Enzymatic Sulfation of Glycosides and Their Corresponding Aglycones by Arylsulfate Sulfotransferase from a Human Intestinal Bacterium

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A novel type of arylsulfate sulfotransferase (ASST) from a predominant human intestinal bacterium catalyzes the stoichiometric transfer of a sulfate group from phenolic sulfate esters to phenols. We clarified that polyphenols were better substrates of this enzyme than the corresponding glycosides. Additionally, a coumarin derivative, esculetin, was sulfated by ASST at the 6-position to give 6-monosulfate. Therefore, ASST is more useful for the preparation of sulfated polyphenols at their specific hydroxyl groups and would play an important role in the metabolism of phenolic compounds in vegetable food and traditional medicines.

Keywords arylsulfate sulfotransferase; esculetin; baicalein; intestinal bacteria; sulfate conjugation

In mammalian tissues, enzymatic sulfation is considered an important reaction in the detoxification of endogenous metabolites and xenobiotics. A number of sulfotransferases (STs) with substrate specificity towards arylamines, phenols, steroids and bile acids, have been investigated.[1-4] An enzyme catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to aliphatic and phenolic hydroxyl groups to form sulfate conjugates. Recently, a similar type of enzyme was also found in the plant, *Flaveria* species.[5,6]

In contrast with the extensive studies of mammalian and plant tissue STs, little information has been reported on enzymatic sulfation in intestinal bacteria. However, arylsulfate sulfotransferase (ASST, E.C. 2.8.2.22) has been characterized from a human intestinal bacterium, *Eubacterium* A-44.[7-9] This enzyme catalyzes the transfer of a sulfate group from phenol sulfate esters, but not from PAPS, to other phenols.

Also, ASST exhibited strict specificity for the position of the hydroxyl groups of polyphenols, such as flavones and tannins.[10-12] These polyphenols, ingested as components of vegetable food and traditional medicines, are usually conjugated in human liver. It is possible that these conjugated sulfates, once excreted *via* the bile, are transferred to other phenolic compounds by intestinal bacterial enzymes. Therefore, ASST may play an important role in the metabolism of sulfate-conjugates of polyphenols.

There have been reported many kinds of naturally occurring glycosides and their aglycones. The present paper describes the enzymatic sulfation of glycosides and related aglycones by ASST, and the position of the sulfated hydroxyl group of esculetin.

MATERIALS AND METHODS

Chemicals 4-Methylumbelliferylsulfate (MUS), *p*-nitrophenylsulfate (PNS), esculin, esculetin, rutin and quercetin were purchased from Sigma Chemical, Co. (U.S.A.). Baicalin and baicalein were from Wako Pure Chemical Industry (Japan). Tyramine was from Nacalai Tesque, Inc. (Japan). All other chemicals were of analytical reagent grade.

Enzyme Preparation and Activity Assay Purification of ASST from *Eubacterium* A-44 was undertaken according to our previous report.[12] An enzyme activity assay was performed using MUS or PNS as a donor and various phenolic test compounds as an acceptor, and the reaction product, 4-methylumbelliferone (MU) or *p*-nitrophenol (PNP), was measured fluorometrically or spectrophotometrically as previously described.[13] Since esculin and esculetin are fluorescent, the activity was then analyzed by HPLC (Chemcosorb 7-ODS-H, column size 4.6 × 150 mm, Chemco, Japan) using PNS as a donor. One unit of enzyme activity was defined as the amount required to catalyze the formation of 1.0 μmol of PNP per min under standard assay conditions.[13] The specific activity of the enzyme was 18.3 units per mg protein in a PNS-tyramine system.

Protein Determination The amount of protein was determined by the method of Lowry *et al.*, using bovine serum albumin as a standard.[14]

Enzymatic Sulfation of Esculetin Esculetin was dissolved in 10% dimethyl sulfoxide (DMSO). A reaction mixture contained 60 ml of 10 mM esculetin, 4 ml of 50 mM PNS, 60 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 26 ml of ASST (162 units). The mixture was incubated for 48 h at 37 °C. The sulfated products were applied to a reverse-phase HPLC (Chemcosorb 7-ODS-H, column size 4.6 × 150 mm, Chemco, Japan). The column was eluted with 10% methanol containing 0.1% trifluoroacetic acid.

Isolation of Sulfated Esculetin The reaction mixture was ultrafiltered by a Diaflo-PM25 (Amicon, U.S.A.) to remove the protein, and lyophilized. The resulting residue was dissolved in 25 ml of water and applied to a Sephadex LH-20 column (2.8 × 77 cm, Pharmacia, Sweden) and eluted with water. Products were collected by monitoring the absorbance at 280 nm and the fluorescence intensity at ex. 365 nm and em. 455 nm.

Structure Determination of Sulfated Esculetin NMR spectra were measured on a JNM-GX 400 spectrometer (JEOL, Japan) in DMSO-d_6 solutions. FAB-MS was

obtained with a JMS-SX 102 spectrometer (JEOL, Japan).

RESULTS

Enzymatic Sulfation of Glycosides and the Corresponding Aglycones Three glycosides from plant origins (Chart 1) and the corresponding aglycones were enzymatically sulfated by ASST (Table I). Rutin and esculin were less effective substrates than tyramine, which is used as an acceptor substrate in the standard assay. However, quercetin and esculetin, which are the related aglycones, were as good acceptors as tyramine. Also, baicalin was sulfated as well as tyramine, and baicalein, which is the aglycone of baicalin, was a markedly good substrate. We already reported the chemical structures of enzymatically sulfated quercetin,[10] showing that ASST catalyzes the position-specific transfer of the sulfate group. Therefore, in the present study we investigated the position of the sulfated hydroxyl group of esculetin.

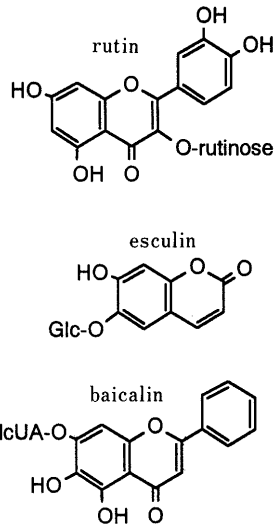

Chart 1. Structure of Rutin, Esculin and Baicalin

Isolation of Sulfated Esculetin The enzymatic sulfation of esculetin reached a plateau in 24 h by monitoring the HPLC pattern when a three-fold molar excess of esculetin over PNS was incubated. After 48 h incubation, 10 μl of the reaction mixture was applied to the HPLC column. PNS was hardly detected and only one product was detected at 17 min, as shown in Fig. 1-B. This sulfated product was isolated by a Sephadex LH-20 column and lyophilized to dryness.

NMR and FAB-MS Prior to analyzing the structures of esculetin sulfate ester (esculetin-S), we measured the ^{13}C-NMR spectrum of esculetin and confirmed the previous assignments of signals (Table II).[15] Also, in the ^{1}H–^{13}C COSY spectrum, 4 proton signals correlated to each carbon peak.

Esculetin-S showed an $(M-H)^-$ ion peak at m/z 257,

TABLE I. Sulfation of Polyphenols

Acceptors	Activity (%)
Tyramine	100[a,b]
Rutin	17.7[a]
Quercetin	234[a]
Esculin	0[b]
Esculetin	87.0[b]
Baicalin	144[a]
Baicalein	3560[a]

Activity for tyramine as an acceptor was taken as 100. *a*) The activities were assayed in a MUS-tyramine system. Final acceptor concentrations were 0.1 mM. *b*) The activities were measured with HPLC using PNS as a donor. Final acceptor concentrations were 2 mM.

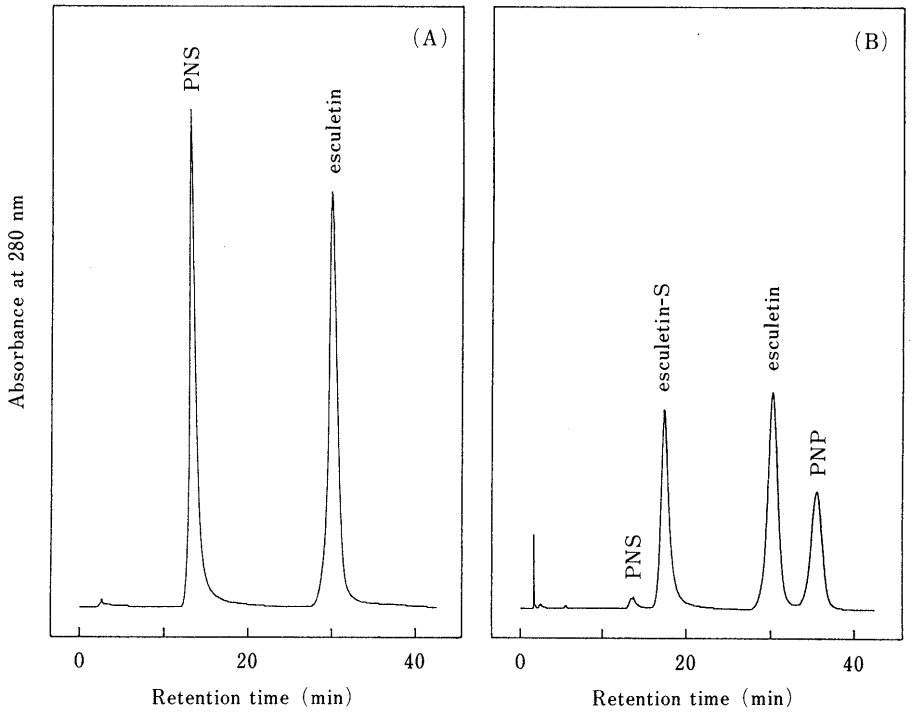

Fig. 1. Isolation of Esculetin-S by Reverse-Phase HPLC

The reaction mixture (150 ml) contained 1.3 mM PNS, 4 mM esculetin, 40 mM Tris–HCl buffer (pH 8.0) and the enzyme (162 units). It was incubated at 37 °C for 0 min (A) and 48 h (B). A 10 μl aliquot of the reaction mixture was injected after millipore filtration. HPLC conditions are described in Materials and Methods.

corresponding to the molecular formula $C_9H_6O_7S$, in negative ion FAB-MS. The ^1H- and ^{13}C-NMR spectra were analyzed with the aid of ^1H–^{13}C and ^1H–^{13}C long-range COSY spectra (Fig. 2), and the quaternary carbon at δ 141.38 (C-6) showed long-range correlation with 5-H (δ 7.20, s) and 8-H (δ 6.27, s), while the quaternary carbon at δ 154.35 (C-9) showed long-range correlation with 4-H (δ 7.68, d, J=9.0 Hz), 5-H (δ 7.20, s) and 8-H (δ 6.27, s). Therefore, the former carbon was assigned to C-6 and the latter to C-9, unambiguously. Some other significant long-range correlations observed are also shown in Fig. 2. Complete assignments of ^1H- and ^{13}C-signals are given in Table II. The ^1H-NMR spectrum of esculetin-S was very similar to that of esculetin (Table II). On the other hand, comparison of the ^{13}C-NMR of esculetin-S with that of esculetin revealed that esculetin-S made a remarkable upfield shift at the signal attributable to C-10

Table II. ^1H- and ^{13}C-NMR Data for Esculetin and Esculetin-S

Position	Esculetin		Esculetin-S	
	δ_H	δ_C	δ_H	δ_C
2	—	160.81 s	—	161.96 s
3	6.16 d (10.0)	111.51 d	5.73 d (9.0)	104.22 d
4	7.86 d (10.0)	144.45 d	7.68 d (9.0)	144.90 d
5	6.97 s	112.36 d	7.20 s	119.40 d
6	—	142.90 s	—	141.38 s
7	—	150.40 s	—	165.82 s
8	6.73 s	102.68 d	6.27 s	104.35 d
9	—	148.52 s	—	154.35 s
10	—	110.78 s	—	104.56 s

δ values in ppm were measured in DMSO-d_6. Multiplicities of carbon signals were indicated as s and d. Coupling constants (Hz) are in parentheses. Signal assignments are based on the analyses of the ^1H–^{13}C and ^1H–^{13}C long-range COSY spectra.

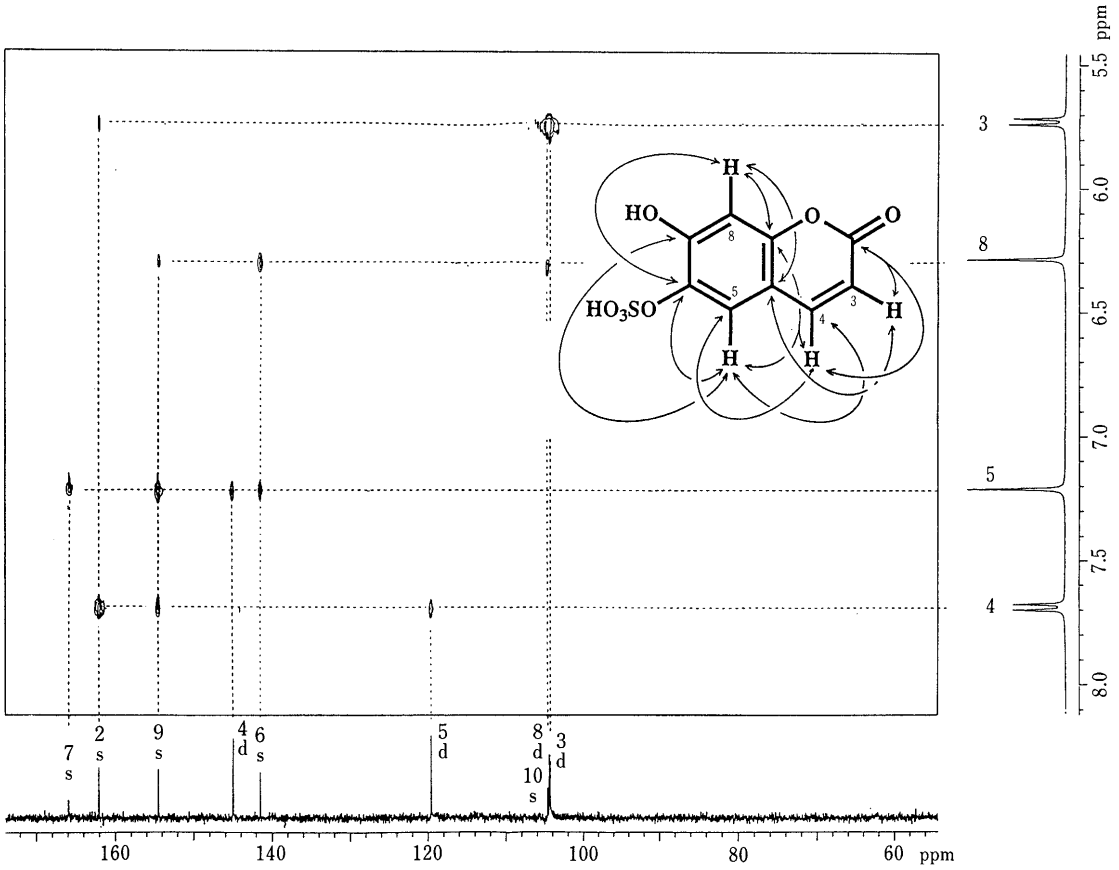

Fig. 2. ^1H–^{13}C Long-Range COSY Spectrum of Esculetin-S in DMSO-d_6

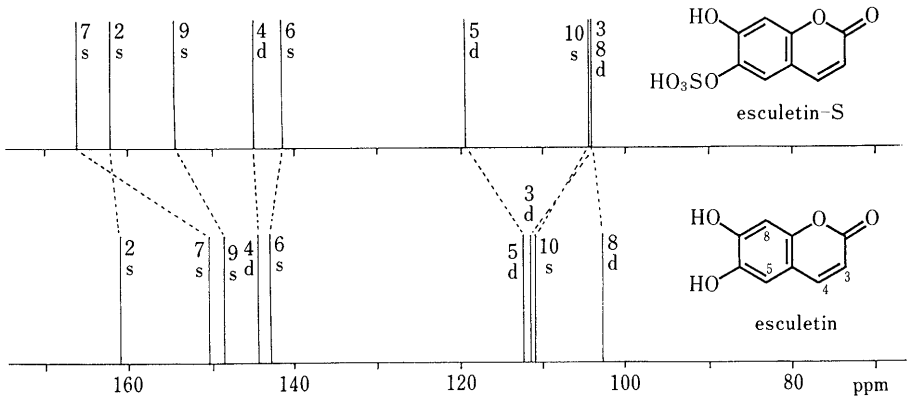

Fig. 3. Comparison of the ^{13}C-NMR Signals of Esculetin with Those of Esculetin-S

(δ 110.78→104.56), and downfield shifts of C-5, C-7 and C-9 (δ: 112.36→119.40, 150.40→165.82 and 148.52→154.35) (Fig. 3). The other signals were essentially the same as those of esculetin. These shifts are consistent with our previous findings[11,13]; since the *O*-sulfate is an electron withdrawing group, electron density decreased in the *ortho* and *para* carbons and a marked downfield shift was observed, and the electron density of the carbon carrying the sulfate group had increased electron density and showed an upfield shift, while the *meta* carbon was almost unaffected. Based on these data, esculetin-S was identified as a 6-*O*-sulfate of esculetin (Fig. 3).

DISCUSSION

Intestinal microflora are composed of numerous bacteria species which produce many kinds of enzymes. Several enzymes from intestinal bacteria have been reported.[16] Among these, β-glycosidase and β-glucuronidases have been well studied. Exogenous β-glycosides or β-glucuronides of polyphenols in vegetable food or traditional medicines are hydrolyzed by bacterial enzymes to aglycones and sugars, which are not hydrolyzable by animal digestive enzymes. The sugars are utilized as nutrients of intestinal bacteria. Also, the aglycones are often metabolized by the corresponding enzymes from other intestinal bacterial sources.

Sodium picosulfate, a laxative, is inactive on its own, but its effect is slowly exhibited after oral administration. The effects of picosulfate are different in animal species and are lost by the simultaneous administration of antibiotics, suggesting that intestinal bacterial enzymes were necessary for its pharmacological effect. We discovered a strain producing sulfotransferase (*Eubacterium rectale* A-44) from the human intestine. Actually, the enzyme ASST catalyzed the transformation of picosulfate to the phenol, the genuine drug.[17] It is clear that this bacterial sulfotransferase plays an important role in human intestinal drug metabolism.

ASST is not a sulfatase, and therefore, some exogenous or endogenous sulfate esters of polyphenols, which are sulfoconjugated in plant or animal liver, might be donor substrates. Also free polyphenols might be acceptor substrates of this enzyme. We have already reported the sulfation by ASST of flavones and tannins as components of vegetable food which are ingested daily or of traditional medicines, and have established the chemical structure of sulfated esters of quercetin, (+)-catechin, isoamylgallate and (−)-epigallocatechin gallate.[10-12] In the present study, esculetin, baicalin and baicalein were also sulfated by ASST. In addition, we demonstrated that all three aglycones examined in the present study were more effectively sulfated by this bacterial enzyme than were the corresponding glycosides. From these findings, aglycones were sulfated remarkably better than glycosides, suggesting that a sugar moiety interferes with the enzymatic sulfation because of hydrophilicity or steric hindrance. Furthermore, it was expected that the ingested glycosides are first hydrolyzed by the corresponding β-glycosidase and then aglycones are sulfated by ASST in the intestine converting them to a more water-soluble form.

Esculin is one of the coumarin derivatives, present in horse chestnut tree, and has been shown to possess antihepatotoxic activity.[18] Esculin has been widely used as the substrate for screening β-glucosidase activity, and many bacterial β-glucosidases can hydrolyze esculin to esculetin.[19] The number of sulfate groups incorporated into esculetin by ASST was shown to be only one, though two phenolic hydroxyl groups are present in esculetin, even if the reaction mixture contained an excess molar amount of PNS as a donor substrate over esculetin (data not shown). Furtheremore, esculin was not sulfated by ASST. The chemical structure of mono-sulfated esculetin was elucidated to be esculetin 6-sulfate by NMR. These results suggested that the 6-hydroxy group was sulfated in esculetin-S.

Baicalin is a constituent of Scutellariae Radix used as a traditional medicine[20] to treat inflammation, suppurative dermatitis, allergic diseases, hyperlipemia and arteriosclerosis. The sulfate ester of baicalin (6-*O*-β-glucopyranuronosyl-baicalein 7-*O*-sulfate) has been isolated from biliary excretions when baicalin was administered orally to rats.[21] Thus, this sulfate ester might be one of the sulfate donors of ASST in the human intestinal tract. In addition, the sulfation activity of baicalein was distinctly higher than that of baicalin by ASST *in vitro*, and baicalein was the best acceptor substrate of all the phenolic compounds which have been tested.[22] From our previous report, the presence of a carboxylic anion has an adverse effect on the enzymatic sulfation of an adjacent hydroxyl group.[11] This fact suggests that the sulfation of baicalein occurs at or near the 7-hydroxyl position by ASST.

In conclusion, we have investigated that the aglycone of polyphenols are better substrates of ASST than related glycosides. Additionally, a coumarin derivative, esculetin, was sulfated by ASST as well as flavones and tannins, and its chemical structure was established. Therefore, bacterial *trans*-sulfation is a more advantageous method for the preparation of sulfated polyphenols at specific positions than chemical sulfation reaction, and could play an important role in the metabolism of phenolic compounds present in vegetable food and herbs.

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