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ENZYMATIC SYNTHESIS OF SULPHATED FLAVONOLS IN *FLAVERIA*

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Key Word Index—*Flaveria bidentis*; *F. chloraefolia*; Compositae; flavonol sulphates; enzymatic synthesis; sulphotransferase.

Abstract—Cell-free extracts of *Flaveria bidentis* and *F. chloraefolia* catalysed the transfer of sulphate groups from 3'-phosphoadenosine-5'-phosphosulphate to the hydroxyl groups of a variety of hydroxylated and *O*-methylated flavonols, but not to flavones or phenylpropanoids. Enzymatic sulphation was more predominant at the 3-hydroxyl group, but not to the exclusion of other hydroxyl substituents on the flavonoid ring. Quercetin was sulphated to yield its mono-, di-, tri- and tetrasulphate esters. This, together with the differences observed in the sulphation of different flavonols by extracts of both *Flaveria* species, suggests the existence of a number of distinct, position-specific sulphotransferases (EC 2.8.2. –). The sulphation reaction was catalysed at an optimum pH of 7.5 in Tris HCl buffer, required SH groups for activity and was stimulated in the presence of divalent cations.

INTRODUCTION

Organic sulphur compounds are known for their ubiquitous occurrence in plants [1]. Recently, a new class of sulphur compounds, the flavonoid sulphates, has been reported to be widespread in a number of plant families [2–4], especially in the Compositae [5, 6] and in such genera as *Brickellia* [7–11] and *Flaveria* [12–17]. Except for the participation of PAPS§ as sulphate donor in the sulphation of phenols [2, 18], nothing is known of the enzymatic synthesis of sulphated flavonoids. This may have been due to the difficulty in separating PAPS from the sulphate ester formed since both compounds are water-soluble ([18] and refs. cited therein).

Very recently, we developed an enzymatic assay for phenol/flavonoid ST (EC 2.8.2.—) activity [19] which made use of TBADP in the formation of ion pairs that rendered the sulphate ester soluble in organic solvents, such as ethyl acetate, while PAPS remained in the aqueous reaction mixture. This paper reports, for the first time, the enzymatic synthesis of sulphated flavonols in *F. bidentis* and *F. chloraefolia* and the characterization of the sulphation reaction.

RESULTS AND DISCUSSION

Cell-free extracts of either *F. bidentis* or *F. chloraefolia* catalysed the sulphation of a number of flavonol substrates, but with different efficiencies (Table 1). Most of the flavonoid sulphate esters formed were identified by co-chromatography with reference compounds (when available), R_f values in different solvent systems, electrophoretic mobility and autoradiography. The combination of chromatographic and electrophoretic properties gave an indication of the number of sulphate groups in the reaction products.

Characterization of the sulphation reaction

The sulphation reaction was linear with time up to 60 min and with protein concentration up to 80 μg at 10 μM quercetin as substrate. The pH optimum was determined in different buffers and was found to be at pH 7.5 in Tris–HCl buffer; however, the enzyme activity dropped by 40% in the presence of phosphate buffer. The protein extract could be stored at −15° in 25 mM Tris HCl buffer, pH 7.5, containing 14 mM 2-ME and 10% glycerol, for at least 2 weeks without loss of activity.

Substrate specificity

Of the various substrates tested for their sulphate acceptor ability (Table 1), flavonol aglycones were generally better acceptors than flavones or phenylpropanoids. Of the simple flavonol derivatives tested, the order of sulphation as catalysed by *F. bidentis* ST was isorhamnetin ≈ quercetin 3'-sulphate > quercetin > myricetin ≈ rhamnetin > kaempferol > patuletin; whereas that of *F. chloraefolia* was rhamnetin > isorhamnetin > kaempferol > eupatin > quercetin > patuletin. Enzymatic sulphation at position 3 seems predominant among flavonols, but not to the exclusion of other hydroxyl substituents on the flavonoid ring. The differences observed in the sulphation of individual substrates, by the two enzyme preparations, seem to indicate the involvement of distinct, position-specific sulphotransferases. It is remarkable to note that, except for quercetin 3'-sulphate, other substituted flavonols were further sulphated but to a much lesser extent (Table 1).

Time-course incubation of the *F. bidentis* enzyme with quercetin (10 μM) and PAPS (2 μM) resulted in the formation of mono-, di-, tri- and traces of tetrasulphate

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§Abbreviations: PAPS, 3'-phosphoadenosine-5'-phosphosulphate; ST, sulphotransferase; TBADP, tetrabutylammonium dihydrogen phosphate.

Table 1. Sulphotransferase activity of cell-free extracts of two *Flaveria* species against different substrates*

Substrate†	*F. bidentis*[a] (% of control)‡	*F. chloraefolia*[b] (% of control)‡	Sulphated products: Major	Sulphated products: Minor
Kaempferol	51	60	3-	mono-
Quercetin	73	36	3-	di-to tetra[a]
Myricetin	62	18	n.d.	
Gossypetin	17	6	n.d.	—
Rhamnetin	57	100	3-	di-[a] mono-[b]
Isorhamnetin	100	86	3-	di-[a]
Tamarixetin	< 5	14	n.d.	n.d.
Patuletin	39	30	3-	—
Eupatin	8	47	3-[b]	
3,7-Dimethylquercetin	26	14	n.d.	—
7,4'-Dimethylquercetin	16	18	n.d.	—
3',4'-Dimethylquercetin	35	15	n.d.	—
Rutin	14	10	n.d.	—
Isorhamnetin 3-SO_4	8	2	n.d.	—
Quercetin 3-SO_4 + patuletin 3-SO_4	18	5	3,7-di[a]	
Quercetin 3'-SO_4	118	5	n.d.	—
Caffeic acid	15	3	n.d.	

*The standard enzyme assay was used as described in the Experimental.

†These substrates were accepted at < 5% of controls in the following descending order: patuletin 3-glucoside, apigenin, luteolin, *p*-coumaric acid, ferulic acid, isoferulic acid and dihydroquercetin.

‡Activity in the control was 3400 and 2000 dpm/mg/min with *F. bidentis* and *F. chloraefolia* enzyme extracts, respectively.

n.d., not determined.

esters (Fig. 1). These results are consistent with the natural occurrence of highly sulphated flavonols in this tissue [14] as compared with *F. chloraefolia* [16, 17], and signify the differences in the STs present in the two plants.

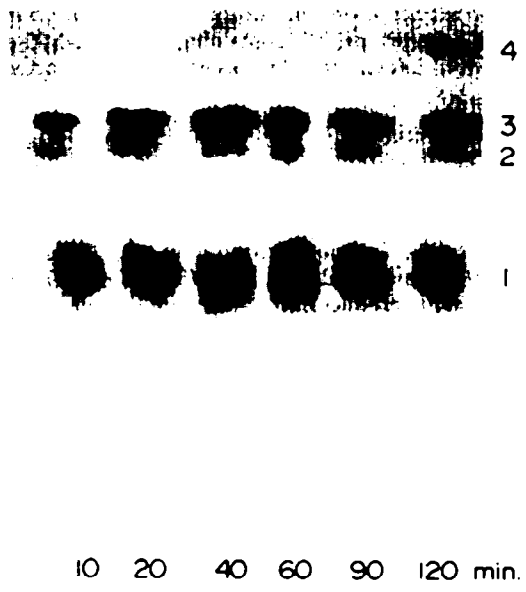

Fig. 1. Photograph of an autoradiogram of the reaction products of *F. bidentis* cell-free extract with quercetin, as substrate, incubated for different time periods. 1-4 indicate mono-, di-, tri- and tetrasulphate esters, respectively.

Effect of divalent cations and SH*-group reagents*

The effect of divalent cations on the sulphation reaction was studied in both *Flaveria* species. Whereas the ST activity of *F. chloraefolia* seems to be stimulated in the presence of 1 mM $MgCl_2$ and was inhibited to various degrees by other divalent cations (Table 2), that of *F. bidentis* was stimulated both by low (1 mM) and high (10 mM) concentrations of all cations tested except Mn^{2+}.

The sulphation reaction was enhanced in the presence of 14 mM 2-ME or 1 mM DTE. However, SH-group

Table 2. Effect of divalent cations on sulphotransferase activity of two *Flaveria* species*

Cation and concentration	Relative activity (% of control): *F. bidentis*	Relative activity (% of control): *F. chloraefolia*
Control (no addition)	100	100
Mg^{2+} (1 mM)	140	142
Mg^{2+} (10 mM)	350	120
Mn^{2+} (1 mM)	100	50
Mn^{2+} (10 mM)	20	5
Ca^{2+} (1 mM)	116	87
Ca^{2+} (10 mM)	180	94
Co^{2+} (1 mM)	215	75
Cu^{2+} (1 mM)	210	72
Zn^{2+} (1 mM)	250	72

*The standard enzyme assay was used as described in the Experimental with quercetin as substrate.

Table 3. Effect of SH-group reagents on the sulphotransferase activity of two *Flaveria* species*

Addition	Concentration (mM)	Relative activity (% of control) *F. bidentis*	*F. chloraefolia*
Control		100	100
2-ME	14	113	145
DTE	1	135	160
N-Ethylmaleimide	10	30	10
+ 2-ME	40	105	95
Phenylmercuriacetate	10	5	7
+ 2-ME	100	45	72
p-Chloromercuribenzoate	10	60	68
+ 2-ME	40	108	97

*The standard enzyme assay was used with quercetin as substrate.

reagents (10 mM) such as *N*-ethylmaleimide or phenylmercuric acetate almost abolished ST activity, as compared with *p*-chloromercuribenzoate which was less inhibitory. Inhibition by SH-group reagents was partially prevented or completely reversed by the addition of various concentrations of 2-ME (Table 3). These results further indicate the differences in properties of the STs of both tissues.

We have demonstrated, for the first time, the existence of a flavonol-specific ST activity in two *Flaveria* species. The results reported here also indicate the presence of a number of distinct, position-specific STs in both tissues. These enzymes are involved in the biosynthesis of flavonol mono-/tetrasulphate esters, which are known to occur naturally in *F. bidentis* [14]. Experiments designed for the purification and characterization of individual enzymes are in progress.

EXPERIMENTAL

Plant material. Seeds of *Flaveria bidentis* var. *angustifolia* O.K. and *F. chloraefolia* A. Gray were germinated in a 1-cm layer of vermiculite on top of potting soil and their growth was maintained under greenhouse conditions.

Chemicals. [^{35}S]PAPS (1.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and cold PAPS from Sigma (St. Louis, MO). TBADH was obtained from Aldrich Chemical Co. (Milwaukee, WI). Most of the flavonoids were from Roth (Karlsruhe, F.R.G.) or Sarsynthese (Bordeaux, France); others were from our laboratory collection. Eupatin and its 3-sulphate were generous gifts from Dr. B. Timmermann, Tucson, Arizona.

Preparation of enzyme extracts. Unless stated otherwise, all steps were carried out at 2–4°. Terminal buds and the first pair of expanded leaves (*ca* 2–3 g) were frozen in liquid N_2, mixed with Polyclar AT (10% w/w) and ground to a fine powder. The latter was homogenized with 0.2 M Tris–HCl buffer, pH 8.0 (1:5, w/v), containing 5 mM EDTA, 10 mM diethylammonium diethyldithiocarbamate and 14 mM 2-ME (buffer A). The homogenate was filtered through nylon mesh and the filtrate centrifuged for 15 min at 15000 *g*. The supernatant was stirred for 15 min with Dowex 1 × 2 (10%, w/v) which had previously been equilibrated with the same buffer, then filtered. One-ml aliquots of the filtrate were passed through a Sephadex G-25 column and the protein was eluted with 25 mM Tris–HCl buffer, pH 8.0, containing 14 mM 2-ME and 10% glycerol (buffer B). The protein collected was directly used as the enzyme source. Protein concn was determined according to ref. [20], using bovine serum albumin as standard.

Enzyme assays and identification of reaction products. The standard assay mixture [19] was used and consisted of 10 μl of 5–10 μM of the flavonoid substrate (in 50% DMSO), 10 μl of 1 μM PAPS (2×10^5 dpm), 10 μl of 5 μM $MgCl_2$ and 70 μl of enzyme protein (*ca* 20–25 μg) in 25 mM Tris HCl buffer, pH 7.8. The enzyme reaction was incubated for 30 min at 30° and was terminated by the addition of 20 μl of 10 mM TBADP. The sulphated products were extracted with 500 μl of EtOAc and an aliquot of the organic phase was counted for radioactivity. The remaining extract was concentrated in N_2 and used for identification of the reaction products.

TLC was carried out on cellulose Avicel using H_2O or *n*-BuOH–HOAc–H_2O (3:1:1) as solvents, or Polyamid 6 using MeOH–H_2O–29% NH_4OH (15:5:1). Electrophoresis was carried out as in ref. [2].

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