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Partial Purification and Some Properties of Flavonol 7-Sulfotransferase from *Flaveria bidentis*[1]

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ABSTRACT

A novel flavonol-specific sulfotransferase was partially purified from the shoot tips of *Flaveria bidentis* var. Angustifolia O.K. (Asteraceae) by chromatography on 3′-phosphoadenosine 5′-phosphate-agarose affinity column and chromatofocusing on Mono P. The latter step resulted in the separation of two isoforms, both of which exhibited expressed specificity for position 7 of quercetin 3,3′- and quercetin 3,4′-disulfate. The 7-sulfotransferase isoforms I and II had a pH optimum of 7.5 in phosphate buffer, apparent pI values of 6.5 and 6.3, and an M_r of 35,000. They had no requirement for divalent cations and were not inhibited by EDTA or SH group reagents. Their K_m values for both the sulfate donor and flavonol acceptor were of the same order of magnitude (0.20–0.46 micromolar). This enzyme, together with the recently reported flavonol 3-, 3′-, and 4′-sulfotransferases from *F. chloraefolia* (L Varin, RK Ibrahim [1989] Plant Physiol 90: 977–981) form the complement involved in the biosynthesis of polysulfated flavonols in this genus. A proposed sequential order for the enzymatic sulfation in both species is described.

Sulfate conjugation of flavonoid compounds has been shown to be widely distributed in plants (9). A recent review (3) lists some 100 flavonoid sulfates from 250 plant species, belonging to 17 dicot and 15 monocot families. Most of these compounds are sulfate esters of common hydroxyflavones or their methyl ethers and, less commonly, of their *O*-glycosides that are sulfated on the flavonoid ring or on the sugar moiety (3). Because of their accumulation in plants growing in saline and marshy habitats, sulfated flavonoids are believed to play a role in sequestering sulfate ions (3). Their solubility in aqueous solution might also facilitate their storage in hydrophilic cellular compartments.

Flaveria spp. (Asteraceae) accumulate a variety of flavonol mono- to tetrasulfates variously substituted at positions 3, 7, 3′, and 4′. We have recently demonstrated the enzymatic sulfation of positions 3, 3′, and 4′ of flavonols in *Flaveria chloraefolia* (14). These reactions were shown to be catalyzed by three distinct, flavonol-specific and position-oriented STs[2].

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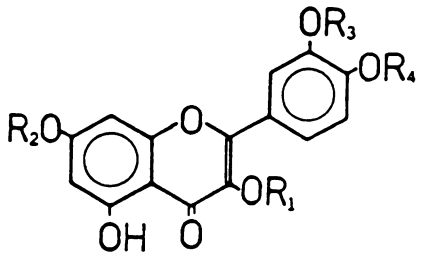
[2] Abbreviations: ST, sulfotransferase; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TBADP, tetrabutylammonium dihydrogen phosphate.

The position specificity of these enzymes is in agreement with the natural occurrence of flavonol 3,3′- and -3,4′-disulfates in this species (1, 3). However, due to the lack of sulfation at position 7 in *F. chloraefolia*, it was not possible to determine the sequence of enzymatic sulfation of flavonol tri- and tetrasulfates. *F. bidentis* accumulates a number of polysulfated flavonols of which several are sulfated at position 7 (Scheme I), and is the only species known to accumulate quercetin 3,7,3′,4′-tetrasulfate (11, 15). Therefore, in order to define the sequence of enzymatic steps leading to the formation of tri- and tetrasulfated flavonols, it was necessary to characterize the flavonol 7-ST of *F. bidentis*.

MATERIALS AND METHODS

Plant Material

Seeds of *Flaveria bidentis* var *Angustifolia* O.K. (Asteraceae) were kindly supplied by Prof. H. R. Juliani, University of Cordoba, Argentina. They were germinated in a 1-cm layer of vermiculite on top of potting soil, and their growth was maintained under greenhouse conditions. Terminal buds and the first pair of expanded leaves were used for enzyme extraction.



R_1	R_2	R_3	R_4	
SO_4	H	H	H	Quercetin 3-sulfate
SO_4	H	OMe	H	Isorhamnetin 3-sulfate
SO_4	SO_4	H	H	Quercetin 3,7-disulfate
SO_4	H	H	SO_4	Quercetin 3,4′-disulfate
SO_4	SO_4	OMe	H	Isorhamnetin 3,7-disulfate
SO_4	SO_4	H	SO_4	Quercetin 3,7,4′-trisulfate
SO_4	SO_4	SO_4	H	Quercetin 3,7,3′-trisulfate
Acetyl	SO_4	SO_4	SO_4	Quercetin 3-acetyl-7,3′,4′-trisulfate
SO_4	SO_4	SO_4	SO_4	Quercetin 3,7,3′,4′-tetrasulfate

Scheme I. Flavonol sulfates of *F. bidentis*.

Chemicals

3′-Phosphoadenosine 5′-phospho-[^{35}S]sulfate (about 1.5–3.0 Ci/mmol) was purchased from NEN (Boston, MA) and diluted with unlabeled PAPS (Sigma, St. Louis, MO) as required. Flavonoid aglycones were obtained from Roth (Karlsruhe, FRG) and Extrasynthese (Bordeaux, France) and were further purified by TLC or HPLC. All flavonol sulfate esters used in this study were from our laboratory collection. TBADP was purchased from Aldrich (Milwaukee, WI) and both PAP-Agarose and polyvinylpolypyrrolidone from Sigma. PD-10 columns, Polybuffer 74, Superose 12, and Mono P columns, as well as the fast protein liquid chromatography system were from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

Buffers

Buffer A, 0.2 M Tris-HCl (pH 8.0) containing 10 mM DTT, 5 mM EDTA, and 10 mM diethylammonium diethyldithiocarbamate; B, 25 mM bis-tris-HCl (pH 6.5); C, 25 mM bis-tris-iminodiacetate (pH 7.1); D, Polybuffer 74-iminodiacetic acid (pH 7.1); E, 0.2 M Tris-HCl (pH 7.5). Buffers B to E also contained 14 mM 2-mercaptoethanol.

Protein Extraction

Shoot tips (about 25 g) were frozen in liquid N_2, mixed with polyvinylpolypyrrolidone (10%, w/w) in order to adsorb phenolics and ground to a fine powder. The latter was homogenized with buffer A (1:3, w/v) and filtered through nylon mesh. The filtrate was centrifuged at 15,000*g* for 15 min and the supernatant stirred with Dowex 1 × 2 (5%, w/v), which had previously been equilibrated with buffer A, then filtered. The filtrate was fractionated with solid ammonium sulfate, and the protein which precipitated between 35 and 75% salt saturation was collected by centrifugation.

Enzyme Purification

The ammonium sulfate pellet was desalted using a PD-10 column preequilibrated with buffer B. The desalted protein was then chromatographed on a PAP-agarose column (1.5 × 12 cm) which had previously been equilibrated with buffer B, and washed with three column volumes of the same buffer. The bound proteins were eluted with 100 mL of a linear salt gradient of 0.0 to 0.7 M NaCl in buffer B. Fractions of 1 mL each were collected and assayed for ST activity using quercetin, quercetin 3-sulfate and quercetin 3,3′-disulfate, as substrates. The active fractions were concentrated by ultrafiltration and desalted on a PD-10 column using buffer C. The desalted proteins were finally chromatographed on a Mono P (HR 5/20) column that was preequilibrated with buffer C. The bound proteins were eluted with buffer D which generated a gradient between pH 7.0 and 4.8. Fractions (1 mL) were collected in 0.3 mL of buffer E, in order to stabilize the enzyme protein before being assayed for activity using quercetin 3,3′-disulfate as substrate.

Sulfotransferase Assay and Identification of Reaction Products

The standard enzyme assay was employed as previously described (14, 16) using labeled PAPS and partially sulfated flavonols as the sulfate donor and acceptor, respectively. The sulfated products were transformed to their ion pairs by the addition of TBADP before extraction with ethyl acetate. The radioactivity in the reaction products was determined by liquid scintillation, and their identity was verified by cochromatography with reference compounds as previously described (14, 16).

Molecular Weight Determination

The partially purified enzymes were applied to a Superose 12 (HR 10/30) column which had previously been calibrated with ribonuclease A (M_r 13,700), chymotrypsinogen A (M_r 25,000), ovalbumin (M_r 45,000), and BSA (M_r 67,000) as reference proteins. The apparent mol wt of each enzyme was estimated by its elution volume from the column.

Protein Estimation

Protein concentration was measured by the method of Bradford (4) using the Bio-Rad dye reagent and BSA as the standard protein.

RESULTS AND DISCUSSION

Purification of Flavonol 7-Sulfotransferase

Preliminary experiments showed that cell-free extracts of *F. bidentis* contained detectable ST activity when assayed with quercetin 3,3′-disulfate as substrate. The fact that the reaction product cochromatographed with quercetin 3,7,3′-trisulfate indicated the presence, in this tissue, of an ST specific for the 7-position of partially sulfated flavonols.

Due to instability of the 7-ST, a purification protocol was developed to allow the recovery of this enzyme free from other contaminating activities, namely the 3, 3′ or 4′-STs (14). Partial purification of the 7-ST was achieved within 8 h after protein extraction by passing the ammonium sulfate pellet through a PD-10 column, followed by chromatography on PAP-agarose. The latter step resulted in a broad peak of enzyme activity which accepted quercetin 3,3′-disulfate and, to a lesser extent, quercetin as substrates (Fig. 1). Further chromatofocusing of the combined active fractions on a Mono P column, revealed three peaks of ST activity (Fig. 2). Peaks 1 and 2, which eluted at pH 6.5 and 6.3, respectively, accepted quercetin 3,3′-disulfate as substrate. Peak 3, which eluted at pH 5.2, contained several unresolved ST activities. Analysis of the reaction products of both peaks 1 and 2 indicated that quercetin 3,7,3′-trisulfate was the only reaction product (Fig. 3), thus these peaks represented two isoforms of the 7-ST. Peak 3, on the other hand, gave rise to the 3-mono- and disulfate ester derivatives when assayed with quercetin as substrate, indicating the presence of a 3-ST as well as other unresolved ST activities. The purification procedure described above resulted in 190- and 125-fold increase in specific activity, with recoveries of 19% and 12%, for peaks 1 and 2, respectively, as compared with the crude extract (Table I).

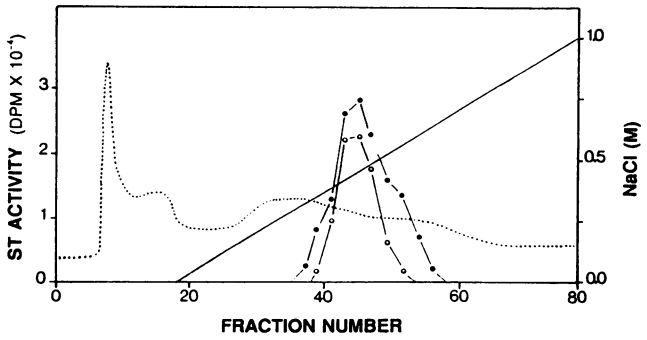


Figure 1. Elution profile of the ST-activities after affinity chromatography on PAP-agarose. The column was preequilibrated with buffer B, and the bound proteins were eluted using a linear salt gradient of 0.0 to 0.7 M NaCl in buffer B. Fractions (1 mL) were collected and assayed for enzyme activity against quercetin (○) and quercetin 3,3′-disulfate (●) as substrates. Protein absorbance was monitored at 280 nm (· · · ·).

Substrate Specificity of the 7-ST

The relative activities of the two 7-ST isozymes when assayed with different flavonoid substrates are shown in Table II. The partially purified 7-STs exhibited strict specificity for position 7 of quercetin 3,3′- and -3,4′-disulfates. In addition, both isoforms accepted isorhamnetin 3-sulfate for further sulfation at position 7. On the other hand, the 7-ST isoforms did not accept quercetin 3-sulfate as substrate, thus indicating the absolute requirement for activity of a sulfated or methoxylated 3′- or 4′-position in addition to a sulfate group at position 3 (Scheme I). Neither of the isozymes exhibited any activity with quercetin, quercetin 3′-sulfate, flavones (apigenin, luteolin), or phenylpropanoids (*p*-coumaric, caffeic or ferulic, acids) as substrates.

The substrate specificity of the 7-ST is consistent with the accumulation, in *F. bidentis,* of isorhamnetin 3,7-disulfate

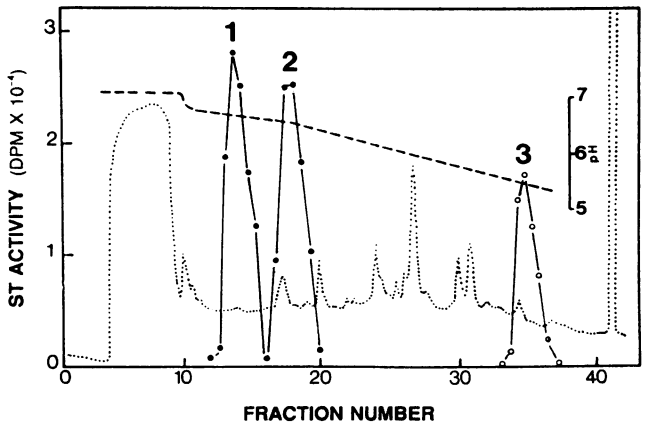


Figure 2. Elution profile of the ST-activities after chromatofocusing on Mono P. The column was preequilibrated with buffer C, and the bound proteins were eluted with buffer D at a flow rate of 0.4 mL/min which generated a gradient between pH 7 and 4.8. Fractions of 1 mL were collected and assayed for enzyme activity against quercetin (○) and quercetin 3,3′-disulfate (●) as substrates. Protein absorbance was monitored at 280 nm (· · · ·).

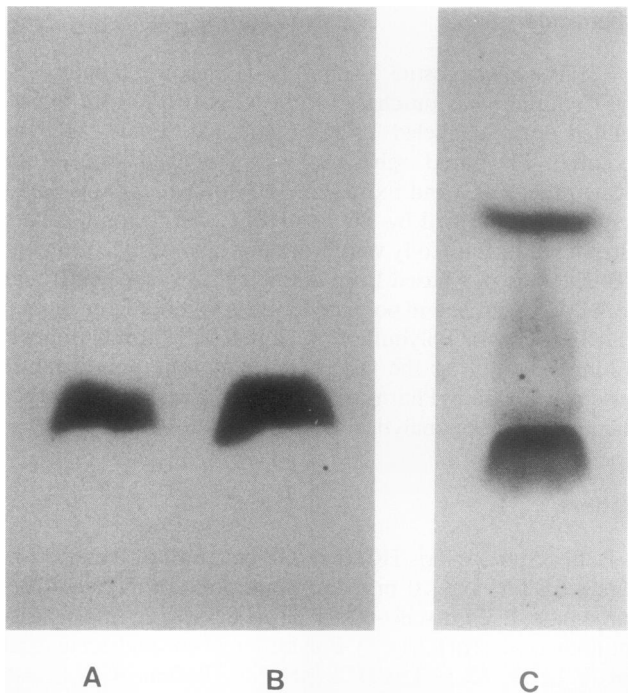


Figure 3. Photograph of an autoradiogram of the chromatographed reaction products of the 7-ST I (A) and II (B) incubated with quercetin 3,3′-disulfate. Spots correspond to quercetin 3,7,3′-trisulfate. *n*-BuOH-HOAc-H_2O (4:1:5, v/v/v) was used to develop the cellulose TLC plate. Lane C is the reaction products of Mono P-purified peak 3 incubated with quercetin: low R_F spot, quercetin disulfate; high R_F spot, quercetin 3-sulfate. *n*-BuOH-HOAc-H_2O (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

(5), as well as quercetin 3,7,3′- and -3,7,4′-trisulfates (6, 7). We propose, therefore, the following designation for this novel enzyme: PAPS:flavonol 3,3′-/3,4′-disulfate 7-ST.

Other Properties

The pH optima of the 7-ST isozymes, when assayed in different buffers over a range of pH 5.5 to 9.5 were found to be 7.5 in phosphate buffer. A change of one unit below or above the pH optimum resulted in a loss of 55 to 60% activity.

Neither of the isozymes was affected by the addition of divalent cations (1–10 mM), nor inhibited by EDTA or SH group reagents when assayed at 1 to 10 mM.

Table I. *Purification of Flaveria bidentis 7-ST I and II*[a]

Purification Step	Total Protein	Specific Activity	Total Activity	Purification	Recovery
	mg	*pkat/mg*	*pkat*	*-fold*	%
Dowex	82	0.051	4.18		100
PAP-agarose	1.88	0.82	1.54	16	37
Mono-P					
7-ST I	0.081	9.69	0.78	190	19
7-ST II	0.078	6.37	0.50	125	12

[a] The sulfotransferase activity was assayed as described in "Materials and Methods" using quercetin 3,3′-disulfate as substrate.

Their apparent mol wt as estimated from their elution volumes on a calibrated Superose 12 column was found to be 35,000.

The apparent K_m values of the 7-ST isozymes were found to be 0.24 and 0.20 μM for the flavonol substrate (at 1.3 μM PAPS), and 0.33 and 0.46 μM for the sulfate donor PAPS (at 1.0 μM quercetin 3,3′-disulfate), respectively. Their activity was inhibited by the flavonol substrate above 1.5 μM. These properties are similar to those previously reported for the 3, 3′, and 4′-STs of *F. chloraefolia* (14). Although the 7-ST isozymes catalysed further sulfation of quercetin 3,3′- and -3,4′-disulfates with equal efficiency (Table II), the physiological significance of two isoforms in the 7-sulfation of flavonols remains to be elucidated. Multiple forms of enzymes involved in phenolic synthesis have been reported, especially those of phenylalanine ammonia-lyase and chalcone synthase (for review, see ref. 13). Further studies as to the regulation of the 7-ST isoforms and their tissue distribution will be required in order to assign their role in *F. bidentis*.

Proposed Sequence for Flavonoid Sulfation in *F. bidentis*

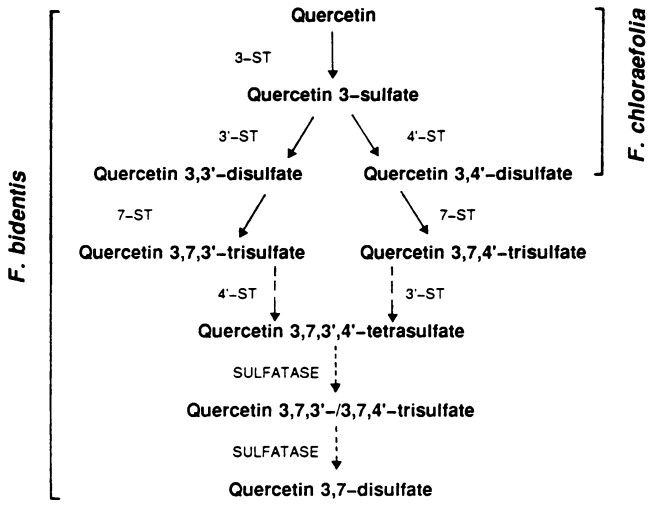
The natural occurrence of flavonol disulfate esters in *F. chloraefolia* (1, 2) and of a variety of flavonol mono- to tetrasulfates in *F. bidentis* (5–7, 11, 15) raises the question as to the sequential order of enzymatic sulfation in both species. The recent discovery of three distinct, position-specific STs in *F. chloraefolia*, namely 3, 3′, and 4′-STs (14) and of the 7-ST in this study constitute the enzyme complement involved in the formation of polysulfated flavonols in both species, and allow us to propose the sulfation sequence depicted in Scheme II.

The first two steps of enzymatic sulfation are probably identical in both species, although quercetin 3,3′-disulfate does not accumulate in detectable amounts in *F. bidentis*. However, further sulfation of quercetin 3,3′- and -3,4′-disulfates by a position-specific 7-ST gives rise to the 3,7,3′- and 3,7,4′-trisulfate ester derivatives; both of which are natural constituents of this species (6, 7). The fact that *F. bidentis* is the only species known to accumulate quercetin 3,7,3′,4′-tetrasulfate (11, 15), implies the existence, in this plant, of two ring B-specific STs which catalyze further sulfation of quercetin 3,7,3′- and -3,7,4′-trisulfates to their tetrasulfate ester derivatives. However, the two latter STs have yet to be characterized in this species.

Table II. *Substrate Specificity of F. bidentis 7-ST I and II*[a]

Substrate[b]	Relative Activity	
	7-ST I	7-ST II
	%	
Quercetin 3,4′-disulfate	100	100
Quercetin 3,3′-disulfate	84	91
Isorhamnetin 3-sulfate	58	60

[a] The standard enzyme assay was used as described in "Materials and Methods" at substrate concentration of 1 μM. [b] Neither of the isozymes accepted quercetin, quercetin 3-sulfate, or quercetin 3′-sulfate as substrates.



Scheme II. Proposed model for the enzymatic synthesis of flavonol sulfate esters in *F. bidentis* and *F. chloraefolia*. Solid arrows indicate enzymes characterized; dotted arrows indicate proposed enzymatic steps.

Very recently, a novel ST from human intestinal bacteria has been reported to sulfate polyphenols including chalcones, xanthones, and flavonols (10). This enzyme utilized *p*-nitrophenyl sulfate or 4-methylumbelliferyl sulfate, rather than PAPS, as the sulfate donor. It is interesting to note that under limiting concentration of the sulfate donor, the bacterial ST transformed quercetin to its -3,3′-disulfate ester, whereas in the presence of an excess of the sulfate donor quercetin 3,7,3′-trisulfate was formed as well (10). Although this work was carried out with a partially purified preparation, it clearly demonstrates the sequential sulfation presumably by position-specific STs and that sulfation of the 7-position is a later step in the biosynthesis of polysulfated compounds, similar to that in *Flaveria*.

Another question that needs to be addressed concerns the biosynthetic origin of quercetin 3,7-disulfate which accounts for about 70% of the flavonoids in *F. bidentis* (15). Since the 7-ST of this species accepts only quercetin 3,3′- and -3,4′-disulfates, but not quercetin 3-sulfate, as substrates (Table II), its involvement in the formation of the 3,7-disulfate must be excluded. Although we have no evidence, as yet, for enzymatic desulfation in plants, it is conceivable that flavonoid-specific arylsulfatases may exist in *Flaveria* in a manner similar to those of animal tissues (12) and microorganisms (8). The action of such sulfatases on quercetin tri- or tetrasulfates may explain the accumulation of quercetin 3,7-disulfate in *F. bidentis* (Scheme II). Work, now in progress, is directed toward the characterization of flavonoid-specific sulfatases in this tissue.

ACKNOWLEDGMENTS

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