of HCl reagent did not cause above 60 nm bathochromic shift of band I in either of the compounds, suggesting the presence of sulfate at position 3. This result was in accordance with the finding in NaOMe. The addition of AcONa caused a bathochromic shift of band II in Y_1 but not in Y_2, so that the hydroxyl group at the 7-position of Y_1 was free and that of Y_2 was sulfated. Further addition of H_3BO_3 did not cause a bathochromic shift of band I in Y_1 and Y_2, indicating that both or one of 3′- and 4′-hydroxyl groups are not free. Because of the presence of the 4′-hydroxyl group of Y_1 and Y_2, it was concluded that the 3′-hydroxyl group in Y_1 and Y_2 was not free. Based on these findings, it seemed that the 3- and 3′-positions of Y_1 were sulfated and the 3-, 3′- and 7-positions of Y_2 were sulfated. Additionally, this conclusion is in accordance with the observation that two or three among the five hydroxyl groups of quercetin were sulfated as determined by fluorometric analysis using MUS as a donor substrate.

The number of sulfate groups on quercetin was also determined by an electrophoretic experiment, because electrophoretic mobility is important in the structural elucidation of sulfated flavonoids. As shown in Fig. 2, Y_2 was

more mobile on electrophoretograms than Y_1 but quercetin was not mobile. The mobilities of Y_1 and Y_2 were 2.5 and 7.0 cm, respectively, under the present conditions.

In conclusion, when quercetin was enzymatically sulfated with excess amounts of PNS, only di- and trisulfate esters, 3,3′-disulfate and 3,3′,7-trisulfate, respectively, were produced (Fig. 3). However, in the case of enzymatic sulfation with equimolar or a two-fold excess of PNS, only the 3,3′-disulfate ester was produced with no monosulfate (data not shown).

Discussion

The flavonoid sulfates have been reported to occur in a number of plant families.[10] 3-Monosulfate of quercetin was found in *Oenanthe crocata*[11] and its 3,7- and 3,4′-disulfates were first reported in *Flaveria bidentis*[12,13] and the 3,3′-disulfate in *Flaveria chloraefolia*.[14] A trisulfate of quercetin, the 3,7,3′-trisulfate, was found in *Flaveria bidentis*.[15] Flavonoid mono- to tetrasulfates occur widely in plants. Recently, Varin *et al.*[16,17] found flavonoid sulfotransferase in *Flaveria chloraefolia* and *Flaveria bidentis*, and succeeded in enzymatically sulfating flavonoids, *e.g.* quercetin, kaempferol, isorhamnetin and so on using PAPS as a donor substrate. Mono- to tetrasulfates of quercetin were produced, sulfated at the 3-, 3,3′-, 3,7,4′- and 3,7,3′,4′-positions. Their results are not in accordance with ours, probably due to the difference of enzyme sources, bacteria and plant, and of substrate specificity.

Sulfate conjugation in animal tissues represents one of the major detoxication mechanisms of foreign phenolic compounds, and it could similarly be argued that sulfation of flavonoids in plant tissues represents a mode of inactivation of harmful products. Actually, sulfation provides water-solubility to otherwise insoluble compounds. Sulfation of hydrophobic substances might be considered as an excretory mechanism, as in the case of glycosylation in plants and animals.

Quercetin orally administered may be enzymatically sulfated in the intestinal bacteria, becoming more water-soluble and less absorbable.

On the other hand, it is known that some flavones, such as quercetin, exhibit mutagenicity *in vitro*,[18] though they are not carcinogenic *in vivo*. Since arylsulfotransferase from human intestinal flora easily sulfates flavones *in vitro*, it is considered that orally administered flavones were sulfated and detoxified in the intestinal tract *in vivo*.

References

1) P. K. Banerjee and A. B. Roy, *Mol. Pharmacol.*, **2**, 55 (1966).
2) W. B. Jakoby, R. D. Sekura, E. S. Lyon, C. J. Marcus, and J. L. Wang, "Enzymatic Basis of Detoxication," ed. by W. B. Jakoby, Academic Press, New York, 1980, pp. 199—228.
3) A. B. Roy, "Sulfation of Drugs and Related Compounds," ed. by G. J. Mulder, CRC Press, Boca Raton, Florida, 1981, pp. 88—130.
4) R. D. Sekura, M. W. Duffel, and W. B. Jakoby, "Methods in Enzymology," Vol. 77, ed. by W. B. Jakoby, Academic Press, New York, 1981, pp. 197—206.
5) L. Varin, D. Barron, and R. K. Ibrahim, *Anal. Biochem.*, **161**, 176 (1987).
6) K. Kobashi, Y. Fukaya, D.-H. Kim, T. Akao, and S. Takebe, *Arch. Biochem. Biophys.*, **245**, 537 (1986).
7) K. Kobashi, D.-H. Kim, and T. Morikawa, *J. Protein. Chem.*, **6**, 237 (1987).
8) D.-H. Kim, L. Konishi, and K. Kobashi, *Biochim. Biophys. Acta*, **872**, 33 (1986).
9) J. B. Harbone, T. J. Mabry, and H. Mabry (eds.), "The Flavonoids," Chapman and Hall, London, 1975, p. 45.
10) J. B. Harbone, *Phytochemistry*, **14**, 1147 (1975).
11) J. B. Harbone and L. King, *Biochem. Syst. Ecol.*, **4**, 111 (1976).
12) L. Varin, D. Barron, and R. K. Ibrahim, *Z. Naturforsch.*, **41C**, 813 (1986).
13) J. L. Cabrera and H. R. Juliani, *Phytochemistry*, **18**, 510 (1979).
14) D. Barron and R. K. Ibrahim, *Phytochemistry*, **26**, 1181 (1987).
15) J. L. Cabrera, H. R. Juliani, and E. G. Gross, *Phytochemistry*, **25**, 1394 (1985).
16) L. Varin, D. Barron, and R. K. Ibrahim, *Anal. Biochem.*, **161**, 176 (1987).
17) L. Varin, D. Barron, and R. K. Ibrahim, *Phytochemistry*, **26**, 135 (1987).
18) M. Nagao, N. Morita, T. Yahagi, M. Shimizu, M. Kuroyanagi, M. Fukuoka, K. Yoshihira, S. Natori, T. Fujino, and T. Sugimura, *Environ. Mutagen.*, **3**, 401 (1986).