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ENZYMOLOGY OF FLAVONOID SULFATION
Purification, characterization and molecular cloning of a number of flavonol sulfotransferases from *Flaveria spp.*

Luc Varin

A thesis

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ABSTRACT

ENZYMOLOGY OF FLAVONOID SULFATION: Purification, characterization and molecular cloning of a number of flavonol sulfotransferases from *Flaveria spp.*

Luc Varin

Concordia University, 1990

Three flavonol sulfotransferases (3-,3'-,4'-STs) from *Flaveria chloraefolia* and a 7-ST from *Flaveria bidentis* were partially purified by a combination of conventional and fast protein liquid chromatography. These novel enzymes exhibited strict position specificity for positions 3 of quercetin, 3' of quercetin 3-sulfate, 4' of quercetin 3-sulfate, and 7 of quercetin 3,3'- or 3,4'-disulfate. None of these four enzymes catalysed the sulfation of flavones or phenylpropanoids.

The four STs were found to have the same molecular weight (ca. 35,000D). However, they displayed different pI values on chromatofocusing. Except for the 3-ST, which exhibited two optima at pH 6.5 and 8.5, the 3'-, 4'-, and 7-STs had an optimum pH of 7.5. The highly purified enzymes exhibited no requirement for divalent cations and were not inhibited by the presence of SH-group reagents. All four enzymes exhibited similar Km values (ca. 0.3 μM) for both the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate and their respective flavonol acceptors.

The 3-ST of *F. chloraefolia* was purified to apparent homogeneity by a procedure which combined gel filtration, hydroxyapatite, affinity, and ion exchange chromatography. The purified enzyme migrated as a single band on SDS-PAGE with an apparent molecular weight of 34,500 D. The pure 3-ST was used for partial amino acid sequencing and to immunize rabbits. The antibodies produced were found to recognize the 3-, 3'-, and 4'-STs suggesting epitope similarities among the different ST proteins.

A *F. chloraefolia* cDNA library was constructed in the expression vector lambda ZAP and was screened with the anti 3-ST antibodies. Two clones corresponding to the 3- and the 4'-STs were isolated and their cDNA inserts sequenced. The two enzymes were found to share 70% and 69% identity at the nucleotide and amino acid levels, respectively. Search for sequence similarities revealed that both enzymes share significant identity with the rat liver hydroxysteroid ST, bovine placenta estrogen ST, and rat liver senescence marker protein 2, thus suggesting a common ancestor gene.

A Suzanne

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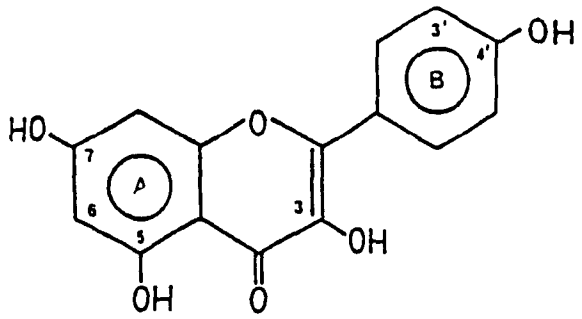
LIST OF ABBREVIATIONS

BAW	*n*-Butanol-acetic acid-water
bis-Tris	*bis*[2-hydroxyethyl]imino-tris[hydroxymethyl]methane
BSA	Bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propane-sulfonic acid
cDNA	Complementary deoxyribonucleic acid
D	Dalton
DEAE	Diethylaminoethyl
DIECA	Diethylammonium diethyldithiocarbamate
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DTT	DL-Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOAc	Ethyl acetate
FPLC	Fast protein liquid chromatography
HOAc	Acetic acid
HPLC	High performance liquid chromatography
IPTG	Isopropyl *B*-D-thioglucoside
MeOH	Methanol
Mes	2-[N-morpholino]ethanesulfonic acid
MEK	Methyl ethyl ketone
mRNA	Messenger ribonucleic acid

NEM	N-ethylmaleimide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAP	3'-phosphoadenosine 5'-phosphosulfate
PAPS	3'-phosphoadenosine 5'-phosphate
PCMB	*p*-chloromercuribenzoic acid
poly(A)$^+$	Polyadenylated
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
R_t	Retention time
SDS	Sodium dodecyl sulfate
ST	Sulfotransferase
TBADP	Tetrabutylammonium dihydrogen phosphate
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris-[hydroxymethyl] aminomethane
UV	Ultraviolet
X-gal	5-bromo -4-chloro -3-indolyl-*B*-D-galactopyranoside

LIST OF GENERIC NAMES

Apigenin	5,7,4'-trihydroxyflavone
Eupalitin	6,7-dimethoxy-3,5,4'-trihydroxyflavone
Eupatin	6,7,4'-trimethoxy-5,3'-dihydroxyflavone
Eupatolitin	6,7-dimethoxy-3,5,3',4'-tetrahydroxyflavone
Galangin	3,5,7-trihydroxyflavone
Gossypetin	3,5,7,8,3',4'-hexahydroxyflavone
Isorhamnetin	3'-methoxy-3,5,7,4'-tetrahydroxyflavone
Kaempferol	3,5,7,4'-tetrahydroxyflavone
Luteolin	5,7,3',4'-tetrahydroxyflavone
Myricetin	3,5,7,3',4',5'-hexahydroxyflavone
Ombuin	7,4'-dimethoxy-3,5,3'-trihydroxyflavone
Patuletin	6-methoxy-3,5,7,3',4'-pentahydroxyflavone
Quercetagetin	3,5,6,7,3',4'-hexahydroxyflavone
Quercetin	3,5,7,3',4'-pentahydroxyflavone
Rhamnetin	7-methoxy-3,5,3',4'-tetrahydroxyflavone
Spinacetin	6,3'-dimethoxy-3,5,7,4'-tetrahydroxyflavone
Tamarixetin	4'-methoxy-3,5,7,3'-tetrahydroxyflavone



Flavonoid numbering system

A. INTRODUCTION

Plants accumulate various natural products in response to environmental stimuli and genetically programmed developmental signals. Of these metabolites, flavonoid compounds are probably the most ubiquitous. The importance of flavonoids in the pigmentation of flowers and protection against uv radiation has been well documented (for review, see Hahlbrock and Scheel 1989). Recently, various flavonoids have been recognized as important stress metabolites that are synthesized in response to microbial attack (Dixon et al. 1983; Darvill and Albersheim 1984; Dixon 1986; Smith and Banks 1986). More recently, a number of flavonoids have been reported to act as signalling molecules for the induction/ inhibition of early nodulation genes in *Rhizobium* (Long 1989), and as regulators of polar auxin transport (Jacobs and Rubery 1988).

Most plants are known to contain the enzyme complement that is involved in the biosynthesis of the basic flavonoid skeleton. On the other hand, the enzymes involved in the modification of flavonoids (*O*-and *C*-methylation, *O*- and *C*-glycosylation, acylation, prenylation and *O*-sulfation) are not evenly distributed, but are mostly restricted to a limited number of species. Most of the latter enzymes have recently been reviewed (Poulton 1981; Hösel 1981; Ebel and Hahlbrock 1982; Heller and Forkmann 1988)

A new class of sulfur compounds, the flavonoid sulfates, has been reported to be of common occurence in a number of plant families, especially the Asteraceae (Harborne 1975; Harborne 1977; Harborne and Williams 1982; Barron et al. 1988). Because of the lack of information on their biosynthesis or the factors regulating their accumulation, the exact role of flavonoid sulfates remains to be elucidated. Apart from their involvment in detoxification of hydroxyl groups, their accumulation in plants growing in saline or marshy habitats seems to suggest a role in the detoxification of excess sulfate. Their solubility in aqueous solution might also facilitate their storage

in hydrophilic cellular compartments as a means of sequestering excess sulfate ions.

In contrast with the extensive studies on the enzymatic sulfation of endogenous metabolites and xenobiotics in animal tissues (for reviews, see Roy 1981; Hobkirk 1985) which led to the purification and characterization of a number of sulfotransferases, almost nothing is known of the sulfation of flavonoids in plants. This situation may be explained by the inherent difficulties in the preparation of specifically substituted flavonoid substrate and reaction products that are required to conduct enzymatic studies, as well as the absence of an adequate assay for flavonoid sulfotransferase. The recent advances in the synthesis of flavonoid sulfates with different substitution patterns (Barron 1987) have rendered possible the study of their enzymatic synthesis. To conduct this study we decided to use *Flaveria bidentis* and *Flaveria chloraefolia* (Asteraceae). Our choice was dictated by the fact that both species are known to accumulate a variety of polysulfated flavonoids (Barron et al. 1988).

In view of the recent discovery of these novel conjugates and the lack of information on their biosynthesis and enzymology, the present study will deal with the following aspects:

1. The phytochemistry and biosynthesis of sulfated flavonoids in *F. bidentis.*

2. The purification and characterization of four novel, position-specific sulfotransferase (STs) that are involved in the biosynthesis of flavonol sulfate esters in *F. chloraefolia* and *F. bidentis.*

3. The purification to homogeneity of *F. chloraefolia* 3-ST and the production and characterization of anti 3-ST antibodies.

4. The isolation and characterization of cDNA clones coding for two flavonoid STs from *F. chloraefolia,* namely the 3- and 4'-STs.

B. REVIEW OF LITERATURE

The aim of this review will be to describe the enzymes which are implicated in flavonol biosynthesis with special emphasis on those which have been purified to homogeneity, fully characterized and/or studied at the molecular level. Our state of knowledge of the biosynthesis of sulfated metabolites will also be reviewed, with reference to the enzymes involved in sulfate activation, and sulfate transfer.

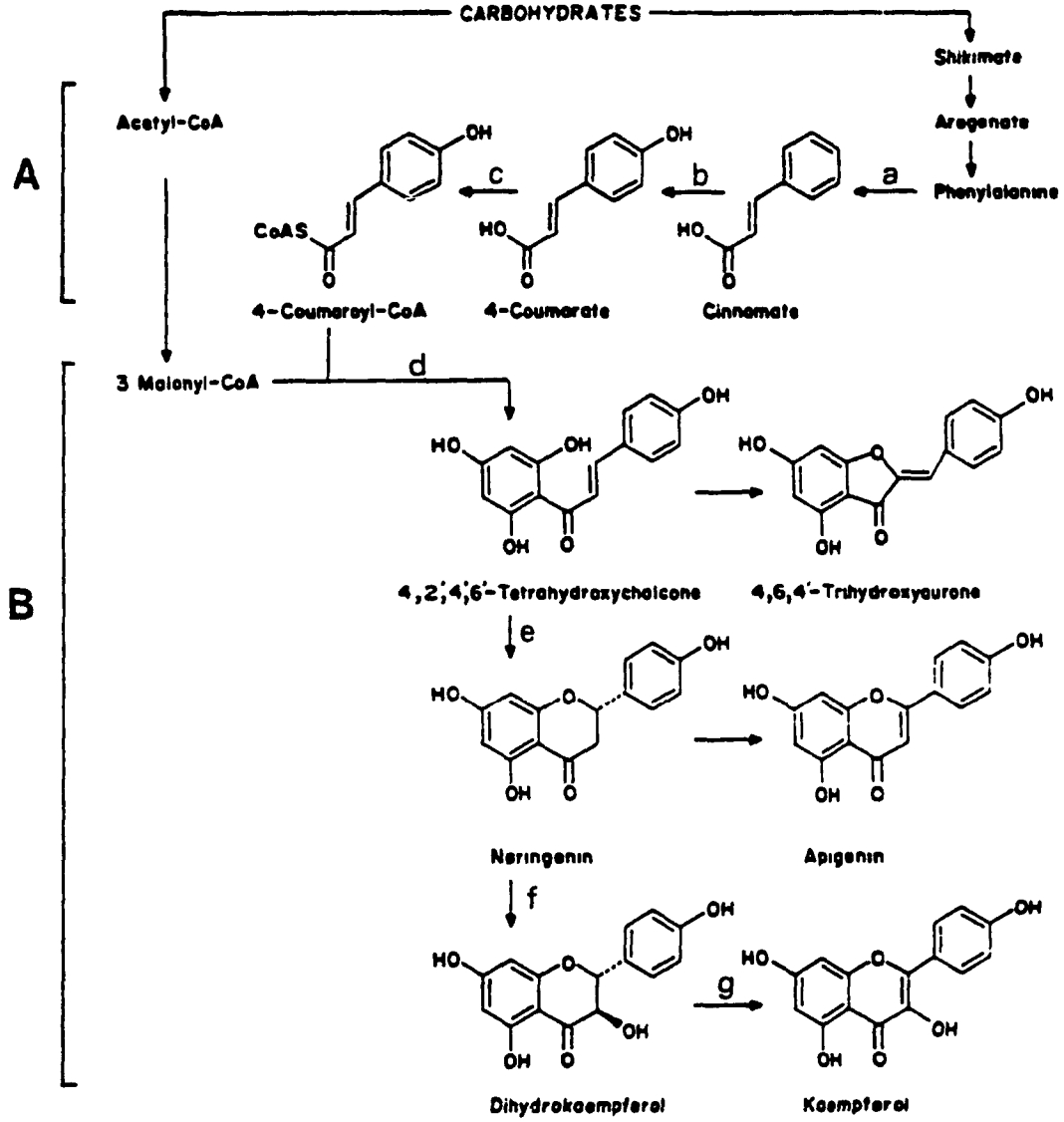
B.1 Biosynthesis of flavonoid compounds

The present review of the enzymes involved in flavonoid biosynthesis will be limited to those involved in the synthesis of flavonol aglycones. A more complete description of the enzymes involved in the biosynthesis of the different flavonoid classes is to be found in the excellent reviews of Hahlbrock (1981), Ebel and Hahlbrock (1982) and Heller and Forkmann (1988).

The flavonoid skeleton is derived from the enzymatic condensation of 3 molecules of malonyl CoA and one molecule of 4- coumaroyl CoA (Scheme 1). Malonyl CoA originates from the carboxylation of acetyl CoA, a reaction catalysed by the enzyme acetyl CoA carboxylase. 4-Coumaroyl CoA is derived from the aromatic amino acid phenylalanine. Its synthesis requires the participation of the first three enzymes of the phenylpropanoid pathway (Scheme 1,A). This pathway generates not only the precursors for flavonoid biosynthesis but also for other phenylpropanoid metabolites such as lignins, and hydroxycinnamoyl derivatives (Gross 1981).

B.1.1 The Phenylpropanoid pathway

The first step in the phenylpropanoid pathway involves the deamination of phenylalanine to *trans*-cinnamic acid (Scheme 1,A,a). This reaction is catalysed by the enzyme phenylalanine ammonia lyase (PAL). Because of its pivotal role between



Scheme 1. Phenylpropanoid and flavonoid pathways
Adapted from Heller and Forkmann 1988

primary and secondary metabolism this enzyme has received considerable attention. PAL has been purified to apparent homogeneity from various plant sources (Hanson and Havir 1981; Kehrel and Wiermann 1985; Belunis and Hrazdina 1988). Furthermore, different forms of this enzyme have been separated from *Phaseolus* (Bolwell et al. 1985; Bell et al. 1986), parsley (Kuhn et al. 1984) and soybean (Grab et al. 1985). PAL has been shown to be induced by light, fungal infection as well as various biotic and abiotic elicitors (Jones 1984).

Trans-cinnamate is then hydroxylated at position 4 by the enzyme cinnamate 4-hydroxylase (Scheme 1,A,b). This enzyme is a cytochrome P-450-dependant mono-oxygenase and has been shown to be associated with the endoplasmic reticulum. The results presented by Fritsch et al. (1986) suggested the presence of different isoenzymes. The cinnamate 4-hydroxylase has never been purified to homogeneity.

Hydroxycinnamic acids are activated by the formation of their CoA ester derivatives. The hydroxycinnamate:CoA ligase (1,A,c) that catalyses the synthesis of the hydroxycinnamate CoA ester utilises ATP as co-substrate and requires Mg^{+2} as cofactor. The enzyme has been purified to homogeneity from various sources (Ebel and Hahlbrock 1982; Luderitz et al. 1982). As is the case for the two previous enzymes, isoenzymes of CoA ligase have been detected in various plant tissues (Ebel and Hahlbrock 1982; Grand et al. 1983)

B.1.2 The flavonoid pathway

Chalcone synthase catalyses the condensation of three acetyl units from malonyl CoA with 4-hydroxycinnamoyl CoA to give naringenin chalcone (Scheme 1,B,d) . Chalcone synthase has been purified from at least 19 angiosperms (for a review, see Stafford 1990) including both dicots and monocots (e.g. Kreuzaler and Hahlbrock 1975; Whitehead and Dixon 1983; Kehrel and Wiermann 1985; Ozeki et al. 1985; Hrazdina et al. 1986; Peters et al. 1988). Two different chalcone synthase activities

have been separated from spinach (Beerhues and Wiermann 1988). They could not be differentiated on the basis of their substrate specificities or general characteristics. Furthermore, their peptide maps indicated considerable homology between the two proteins.

The enzyme chalcone isomerase (Scheme 1,B,e) catalyses the stereospecific formation of (2S)-flavanones from their corresponding chalcones (Heller et al. 1979). The enzyme has been purified to homogeneity from *Phaseolus* (Robbins and Dixon 1984) and soybean (Bednar and Hadcock 1988). Two isoenzymes have been detected in *Petunia* (van Weely et al. 1983).

Hydroxylation of naringenin at position 3 to give rise to a dihydroflavonol (Scheme 1,B,f) is catalysed by the enzyme flavanone 3-hydroxylase (Forkmann et al 1980). It requires the presence of 2-oxoglutarate, Fe^{+2} and ascorbate as cofactors. The enzyme has been purified to apparent homogeneity from *Petunia* flowers (Britsch and Grisebach, 1986; Britsch 1990).

Enzymic conversion of dihydroflavonols to flavonols has first been detected in parsley and is catalysed by the enzyme flavonol synthase (Scheme 1,B,g). It was shown to be a soluble 2-oxoglutarate-dependant dioxygenase (Britsch et al. 1981). Since first reported, its presence has been detected in other plant tissues but it has not been purified to homogeneity (Spribille and Forkmann 1984; Forkmann et al. 1986).

B.1.3 Flavonoid modification

Apart from the hydroxyl groups at positions 5, 7 and 4' which are derived from the assembly of the flavonoid precursors, other positions can be hydroxylated by position specific hydroxylases. Other modifications of the flavonoid skeleton include: *O*-methylation, *C*-/*O*-glycosylation, acylation, prenylation, and *O*-sulphation.

Of the different reactions involving modification of flavonoid compounds, *O*-

methylation and *O*-glycosylation have received most attention. Several position-specific enzymes catalysing both reactions have been characterized. An exhaustive account of the characterized flavonoid methyltransferases and glycosyltransferases, is to be found in the excellent review on the biosynthesis of flavonoids by Heller and Forkmann (1988).

Several enzyme systems have been characterized where the modification of the flavonoid skeleton proceeds via a defined sequential order. Of particular interest is the characterization of the enzymes involved in the sequential methylation, glucosylation and hydroxylation of quercetin to give rise to penta-O-methylquercetagetin glucoside in *Chrysosplenium americanum* (for review, see Ibrahim et al. 1986, 1987). In *Pisum*, the formation of flavonol 3-O-triglucoside was shown to involve three sequential glucosylation reactions, each of which is catalysed by a distinct O-glucosyltransferase (Jourdan and Mansell 1982). Other systems, where highly specific enzymes for a given position and/or substrate are involved, have also been reported (Jonsson et al. 1982, 1984; Teusch 1986; Teusch et al. 1986a,b).

In contrast with the extensive studies on flavonoid methyltransferases and glycosyltransferases, nothing is known of the enzymatic sulfation of flavonoids in plants, except for two preliminary reports on the incorporation of [^{35}S] sodium sulfate into flavonoid sulfates of *Zostera* (Nissen and Benson 1964) and *Flaveria* (Cabrera and Juliani 1982) tissue.

This situation may be explained by the inherent difficulties in the preparation of specifically substituted flavonoid substrates and reaction products that are required to conduct enzymatic studies, as well as the absence of an adequate assay for flavonoid ST activity. The recent advances in the chemical synthesis of flavonoid sulfates with different substitution patterns (Barron 1987) made it possible to acquire a number of authentic compounds for use in enzyme studies.

B.2 Flavonoid sulfates

Although the occurrence of the first flavonol sulfate has been reported as early as 1937 (Kawaguchi and Kim 1937), it was not until the publication of the first review on flavonoid sulfates (Harborne 1975) that they were considered of common occurence in a number of plant families. In a subsequent review on flavonoid sulfates, 250 plant species belonging to 17 dicot and 15 monocot families are reported to accumulate close to 100 flavonoid sulfates (Barron et al. 1988). Most of the known compounds are sulfate esters of common hydroxyflavones and hydroxyflavonols or their methyl ethers, as well as some O-glycosides sulfated on the flavonoid ring or on the sugar moiety. Of particular interest for the present review is the occurence of polysulfated flavonoids as natural constituents. Of twelve polysulfated flavonols identified to date, six were purified from the genus *Flaveria* (Asteraceae) (Barron et al. 1988). *F. bidentis* is the only known species to accumulate a flavonol tetrasulfate (Pereyra de Santiago and Juliani 1972). By comparison, seven polysulfated flavones have been isolated, all of which are disulfate esters. No tri- or tetrasulfated flavones have yet been identified.

B.3 Regulation of flavonoid synthesis

Flavonoid compounds seem to be involved in several functions in plant tissues. They are responsible for the color of flowers and fruits, as well as protection against uv radiation. Although constitutive in members of the Leguminosae (Ingham 1983), many plants synthesize isoflavonoid phytoalexins when challenged by fungal infection or wounding (McClure 1975; Hahlbrock and Grisebach 1979; Hahlbrock 1981; Dixon 1986; Ebel 1986). In addition, various flavones have recently been recognized as extracellular signal molecules involved in the induction of *Rhizobium* nodulation genes when interacting with its legume host (Long 1989). The synthesis and accumulation of many of these classes of flavonoids are both cell type specific and under the

control of developmental cues and/or external stimuli. While considerable studies have recently involved the induction of the expression of genes encoding flavonoid enzymes, little is known concerning the endogenous factors that modulate flavonoid biosynthesis during plant development.

The best studied case of regulation is the photocontrol of flavonoid synthesis (for a review see, Beggs et al. 1986). Hahlbrock and co-workers have demonstrated the photocontrol of flavonoid biosynthesis in parsley cell culture. In this system, the transient increase in the transcription rates of the genes coding for PAL and CHS correlated with the appearance of the enzymes and of the flavonoid products (Hahlbrock and Scheel 1989).

In leguminous plants, isoflavonoids are often formed in response to both biotic and abiotic stress (for a review see, Darvill and Albersheim 1984; Smith and Banks 1986). The most extensively studied system of stress-induced isoflavone phytoalexin biosynthesis is the response to microbial infection. As with the case of light induction, the formation of isoflavonoids correlated with the increase in the mRNAs and the enzymes involved in their biosynthesis (Dixon 1986).

Recent studies of the promoter architecture of the chalcone synthase gene in transgenic tobacco plants have revealed the presence of *cis*-acting elements required to establish temporal and tissue-specific control of flavonoid biosynthesis during development and in reponse to environmental stimuli (Schmid et al. 1990). Among the stimuli tested were wounding, fungal infection and uv irradiation. Glucuronidase gene reporter assay of the activities of mutated promoters in electroporated protoplasts has permitted the identification of a region of the bean CHS promoter required for elicitor function (Dron et al. 1988). In addition, this assay allowed the identification of the *cis*-acting elements involved in uv induction of parsley and *Antirrhinum* CHS promoters (Lipphardt et al. 1988; Schulze-Lefert et al. 1989). Other aspects of the enzymatic regulation of flavonoid biosynthesis which received less attention include:

cellular compartmentation of the flavonoid synthesizing enzymes, their substrates, and the products of their reactions; regulation of enzyme activities by substrate and product inhibition, reversible and irreversible inactivation, and protein degradation; channeling of intermediates through an organized multi-enzyme complex. These aspects have been adequately discussed in a recent monograph (Stafford 1990).

There is an evident lack of knowledge of the role of sulfated flavonoids in plants. Their presence in a number of species inhabiting saline or marshy environments suggests that they might be involved in salt stress adaptation. Other possible roles include the sequestering of sulfate ions, or the modification of flavonoid solubility for possible storage in hydrophilic cellular compartments. Additional research on their tissue distribution and cellular localization, as well as studies on the regulation of the enzymes involved in their biosynthesis should allow a better understanding of their role.

B.3.1 Cloned genes of the phenylpropanoid and flavonoid pathways

Since the regulation of flavonoid synthesis is under developmental and environmental control, it represents an excellent system for the analysis of the molecular mechanisms involved in plant gene expression. The recent advances in the development of gene transfer systems into higher plants made it possible to clone a number of genes coding for enzymes involved in the biosynthesis of flavonoids.

Table 1 lists the cloned genes of the phenylpropanoid and flavonoid pathways. As can be seen, a limited number of genes coding for enzymes involved in the modification of the flavonoid skeleton have been isolated. The improvements in protein purification and microsequencing techniques, as well as the development of cDNA library screening protocols using monospecific antibodies have facilitated the isolation of genes coding for proteins present in very small quantities. We expect to witness, in the near future, an increasing number of reports on the isolation of genes

TABLE. 1 CLONED GENES OF PHENYLPROPANOID AND FLAVONOID PATHWAY[1]

Gene product[2]	Plant source	Reference
PAL	Petroselium hortense	Kuhn et al. 1984
	Petroselium crispum	Scheel et al. 1988
	Phaseolus vulgaris	Edwards et al. 1985
		Cramer et al. 1988
4-CL	Petroselium crispum	Douglas et al. 1987
CHS	Petroselium crispum	Hermann et al. 1988
	Antirrhinum majus	Sommer & Saedler 1986
	Zea mays	Wienand et al. 1986
	Petunia hybrida	Koes et al. 1986, 1987
	Phaseolus vulgaris	Ryder et al. 1987
	Ranunculus acer	Niesbach-Klosgen et al.
	Hordeum vulgare	" 1987
	Magnolia liliflora	"
	Arabidopsis thaliana	Feinbaum & Ausubel 1988
CHI	Phaseolus vulgaris	Mehdy & Lamb 1987
	Petunia hybrida	van Thunen et al. 1988
UFGT	Zea Mays	Fedoroff et al. 1984
		Dooner et al. 1985

[1] Table modified from Mol et al. (1988)

[2] Abbreviations: PAL, phenylalanine ammonia lyase; 4-CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; UFGT, UDP glucose:flavonoid 3-O-glucosyltransferase

coding for other flavonoid modifying enzymes.

B.4 The sulfation reaction

As early as 1977 it has been suggested that the sulfate transfer to the flavonoid hydroxyl group was catalysed by a phenol sulfotransferase utilising 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfate donor, in a manner similar to the extensively studied reaction in animal tissue (Harborne 1977). The following sections will review the enzymes involved in sulfate activation and sulfate transfer. Because of the paucity of reports on sulfotransferases that were characterized from plant tissues, a review of the sulfotransferases from animal tissues is included for comparative purposes.

B.4.1 Sulfate activation

Between 1950 and 1958, the chemical structure and biosynthesis of the cosubstrate for sulfate transfer were elucidated. It was established that the active form of sulfate was PAPS. Robbins and Lipmann (1958) found that PAPS was synthesized by a two step process, catalysed by the enzymes ATP sulfurylase and APS kinase. The two activating reactions are:

$$ATP + SO_4^{2-} \longrightarrow APS + PP_i \qquad (1)$$

$$APS + ATP \longrightarrow PAPS + ADP \qquad (2)$$

The initial step in the biosynthesis of PAPS is the formation of adenosine 5'-phosphosulfate (APS) from ATP and inorganic sulfate (1). The enzyme ATP sulfurylase which catalyses this reaction has been characterized in bacteria (Varma and Nicholas 1971; Akagi and Campbell 1962), yeast (Robbins and Lipmann 1958), filamentous fungi (Tweedie and Segel 1971), rat liver (Burnell and Roy 1978) and from the higher plants, spinach and corn (Shaw and Anderson 1972; Onajobi et al.1973).

The second step in the biosynthesis of PAPS involves the phosphorylation of

APS at the 3'-position, and is catalysed by the enzyme APS kinase (2). This enzyme has been purified from yeast (Robbins and Lipmann 1958), and spinach (Burnell and Anderson 1973).

Both ATP sulfurylase and APS kinase isolated from spinach leaves were found to be associated with the chloroplast fraction and were recovered in the supernatant after sonication, suggesting that the synthesis of PAPS is localized in the chloroplast stroma (Shaw and Anderson 1972; Burnell and Anderson 1973).

B.4.2 Sulfotransferases of plant tissues

If we exclude the APS- and PAPS-dependant STs which use thiols as acceptors and are involved in assimilatory sulfate reduction, only two reports have recently appeared on the isolation of STs involved in the biosynthesis of plant secondary metabolites.

Glendening and Poulton (1988) demonstrated the presence of a sulfotransferase activity catalysing the sulfation of desulfobenzylglucosinolate to benzylglucosinolate by a cell-free extract of cress (*Lepidium sativum*) seedlings. The enzyme used PAPS as a sulfate donor, with Km values of 0.23 and 1 millimolar for the sulfate acceptor and donor, respectively. The optimum ST activity was at pH 9.0, was activated by magnesium and manganese ions and was inhibited by SH-group reagents.

Another ST involved in glucosinolate biosynthesis was partially purified from *Brassica juncea* (Jain et al. 1990). This enzyme exhibited maximum activity at pH 8.5-9.0, had no requirement for divalent cations and was inhibited by SH-group reagents. The enzyme had an apparent pI of 4.84 and a molecular weight of 44,000D. The Km values for desulphobenzylglucosinolate and PAPS were 2.3 and 0.78 μM, respectively. The higher degree of purification of the *Brassica* ST (200-fold) might explain the lower apparent Km for both substrates when compared with the cress enzyme.

B.4.3 Sulfotransferases of animal tissues

A considerable number of STs have been characterized with different specificities towards phenols, arylamines, hydroxysteroids, estrone and bile salts. In animal tissues, two major biosynthetic routes result in the modification of the reactive hydroxyl groups: glucuronidation and sulfation (Jakoby et al. 1980). As a detoxification process, sulfation facilitates metabolite excretion by the formation of highly ionized organic sulfates that are more soluble in aqueous solution. However, the role of animal STs should not only be restricted to excretion processes. The involvment of steroid, glucosaminoglycans, sulfolipids, and protein-tyrosine STs in metabolic pathways has been demonstrated. Although the study of animal STs has received considerable attention their broad, and often overlapping, substrate specificities have rendered the evaluation of their number as well as their classification a difficult task.

In the following section, the enzymes which have been purified to homogeneity and fully characterized will be presented. Table 2 summarizes the major characteristics of the purified animal STs. Some of the enzymes listed with specificity for one substrate might, in fact, exhibit broader substrate specificity. For example, it has been proposed by Barnes et al. (1989) that the Bile acid ST I may be the same as the hydroxysteroid II that was previously purified by Lyon and Jakoby (1980).

Most of these enzymes have been purified by conventional purification techniques. In only few cases, the use of affinity supports was employed to improve the purification. Except for the rat liver phenol ST purified by Borchardt and Schasteen (1982), which has a molecular weight of 70,000D, the molecular weight of the denatured enzymes varies between 28,000 and 38,000D. In most cases, the native enzymes seem to exist as dimers or even multimers. The pH optima were also variable, ranging from 5.5 to 9.0, with one enzyme exhibiting two pH optima at 5.5 and 7.5 (Lyon and Jakoby 1980). Most STs were activated by magnesium ions, but in very few cases was the presence of divalent cations required for ST activity. When tested,

Table 2. Sulfotransferases from animal tissues

Source of enzyme	Classification	Substrate	pH Optimum	pI	Effect of cations	Effect of SH-group reagents	Mol Wt native (Dalton)	Mol Wt SDS (Dalton)	Km PAPS (μM)	Km Sulfate acceptor (μM)	Reference
Human liver	Phenol-ST	p-nitrophenol	7.5	n.d.	activated by Mg	inhibited by NEM	68,000	32,000	2.0	4.0	Falany et al. 1990
Rat liver	Phenol-ST I	ß-naphtol	6.5	8.05	n.d.	n.d.	65,000	35,000	6.5	60.0	Sekura and Jakoby 1979
Rat liver	Phenol-ST II	ß-naphtol	6.5	n.d.	n.d	n.d.	65,000	35,000	12.0	90.0	Sekura and Jakoby 1979
Rat liver	Phenol-ST III	N-OH-acetyl-aminofluo-rene	6.3	5.66	activated by Mg	inhibited by NEM	68,000	38,000	n.d.	n.d	Wu and Straub 1976
Rat liver	Phenol-ST	p-nitrophenol	5.5-6.4	n.d.	n.d.	n.d.	70,000	70,000	2.5	3.6	Borchart and Schasteen 1982
Human brain	M-pST	dopamine	7.0	n.d	none	n.d.	250,000	n.d.	0.35	2.9	Whittemore et al. 1985
Rat liver	Hydroxysteroid ST I	dehydroepi-androsterone	5.5-6.1	7.85	n.d.	n.d.	290,000	32,000	47.0	24.0	Marcus et al. 1980
Rat liver	Hydroxysteroid ST II	dehydroepi-androsterone	5.5, 7.5	5.0	activated by Mg	n.d.	180,000	28,000	12.0	4.0	Lyon and Jakoby 1980
Human liver	Hydroxysteroid ST	dehydroepi-androsterone	7.5	n.d.	activated by Mg	n.d.	68,000	35,000	1.6	1.6	Falany et al. 1989
Human adrenal	Hydroxysteroid ST	dehydroepi-androsterone	7.5	n.d.	activated by Mg	inhibited by PCMB	68,000	34,500	n.d.	n.d.	Adams and McDonald 1979
Rat liver	Bile acid ST	taurо-lithocholate	5.5-7.5	5.3	activated by Mg	n d.	30,000 60,000	30,000	43.0	0.24	Barnes et al. 1989

Table 2. Sulfotransferases from animal tissues (continued)

Source of enzyme	Classification	Substrate	pH Optimum	pI	Effect of cations	Effect of SH-group reagents	Mol Wt native (Dalton)	Mol Wt SDS (Dalton)	Km PAPS (μM)	Km Sulfate acceptor (μM)	Reference
Human liver	Bile acid ST	glyco-lithocholate	6.5	5.2	activated by Mg	inhibited PCMB	67,000	n.d.	0.7	2.0	Chen and Segel 1985
Rat liver	Bile acid ST + phenolic steroid ST	tauro-lithocholate	7.0	5.6	activated by Mg	n.d.	60,000 - 70,000	30,000 - 35,000	n.d.	n.d.	Takikawa et al. 1986
Rat kidney	Bile acid ST	tauro-lithocholate	7.0	5.8	required	inhibited PCMB	80,000	n.d.	2.0	40.0	Chen et al. 1978
Bovine placenta	Phenolic steroid ST	estrone	n.d	n.d.	n.d.	n.d	70,000	36,000	n.d.	n.d	Moore et al. 1988
Porcine endometrium	Phenolic steroid ST	estrone	7.6 - 7.8	6.1	required	n.d.	31,000	31,000	24.0	24.0	Brooks et al. 1987
Rat liver	Glucocorticoid ST	gluco-corticoid	6.0	n.d	activated by Mg	inhibited PCMB	68,000	n.d.	6.78	6.48	Singer and Bruns 1980

the STs were found to be sensitive to the presence of SH- group reagents suggesting the involvment of a sulfhydryl group in catalysis.

A novel type of sulfotransferase has recently been purified from a human intestinal bacteria (Kobashi et al. 1987). This enzyme transferred the sulfate group from phenol sulfate esters, rather than PAPS, to other phenols. This enzyme is of interest for this review because polyphenols such as chalcones, xanthones and other flavonoids were found to be sulfated by the bacterial enzyme (Koizumi et al. 1990). In presence of an excess of the sulfate donor, quercetin was sulfated to its 3,3'-di- and 3,7,3'-trisulfate derivatives. Under limiting concentration of the sulfate donor only the 3,3'-disulfate was formed, suggesting a sequential sulfation where the 7- position is the last one to be sulfated. Although this work was carried out with a partially purified preparation, it will be interesting to see if the sulfation at different positions is catalysed by the same enzyme or by enzymes with different position specificities.

B.4.4 Cloned genes of animal STs.

The cDNA of two rat liver hydroxysteroid STs (STa and ST 40) and of the bovine placenta oestrogen ST have been cloned (Ogura et al. 1989,1990; Nash et al. 1988). The deduced amino acid sequence of the two rat liver enzymes consists of 284 amino acids, whereas that of the oestrogen ST was found to code for a protein containing 295 amino acids. Ninety-four percent homology was found between the nucleotide sequences of the two hydroxysteroid ST clones suggesting that they might code for isoenzymes. The deduced amino acid sequence of the STa cDNA clone has 73.7% sequence identity with that of a rat liver senescence marker protein (SMP 2) (Ogura et al. 1990). SMP 2 is a rat liver protein whose synthesis is regulated by both androgens and aging (Chatterjee et al. 1987). No sequence comparison of the hydroxysteroid ST clones with the oestrogen ST clone was reported.

C. MATERIAL AND METHODS

C.1 Biologicals and chemicals

C.1.1 Plant material

Seeds of *Flaveria chloraefolia* A. Gray (Asteraceae) were kindly provided by Dr. A. M. Powell, Sul Ross State University, Alpine, Texas. Seeds of *Flaveria bidentis* var angustifolia O.K. (Asteraceae) were obtained from Dr. H.R. Juliani, University of Cordoba, Argentina. Seeds of both species were germinated in vermiculite on top of potting soil, and their growth was maintained under greenhouse conditions. Both species (Fig. 1) were further propagated from cuttings.

Our choice of *F. bidentis* and *F. chloraefolia* was dictated by the fact that both species accumulate a variety of polysulfated flavonoids (Table 3). *F. chloraefolia* accumulates mono- and disulfates of quercetin and patuletin, whereas *F. bidentis* accumulates quercetin mono- to tetrasulfates. An interesting difference between the two species is the absence in *F. chloraefolia* of sulfation at position 7, while several flavonoid sulfates in *F. bidentis* are sulfated at this position.

C.1.2 Bacterial strains

E. coli BB4: supF58, supE44,hsdR514 (r_k^-, m_k^-), galK2, galT22, trpR55, metB1, tonA, lambda$^-$, Δ(arg-lac) U169 [f', proAB, $lacI^qZ\Delta M15$, Tn10(tet^r)].

E. coli XL1-blue: endA1, hsdR17 (r_k^-, m_k^-), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, (lac-), [f', proAB, $lacI^q$ ZΔM15, Tn10(tet^r)].

pSTH1: *E. coli* Xl1-blue harboring pBluescript containing the alcohol dehydrogenase cDNA from potato (Marineau et al. 1987).

C.1.3 Phages

lambda ZAP: sbhI 1 chiA131 , T amp ColE1 ori lacZ' T3 promoter-polycloning site- t7 promoter I> srI 3 clts857,srI 4,nin5,srI 5,Sam100

Figure 1. Photograph of Flaveria bidentis (A) and Flaveria chloraefolia (B)

TABLE 3

The flavonol sulfates of

Flaveria bidentis and Flaveria chloraefolia

Flaveria bidentis	Flaveria chloraefolia
Quercetin 3-sulfate	Quercetin 3-sulfate
Isorhamnetin 3-sulfate	Patuletin 3-sulfate
Quercetin 3,4'-disulfate	Ombuin 3-sulfate
Quercetin 3,7-disulfate	Eupalitin 3-sulfate
Isorhamnetin 3,7-disulfate	Spinacetin 3-sulfate
Quercetin 3,7,3'-trisulfate	Eupatolitin 3-sulfate
Quercetin 3,7,4'-trisulfate	Eupatin 3-sulfate
Quercetin 3-acetyl-7,3',4'-trisulfate	6-methoxykaempferol 3-sulfate
Quercetin 3,7,3',4'-tetrasulfate	Quercetin 3,3'-disulfate
	Patuletin 3,3'-disulfate
	Quercetin 3,4'-disulfate

C.1.4 Chemicals

3'-Phosphoadenosine 5'-phospho-[^{35}S]sulfate and adenosine 5'-phospho-[^{35}S]sulfate were purchased from New England Nuclear (Boston, MA). ATP[^{35}S] was from Amersham (Mississauga, Ontario). Sephadex G-10, G-25, and G-100, Sephacryl S-200, Sepharose CL-4B, Superose 12 HR 10/30, Mono P HR 5/20, Mono Q HR 5/5 columns; Polybuffer 74, T7 DNA polymerase, T4 DNA polymerase, *E. coli* Polymerase 1, T4 DNA ligase, EcoRI methylase and Eco R1; the FPLC system; the PhastSystem, pre-cast SDS-PAGE gels, and protein silver stain kit were from Pharmacia (Uppsala, Sweden). Lambda ZAP, picoBLUE immunodetection kit, and Gigapack packaging kit were purchased from Stratagene (San Diego, CA). The cDNA library was constructed using the cDNA synthesis kit from Bethesda Research laboratories (Gaithersburg, MD). Unlabelled PAPS, PAP, and PAP-agarose were obtained from Sigma chemicals (St. Louis, MO). Dowex 1X2, and protein dye reagent were purchased from Bio-Rad Labs (Richmond, CA). Flavonoid aglycones were purchased from Roth (Karlsruhe, FRG) or Extrasynthese (Bordeaux, France) and were further purified by TLC or HPLC. Quercetin 3-acetyl- 7,3',4'- trisulfate was a generous gift from Prof. H.R. Juliani, (Cordoba, Argentina). All the other flavonoid esters used in this study were from our laboratory collection. These compounds were synthesized by previously described methods (Barron and Ibrahim 1987a, 1988a, 1988b) and their identity was confirmed by spectroscopic methods. All other chemicals were of analytical grade.

C.2 Phytochemistry and biosynthesis

C.2.1 Flavonoid extraction and purification

Fresh leaves, taken from 3-4 month old plants, were frozen in liquid N_2, ground to a fine powder, and extracted three times with 50% aqueous MeOH at room temperature. The combined extracts were concentrated under reduced pressure at

30°C . Partial purification of sulfated flavonoids was carried out on a Sephadex G-10 column using water as eluent. Eluted fractions were analyzed by chromatography on cellulose TLC plates using *n*-BuOH-HOAc-H_2O (6:2:2,v/v/v) as solvent system, followed by visualization in uv light. The flavonoid-containing fractions were combined, concentrated and chromatographed on semi-preparative TLC plates using the same solvent system. Bands of individual flavonoids were eluted in 50% aqueous MeOH, concentrated and further purified by HPLC using a MicroBondapak C_{18} column (particle size, 10 μm). Separation was achieved according to Harborne (1984) after some modification. Isocratic elution with 60% solvent A (10mM aqueous TBADP) and 40% solvent B (MeOH-HOAc-H_2O, 18:1:1, v/v/v) was used for 10 min, proceeding to 50% A and 50% B in 40 min and finally to 40% A and 60% B in 10 min at a flow rate of 1ml/min.

C.2.2 Flavonoid identification

Identification of individual flavonoids was carried out by determining their uv absorption spectra, using spectral shift reagents (Markham 1982), co-chromatography with reference compounds (when available) on cellulose and polyamide TLC plates, using *n*-BuOH-HOAc-H_2O (6:2:2 v/v/v), 15% aqueous HOAc and MeOH-H_2O (3:2) as solvent systems and visualization under uv light (360 nm). Identification of the aglycone moieties of different flavonoids was carried out after hydrolysis with 2N HCl at 95°C for 10 min, followed by uv spectral analysis and co-chromatography with reference compounds. Hydrolysis with arylsulfatase was used to detect the presence of sulfate groups esterified with flavonoid compounds. Acid or enzyme hydrolysates were co-chromatographed with reference compounds on Polyamide DC 6.6 TLC plates, using benzene-methyl ethyl ketone-MeOH (8:1:1 and 6:2:2) as solvent systems. Estimation of the electrophoretic mobility of the individual flavonoids was performed on Whatman No. 3 chromatographic paper in a formic acid- acetic acid- water

(43:147:1820 v/v/v) mixture, pH 2.2 at 250 Volts and 8 mA for 3 hours.

C.2.3 Flavonoid content of different organs

Aqueous methanolic extracts (1:1, v/v) of buds, stems or leaves were analyzed for their total flavonoid content by measuring their absorbance at 340 nm, and for their flavonoid composition by HPLC. Individual peaks were integrated and the quantity of each flavonoid compound was calculated from total absorbance values, using an average molar extinction coefficient of 20,000.

C.2.4 Isotopic experiments

Excised organs, or leaf disks secured from the first pair of expanded leaves of 3 week-old seedlings, were incubated with the labelled compounds in the presence or absence of non-labelled precursors, in the light (ca. 250 f.c.). At the end of the metabolic period, the tissue was thoroughly rinsed with water and homogenized in 50% aqueous methanol. Aliquots of the methanolic extracts were counted for total radioactivity by liquid scintillation. The remainder of the extracts were chromatographed on cellulose TLC plates, then autoradiographed. Individual flavonoids were scraped off the plate, mixed with Cab-O-Sil (colloidal silica) and counted for radioactivity.

C.3 Enzymology

C.3.1 Buffers

The following buffers were used: A, 200 mM Tris-HCl (pH 7.5) containing 10 mM DTT, 5mM EDTA and 10 mM diethylammonium diethyldithiocarbamate; B, 50 mM Tris-HCl (pH 7.5); C, 25 mM *bis*-Tris-HCl (pH 6.5); D, 25 mM *bis*-Tris-iminodiacetate (pH 7.1); E, Polybuffer 74 (1:10 dilution)-iminodiacetate (pH 4.8); F, 200 mM Tris-HCl (pH 7.5); G, 0.2 M phosphate (pH 7.5); H, 20 mM phosphate (pH 6.8). Buffers B to H contained 14 mM 2-mercaptoethanol. Other buffers: TBS, 25

mM Tris-HCl (pH 7.5), 150 mM NaCl; TTBS, 0.5% Tween 20 in TBS; PBS, 100 mM phosphate (pH 7.5) and 150 mM NaCl; TPBS, 0.5% Tween 20 in PBS.

C.3.2 Characterization of 3-, 3'-, and 4'-STs from *F. chloraefolia*

C.3.2.1 Protein extraction

Unless otherwise stated, terminal buds and the first pair of expanded leaves were used for enzyme extraction. All steps were performed at 4°C. The plant tissue (ca. 50 g) was mixed with PVPP (5:1, w/w) and homogenized in a Waring blender for one minute at full speed in ice cold buffer A (1:3, w/v). The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 27000g for 20 min. The supernatant was stirred with Dowex 1X2 (5%, w/v) which had previously been equilibrated in the same buffer, then filtered through glass wool. The filtrate was fractionated with solid ammonium sulfate, and the protein which precipitated between 35 and 75% salt saturation was collected by centrifugation.

C.3.2.2 Chromatography on Sephacryl S-200

The protein pellet was suspended in 20 mL of buffer B and chromatographed on a Sephacryl S-200 column (3 X 65 cm) preequilibrated with the same buffer. The column was developed with buffer B, and 3-ml fractions were collected for ST assay using quercetin and quercetin 3-sulfate as substrates. The active fractions were pooled and concentrated by ultrafiltration using an Amicon cell equipped with a PM 10 membrane.

C.3.2.3 Chromatography on PAP-agarose

The concentrated active fractions recovered from the G-100 column were then chromatographed on a PD-10 column preequilibrated with buffer C. The eluted protein was applied onto a PAP-agarose column (1.5 X 12 cm) that was preequilibrated

with buffer C, and washed with three column volumes of the same buffer. The bound proteins were eluted with a linear salt gradient of 0.0 to 1M NaCl in buffer C. Fractions of one ml each were collected and assayed for ST activity using quercetin and quercetin 3-sulfate as substrates. The active fractions were pooled and concentrated by ultrafiltration as in step C.3.2.2.

C.3.2.4 Chromatofocusing on Mono P

The ST proteins previously purified on PAP-agarose were desalted by gel filtration on a PD-10 column preequilibrated with buffer D. The desalted proteins were chromatographed on a Mono P column (HR 5/20) that was previously equilibrated with buffer D. The bound proteins were eluted with buffer E at a flow rate of 0.4 ml/min, which generated a gradient between pH 7.0 and 4.8. Fractions of one ml each were collected in 0.3 ml of buffer F to restore enzyme activity. The individual fractions were assayed for ST activity against both quercetin and quercetin 3-sulfate.

C.3.3 Characterization of 7-ST from *F. bidentis*

C.3.3.1 Preparation of crude extract

The extraction procedure described in section C.3.2.1 was used with the following modifications. The plant material (ca. 25g) was frozen in liquid N_2, mixed with polyclar AT (10% w/w), and ground to a fine powder. The latter was homogenized with buffer A adjusted to pH 8.0.

C.3.3.2 Chromatography on PAP-agarose

The protein which precipitated between 35 and 75% salt saturation was desalted by chromatography through a PD-10 column preequilibrated with buffer C. The desalted proteins were chromatographed on a PAP-εgarose column (1.5 X 12 cm) that was preequilibrated with buffer C and washed with three column volumes. The

bound proteins were eluted with a 100 ml linear salt gradient of 0.0 to 0.7M NaCl in buffer C. Fractions of one 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 pooled and concentrated by ultrafiltration as in step C.3.2.2.

C.3.3.3 Chromatofocusing on Mono P

The PAP-agarose purified proteins were desalted on a PD-10 column preequilibrated with buffer D. The desalted proteins were chromatographed on a Mono P column (HR 5/20) that was preequilibrated with buffer D. The conditions used in this purification step were the same as described in section C.3.2.4, except that one ml fractions were collected in 0.3 ml of buffer G to restore enzyme activity. The individual fractions were assayed with quercetin 3,3'-disulfate as substrate.

C.3.4 Purification of the 3-ST of *F. chloraefolia*

C.3.4.1 Preparation of crude extract

Crude protein extract was prepared as described in step C.3.2.1, but using 200 g of plant material.

C.3.4.2 Chromatography on Sephadex G-100

The protein pellet was suspended in 40 mL of buffer B and chromatographed on a Sephadex G-100 column (5 X 120cm) that was preequilibrated with the same buffer. The column was developed with buffer B, and 5-ml fractions were collected for ST assay using quercetin as substrate.

C.3.4.3 Chromatography on DEAE-sephacel

The pooled fractions from the G-100 column were applied on a DEAE-sephacel column preequilibrated with buffer B. The column was washed with 5 volumes of the

same buffer, and the bound 3-ST was eluted with a linear salt gradient (300 ml) of 0.0 to 0.3 M NaCl in buffer B. Fractions of 3 ml each were collected and assayed for 3-ST activity using quercetin as substrate.

C.3.4.4 Chromatography on hydroxyapatite

The DEAE-sephacel purified 3-ST was chromatographed on hydroxyapatite column that was preequilibrated in buffer H and washed with 5 bed volumes of the same buffer. The column was developed with a linear gradient (300 ml) of 20 to 200 mM phosphate in buffer H. Fractions of 3 ml each were collected and assayed for 3-ST activity against quercetin. The active fractions were pooled and concentrated as in section C.3.2.2

C.3.4.5 Chromatography on PAP-agarose

The hydroxyapatite purified 3-ST was chromatographed on PAP-agarose as described in section C.3.2.3. The purified protein was then concentrated and desalted in buffer B using the Amicon ultrafiltration cell in order to minimize protein loss.

C.3.4.6 Chromatography on Mono Q

Further purification of the 3-ST enzyme protein was achieved by high performance ion-exchange chromatography on Mono Q HR 5/5 column (5 X 50 mm I.D.). The protein was applied to the column that was preequilibrated in buffer B and washed with 5 column volumes of the same buffer. The 3-ST enzyme activity was eluted using a linear salt gradient (30 ml) of 0.0 to 0.2 M NaCl in buffer B, at a flow rate of 0.5 ml/min. Fractions of one ml each were collected and assayed for 3-ST activity using quercetin as substrate. Other substrates were also assayed in order to verify the purity of the enzyme preparation.

C.3.5 Sulfotransferase assay and identification of reaction products.

The standard enzyme assay contained 0.1 nmol of the flavonoid substrate, 0.1 nmol [^{35}S] PAPS (containing 220,000 dpm) and up to 60 μg protein in a final volume of 100 μl. The reaction was started by addition of the enzyme protein, and was incubated at 30°C for 10 min. The reaction was stopped by the successive addition of 20 μl 2.5% acetic acid and 20 μl 0.1 M TBADP. The mixture was extracted with 250 μl of ethyl acetate and the organic layer was separated by centrifugation. An aliquot (ca. 100 μl) of the ethyl acetate extract was counted for radioactivity in a toluene-based liquid scintillation fluid for the determination of total sulfating activity. The remaining organic layer was concentrated in a stream of N_2 and used for identification of the reaction products by co-chromatography with reference compounds. TLC was carried out on Avicel cellulose using H_2O, or *n*-BuOH-HOAc-H_2O (3:1:1 or 4:1:5, v/v/v) as solvents. Developed chromatograms were visualized in uv light (360 nm) and then autoradiographed on X-ray film.

C.3.6 Definition of enzyme units

Enzyme units are expressed in Katals as recommended by the International Union of Biochemists (NC-IUB 1979). One Katal is defined as the amount of enzyme that catalyzes the conversion of one mole of substrate per second under the assay conditions.

C.3.7 Protein determination

Protein was measured by the method of Bradford (1976) using the dye reagent concentrate and the assay procedure from Bio-Rad. Bovine serum albumin was used as the standard protein.

C.3.8 Molecular weight determination

The molecular weight of the different enzymes was estimated by gel filtration on Superose 12 using an FPLC system. The column was equilibrated in 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl and 10 mM 2-mercaptoethanol. The reference proteins used to calibrate the column were bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), chymotrypsinogen A (M_r 25,000), and ribonuclease A (M_r 13,700). The void volume was measured by the elution of a sample of blue dextran 2000. The reference and sample proteins were applied individualy in a volume of 0.5 ml using the manual V-7 injector of the Pharmacia FPLC system. The proteins were eluted at a flow rate of 0.2 ml/min. A linear relationship between elution volume and log of the molecular weight of reference proteins was observed. The K_{av} of each ST was calculated using the formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where V_t = total volume of the column, V_o = void volume, and V_e = elution volume.

C.3.9 SDS-Polyacrylamide gel electrophoresis

Protein extracts at different stages of purification were analysed by gel electrophoresis under denaturing conditions according to the method of Laemmli (1970) using 12% acrylamide gels. The molecular weight of the polypeptide present in the purified enzyme fraction was determined from a graph of migration distance versus log of molecular weight of standard polypeptides. Alternatively, the proteins were electrophoresed on 12% acrylamide gel using the PhastSystem (Pharmacia) according to the manufacturer's instructions. The proteins were visualized by Coomassie

blue or silver staining.

C.3.10 Native-Polyacrylamide gel electrophoresis

Protein extracts were analysed by gel electrophoresis under non denaturing conditions according to the method of Davis (1964) using 7% acrylamide gels. Following electrophoresis, the gel was sliced in 2 mm bands. The proteins were eluted from the gel slices by incubation in 0.2 M *bis*-Tris (pH 6.5) containing 5 mM DTT and assayed for ST activity using the standard assay. Alternatively, the proteins were visualized by Coomassie blue staining.

C.3.11 Amino acid sequence analysis

Reduction and alkylation of sulfotransferase were performed as described by Andrews and Dixon (1987). Trypsin digestion was performed in 0.1M ammonium bicarbonate (pH 8.2) containing 0.1M calcium chloride for 16 hours at room temperature with an enzyme to substrate ratio of 1:100. The peptides generated by trypsin cleavage were isolated by HPLC using a Vydac C_{18} column (2.1 X 150mm) and eluted with a linear 0-60% acetonitrile-*iso*-propanol 1:1 (v/v) gradient in 0.1% trifluoroacetic acid at a flow rate of 150 μl/min. The eluted peptides were detected at 210, 277, and 292 nm. Peptide sequences were determined by automated Edman degradation with an Applied Biosystems 477A gas-phase sequencer. Phenylthiohydantoin derivatives of amino acids were identified using an Applied Biosystems 120A PTH analyzer.

C.4 Immunological methods

C.4.1 Antibody production

New Zealand rabbits were immunized according to the following schedule: 80 μg of Mono Q purified 3-ST in complete Freund's adjuvant (1:1, v/v) was injected subcutaneously at day 0; 80 μg of similar aliquots in incomplete Freund's adjuvant

(1:1, v/v) were administered subcutaneously at days 21, 42, and 63. The rabbits were bled from the marginal ear vein before immunization (pre-immune serum), and prior to injections, at days 21, 42, and 63. Sera were separated from red blood cells by clotting red cells overnight at 4°C, followed by centrifugation at 10,000g in an IEC clinical centrifuge. The serum titer was verified by ELISA. At day 87, the rabbits were anaesthesized and their blood collected by cardiac puncture.

C.4.2 Immunoblotting

After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes according to the Bio-Rad Mini Trans-Blot apparatus protocol. Briefly, after completion of electrophoresis the gels were equilibrated for 5 minutes in transfer buffer (25 mM Tris and 192 mM glycine in 20% MeOH, pH 8.3), then the transblotting sandwich was assembled and the proteins electrotransferred to the membranes at 90 V for 60 min. Alternatively, the proteins were transferred to Immobilon membranes according to the method of Matsudaira (1987) using 10 mM CAPS-NaOH in 10% methanol (pH 11) as transfer buffer. The proteins were electrotransferred at 90 V for 15 min. Blots were developed with either anti-3-ST (1:2000), or non immune serum (1:2000) as primary antibody, and with alkaline phosphatase conjugated anti-rabbit antibody as secondary antibody, and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as substrate. Blotting was carried out using the Bio-Rad immunodetection kit. Blots were stained for total protein by incubation in 0.1% amido black in 10% methanol and 2% acetic acid.

C.4.3 ELISA

The antigen was added to individual wells (50 μl/ well) of Immulon II round bottomed EIA plates at a dilution of 1 μg/ml in carbonate buffer (pH 9.4). The plates were incubated overnight at 4°C. Unadsorbed proteins were removed by washing

with PBS buffer, and the remaining sites were blocked with 3% BSA in PBS. Serial dilutions of the anti-3-ST serum were then added to the wells and incubated for 1 hour at room temperature. The plates were then subjected to 3 washes with TPBS. Goat anti-rabbit heavy chain antibodies coupled to peroxidase were added at the recommended dilution. After one hour incubation at room temperature, the plates were washed 3 times with TPBS. Freshly prepared enzyme substrate (100 μl) was added to each well. This consisted of 400 μg/ml *o*-phenylenediamine and 40 μl/100ml 30% H_2O_2 in phosphate-citrate buffer (pH 5.0). The reaction was stopped by the addition of 25 μl of 2.5 M H_2SO_4. Absorbance was read at 492 nm using a Bio-Tek Industries ELISA plate reader.

C.5 Molecular cloning

C.5.1 Buffers

TE: 10 mM Tris-HCl (pH 7.5), 1mM EDTA

TAE: 40 mM Tris-acetate (pH 7.8), 5 mM NaOAc, 1 mM EDTA

SM: 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM $MgSO_4$, 0.05% Gelatin

C.5.2 Growth media for bacteria and bacteriophages

LB: (per liter), Bacto-tryptone 10 g, NaCl 5 g, Bacto yeast extract 10 g,

for plates: Bacto-agar: 15 g

NZY: (per liter),Nz amine 10 g, NaCl 5 g, Bacto yeast extract 5 g,

Casamino acids 1 g, $MgSO_4.7H_2O$ 2 g,

For plates: Bacto-agar 15 g

For top agar: Agarose 6.5 g

TB-MM: (per 490 ml), Bacto tryptone 5 g, NaCl 2.5 g

Autoclave and add 5 ml of sterile 1 M $MgSO_4$ plus 5 ml of sterile 20% maltose.

2xYT: (per liter), Bacto tryptone 16 g, Bacto yeast extract 10 g, NaCl 5 g

C.5.3 Construction of *F. chloraefolia* cDNA library.

C.5.3.1 Extraction of total RNA

Total RNA was extracted by the method of Logemann et al. (1987). Briefly, the plant material was frozen in liquid nitrogen, and reduced to a fine powder. The latter was homogenized in buffer containing 8 M guanidine-HCl, 20 mM Mes, 20 mM EDTA and 50 mM 2-mercaptoethanol, pH 7.0. The buffer extract was centrifuged (12,000g) at 4°C for 10 minutes and the supernatant was filtered through miracloth. One volume of a mixture of phenol-chloroform-*iso*-amyl alcohol (24:23:1, v/v/v) was added to the filtrate. Following centrifugation at 12,000g for 15 minutes, the RNA containing aqueous phase was collected and mixed with precooled 0.7 vol of ethanol and 0.2 volume of 0.1M acetic acid. The precipitated RNAs were pelleted at 12,000g for 10 minutes and washed twice with sterile 3M sodium acetate, pH 5.2. Finally, the salt was removed by a wash with 70% ethanol and the RNAs were dissolved in sterile H_2O.

C.5.3.2 Purification of polyA$^+$ mRNAs

Poly(A)$^+$ mRNAs were isolated by chromatography on oligo dT-cellulose according to a previously described method (Aviv and Leder 1972).

C.5.3.3 cDNA synthesis

Double stranded cDNAs were prepared according to the procedure of Gubler and Hoffman (1983) using the BRL cDNA synthesis system. Briefly, the first strand of cDNA was synthesized from 5 μg of PolyA$^+$ mRNA incubated with an oligo(dT) primer and murine reverse transcriptase in presence of dNTPs. The second strand of cDNA was synthesized by DNA polymerase I in presence of RNAse H. The

RNAse H produces nicks in the mRNA strand of the hybrid DNA:RNA creating several RNA primers that are used by the *E. coli* DNA polymerase I for the synthesis of the second strand. After a standard Klenow reaction (Maniatis et al. 1989), the double stranded cDNA was methylated with Eco R1 methylase, to protect the cDNA internal Eco R1 sites. Following ligation with EcoR1 linkers, the EcoR1 digested cDNA was fractionated by gel filtration through a Sepharose CL-4B column to remove the remnants of the digested linkers and to eliminate cDNA molecules smaller than 500 base pair in length. The cDNA was ligated to the EcoR1 cut and phosphatased arms of the expression vector lambda Zap. The ligated lambda DNA was packaged using the Gigapack packaging kit and the titer of the library was determined.

C.5.3.4 *E. coli* BB4 infection

BB4 cells were grown overnight in TB-MM supplemented with 10 μg/ml tetracycline. After centrifugation at 1000 g for 10 min, the BB4 cells were gently resuspended in 10 mM $MgSO_4$ to a final dilution of OD_{600} = 0.5. Ten μl of lambda phage solution, diluted serially in SM buffer, was added to 200 μl of diluted BB4 cells and the mixture was incubated at 37°C for 15 minutes. At the end of the incubation period, the infected BB4 cells were mixed with 2.5 ml of premelted NZY top agarose (45°C), and were spread immediately on top of NZY 82 mm plate. The plates were incubated at 37°C until the plaques were clearly visible (5-8 hours). The titer of the library was determined using BB4 host cells infected with serial dilutions of the packaged lambda ZAP phages, as described above, but with the addition of 50 μl of 0.5 M IPTG and 50 μl of X-Gal (250 mg/ml diluted in DMF) to the top agarose. For titer determination, the plaques which showed the blue color were substracted from the clear ones.

C.5.3.5 cDNA library screening

The library was screened according to the picoBLUE immunodetection kit manual

(Stratagene, CA). Briefly, the infected BB4 cells were plated at a density of 7,000 pfu per 82 mm plates as described in section C.5.3.4. The plates were incubated at 42°C until the plaques started to be visible (3-4 hours). IPTG-treated nitrocellulose filters were applied on top of the agar and the plates were incubated at 37°C for 3.5 hours. At the end of the incubation period, the filters were removed, replaced with fresh ones, and the plates were incubated for another 4 hours. The second nitrocellulose filters were carefully removed and combined with the first one. The plates were stored at 4°C awaiting for plaque purification. The nitrocellulose filters were immersed in TBTS and the sulfotransferase clones were identified by screening the duplicate filters with the previously prepared anti-3-ST antibodies (See section C.4.2).

C.5.3.6 Plaque purification

The plaques giving a positive signal were cored from the top agarose and transferred to a sterile microfuge tube containing 500 μl of SM buffer and 20 μl of chloroform. The microfuge tube was stored overnight at 4°C and this phage stock was submitted to several rounds of purifications, as described in section C.5.3.5, until all plaques were positives.

C.5.3.7 pBLUESCRIPT excision

After plaque purification, plasmids containing putative sulfotransferase clones were excised from purified lambda Zap phage stocks, as described in the lambda Zap cloning manual (Stratagene, CA), using the M13K08 helper phage for co-infection.

C.5.4 cDNA clone characterization

C.5.4.1 Plasmid DNA isolation

Plasmid DNA was prepared by the centrifugation of 1.5 ml of overnight-grown bacterial culture for 30 seconds in an Eppendorf microfuge. The supernatant was

removed by aspiration and the bacterial pellet was resuspended in 0.4 ml of 100 mM Tris-HCl, 100 mM EDTA, and 0.4% Triton X-100. Lysozyme solution of 25 μl (10 mg/ml) was added and the tube placed in a boiling water bath for 40 seconds. The bacterial lysate was centrifuged for 15 minutes. The supernatant was removed and the DNA was precipitated by the addition of two volumes of EtOH-AmOAc. After centrifugation for 5 minutes, the supernatant was removed by suction and the pellet redissolved in 0.3 ml of 0.3 M NaOAc by vortexing. The solution was centrifuged for 5 minutes and the supernatant was re-precipitated by the addition of two volumes of EtOH-AmOAc. The pellet was finally washed with 70% ethanol and redissolved in 20 μl of TE buffer.

C.5.4.2 Restriction enzyme digestion

Restriction enzymes were used as recommended by the suppliers. One unit of restriction enzyme was used per microgam of DNA and the reaction was allowed to proceed for three hours at the recommended temperature. At the end of the reaction time, the DNA was either precipitated with ethanol or loaded directly on the gel for electrophoresis.

C.5.4.3 Agarose gel electrophoresis

Agarose gels were prepared by melting agarose to a concentration of 1% in TAE buffer, and electrophoresis was run at 100 V. The DNA was visualized by staining with 1 μg/ml ethidium bromide for 15 minutes and the gel photographed. pBR322 plasmid digested with Hinf I or lambda DNA digested with EcoR1 were used as molecular weight markers.

C.5.4.4 ST expression in *E. coli*

E. coli cells carrying the plasmid of interest were grown in 3 mls of LB medium

containing ampicillin (50 μg/ml) until it reached an $O.D._{600}$ of 0.6, after which the cells were centrifuged at 3,000g for 5 minutes. The pellet was resuspended in 5 ml of fresh medium and divided in two aliquots. IPTG (250 mM) was added to one of the aliquots to a final concentration of 1 mM, and the cells were incubated for 3 hours at 37°C. At the end of the incubation period, the cells were centrifuged for 5 minutes at 3,000g and the pellet redissolved in one ml of ice cold buffer containing 50 mM Tris-HCl (pH 7.5) and 14 mM 2-mercaptoethanol. The cells were lysed by sonication and the cell extract clarified by centrifugation. The ST assay was performed as described in section C.3.5 using quercetin, quercetin 3-sulfate, isorhamnetin 3-sulfate, tamarixetin 3-sulfate, and kaempferol 3-sulfate, as substrates.

C.5.5 DNA sequencing

C.5.5.1. DNA template preparation

Plasmid DNA was prepared using the mini preparation described in section C.5.4.2 and the DNA was further purified as follows. The volume of the mini-preparation (20 μl) was increased to 100 μl to which 2 μl of RNAse (10 mg/ml) was added. The mixture was incubated for 1 hour at 37°C, after which 5 μl of proteinase K (10 mg/ml) was added and the solution was incubated for another hour at 37°C. The DNA preparation was extracted twice with phenol-chloroform (1:1, v/v), once with chloroform-*iso*-amyl alcohol (23:1, v/v) and was finally precipitated with ethanol. The DNA pellet was redissolved in 8 μl of TE buffer to which 2 μl of 2M NaOH was added to denature the DNA. The denatured DNA was allowed to stand for 10 min at room temperature then precipitated with ethanol. The denatured DNA pellet was redissolved in 4 μl of TE buffer plus 2 μl of H_2O. Two μl of 5X Sequenase buffer and 2 μl of appropriate oligonucleotide primer were added to the denatured DNA.

C.5.5.2 Labelling reaction

The cDNA was sequenced on both strands using double stranded template by the dideoxy chain termination method using the T7 polymerase and oligonucleotide primers (Sanger et al. 1977).

C.5.5.3 Oligonucleotide synthesis

The oligonucleotide primers were synthesized using a Pharmacia automated DNA synthesizer employing the deoxynucleoside 3'-phosphoramidites chemistry (Caruthers 1985).

C.5.5.4 Sequencing gels

The sequencing gels contained either 4% or 5% acrylamide with a ratio of acrylamide to *bis*-acrylamide of 20:1 (w/w) and were made in 2 x TBE buffer containing 8 M urea. The gels were pre-electrophoresed for 30 minutes prior to sample loading. The DNA was denatured at 75°C for 2 minutes and cooled rapidly on ice before loading. The gels were run at 37 W constant power. An average of 450 nucleotides could be resolved per reaction by using a combination of 4% and 5% resolving gels.

C.5.6 DNA sequence analysis

Comparisons of pFST3 and pFST4' cDNAs nucleotide and deduced amino acid sequences with GenBank (release 55) and National Biochemical Research Foundation (NBRF release 18) sequence libraries were performed using the FASTA program package (Pearson and Lipman 1988).

Hydropathy profiles of pFST3 and pFST4' deduced amino acid sequences were calculated according to the method of Kyte and Doolittle (1982). The hydrophobicity of each amino acid was averaged over a 9 amino acids window and plotted to the middle residue. Positive and negative values on the graph indicate hydrophobic and

hydrophilic regions of the proteins, respectively.

D. RESULTS

D.1 Phytochemistry and biosynthesis

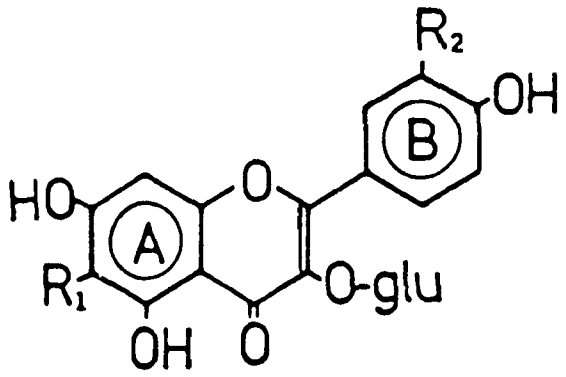
D.1.1 Characterization of the flavonoid constituents of *F. bidentis*.

The chromatographic, electrophoretic and UV-spectral characteristics of the flavonoids isolated from *F. bidentis* are summarized in Figure 2 and Tables 4 and 5. Flavonol sulfate esters could be distinguished from flavonol glucosides by their: (a) low R_f values in organic as compared with aqueous solvents, especially those of highly sulfated compounds; (b) electrophoretic mobility towards the anode (Table 4); (c) susceptibility for hydrolysis with aryl sulfatase, except for the sulfate group at position 3 (Barron et al. 1986).

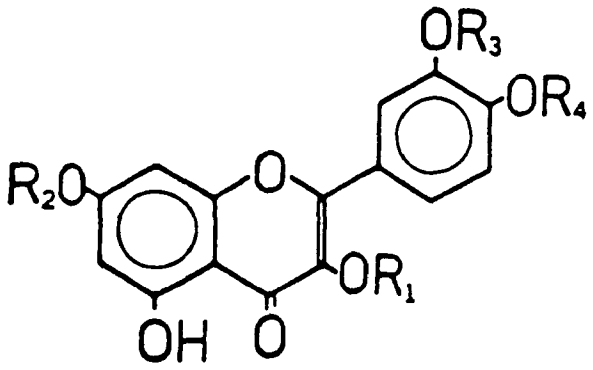
Flavonoid aglycones were identified after acid or enzymatic hydrolysis of the parent flavonol derivative by their uv spectra using spectral shift reagents (Markham 1982), fluorescence in uv light (360 nm) and co-chromatography with reference compounds. The number of sulfate groups in sulfated flavonoids was determined by their electrophoretic mobilities relative to quercetin 3-sulfate (Table 4) and R_t on HPLC of their TBADP derivatives (Table 4, Figure 3). The position of sulfation was determined from the uv spectral shifts observed after the addition of specific reagents (Table 5), and considering the resistance of flavonol 3-sulfates to hydrolysis with aryl sulfatase (Barron et al. 1986).

Among the different flavonoids characterized in *F. bidentis* (Table 4) patuletin 3-glucoside, 6-methoxykaempferol 3-glucoside, and quercetin 3,7-disulfate are reported for the first time in this tissue. Due to the less common occurrence of the two latter compounds, further evidence of their identity is given below.

6-Methoxykaempferol 3-glucoside: This compound exhibited a shift of 15 nm of band I in the presence of $AlCl_3$ + HCl (Table 5) indicating substitution at position 3. However, the low magnitude of that shift suggested a 6-methoxy substitution (Mears and Mabry 1972). Furthermore, there was 25 nm shift of band I in presence of



R_1	R_2	
H	H	Kaempferol 3-glucoside
OMe	H	6-OMe-kaempferol 3-glucoside
OMe	OH	Patuletin 3-glucoside



R_1	R_2	R_3	R_4	
SO_3	H	H	H	Quercetin 3-sulfate
SO_3	H	Me	H	Isorhamnetin 3-sulfate
SO_3	SO_3	H	H	Quercetin 3,7-disulfate
SO_3	H	H	SO_3	Quercetin 3,4'-disulfate
SO_3	SO_3	Me	H	Isorhamnetin 3,7-disulfate
SO_3	SO_3	H	SO	Quercetin 3,7,4'-trisulfate
SO_3	SO_3	SO_3	H	Quercetin 3,7,3'-trisulfate
Acetyl	SO_3	SO_3	SO_3	Quercetin 3-acetyl-7,3',4'-trisulfatte
SO_3	SO_3	SO_3	So_3	Quercetin 3,7,3',4'-tetrasulfate

Figure 2. Structural formulae of the glucosylated (top) and sulfated (bottom) flavonol constituents of F. bidentis

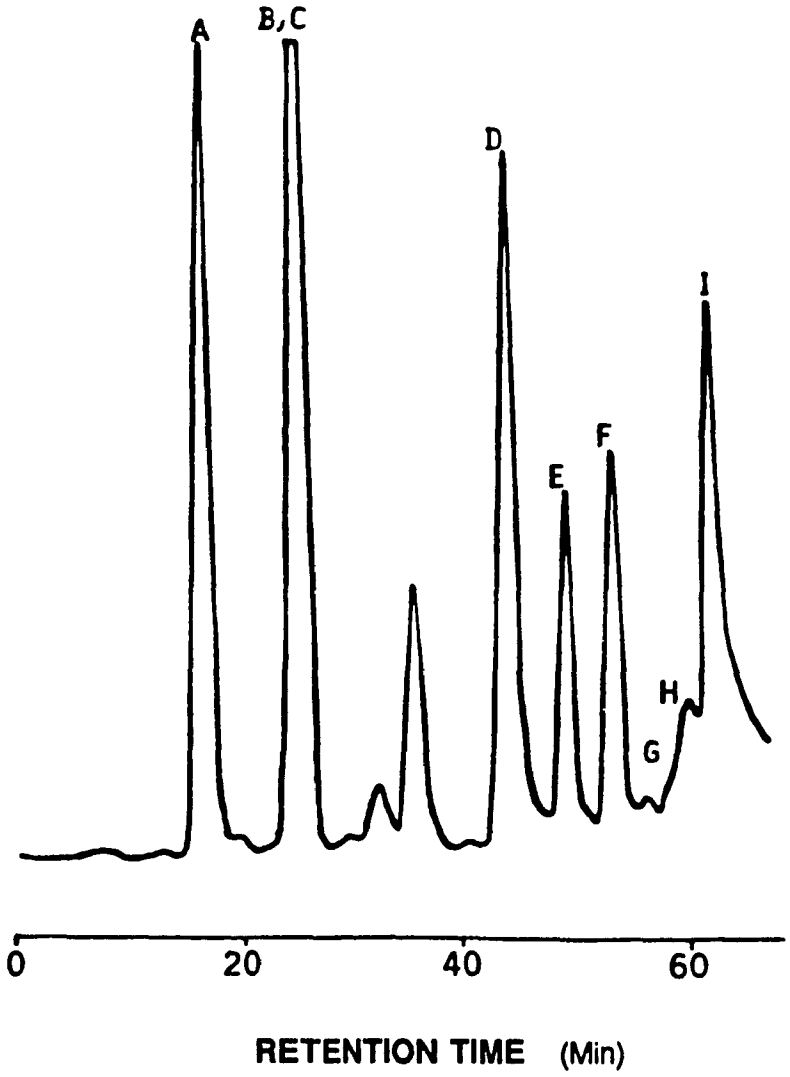


Figure 3. HPLC separation of the flavonol glucosides and flavonol sulfate esters of F. bidentis shoots on a Microbondapak C_{18} column as described in Section C.2.1. A, patuletin 3-glucoside; B, kaempferol 3-glucoside; C, 6-methoxykaempferol 3-glucoside; D, quercetin 3-sulfate; E, isorhamnetin 3-sulfate; F, quercetin 3,7-disulfate; G, isorhamnetin 3,7-disulfate; H, quercetin 3-acetyl, 7,3',4'-trisulfate; I, quercetin 3,7,3',4'-tetrasulfate.

TABLE 4

Characteristics of Flaveria bidentis flavonoids

Flavonoid compound	R_f value BAW[1]	R_f value H_2O	R_t (min)	Electrophoretic mobility[2]	Relative amount (%)[3]
6-Methoxy-kaempferol 3-glucoside	0.70	-	27	0.8	17
Kaempferol 3-glucoside	0.67	-	26	0.8	13
Patuletin 3-glucoside	0.52	-	16	0.7	19
Isorhamnetin 3-sulfate	0.45	0.45	49	1.0	7.5
Quercetin 3-sulfate	0.36	0.40	44	1.0	19
Isorhamnetin 3,7-disulfate	0.27	0.82	56	1.8	0.5
Quercetin 3,7-disulfate	0.20	0.84	53	1.9	9
Quercetin 3-acetyl-3,7,3'-trisulfate	0.11	0.88	59	2.7	3
Quercetin 3,7,3',4'-tetrasulfate	0.05	0.94	62	3.1	12

[1] BuOH-HOAc-H_2O (6:2:2, v/v/v).
[2] Relative to quercetin 3-sulfate.
[3] Based on integrated peak areas by HPLC analysis of 3-4 month-old shoots and TLC separation of kaempferol 3-glucoside and 6-methoxykaempferol 3-glucoside.

TABLE 5

UV spectral characteristics of F. bidentis flavonoids

Flavonoid compound	MeOH	$AlCl_3$	$AlCl_3$ + HCl	NaOAc	NaOAc+ boric acid	MeOH + HCl
6-Methoxy-Kaempferol 3-glucoside	335 270	385 277 300sh	350 277 295sh	360 270	335 270	335 270
Kaempferol 3-glucoside	345 265	395 300sh	395 300sh	360 270	347 265	345 265
Patuletin 3-glucoside	353 257	430 275	370 265	382 270	380 265	353 257
Isorhamnetin 3-sulfate	347 252	395 365sh 272sh	360 390sh 272sh	357 267	350 265sh	365 252
Quercetin 3-sulfate	350 257	395 275	365 268	407 267	388 267	367 257
Isorhamnetin 3,7-disulfate	350 253	397 365sh	392 355sh	355 253	353 253	365 253
Quercetin 3,7-disulfate	355 255	405 272	395 365sh	355 255	375 257	365 255
Quercetin 3-acetyl-3,7,3'-trisulfate	345 267 245sh	392 340 272	392 340 272	395 267	350 267	370 267

sh = shoulder

NaOAc, but none with NaOAc + H_3BO_3, indicating a free 4'-OH and the absence of an *o*-dihydroxy function, respectively. The presence of a C-8 proton (6.25 ppm), as well as a methoxyl group (3.7 ppm) in the ^1H NMR spectrum (DMSO-d_6), confirmed the presence of a 6-methoxy substitution. Furthermore, ring B protons appeared as two groups, of two equivalent protons each (δ +6.84 and 7.97 ppm), coupled together with an *ortho* coupling (δ=8.8Hz) which is characteristic of a disubstitution in 1',4', indicating a kaempferol derivative. A 7.7 Hz coupling constant was observed between H-1" (δ =5.37ppm) and H-2" of the sugar moiety, indicating a ß-linked sugar. Acid hydrolysis yielded glucose and 6-methoxykaempferol. The fact that the latter aglycone did not chromatograph with 8-methoxykaempferol provided further, though indirect, evidence of being the 6-substituted analog.

Quercetin 3,7-disulfate: The electrophoretic mobility of this compound (Table 4) suggested a disulfate ester which on hydrolysis with sulfatase gave quercetin 3-sulfate, and on acid hydrolysis gave quercetin. The spectral shift of the disulfate ester in presence of HCl indicated 3-sulfation, whereas the absence of a NaOAc shift in band II suggested 7-sulfation. Furthermore, a 22 nm shift of band I in presence of NaOAc + H_3BO_3 demonstrated the presence of an *o*-dihydroxy function on ring B. This compound was tentatively identified as quercetin 3,7-disulfate, since it did not co-chromatographed with either the 3,3'- or 3,4'- analogs.

D.1.2 Flavonoid content of different organs.

Quantitative analysis of various flavonoids in different organs of 3-4 week-old seedlings (Table 6) indicated that the highest amounts of individual, as well as total flavonoids were found in the buds > shoots > leaves. On the other hand, the root system of the seedlings was conspicuous by the absence of both glucosylated and sulfated flavonols. The fact that the leaf tissue constitutes the major part (both weight and surface area) of the shoot system, suggests that the leaf is the principal

TABLE 6

Flavonoid content of different organs

of *Flaveria bidentis* seedlings[1]

Flavonoid[2] compound	nmol per gram fresh weight		
	Bud	Stem	Leaf
K 3-glucoside + 6-OMeK 3-glucoside[3]	111	10.4	3.64
P 3-glucoside	162	93.5	7.81
Q 3-sulfate	147	24.7	2.60
IsoR 3-sulfate	38.1	33.6	4.17
Q 3,7-disulfate	1540	686	354
IsoR 3,7-disulfate	19	13.8	6.51
Q 3,7,3',4'-tetrasulfate	43.3	13.9	2.60

[1] Aqueous methanolic extracts of 3 week-old seedlings were analysed by HPLC as described in Section C.2.3.

[2] IsoR, isorhamnetin; K, kaempferol; P, patuletin; Q, quercetin.

[3] K 3-glucoside and 6-OMeK 3-glucoside could not be resolved on HPLC.
Quercetin 3-acetyl-7,3',4'-trisulfate was absent in the seedlings.

site of flavonoid synthesis and accumulation.

It is interesting to note that flavonol glucosides in the seedlings amounted to < 10% of total flavonoids (Table 6) as compared with approximately 50% in mature shoots (Table 4). Quercetin 3,7-disulfate represented the major constituent in the seedlings and amounted to 50-70% of total flavonoids. However, in mature shoots quercetin 3-sulfate, -3,7-disulfate and -3,7,3',4'-tetrasulfate were the major sulfate esters, even though they represented only 40% of total flavonoids (Table 4).

D.1.3 Biosynthesis of flavonoids from labelled precursors

Figure 4 shows the time course for the incorporation of [^{3}H]cinnamate (Fig. 4A) and [^{35}S]sulfate (Fig. 4B) into individual flavonoids, as well as the total incorporation of either precursor in the total methanolic extract (insert) of leaf disks. Whereas the rate of incorporation of either precursor was almost linear with time, the order of labeling of the various flavonoid derivatives was different. Comparison of the profiles of glucosylated and sulfated flavonols shows that the cinnamate label was found mostly in flavonol glucosides, as compared with the sulfate esters, with a ratio of 13 to 1, after 3-hour incubation with the labelled precursor (Fig. 4A). The incorporation of label from [^{35}S]sulfate was, as expected, restricted to flavonol sulfates, and was proportional to the number of sulfate ester linkages of individual compounds (Fig. 4B).

When leaf disks were pulsed for 30 min in either precursor followed by a 3-hr chase in water, similar results were obtained (Fig. 5A, 5B) except for a drop in radioactivity of quercetin 3-sulfate and quercetin 3,7-disulfate. The latter compounds seem to have been metabolized to the higher order of sulfated flavonols (Fig. 5B).

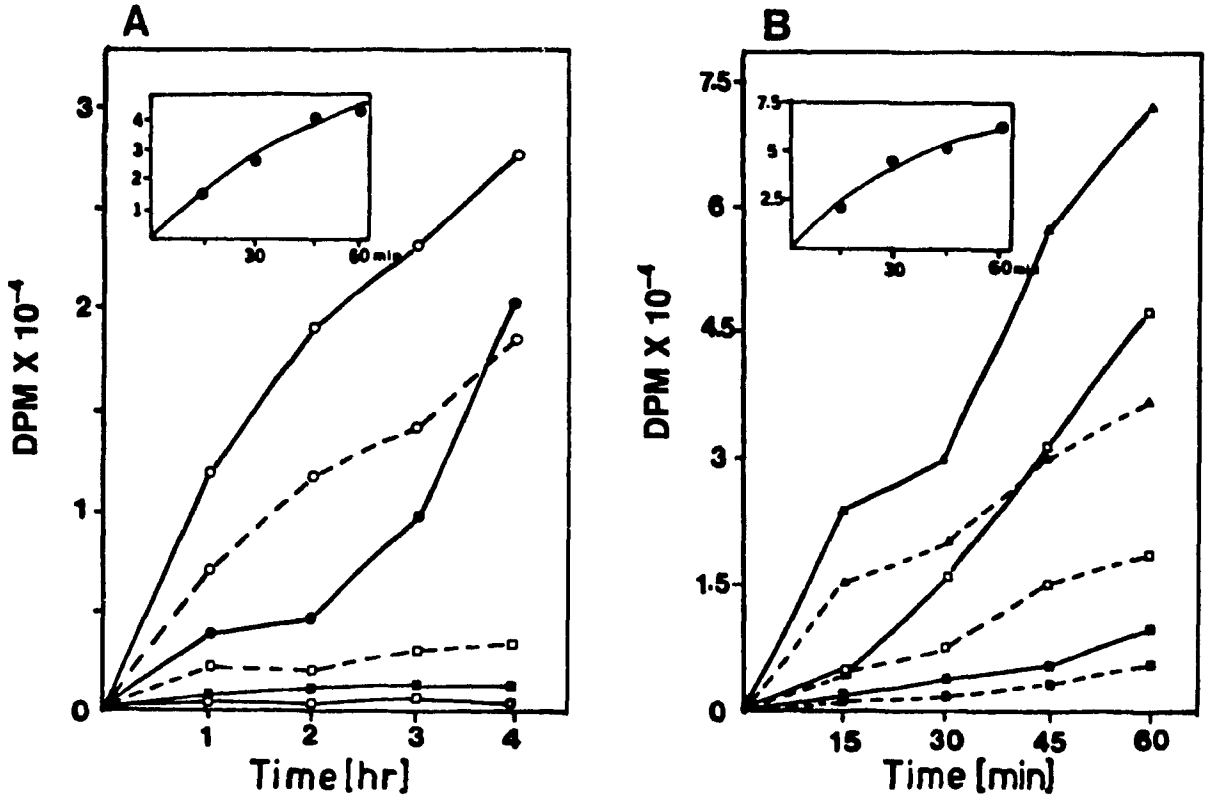


Figure 4. Incorporation of label of [^{3}H]cinnamate (A) or [^{35}S]sulfate (B) into total MeOH extracts (inserts) and the individual flavonoid constituents of the first pair of seedling leaves of F. bidentis at the times indicated. Leaf disks were administered 3.7 X 10^{4} Bq cinnamate (4.03 X 10^{2} GBq/mMol) or sulfate (1.59 X 10^{3} GBq/mg). IsoR, isorhamnetin; K, kaempferol; Q, quercetin; P, patuletin.
(--o--), K 3-glu; (—o—), 6-MeO-K 3-glu; (—•—), P 3-glu; (--▪--), IsoR 3-sulfate; (—▪—), IsoR 3,7-disulfate; (--□--), Q 3-sulfate; (—□—), Q 3,7-disulfate; (--▲--) Q 3-acetyl-7,3',4'-trisulfate; (—▲—), Q 3,7,3',4'-tetrasulfate.

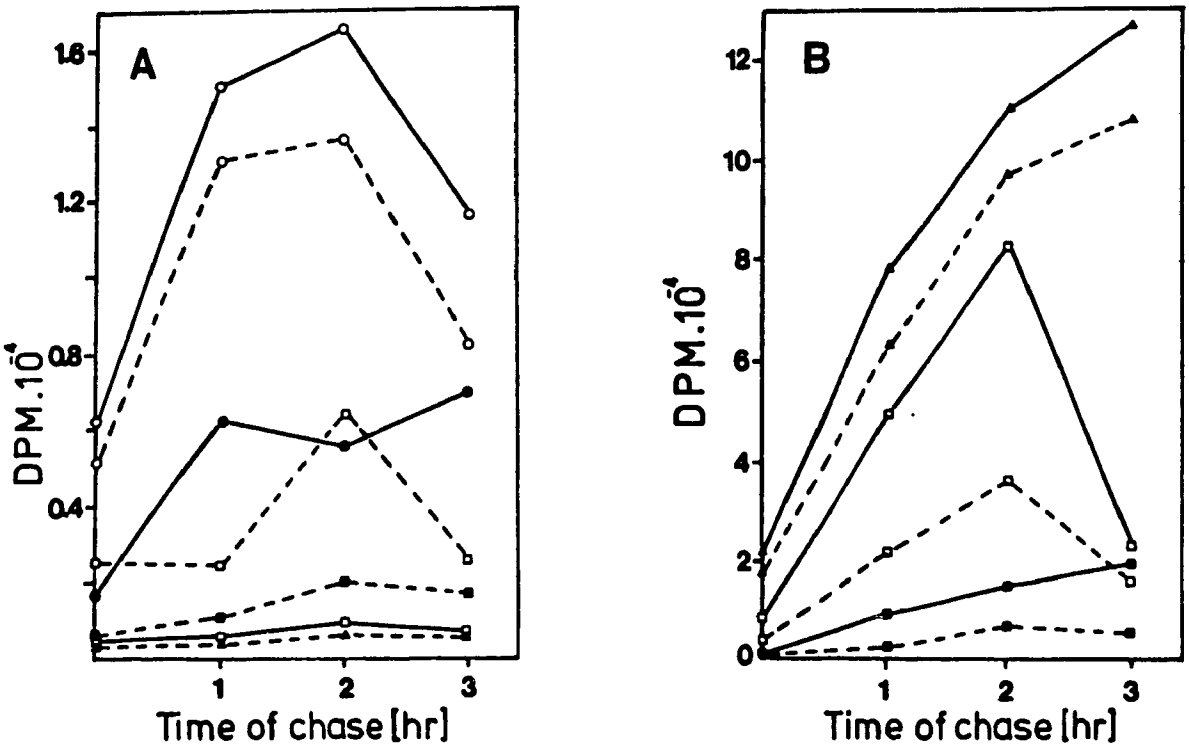


Figure 5. Incorporation of label of [^{3}H]cinnamate (A) or [^{35}S]sulfate (B) into individual flavonoid constituents of the first pair of seedling leaves of F. bidentis , after 30 minute pulse with either label precursor followed by a 3-hr chase in water. Leaf disks were administered 3.7 X 10^{4} Bq cinnamate (4.03 X 10^{2} GBq/mMol) or sulfate (1.59 X 10^{3} GBq/mg). IsoR, isorhamnetin; K, kaempferol; Q, quercetin; P, patuletin.
(--o--), K 3-glu; (—o—), 6-MeO-K 3-glu; (—•—), P 3-glu;
(--▪--), IsoR 3-sulfate; (—▪—), IsoR 3,7-disulfate;
(--□--), Q 3-sulfate; (—□—), Q 3,7-disulfate;
(--▲--) Q 3-acetyl-7,3',4'-trisulfate;
(—▲—), Q 3,7,3',4'-tetrasulfate.

D. 2 Enzymology of the sulfation reaction

D.2.1 Sulfotransferase assay

The enzyme assay for phenol ST is based on incubation of the enzyme protein together with [^{35}S]PAPS as the sulfate donor and the appropriate phenolic acceptor (Fig. 6). The separation of the sulfate ester formed, from the sulfate donor, is difficult to achieve since both are soluble in aqueous media. The commonly used methods of separation (for review, see Roy 1981) were found to be either inaccurate or time consumming, especially for routine use in enzyme purification on different columns. The enzyme assay that we developed made use of TBADP, which forms an ion pair with the flavonol sulfate ester, thus rendering the molecule soluble in organic solvent such as ethyl acetate, whereas PAPS remains in the aqueous layer. For the determination of the sulfating activity, an aliquot of the organic layer was counted for radioactivity in a toluene based liquid scintillation fluid. In order to demonstrate the reliability of the enzyme assay, its efficiency and accuracy were verified.

D.2.1.1 Ion-pairing method

The addition of TBADP to the acidified (pH 4) assay mixture resulted in the formation of ion-pairs with the flavonoid sulfate esters, and rendered them soluble in ethyl acetate, whereas PAPS remained in the aqueous medium (Fig. 7). A final concentration of 10 mM TBADP was found adequate for the separation of the sulfated reaction products (Fig. 7A and C) from the sulfate donor (Fig. 7B and D). It is remarkable to note that PAPS was not found in the ethyl acetate layer, nor did any of the sulfated flavonoids remained in the aqueous layer.

D.2.1.2 Efficiency of the assay in recovering mono- to tetrasulfated flavonoids.

In order to demonstrate the efficiency of the assay to recover sulfated flavonoids

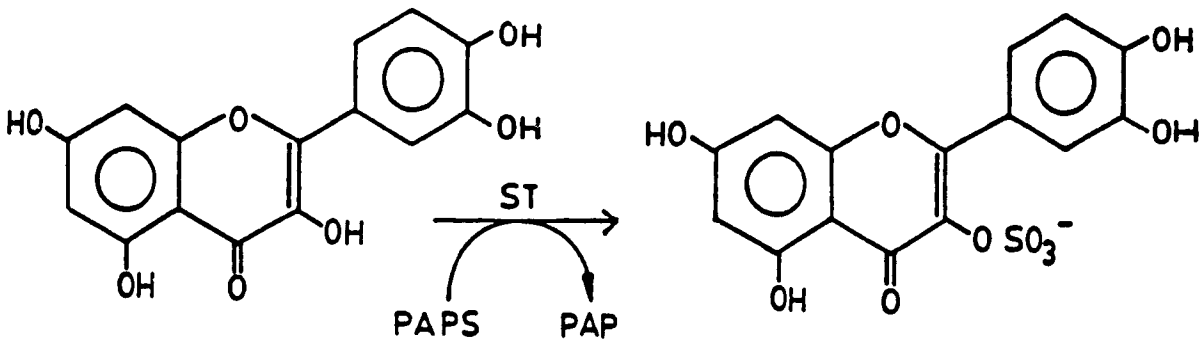


Figure 6. Enzymatic sulfation of quercetin to quercetin 3-sulfate

A B C D

Figure 7. Photograph of an autoradiogram of the chromatographed ethyl acetate (A,C) and aqueous (B,D) layers of *F. bidentis* ST activity with quercetin (A,B) and isorhamnetin (C,D) as substrates. (A and C) co-chromatographed with quercetin 3-sulfate and isorhamnetin 3-sulfate, respectively; (B and D) with PAPS. BAW (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

from the aqueous medium, known amounts of mono- to tetrasulfate esters were added to one ml of the assay buffer (Table 7). To the latter, HOAc and TBADP were added and the mixture was extracted with one ml of ethyl acetate. The absorbance of the organic layer was then measured spectrophotometrically in order to determine the amount of flavonoid sulfate recovered. Table 7 shows that, regardless of the level of sulfation of the flavonoids tested, the percentage recovery ranged between 88.7 and 95.6%.

D.2.1.3 Reproducibility of the enzyme assay.

Table 8 shows that when the sulfotransferase assay was tested using different flavonoid substrates, the standard deviation in the activities determined was found to vary between 1.75 to 3.27% of the mean enzyme activity. The efficiency and the reproducibility of the flavonol ST assay allowed us to study the enzymology of flavonoid sulfation in *Flaveria spp.*

D.2.2 Demonstration of ST activity in *F. bidentis* and *F. chloraefolia*

Preliminary study of the sulfation reaction was conducted with crude enzyme preparations which were desalted on Sephadex G-25 and using the previously described enzyme assay. Figure 8 is a photograph of the autoradiographed reaction products of the *F. chloraefolia* enzyme preparation that were assayed against kaempferol, quercetin, rhamnetin, isorhamnetin, patuletin, and eupatin. All these flavonol aglycones were converted to their 3-monosulfate derivatives indicating the presence, in this plant, of a ST specific for position 3 of flavonol aglycones. Despite the fact that *F. chloraefolia* contains flavonol disulfates, very little if any disulfates was formed when the crude extract was incubated with any flavonol aglycone. By contrast, time course incubation of the *F. bidentis* enzyme with quercetin and PAPS resulted in the formation of mono-, di-, tri- and traces of the tetrasulfate ester derivatives (Fig. 9). These

TABLE 7

Percent recovery of quercetin mono- to tetrasulfates from the aqueous medium after addition of HOAc and TBADP

Compound[1]	% Recovery[2]	S.D.[3]
Quercetin 3-sulfate	93.12	3.12
Quercetin 3,3'-disulfate	90.73	2.59
Quercetin 3,7,4'-trisulfate	95.61	3.67
Quercetin 3,7,3',4'-tetrasulfate	88.72	1.27

[1] Concentrations used were 120-250 μM.
[2] Detremined as described in Section C.3.5
[3] Per cent of the mean, *n*=3.

TABLE 8

Reproducibility of the flavonoid sulfotransferase assay[1]

Substrate	Mean activity[2]	S.D.[3]
Kaempferol	4360	1.75
Quercetin	4880	2.35
Isorhamnetin	14750	1.94
Patuletin	2450	3.27

[1] Using a cell-free extract of F. bidentis and the standard assay described in section C.3.5.

[2] DPM/assay corrected for background activity of the controls which amounted to approximatively 120 DPM.

[3] Percent of the mean, n=5.

A B C D E

Figure 8. Photograph of an autoradiogram of the chromatographed reaction products of F. chloraefolia ST activity with the flavonol substrates: A, quercetin; B, rhamnetin; C, isorhamnetin, D, patuletin; E, eupatin. Water was used to develop the cellulose TLC plate.

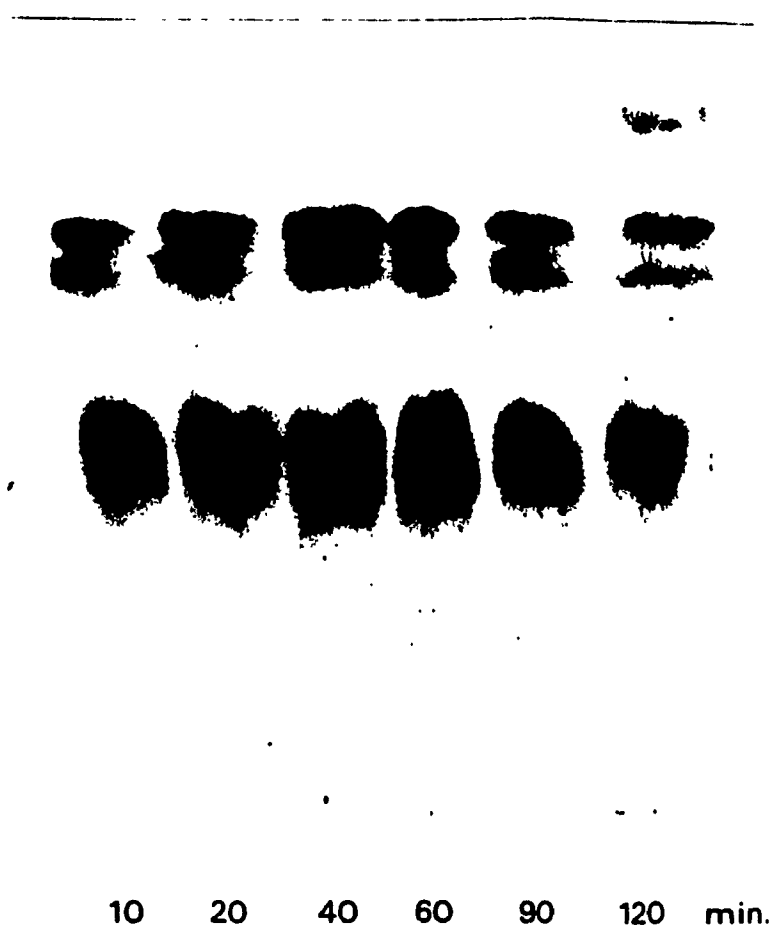


Figure 9. Photograph of an autoradiogram of the reaction product of F. bidentis cell-free extract with quercetin, as substrate, incubated for different time periods. Electrophoresis of the reaction products on No. 3 Whatman paper was conducted as described in Section C.2.2.

results are consistent with the natural occurrence of highly sulfated flavonols in this tissue (Cabrera and Juliani 1976) and suggested the existence of several, position-specific STs. The reports on the accumulation of polysulfated flavonols in both *F. chloraefolia* (Barron 1987) and *F. bidentis* (Cabrera and Juliani 1976, 1977, 1979, 1985), as well as the preliminary results of our study of the sulfation reaction prompted us to isolate the individual STs from both species.

D.2.3 Characterization of the 3-, 3'-, and 4'-STs from *F. chloraefolia*

Three distinct, position-specific flavonol STs were partially purified from *F. chloraefolia* by ammonium sulfate precipitation and successive chromatography on Sephacryl S-200, PAP-agarose, and chromatofocusing on Mono P columns. Figure 10A shows that when the Sephacryl S-200 fractions were assayed against quercetin and quercetin 3-sulfate, the ST activities accepting both substrates coeluted as a discrete peak. Further chromatography of the active fractions on PAP-agarose resulted in partial, but incomplete, separation of the 3-ST from the 3'- and 4'- ST activities (Fig. 10B). However, the use of this column improved considerably the purification of the STs (Table 9), since it eliminated most contaminating proteins. Further purification of the active fractions by chromatofocusing on Mono P revealed four ST activity peaks (Fig. 11). Two of the latter (peaks 1 and 2) accepted quercetin as substrate, whereas the other two (peaks 3 and 4) were active against quercetin 3-sulfate. Analysis of the reaction products revealed that peak 1, which eluted at pH 5.7, contained both the 3- and the 3'-ST activities (Fig. 12 lane D). Attempts of further chromatography of this peak were unsuccessful due to instability of the ST activities. Peak 2, which eluted at pH 5.4, accepted quercetin as substrate and gave quercetin 3-sulfate as the only reaction product (Fig. 12 lane F), thus identified as the 3-ST. Both peaks 3 and 4, which eluted at pH 6.0 and 5.1, respectively, accepted quercetin 3-sulfate as substrate and gave rise to the 3,3'- and 3,4'-disulfate esters,

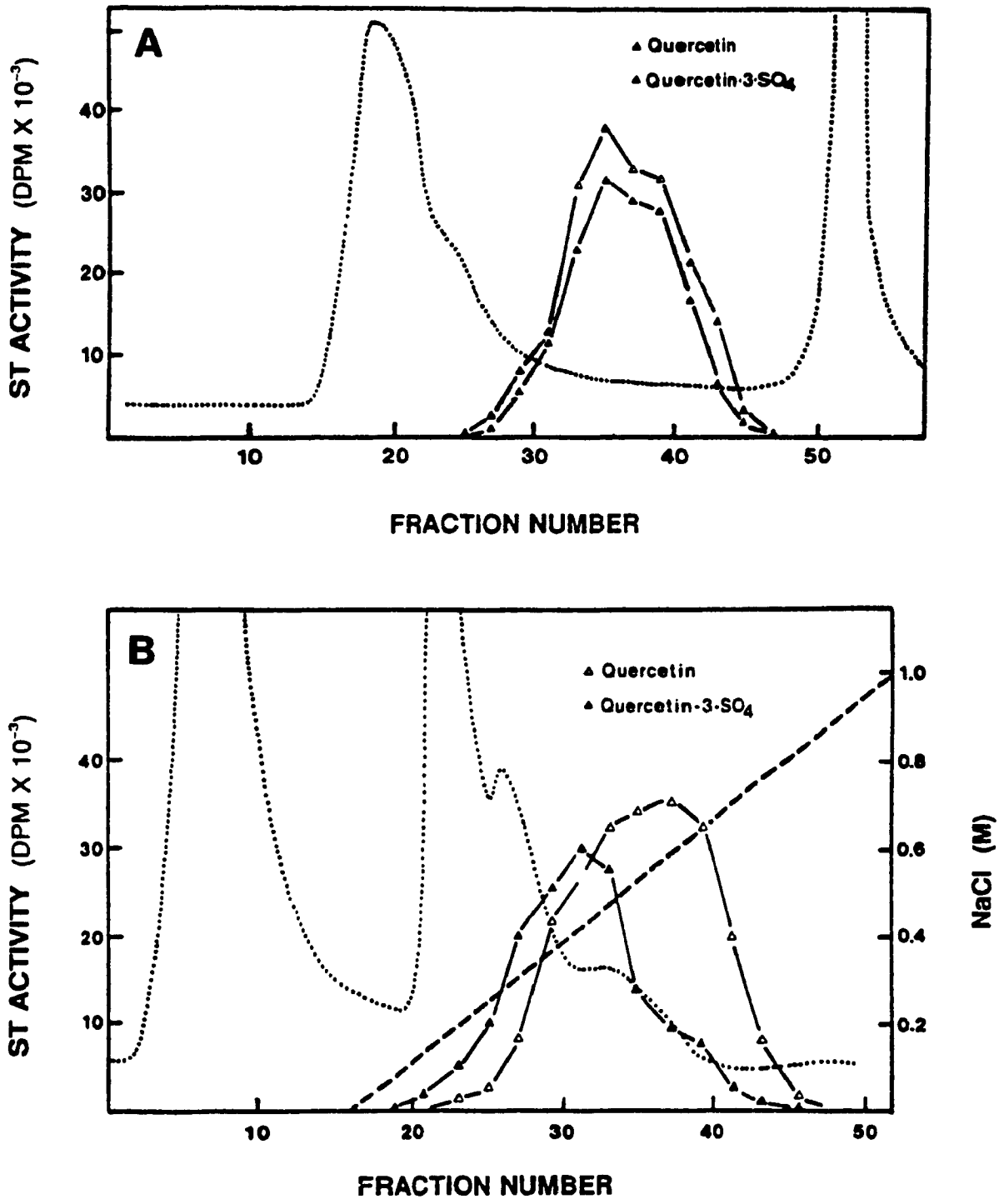


Figure 10.A)Elution profile of F. chloraefolia ST activities after gel filtration on Sephacryl S-200 as described in Section 3.2.2. B) Elution profile of the ST activities after chromatography on PAP-agarose as described in Section C.3.2.3. The fractions were assayed for enzyme activity against quercetin (——△——) and quercetin 3-sulfate (——▲——) as substrates. Protein absorbance was monitored at 280 nm (.....)

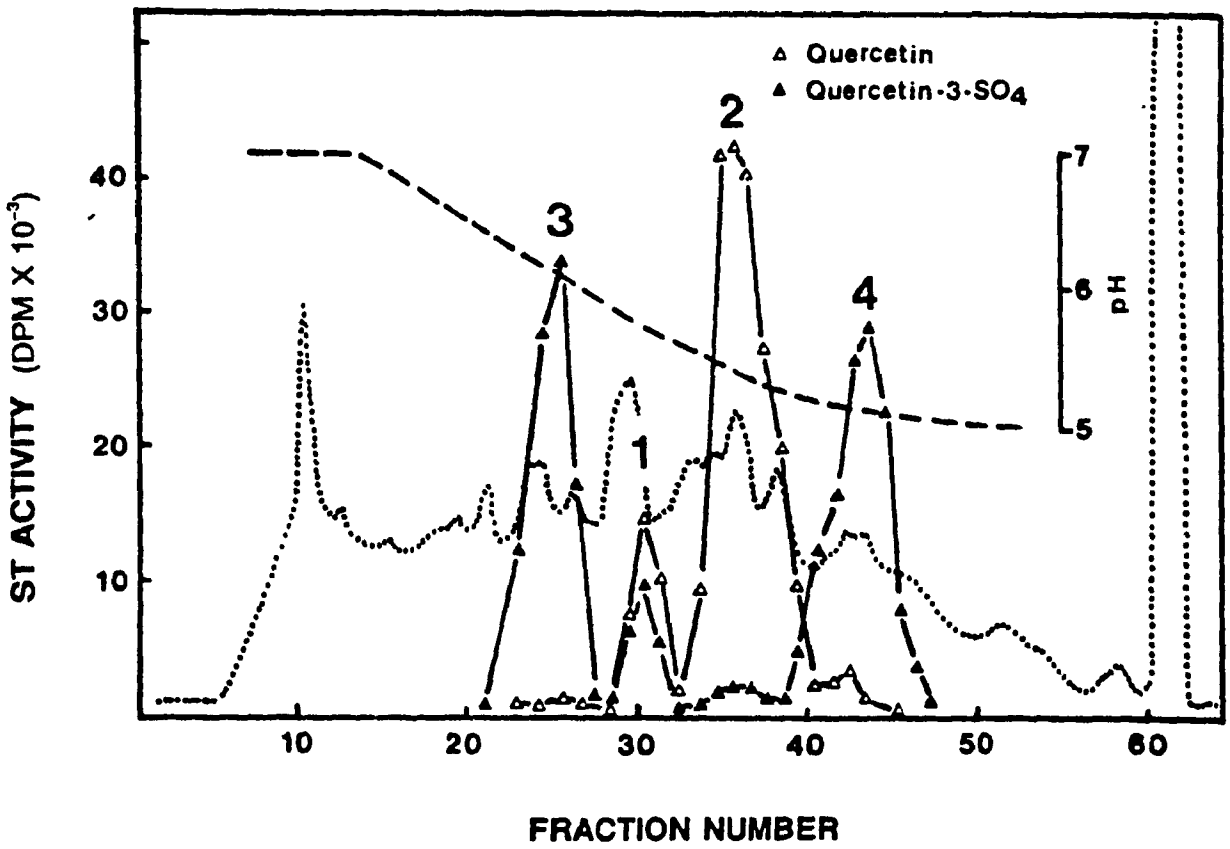


Figure 11. Elution profile of F. chloraefolia ST activities after chromatofocusing on Mono P as described in section C.3.2.4. The fractions were assayed with quercetin (—Δ—) and quercetin 3-sulfate (——▲——) as substrates. Protein absobance was monitored at 280 nm (.....).

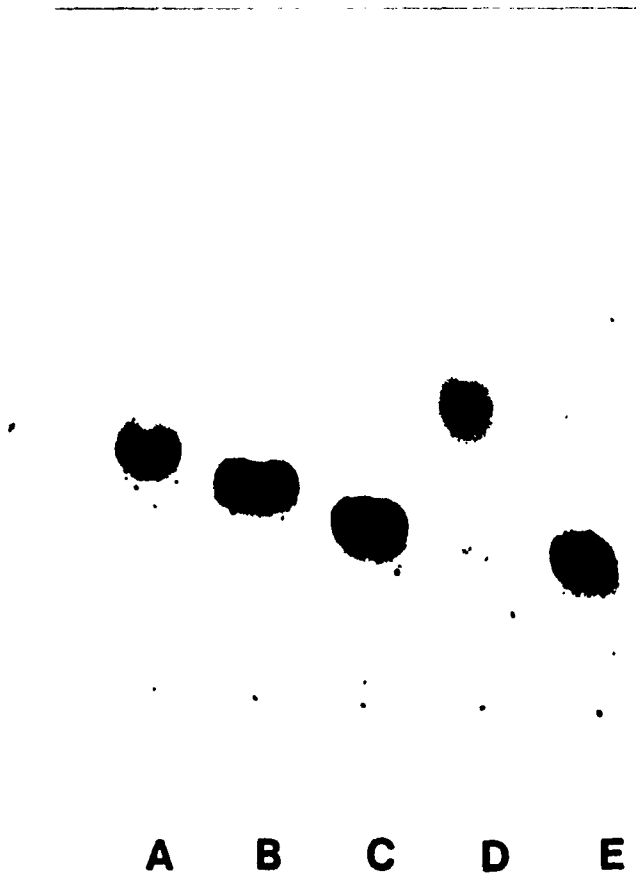


Figure 12. Photograph of an autoradiogram of the chromatographed reaction products of F. chloraefolia 4'-ST with: A, isorhamnetin 3-sulfate; C, quercetin 3-sulfate. 3'-ST with: B. tamarixetin 3-sulfate; E, quercetin 3-sulfate. Agregated 3-and 3'-STs with: D, quercetin. BAW (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

TABLE 9

Purification of *Flaveria chloraefolia* sulfotransferases[1]

Purification step	Total protein (mg)	Specific activity (pKat/mg)	Total activity (pKat)	Purification (-fold)	% Recovery
Dowex					
3-ST	187	0.079	14.80	-	100
3' + 4'-ST	187	0.065	12.16	-	100
S-200					
3-ST	15.2	0.808	12.29	10	83
3' +4'-ST	15.2	0.647	9.83	10	81
PAP-agarose					
3-ST	0.8	9.328	7.46	118	50
3' + 4'-ST	0.8	6.413	5.13	99	42
Mono-P					
3-ST	0.029	43.47	1.26	549	9
3'-ST	0.028	41.17	1.15	633	10
4'-ST	0.044	32.71	1.44	503	12

[1] The sulfotransferase activities were assayed as described in section C.3.5 utilising quercetin as substrate for the 3-ST, and quercetin 3-sulfate for the 3'- and 4'-STs.

respectively, thus representing the 3'- and 4'-ST activities (Fig. 12 lane E, C). This purification protocol resulted in 550-, 630-, and 500-fold increase in specific activities of the 3-, 3'-, and 4'-STs, respectively, with recoveries of 9 to 12% as compared with the crude extract (Table 9). Examination of enzyme preparations after SDS-PAGE revealed that the purified STs were still contaminated with several protein contaminants (Fig. 13).

D.2.3.1 Substrate specificity of *F. chloraefolia* sulfotransferases

Table 10 summarizes the relative activities of the three enzymes when assayed against different substrates. The 3-ST exhibited strict specificity for position 3 of several flavonols in the following order of decreasing activity, with rhamnetin > isorhamnetin > quercetin > patuletin > kaempferol, as sulfate acceptors. However, it accepted neither flavonols with additional hydroxyl groups at position 6 (quercetagetin), 8 (gossypetin) or 5' (myricetin), nor flavonols lacking ring-B hydroxylation (e.g. galangin). The accumulation of quercetin 3-sulfate and patuletin 3-sulfate in *F. chloraefolia* (Barron et al. 1986) as well as the common occurence of flavonol 3-sulfates in plants (Barron et al. 1988) suggest that 3-sulfation is the first step in the enzymatic synthesis of polysulfated flavonols.

The 3'-ST accepted the following flavonols in a decreasing order of relative activity, with quercetin 3-sulfate > patuletin 3-sulfate > tamarixetin 3-sulfate, for further sulfation at the 3'- position (Table 10, Fig. 12, lanes B,E). It did not accept kaempferol 3-sulfate, isorhamnetin 3-sulfate, or any of the flavonol aglycones tested.

The 4'-ST accepted quercetin 3-sulfate > kaempferol 3-sulfate > isorhamnetin 3-sulfate > patuletin 3-sulfate for further sulfation at 4'-position (Table 10, Fig. 12, lane A, C). It did not react with either tamarixetin 3-sulfate or any of the flavonol aglycones. The specificity of the two latter enzymes is consistent with the accumulation, in *F. chloraefolia,* of quercetin 3,3'-, patuletin 3,3'- and quercetin 3,4'-disulfates (Barron

TABLE 10

Substrate specificity of *Flaveria chloraefolia* STs[1]

Substrate[3]	Relative activity (%)[2]		
	3-ST	3'-ST	4'-ST
Rhamnetin	100	-	-
Isorhamnetin	94	-	-
Quercetin	58	-	-
Patuletin	52	-	-
Kaempferol	48	-	-
Ombuin	37	-	-
Tamarixetin	31	-	-
Quercetin 3-SO_4	-	100	100
Patuletin 3-SO_4	-	58	12
Tamarixetin 3-SO_4	-	33	0
Kaempferol 3-SO_4	-	0	45
Isorhamnetin 3-SO_4	-	0	38

[1] The standard enzyme assay was used as described in Section C.3.5, at a substrate concentration of 1μM.

[2] The maximum activities (100%) observed were 65, 39 and 32 pKat/mg for the 3-, 3'- and 4'- STs, respectively.

[3] The following substrates were utilized as sulfate acceptors at <5% of the controls: myricetin, quercetagetin, dihydroquercetin, and galangin (for the 3-ST); apigenin, luteolin, caffeic acid, ferulic acid and p-coumaric acid (for the 3'- and 4'-STs).

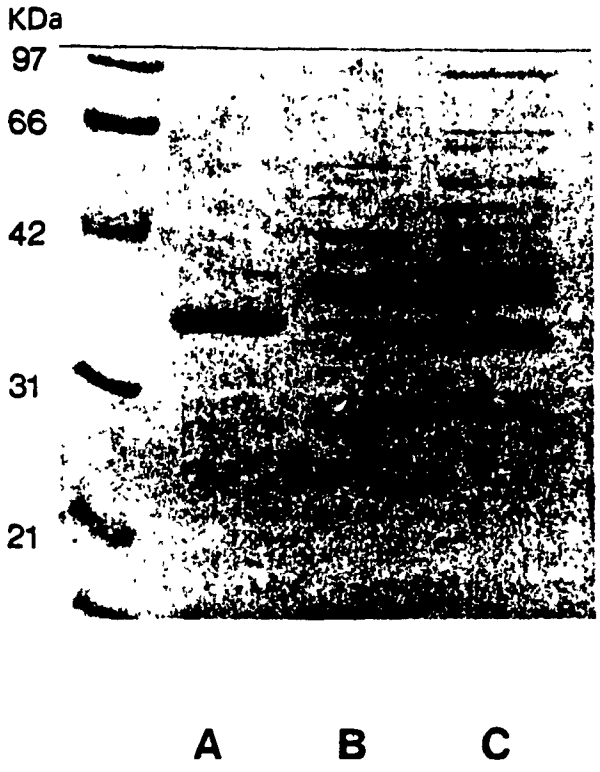


Figure 13. SDS-PAGE of partially purified enzyme preparations (ca. 5 μg) of 3- (A), 3'- (B), and 4'- (C) STs. Electrophoresis was performed as described in section C.3.9. Proteins were visualized by Coomassie blue staining. Numbers on the left correspond to molecular weight markers.

and Ibrahim 1987b) and suggests that sulfation at either of these two positions is the second step in the biosynthesis of disulfate esters in this tissue.

None of the three enzymes described above exhibited any activity with phenylpropanoids, flavones, or dihydroflavonols, thus indicating their strict specificity for flavonol aglycones or their partially sulfated esters.

When [^{35}S] APS was used as the sulfate donor with the standard enzyme assay, no flavonol sulfation was observed with either of the ST preparations, thus demonstrating the strict requirement for the sulfate donor PAPS by the *F. chloraefolia* STs.

D.2.3.2 Other properties

The three flavonol STs were found to have similar molecular weights (ca. 35,000D) on a calibrated Superose 12 column using an FPLC system (Table 11). However they displayed different pI values (Table 11) which allowed their separation by chromatofocusing.

The S I activity of each enzyme was measured against its best substrate in presence of histidine-HCl, *bis*-Tris-HCl, Tris-HCl, phosphate,and glycine-NaOH buffers over the pH range 5.5 to 9.5. Except for the 3-ST, which exhibited two pH optima at pH 6.5 in *bis*-Tris and 8.5 in Tris buffers, the 3'- and 4'-STs had an optimum pH of 7.5 in Tris buffer (Table 11). The pH behavior of the 3-ST has also been reported for a phenol ST (Lyon and Jakoby 1980) and was attributed to the requirement for catalysis of a specific ionized form of the substrate, or to the presence of a specific ionizing group which facilitates catalysis. Another interesting feature of the sulfation system of *F. chloraefolia* is the complete inhibition of the 4'-ST activity in the presence of phosphate.

The highly purified enzymes exhibited no requirement for divalent cations and were not inhibited by EDTA at concentrations up to 10 mM. Furthermore, their activities were not affected by the presence of the SH-group reagents, *p*-

TABLE 11

Properties of *Flaveria* sulfotransferases

Property	3-ST	3'-ST	4'-ST
Km Flavonoid (μM)[1]	0.2	0.29	0.36
Km PAPS (μM)	0.2	0.35	0.38
pH optimum	6.0, 8.5	7.5	7.5
Apparent pI	5.4	6.0	5.1
Apparent mol wt (D)	35,000	35,000	35,000

[1] With quercetin as substrate for the 3-ST and quercetin-3-sulfate for the 3'- and the 4'-STs

chloromercuribenzoate, iodoacetate, or iodoacetamide, when assayed at 1 to 10 mM concentration.

When the flavonoid substrate was varied at constant PAPS concentration, Michaelis-Menten kinetics were observed below Km for the three STs of *F. chloraefolia*, whereas higher flavonol concentration resulted in substrate inhibition (Fig. 14). Substrate inhibition may be due to the binding of the substrate to the wrong form of the enzyme resulting in the formation of a non-productive dead-end complex. The Km values of the 3-ST for quercetin and of the 3'- and 4'-STs for quercetin 3-sulfate were found to be 0.28 μM, 0.35 μM, and 0.38 μM, respectively (Table 11).

Substrate inhibition was not observed when PAPS was the variable substrate (Fig. 14). The Km values of the 3-, 3'-, and 4'-STs for PAPS were found to be 0.25, 0.29 and 0.36 μM, respectively (Table 11).

D.2.4 The STs of *Flaveria bidentis*

In order to define the number of STs present in this species, flavonol aglycones as well as mono- to trisulfated derivatives of quercetin were assayed with the crude enzyme preparation. Although the 3-ST activity was easily detected when assayed with flavonol aglycones, those accepting partially sulfated flavonols were very low, except when assayed with quercetin 3,3'-disulfate. The product of the latter reaction co-chromatographed with quercetin 3,7,3'-trisulfate. These observations suggested the presence in *F. bidentis* of a ST specific for position 7 of partially sulfated flavonols, as well as other unresolved ST activities. In order to elucidate the sequence of sulfation reactions leading to the formation of tetrasulfated flavonols, the 7-ST of *F. bidentis* was further purified and characterized.

D.2.4.1 Partial purification of *F. bidentis* 7-STs

The ST activities of *F. bidentis* were found to be very unstable. To prevent ST

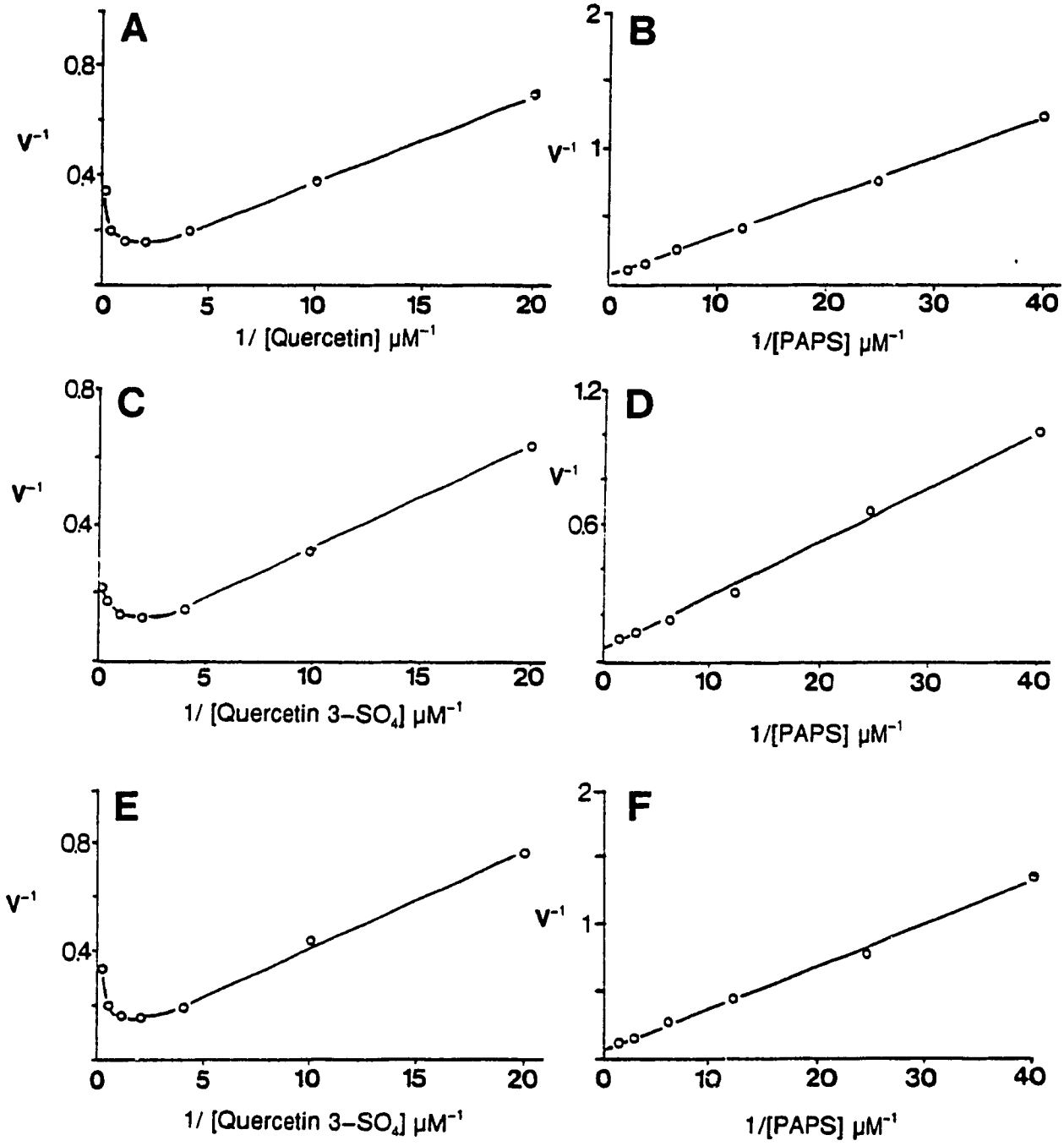


Figure 14. Initial velocities 1/V versus 1/flavonol and 1/PAPS for the 3- (A,B), 3'- (C,D), and 4'-STs (E,F). (A,C,E), 1/V versus 1/Flavonol at constant PAPS concentration of 1.0 μM. (B,D,F), 1/V versus 1/PAPS at constant flavonol concentration of 1.0 μM. Velocities are expressed in pKat/mg protein. The assay conditions were as described in section C.3.5.

inactivation, several modifications of the purification procedure and of the protein storage conditions were attempted without success. In order to characterize the 7-ST, a purification procedure was developed which permitted the recovery of this enzyme free from contaminating activities in the minimum of time. In fact, the 7-ST preparation was obtained after 8 hours from protein extraction.

F. bidentis STs were extracted and partially purified as described in section C.3.3. Chromatography on PAP-agarose resulted in a single broad peak which accepted quercetin 3,3'-disulfate, and to a lesser extent, quercetin as substrates (Fig. 15A). Further chromatography of the combined active fractions by chromatofocusing on Mono P revealed three ST activity peaks (Fig. 15B). Two of the latter (peaks 1 and 2) which eluted at pH 6.5 and 6.3, respectively, accepted quercetin 3,3'-disulfate as substrate, whereas peak 3, which eluted at pH 5.2 was found to contain several unresolved ST activities. Analysis of the reaction products revealed that both peaks 1 and 2 accepted quercetin 3,3'-disulfate and gave rise to quercetin 3,7,3'-trisulfate as the only reaction product, thus representing two isoforms (I and II) of the 7-ST (Fig. 16). Peak 3 gave rise to mono- and disulfated derivatives when assayed with quercetin as substrate (Fig. 16). The high instability of the ST(s) present in the latter peak prevented any attempt of further purification. However, the formation of quercetin 3-sulfate as the only sulfate ester suggested that peak 3 contained a flavonol 3-ST, in addition to other uncharacterized ST activities. The above purification procedure 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 12). As expected, the partially purified peaks 1 and 2 were found to contain several protein bands after SDS-PAGE (Fig. 17).

D.2.4.2 Substrate specificity of *F. bidentis* 7-ST I and II

Table 13 summarizes the relative activities of the two 7-ST isoenzymes when

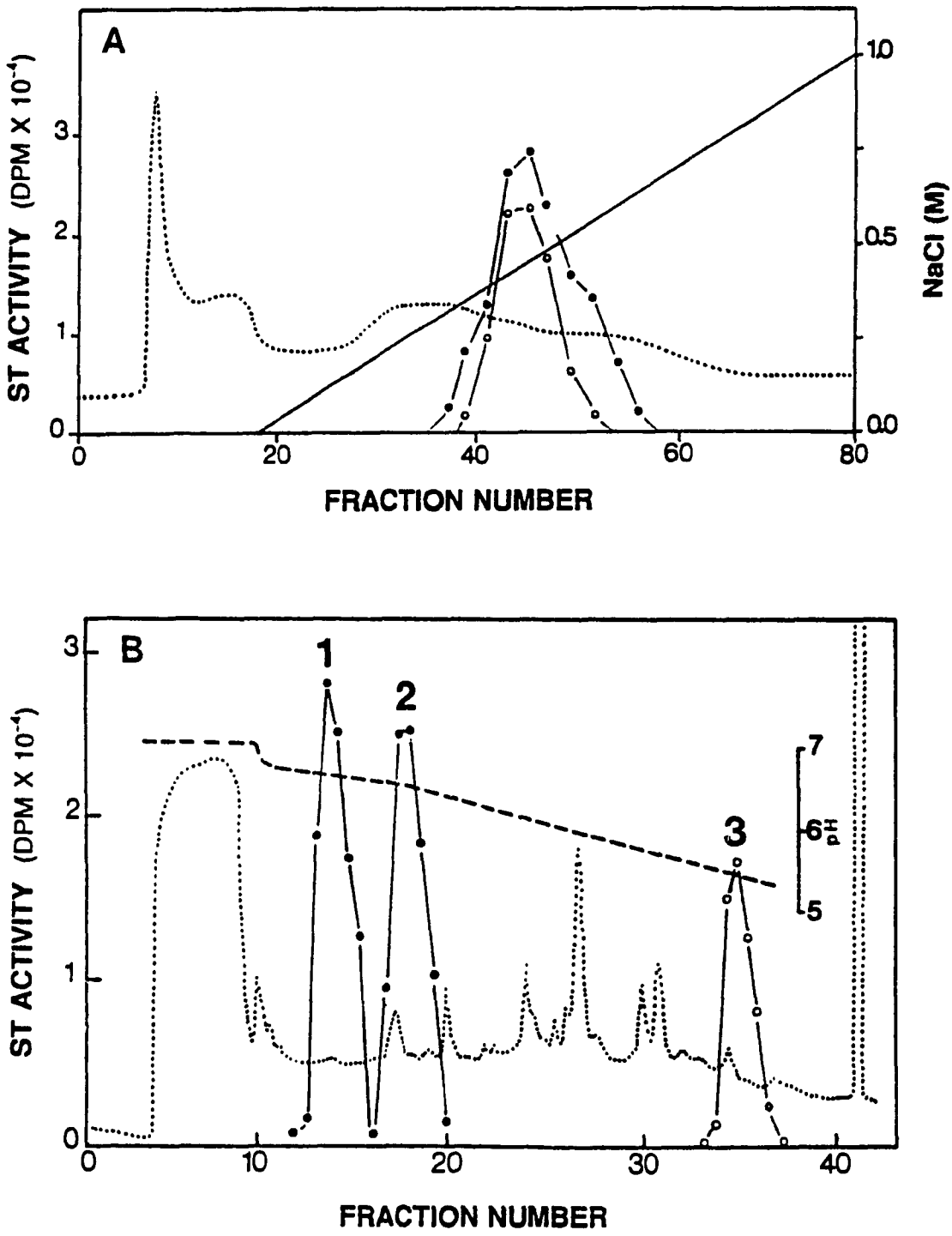


Figure 15. A) Elution profile of F. bidentis 7-ST activity after chromatography on PAP-agarose as described in section C.3.3.2. B) Elution profile of the 7-ST activity after chromatofocusing on Mono P as described in section C.3.3.3. The fractions were assayed with quercetin (——○——) and quercetin 3,3'-disulfate (——●——) as substrates. Protein absorbance was monitored at 280 nm (.....).

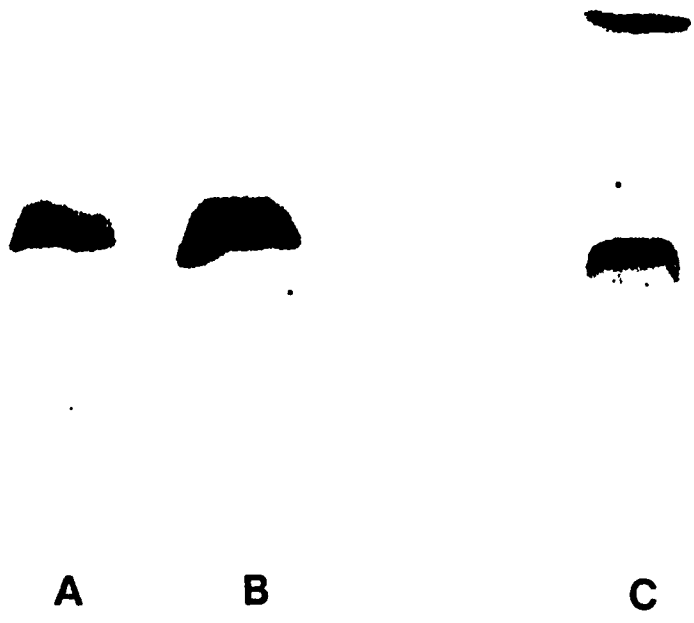


Figure 16. Photograph of an autoradiogram of the chromatographed reaction products of F. bidentis 7-ST I (A) and II (B) incubated with quercetin 3,3'-disulfate. Spots correspond to quercetin 3,7,3'-trisulfate. BAW (4:1:5, v/v/v) was used to develop the cellulose TLC plate. Lane C is the reaction products of Mono P-purified peak 3 incubated with quercetin: low R_f spot, quercetin disulfate; high R_f spot, quercetin 3-sulfate. BAW (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

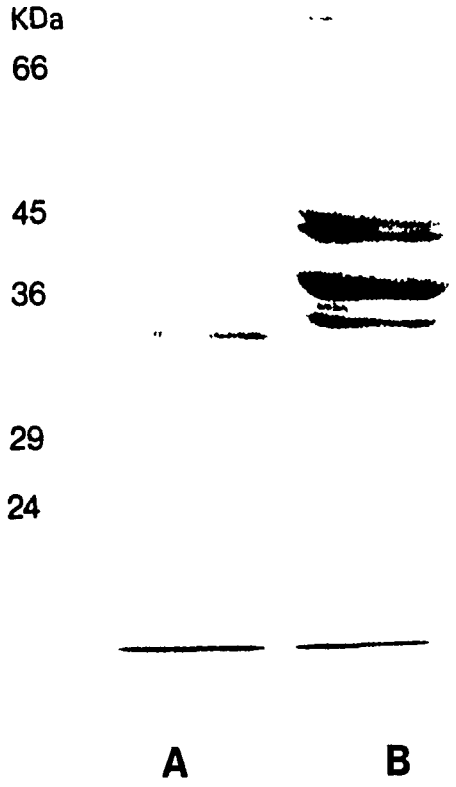


Figure 17. SDS-PAGE of partially purified (A), 7-ST I (ca. 5μg), and (B), 7-ST II (ca. 25 μg). Electrophoresis was performed as described in section C.3.9. Proteins were visualized by Coomassie staining. Numbers on the left correspond to molecular weight markers.

TABLE 12

Purification of *Flaveria bidentis* 7-ST I and II[1]

Purification step	Total protein (mg)	Specific activity (pKat/mg)	Total activity (pKat)	Purification (-fold)	% Recovery
Dowex	82	0.051	4.18	-	100
PAP-agarose	1.88	0.82	1.54	16	37
Mono-P					
7-ST 1	0.081	9.69	0.78	190	19
7-ST 2	0.078	6.37	0.50	125	12

[1] The sulfotransferase activities were assayed as described in the section C.3.5 using quercetin 3-3'-disulfate as substrate.

TABLE 13

Substrate specificity of *F. bidentis* 7-ST I and II[1]

Substrate	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
Quercetin 3,7-disulfate	0	0
Quercetin 3-sulfate	0	0
Quercetin 3'-sulfate	0	0
Quercetin	0	0

[1] The standard enzyme assay was used as described in Section C.3.5, at a substrate concentration of 1μM.

assayed against different substrates. The partially purified 7-STs were shown to possess 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 the 7-position. On the other hand, they did not accept quercetin 3-sulfate as substrate, thus indicating the absolute requirement for activity of a sulfate group at position 3, in addition to another sulfated or methoxylated 3'- or 4'-position. Neither of the two isoenzymes described here exhibited any activity with quercetin, quercetin 3-sulfate, quercetin 3'-sulfate, flavones or phenylpropanoids as substrates. The substrate specificity of the 7-ST charged isoenzymes is consistent with the accumulation, in *F. bidentis*, of isorhamnetin 3,7-disulfate (Cabrera and Juliani 1977) as well as quercetin 3,7,3'- and -3,7,4'-trisulfates (Cabrera and Juliani 1979; Cabrera et al. 1985).

D.2.4.3 Other properties.

The 7-ST I and II were assayed with quercetin 3,3'-disulfate in presence of histidine-HCl, *bis*-Tris-HCl, Tris-HCl, phosphate, and glycine-NaOH buffers over the pH range 5.5 to 9.5. Both isoenzymes exhibited maximum ST activity in phosphate buffer at pH 7.5 (Table 14). A change of one unit above or below the pH optimum resulted in a loss of 60 and 55% activity for the 7-ST I and II, respectively. Neither of the charged isoenzymes was affected by the addition of divalent cations, nor inhibited by EDTA, or SH-group reagents.

The apparent molecular weights of the 7-ST I and II were estimated by gel filtration. Both isoenzymes eluted from a calibrated Superose 12 column at a volume corresponding to a molecular weight of approximately 35,000 D.

Apparent kinetic parameters were evaluated using PAPS at fixed saturating concentration (1.0 μM) and quercetin 3,3'-disulfate as variable substrate (Fig. 18). Michaelis-Menten kinetics with flavonol was observed until a concentration of 1.5 μM at which point substrate inhibition was evident. The apparent Km values of the

TABLE 14

Properties of *F. bidentis* ST I and II

Property	7-ST I	7-ST II
Km Flavonoid (μM)[1]	0.24	0.20
Km PAPS (μM)	0.33	0.46
pH optimum	7.5	7.5
Apparent pI	6.5	6.3
Apparent mol wt (D)	35,000	35,000

[1] With quercetin 3,3'-disulfate as substrate

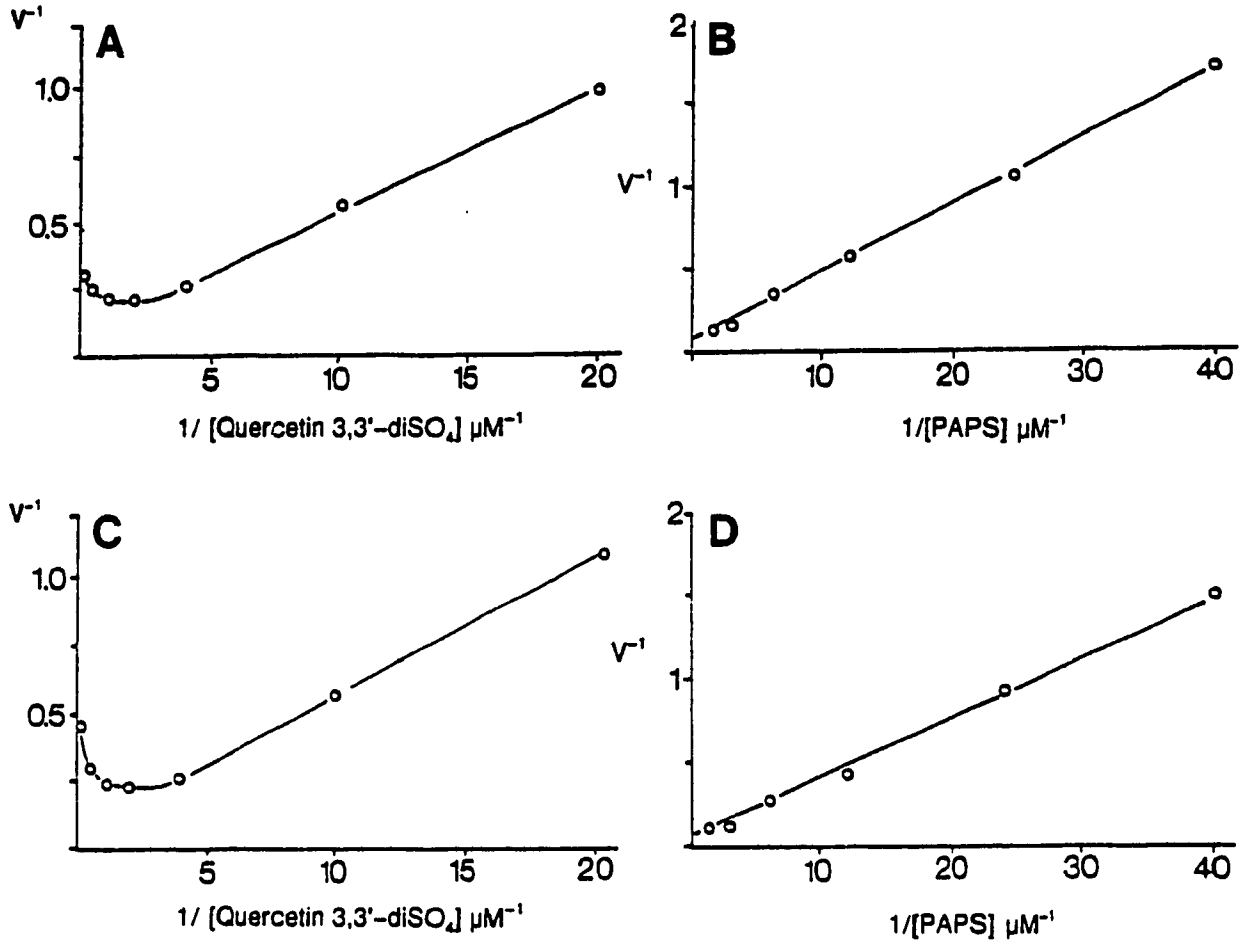


Figure 18. Initial velocities 1/V versus 1/Flavonol (A,C), and 1/PAPS (B,D) for the 7-ST I (A,B), and II (C,D). The constant flavonol concentration was 1.0 µM. The constant PAPS concentration was 1.0 µM. Velocities are expressed in pKat/mg protein. The assay conditions were as described in section C.3.5.

7ST I and II for the flavonol substrate were found to be 0.24 μM and 0.20 μM, respectively. The reaction was saturable when PAPS was the variable substrate without apparent substrate inhibition. The apparent Km values of the 7ST I and II for the sulfate donor, PAPS were found to be 0.33 μM and 0.46 μM, respectively (Fig. 18).

Table 14 summarizes the general properties of the 7ST I and II. Apart from differences in pI, both enzymes were found to have identical properties, which raises the question as to the significance of two 7-ST isoforms in *F. bidentis.*

D.2.5 Purification to homogeneity of *F. chloraefolia* 3-ST

The fact that flavonol 3-sulfates are of common occurrence in plants, and that sulfation at position 3 represents the first step in the biosynthesis of polysulfated flavonols, prompted us to develop a protocol which allows the purification of a homogenous 3-ST in sufficient quantity for the production of polyclonal antibodies. The antibodies will be used to study the serological relationship with other STs, and to isolate a cDNA clone coding for the 3-ST.

The 3-ST of *F. chloraefolia* was purified by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, DEAE-sephacel, hydroxyapatite and affinity chromatography on PAP-agarose (Fig. 19, 20). At this stage of purification, the enzyme preparation was still contaminated with the 3'- and 4'-ST activities. The two latter activities were removed by ion-exchange chromatography on a Mono Q column using the FPLC system. The 3-ST activity was recovered as a single peak which eluted with 90 mM NaCl. The enzyme activity peak was found to coincide with the major protein peak (Fig. 21). At this stage of purification the 3-ST was found to be free from other contaminating ST activities.

A summary of the 3-ST purification protocol is shown in Table 15, representing one typical experiment. On the average, about 2000-fold increase in specific activity

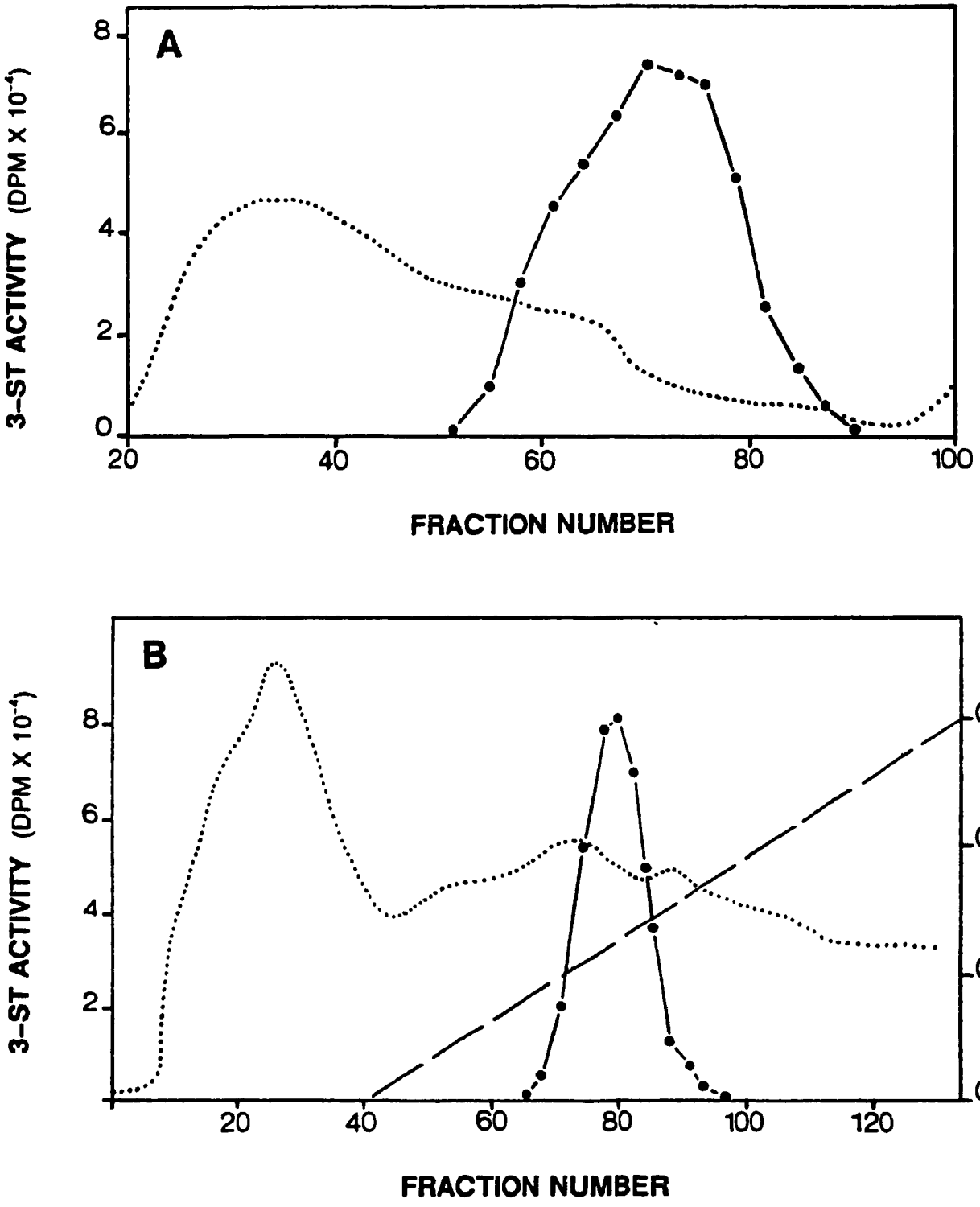


Figure 19. A) Elution profile of F. chloraefolia 3-ST activity after gel filtration on Sephadex G-100 as described in section C.3.4.2. B) Elution profile of the 3-ST activity after chromatography on DEAE-Sephacel as described in section C.3.4.3. The fractions were assayed with quercetin as substrate. Protein absorbance was monitored at 280 nm (.....).

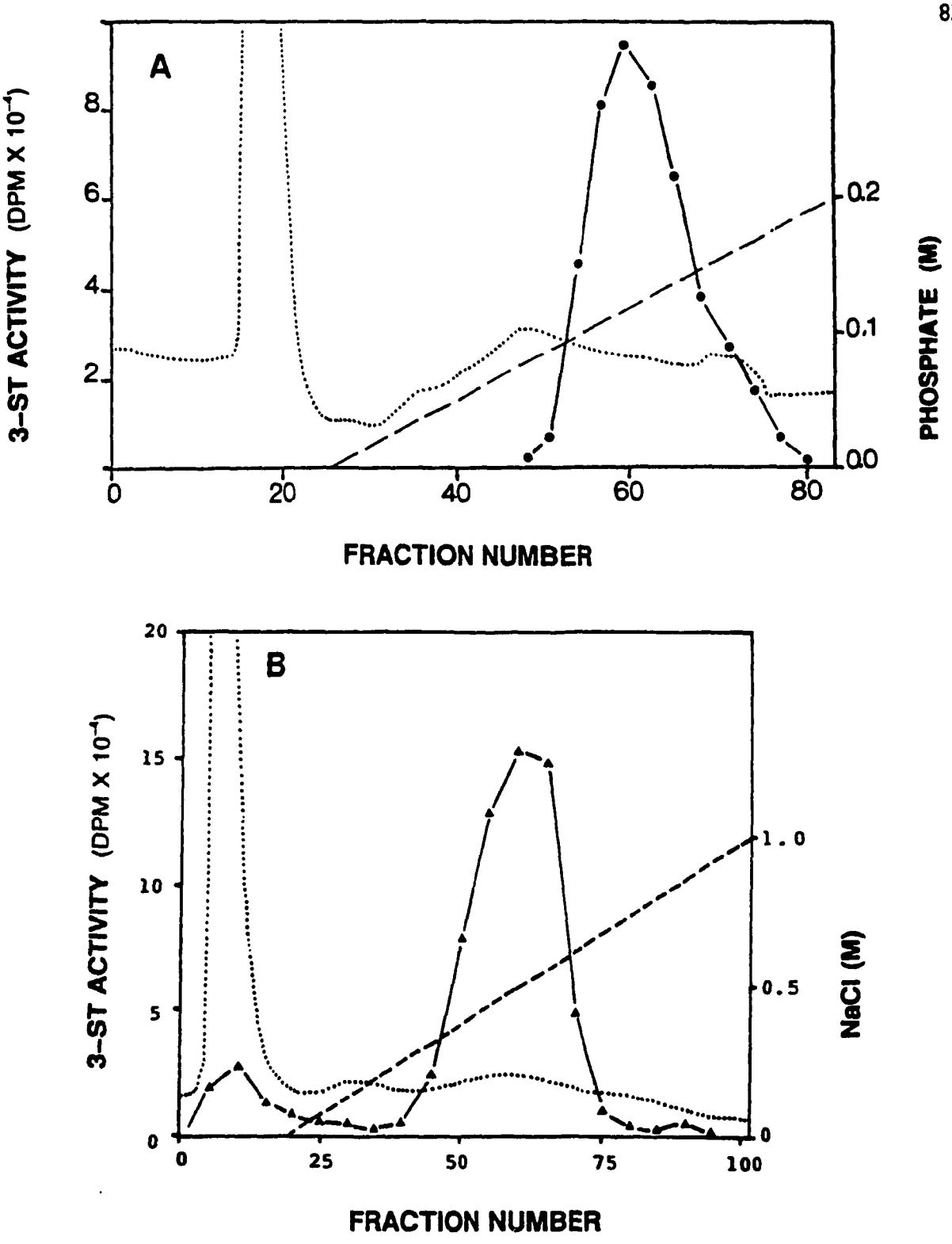


Figure 20. A) Elution profile of F. chloraefolia 3-ST activity after chromatography on hydroxyapatite as described in section C.3.4.4. B) Elution profile of the 3-ST following chromatography on PAP-agarose as described in section C.3.4.5. The fractions were assayed with quercetin as substrate. Protein absorbance was monitored at 280 nm (.....).

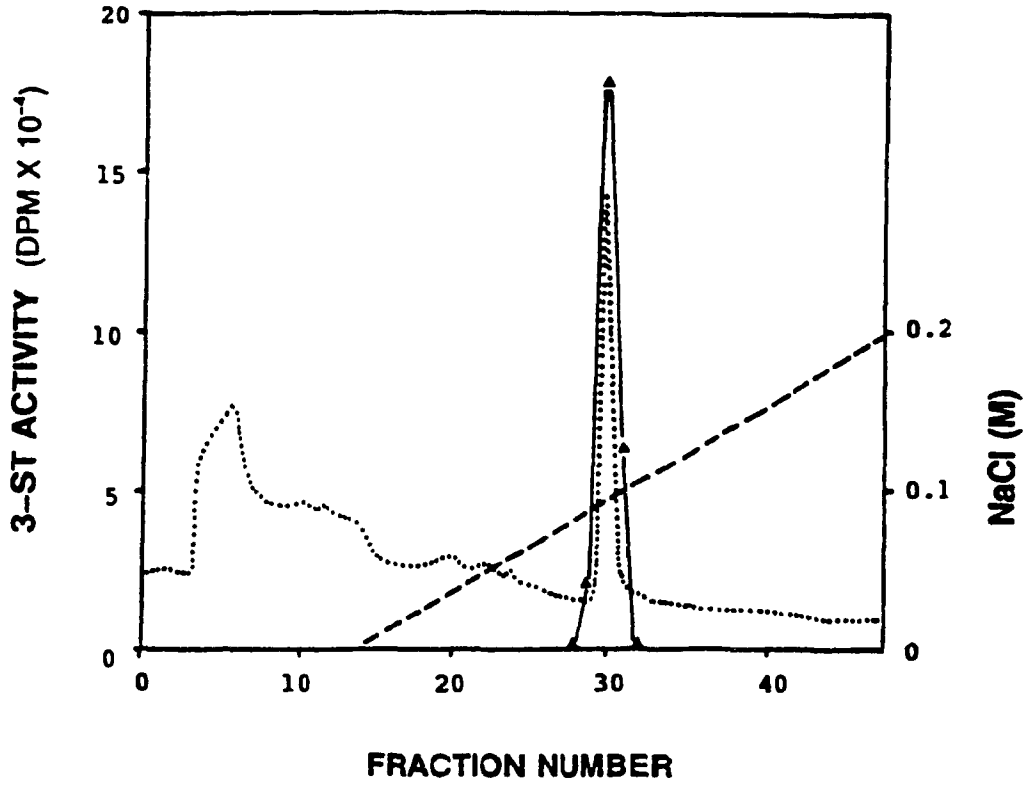


Figure 21. Elution profile of F. chloraefolia 3-ST activity after chromatography on Mono Q as described in section C.3.4.6. The fractions were assayed with quercetin as substrate. Protein absorbance was monitored at 280 nm (.....).

TABLE 15

Purification of *F. chloraefolia* 3-ST[1]

Purification step	Total protein (mg)	Specific activity (pKat/mg)	Total activity (pKat)	Purification fold	% Recovery
Dowex	1087	0.68	739	-	100
G-100	106	7.19	766	10.6	106
DEAE-sephacel	25	26.86	671	39.5	91
Hydroxyapatite	3.7	56.91	208	83.7	28
PAP-agarose	0.425	413.2	176	607.6	24
Mono Q	0.050	1356.8	68	1994	9.2

[1] The 3-sulfotransferase was assayed as described in section C.3.5 utilising quercetin as substrate.

could be obtained with a recovery of about 9%. The final preparation had a specific activity of 1.4 nKat/mg.

D.2.5.1 Criteria for homogeneity

The typical Coomassie blue staining pattern obtained at different stages of purification is illustrated in Figure 22. The Mono Q-purified protein migrated as a single band with an apparent molecular weight of 34,500 D. This value was consistent with the molecular weight of the native enzyme as determined by gel filtration.

To further demonstrate that the band observed on SDS-PAGE corresponded to the 3-ST, the Mono Q-purified enzyme was electrophoresed under non-denaturing conditions. A portion of the gel was used for protein visualization, whereas the remaining gel was sliced in 2 mm bands, and the enzyme protein was eluted and assayed for 3-ST activity using quercetin as substrate. Figure 23A shows a Coomassie blue stained preparation of the 3-ST after native-PAGE, where the stained protein band coincided with the enzyme activity (Figure 23B). Furthermore, when the eluted fraction corresponding to maximum 3-ST activity was subjected to SDS-PAGE, a single band was observed migrating at an apparent molecular weight of 34,500 (Fig. 23C)

D.2.5.2 Peptide purification and sequencing

The Mono Q-purified 3-ST (ca. 5 μg) was submitted to Edman degradation in order to obtain N-terminal sequence information. The N-terminal amino acid cleavage reaction did not release any free amino acid, suggesting that the N-terminal was blocked. To overcome this problem, the purified 3-ST (ca 30 μg) was cleaved with trypsin to obtain internal sequence information. Tryptic peptides were fractionated by reverse-phase HPLC on a Vydac c_{18} microbore column (Fig. 24). Two peptides, I and II, were selected for the symmetry of their respective peaks and for their

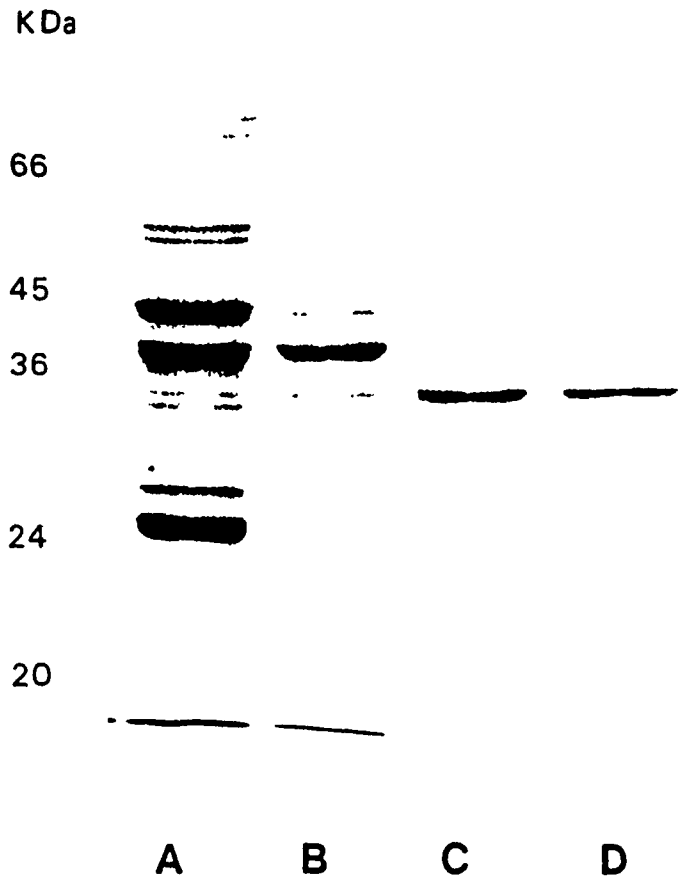


Figure 22. SDS-PAGE of fractions collected during purification of F. chloraefolia 3-ST. Lanes A) DEAE-sephacel (ca. 10 μg), B) Hydroxyapatite (ca. 10 μg), C) PAP-agarose (ca. 4 μg), D) Mono Q (ca. 1 μg). Electrophoresis was performed as described in section C.3.9. Proteins were visualized by Coomassie blue staining. Numbers on the left correspond to molecular weight markers.

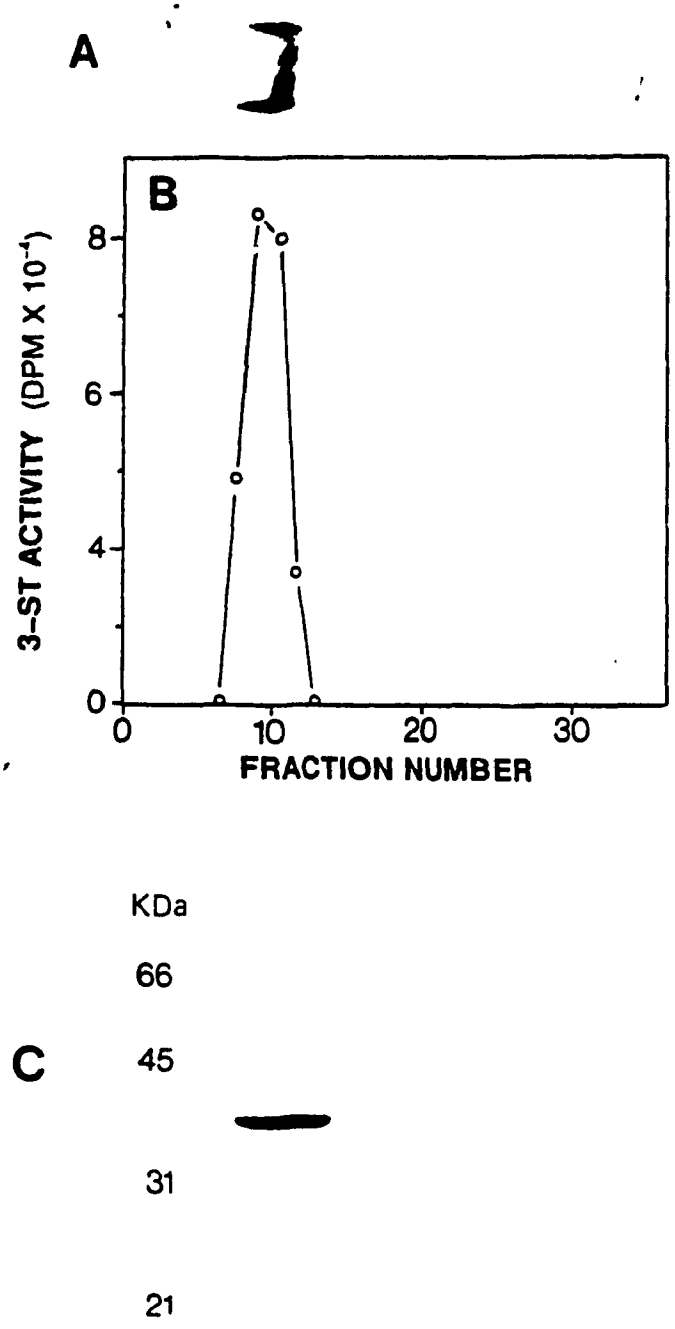


Figure 23. Mono Q-purified 3-ST was subjected to native-PAGE as described in section C.3.10. A) One lane containing 2 μg of purifed protein was stained with Coomassie blue. B) Activity profile of the eluted protein from the sliced gel (2mm). C) The most active fraction (ca. 1μg) was subjected to 12% SDS-PAGE on the Phastsystem. The protein band was revealed by silver staining.

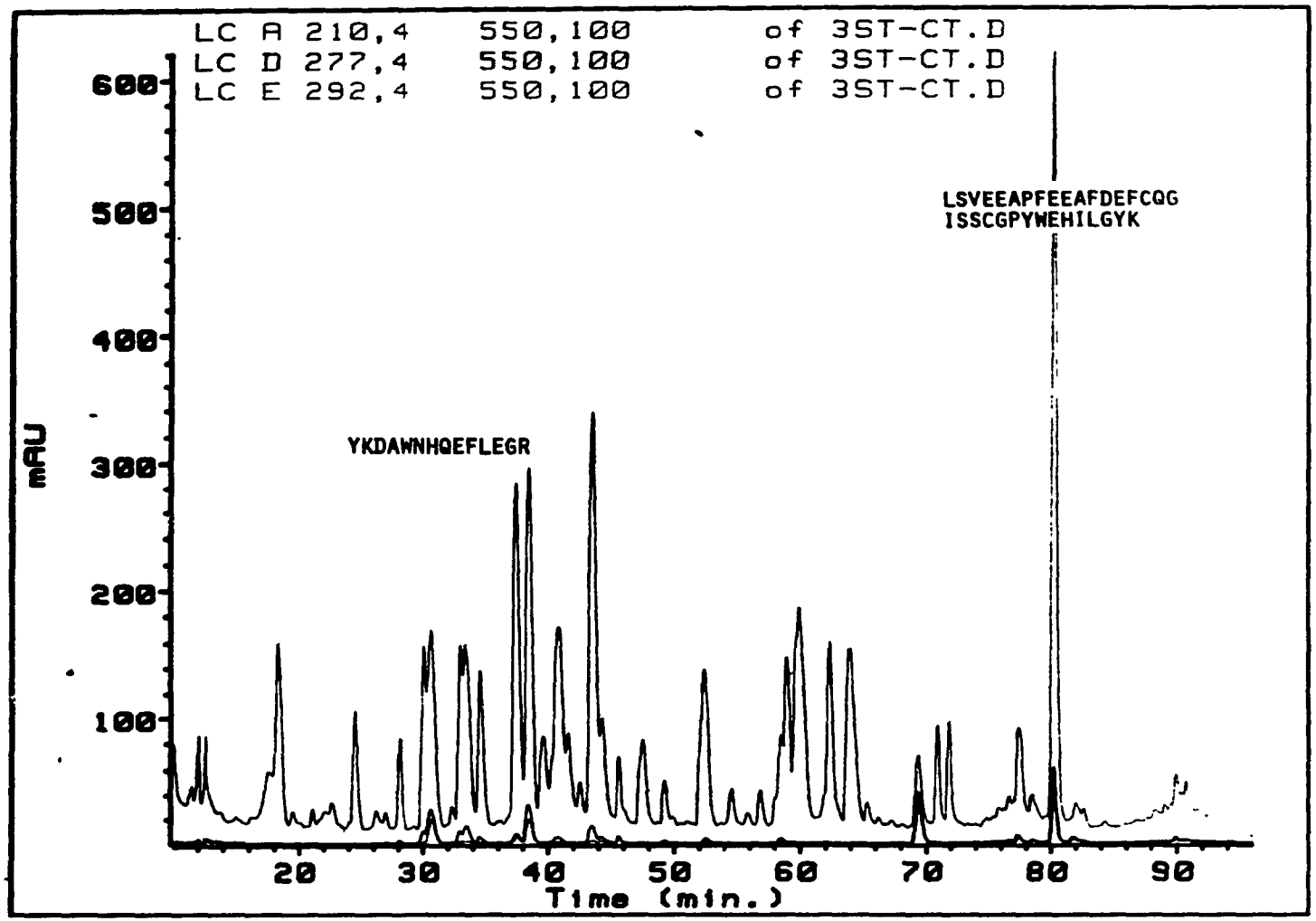


Figure 24. HPLC–tryptic profile of purified 3–ST. Mono Q–purified 3–ST was subjected to tryptic cleavage and the fragments were purified by HPLC on a Vydac C_{18} column as described in section C.3.11.

absorbance at 292 nm suggesting the presence of a tryptophan residue. The sequences of both peptide are shown in Tables 16 and 17, respectively. Peptide I was found to be composed of 14 amino acids, while peptide II contained 33 amino acids. The amino acid released at cycle 26 of peptide II could not be identified. Based on the absorption of this peptide at 292 nm, a tryptophan residue was tentatively assigned to this position. Both peptides end with basic amino acids as expected for tryptic peptides.

D.2.6 Immunological studies

D.2.6.1 Specificity of the anti-3-ST antibodies

Following the immunization protocol described in section C.4.1, the animals were sacrificed and the serum collected. The specificity of the serum for the 3-ST was verified by Western blot. Samples of *F. chloraefolia* crude protein extract (ca. 25 μg) and purified 3-ST preparation (ca. 1 μg) were transferred to nitrocellulose after separation by SDS-PAGE (Fig. 25). Immunodetection of the blotted proteins revealed that the anti-3-ST immune serum reacted specifically with a protein migrating at the level of the purified 3-ST (Fig. 25). No protein band could be detected when the blots were incubated with the pre-immune serum.

D.2.6.2 Cross reactivity of anti 3-ST antibodies with other STs of *F. chloraefolia*

We investigated the extent to which the 3-ST antisera cross-react with the other STs of *F. chloraefolia*. The Mono P-purified 3-, 3'-, and 4'-STs were electrophoresed and blotted on nitrocellulose and incubated with the anti-3-ST antibodies. Figure 26 shows that the antibodies recognized also the 3'- and the 4'-STs. The migration behavior of the immunodetected proteins corresponded to their expected molecular weights.

TABLE 16

PEPTIDE SEQUENCE I

Cycle	Residue	pmole[1]	Absolute[2] yield	Background[3]	Lag%[4]
1	TYR	231.6	231.6	0	2
2	LYS	212.2	213.3	1	1.4
3	ASP	99.8	100.4	O.6	7
4	ALA	181.2	184.5	3.2	1.9
5	TRP	46.6	66.1	19.5	2
6	ASN	87.8	90.2	2.4	3.7
7	HIS	36	37.3	1.3	3.7
8	GLN	85.5	88.2	2.7	3.4
9	GLU	51.4	65.9	14.5	1.4
10	PHE	63.2	68.1	4.9	2.6
11	LEU	74.2	80.1	5.9	1.6
12	GLU	37.4	45.6	8.2	1.5
13	GLY	51.7	56.6	4.9	2.6
14	ARG	17.1	17.1	0	4.3

[1] pmole = Absolute yield - Background
[2] Absolute yield = Yield of residue at cycle N
[3] Background = Yield of residue at cycle N-1
[4] Lag% = 100 X ((yield of residue at cycle N+1 - Bkg)/(Abs - Bkg))/ (N+1)

TABLE 17

PEPTIDE SEQUENCE II

Cycle	Residue[1]	pmole[2]	Absolute[3] yield	Background[4]	Lag%[5]
1	LEU	89.8	89.8	0	4.2
2	SER	49.3	49.3	0	1.9
3	VAL	54.5	54.8	0.3	1.3
4	GLU	39.1	39.5	0.4	nd
5	GLU	40.8	41.2	0.4	2.1
6	ALA	59.6	59.6	0	1.3
7	PRO	57.2	57.9	0.7	3.2
8	PHE	60.7	61	0.3	2.4
9	GLU	34	35.7	1.7	nd
10	GLU	33.1	34.8	1.7	2.1
11	ALA	35	36.3	1.3	2
12	PHE	41.7	42.9	1.2	1.9
13	ASP	19.8	19.8	0	1.9
14	GLU	10.6	12.6	2	2.6
15	PHE	27	30.3	3.3	2.9
16	CYS	0	0	0	nd
17	GLN	19	19	0	2.6
18	GLY	18.4	19.7	1.3	1.9
19	ILE	20.5	21.8	1.2	2.1
20	SER	9.7	12	2.3	nd
21	SER	12.4	14.6	2.3	10.8
22	CYS	0	0	0	nd
23	GLY	9.9	11.8	1.9	1.4
24	PRO	8.5	8.5	0	2.7
25	TYR	4.4	4.4	0	2.7
26	nd	nd	nd	nd	nd
27	GLU	1.6	2.1	0.4	2
28	(HIS)	0	0	0	nd
29	ILE	3.3	4.1	0.8	3.1
30	LYS	4.4	4.4	0	2.5
31	GLY	2.7	4	1.3	2.1
32	TYR	1.1	1.7	0.7	1.8
33	(LYS)	0	0	0	nd

[1] An identified residue in parantheses indicate a low confidence result.
[2] pmole = Absolute yield - Background
[3] Absolute yield = Yield of residue at cycle N
[4] Background = Yield of residue at cycle N-1
[5] Lag% = 100 X ((yield of residue at cycle N+1 - Bkg)/(Abs - Bkg))/ (N+1)

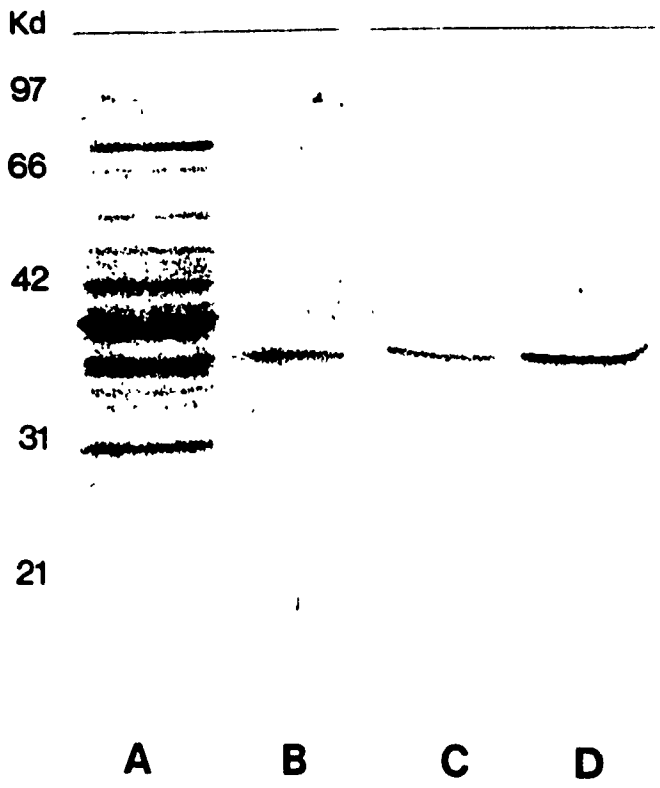


Figure 25. Western blot: Analysis of anti-3-ST antibodies. Hydroxyapatite- (A,C) and Mono Q-purified (B,D) 3-ST were electrophoresed and electrotransferred to nitrocellulose as described in sections C.3.9 and C.4.2. Lanes A and B were stained with amido black for protein detection. Lanes C and D were immunodetected with the anti-3-ST antibodies as described in section C.4.2. Numbers on the left correspond to molecular weight markers.

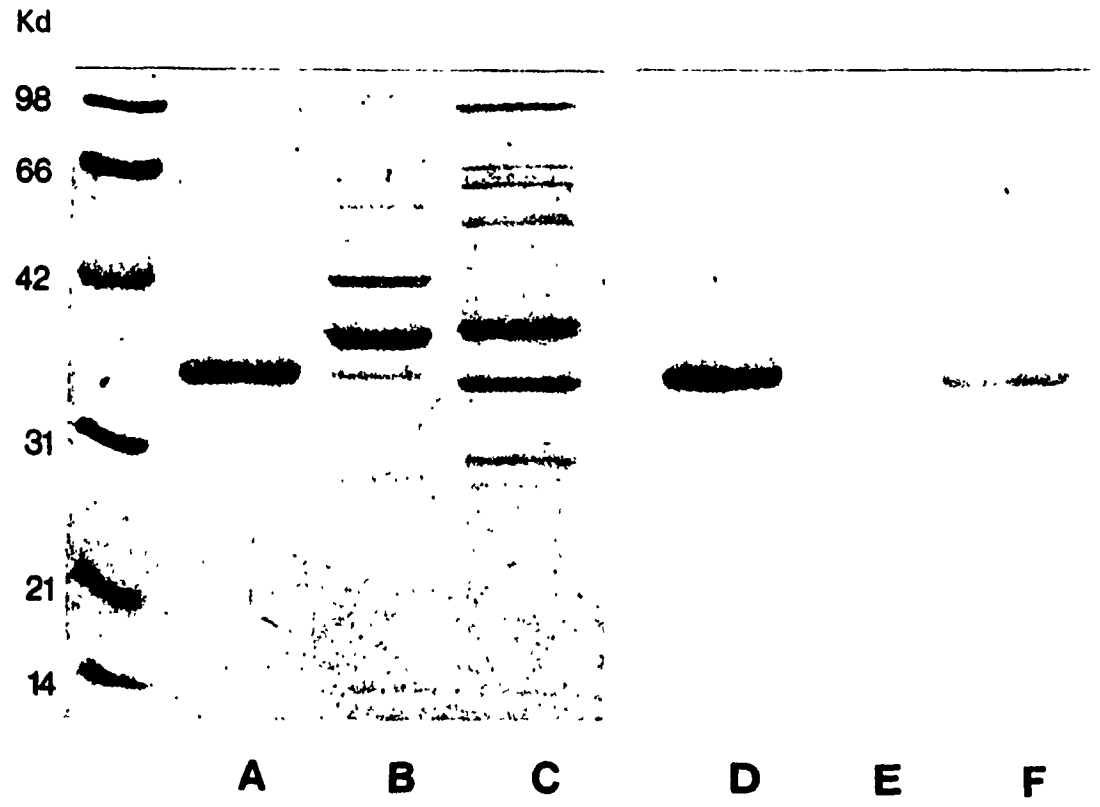


Figure 26. Western blot: Cross reactivity of the anti-3-ST antibodies with the 3- (A,D), 3'- (B,E), and 4'-STs (C,F) were electrophoresed and electrotransferred to nitrocellulose as described in sections C.3.9 and C.4.2. Lanes A,B, and C were stained with amido black for protein detection. Lanes D,E, and F were immunodetected with the anti-3-ST antibodies as described in section C.4.2. Numbers on the left correspond to molecular weight markers.

D.3 Molecular cloning

D.3.1 Isolation of *F. chloraefolia* ST cDNA clones

Poly(A)$^+$ mRNAs isolated from terminal buds of *F. chloraefolia* were used to construct a cDNA library in the expression vector lambda ZAP. The cDNA library was found to contain 4.0 x 10^6 pfu with less than 1% non-recombinants. About 320,000 plaques of the cDNA library were screened with the anti-3-ST antibodies, and 49 positive clones were isolated. Plasmids containing the cDNA inserts were excised from the purified phage by superinfection with the helper phage M13K08. Plasmid DNA from the positive clones was purified and the size of the cDNA insert was determined by digestion with EcoR1. Two patterns of digestion were obtained (Fig. 27). The first one (Group I) was present in 6 of the 49 clones and consisted of one fragment of about 1200 base pairs. The second pattern (Group II) was present in 40 of the 49 positive clones and generated two fragments of 320 and 950 base pairs, suggesting the presence of an internal EcoR1 site in the cloned cDNA. The remaining three clones contained inserts bearing no relationship with one another, and varying in size between 450 and 700 base pairs in length.

In order to demonstrate that our cDNA clones contained sequences coding for flavonol STs, we expressed the cDNA in *E. coli.* Only one of the 6 clones belonging to the Group I demonstrated ST activity in *E. coli.* This clone accepted quercetin but did not accept partially sulfated flavonols. The reaction product co-chromatographed with quercetin 3-sulfate (Fig. 28B). On the other hand, cells transformed with the control plasmid pSTH1 contained no measurable 3-ST activity. These results strongly suggest that one clone of Group I contains a cDNA coding for the 3-ST enzyme and was tentatively identified as pFST3.

Partial sequencing of each of the Group I clones was determined by double stranded dideoxy sequencing. All six clones were found to be of different length, but having the same nucleotide sequence. The longest clone (pFST3) was the one exhibiting

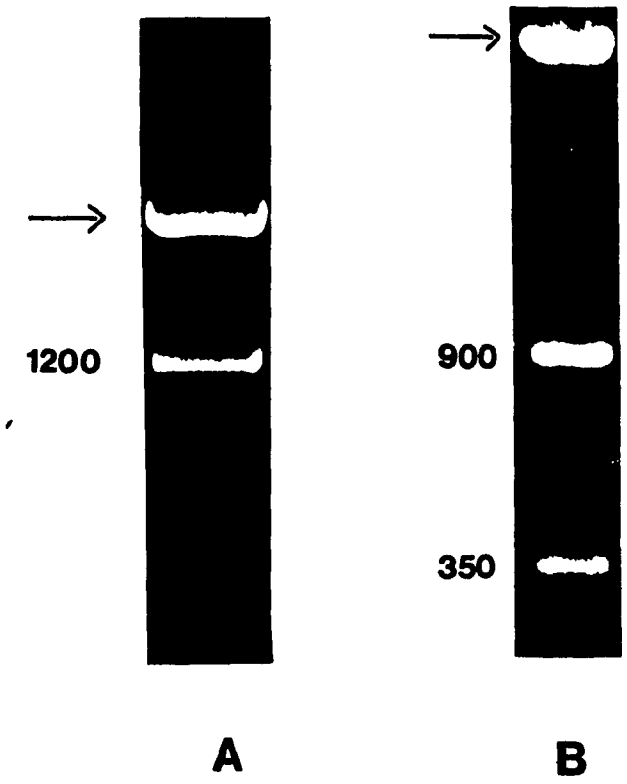


Figure 27. Photographs of EcoR1 digested pFST3 (A) and pFST4'(B). The digestion mixtures were electrophoresed on 1% agarose gels and the DNA visualized as described in section C.5.4.3. Numbers on the left correspond to the approximate size of the DNA fragments.
—>, linearized pBLUESCRIPT.

3-ST activity in *E. coli*. The remaining clones of Group I were missing between 48 and 93 nucleotides at their 5' end. Protein extract from IPTG-induced pFST3 was electrophoresed and transferred onto nitrocellulose membranes for immunodetection with the anti-3-ST antibodies. One protein band corresponding to a molecular weight of 43,000D was detected (Fig. 28A). The presence of this band correlates well with the expected molecular weight of pFST3 fusion protein. No band was detected in the protein extract of *E. coli* cells harboring the pSTH1 control plasmid.

The substrate specificity of the second group of clones was also studied and all of which were found to accept quercetin 3-sulfate as substrate. None of the Group II clones accepted quercetin as substrate. To confirm the identity of Group II clones as coding for the 3'- or 4'-STs, several substrates were tested. All the clones of the Group II accepted kaempferol 3-sulfate and isorhamnetin 3-sulfate, but did not accept tamarixetin 3-sulfate. The enzyme reaction products co-chromatographed with the 3,4'-disulfate derivatives (Fig. 29B). Cells transformed with the control plasmid pSTH1 contained no measurable ST activity when assayed with quercetin 3-sulfate. These results clearly suggest that the Group II clones are coding for the 4'-ST. Five clones belonging to Group II were partially sequenced by double stranded dideoxy sequencing. The sequence of two clones were identical, whereas the remaining three had identical sequence although they differed in length. One of the two longest clones was used for full characterization and was tentatively identified as pFST4'.

A protein extract from IPTG-induced pFST4' clone was electrophoresed by SDS-PAGE and transferred to nitrocellulose membranes for immunodetection with the anti 3-ST antibodies. A band was revealed migrating at a molecular weight of 32,000 D (Fig. 29A). The presence of this band was not detected in a protein extract of *E. coli* cells harboring the pSTH1 control plasmid. The observed molecular weight is lower than the 34,500 D obtained for the 3-ST by SDS-PAGE, and the 43,000 D expected molecular weight of the fusion protein. The difference between the observed

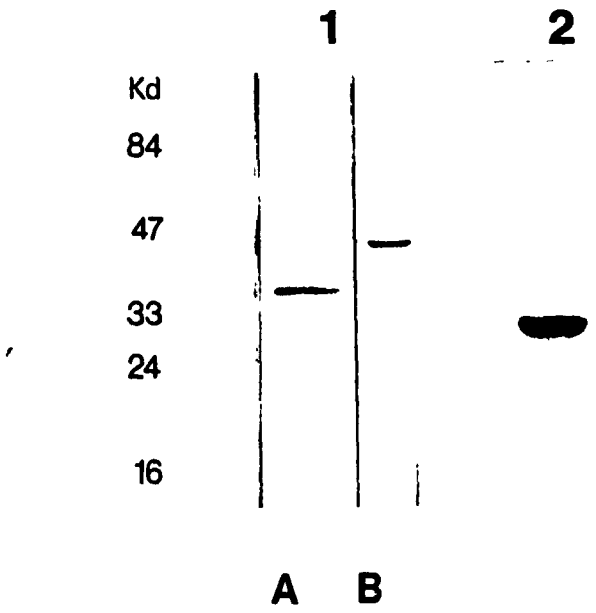


Figure 28. 1) Western blot: Hydroxyapatite purified 3-ST (A) and a protein extract from pFST3 (B) were electrophoresed, electrotransferred to Immobilon, and immunodetected as described in sections C.3.9 and C.4.2. Numbers on the left correspond to molecular weight markers.
2) Photograph of an autoradiogram of the chromatographed reaction product of a pFST3 protein extract incubated with quercetin. Reaction product corresponds with quercetin 3-sulfate. BAW (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

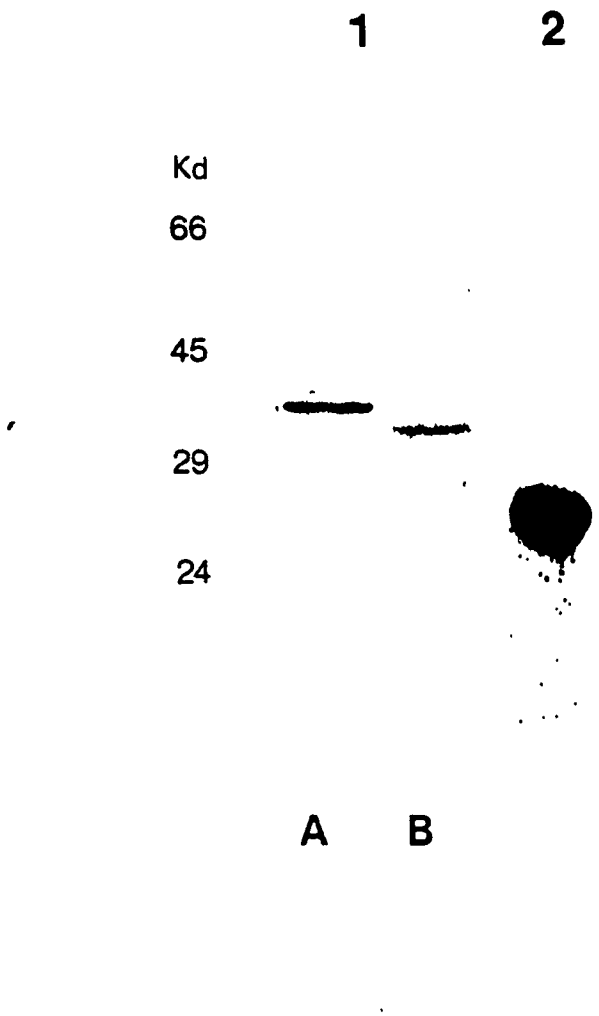


Figure 29. 1) Western blot; Hydroxyapatite purified 3-ST (A) and a protein extract from pFST4' (B) were electrophoresed, electrotransferred to Immobilon, and immunodetected as described in sections C.3.9 and C.4.2. Numbers on the left correspond to molecular weight markers.
2) Photograph of an autoradiogram of the chromatographed reaction product of a pFST4' protein extract incubated with quercetin 3-sulfate. Reaction product corresponds to quercetin 3,4'-disulfate. BAW (6:2:2, v/v/v) was used to develop the cellulose TLC plate.

and expected molecular weights may reflect the influence of the fusion protein primary structure on the amount of SDS it can bind (De Jong et al. 1978). It may have also been due to the proteolytic cleavage at an exposed site of the immunodetected protein, thus generating the smaller molecular weight fragment.

D.3.2 Deduced primary structure of *F. chloraefolia* 3-ST

The complete nucleotide sequence of pFST3 was determined for both strands. The results displayed in Figure 30 show that the pFST3 cDNA contained a 1153 base-pair cDNA insert with a single long ORF begining at nucleotide 19 and terminating at nucleotide 954. The methionine initiation codon is not surrounded by the AACAATGGC consensus sequence, which is commonly associated with ATG translation initiation codons in plant genes (Luetcke et al. 1987). A 204-base pair non-coding sequence separates the stop codon from the 10 nucleotide polyadenylic acid tail at the 3' end. The ORF of the cloned cDNA (pFST3) encodes a putative protein of 311 amino acid residues with a calculated molecular weight of 36,442 D. The calculated molecular weight is in close agreement with the molecular weight of the 3-ST determined by gel filtration and by SDS-PAGE. That pFST3 encodes the 3-ST is further confirmed by the presence in the deduced amino acid sequence of segments matching the sequences determined for the two peptide fragments of the purified 3-ST (Fig. 30). The deduced polypeptide contains 56 basic and 47 acidic residues and is made of 33.1% charged and 33.8% apolar amino acids. It is interesting to note that the 5 other clones of Group I were found to miss between 10 to 25 amino acids at the N terminal part of the protein, which may explain their catalytic inactivity.

D.3.3 Deduced primary structure of *F. chloraefolia* 4'-ST

Both strands of pFST4' cDNA insert were sequenced by the double stranded dideoxy chain termination reaction (Fig. 31). pFST4' was found to contain a 1242

```
                                    M   E   D   I   I   K   T   L   P   Q   H
1       GTATCATTTCGATCAATT          ATG GAA GAT ATT ATC AAA ACA CTT CCA CAA CAC
          T   C   S   F   L   K   H   R   F   T   L   Y   K   Y   K   D
52      ACT TGT AGC TTT CTG AAA CAC AGG TTC ACC TTG TAC AAG TAT AAA GAC
          A   W   N   H   Q   E   F   L   E   G   R   I   L   S   E   Q
100     GCC TGG AAT CAT CAG GAG TTT CTT GAA GGA CGA ATT TTA TCT GAA CAA
          K   F   K   A   H   P   N   D   V   F   L   A   S   Y   P   K
149     AAG TTC AAG GCT CAT CCC AAT GAT GTA TTT CTC GCT AGT TAT CCC AAA
          S   G   T   T   W   L   K   A   W   I   C   I   I   T   R   E
197     AGT GGC ACA ACA TGG CTA AAA GCT TGG ATT TGC ATC ATA ACG CGA GAA
          K   F   D   D   S   T   S   P   L   L   T   T   M   P   H   D
245     AAG TTT GAT GAT TCC ACA AGT CCT TTG CTC ACA ACC ATG CCT CAT GAT
          C   I   P   L   L   E   K   D   L   E   K   I   Q   E   N   Q
293     TGC ATT CCT CTC CTA GAG AAA GAC CTT GAA AAA ATT CAA GAA AAC CAA
          R   N   S   L   Y   T   P   I   S   T   H   F   H   Y   K   S
341     AGG AAC TCG CTC TAC ACA CCC ATC TCC ACA CAC TTT CAT TAC AAA TCC
          L   P   E   S   A   R   T   S   N   C   K   I   V   Y   I   Y
389     CTA CCG GAG TCG GCC CGA ACA TCA AAC TGC AAG ATA GTT TAC ATA TAC
          R   N   M   K   D   V   I   V   S   Y   Y   H   F   L   R   Q
437     CGG AAC ATG AAA GAT GTC ATT GTT TCT TAT TAC CAT TTC CTG AGA CAG
          I   V   K   L   S   V   E   E   A   P   F   E   E   A   F   D
485     ATA GTT AAA CTA TCT GTG GAA GAG GCC CCA TTT GAG GAG GCT TTT GAT
          E   F   C   Q   G   I   S   S   C   G   P   Y   W   E   H   I
533     GAG TTT TGT CAG GGT ATT TCA AGT TGT GGA CCA TAT TGG GAA CAC ATC
          K   G   Y   W   K   A   S   L   E   K   P   E   I   F   L   F
581     AAG GGA TAC TGG AAA GCA AGC TTG GAG AAG CCG GAG ATA TTT CTT TTC
          L   K   Y   E   D   M   K   K   D   P   V   P   S   V   K   K
629     TTG AAA TAC GAA GAC ATG AAA AAG GAT CCG GTA CCA AGT GTG AAG AAA
          L   A   D   F   I   G   H   P   F   T   P   K   E   E   E   A
677     CTT GCG GAT TTC ATT GGG CAT CCC TTT ACA CCC AAA GAA GAG GAA GCG
          G   V   I   E   D   I   V   K   L   C   S   F   E   K   L   S
725     GGT GTG ATA GAA GAT ATT GTA AAG TTA TGT AGT TTT GAG AAA TTG AGC
          S   L   E   V   N   K   S   G   M   H   R   P   E   E   A   H
773     AGC TTA GAA GTA AAC AAA AGT GGC ATG CAT CGT CCC GAA GAA GCT CAT
          S   I   E   N   R   L   Y   F   R   K   G   K   D   G   D   W
821     TCC ATT GAA AAC CGG CTT TAC TTC AGG AAG GGT AAG GAT GGA GAT TGG
          K   N   Y   F   T   D   E   M   T   Q   K   I   D   K   L   I
869     AAG AAC TAC TTC ACT GAT GAG ATG ACT CAG AAA ATA GAC AAA CTG ATC
          D   E   K   L   G   A   T   G   L   V   L   K   *
917     GAC GAG AAA CTG GGT GCC ACT GGT TTA GTT CTA AAA TGA AAGGCTTCAAG
967     ATGCTATCTAGTTGAGAGCAAAACTTGATTTCAAACTATCTTTCATTAGTCTTGTTTCAAATA
1030    ATTGAGGCCGATGTCTCTTTTCCATTCTTTCTTTTCATGCATCATTATGTTATGTAATTTAGT
1093    TTGGGTTTATGTTATTTTGTTGCATGTCTTATTAATAATAATTGTTTTCATTGTTGGAAAAAA
1156    AAAA
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Figure 30. Nucleotide and deduced amino acid sequences of pFST3. The boxed regions match with the peptide I and II of the 3-ST.

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1       GTTTTCTCTTCCTTGCAGTACCAGTAGCCACTAGCAATCATATATTCATATATATATAT
          M   E   T   T   K   T   Q   F   E   S   M   A   E   M   I
60      ATG GAA ACT ACA AAA ACC CAG TTT GAG TCC ATG GCA GAA ATG ATC
          K   K   L   P   Q   H   T   C   S   S   L   K   G   R   I
105     AAA AAA CTT CCA CAA CAT ACT TGT AGT TCG TTG AAA GGC AGG ATC
          T   L   Y   K   Y   Q   D   F   W   G   L   Q   N   N   I
150     ACG TTA TAT AAG TAT CAA GAC TTT TGG GGT CTT CAG AAC AAC ATT
          E   G   A   I   L   A   Q   Q   S   F   K   A   R   P   D
195     GAA GGA GCA ATT CTT GCT CAA CAA AGT TTC AAG GCT CGA CCA GAT
          D   V   F   L   C   S   Y   P   K   S   G   T   T   W   L
240     GAT GTA TTT CTA TGC AGT TAT CCC AAA AGT GGC ACA ACA TGG CTA
          K   A   L   A   Y   A   I   V   T   R   E   K   F   D   E
285     AAG GCC TTG GCT TAT GCC ATT GTA ACC CGA GAA AAG TTT GAT GAA
          F   T   S   P   L   L   T   N   I   P   H   N   C   I   P
330     TTC ACG AGT CCG TTG CTC ACG AAC ATT CCT CAT AAT TGC ATT CCC
          Y   I   E   K   D   L   K   K   I   V   E   N   Q   N   N
375     TAC ATT GAG AAA GAC CTT AAA AAA ATA GTT GAA AAC CAA AAT AAC
          S   C   F   T   P   M   A   T   H   M   P   Y   H   V   L
420     TCA TGC TTC ACA CCC ATG GCT ACA CAC ATG CCC TAC CAT GTT CTA
          P   K   S   I   L   A   L   N   C   K   M   V   Y   I   Y
465     CCA AAA TCC ATT CTA GCT TTA AAC TGC AAG ATG GTT TAC ATA TAT
          R   N   I   K   D   V   I   V   S   F   Y   H   F   G   R
510     CGC AAT ATA AAA GAT GTC ATA GTT TCT TTT TAC CAT TTT GGG AGA
          E   I   T   K   L   P   L   E   D   A   P   F   E   E   A
555     GAA ATA ACA AAA CTA CCC TTG GAA GAT GCC CCA TTT GAG GAG GCA
          F   D   E   F   Y   H   G   I   S   Q   F   G   P   Y   W
600     TTT GAC GAG TTC TAT CAT GGC ATT TCC CAA TTC GGA CCA TAT TGG
          D   H   L   L   G   Y   W   K   A   S   L   E   R   P   E
645     GAT CAT CTA TTG GGA TAT TGG AAA GCA AGC TTG GAA AGG CCA GAG
          V   I   L   F   L   K   Y   E   D   V   K   K   D   P   T
690     GTA ATT TTA TTC TTG AAA TAT GAA GAT GTG AAA AAG GAT CCT ACA
          S   N   V   K   R   L   A   E   F   I   G   Y   P   F   T
735     AGT AAT GTA AAG AGA CTT GCG GAG TTC ATT GGG TAT CCA TTT ACA
          F   E   E   E   K   E   G   V   I   E   S   I   I   K   L
780     TTT GAA GAA GAG AAA GAA GGT GTG ATA GAA AGC ATC ATA AAG TTG
          C   S   F   E   N   L   S   N   L   E   V   N   K   S   G
825     TGT AGT TTC GAG AAT TTG AGC AAC TTA GAA GTA AAC AAA AGT GGT
          N   S   K   G   F   L   P   I   E   N   R   L   Y   F   R
870     AAT TCA AAA GGA TTT CTT CCA ATT GAA AAT CGA CTT TAC TTC AGG
          K   A   K   D   G   D   W   K   N   Y   F   T   D   E   M
915     AAG GCT AAG GAC GGA GAT TGG AAG AAC TAC TTC ACG GAT GAA ATG
          T   E   K   I   D   K   L   I   D   E   K   L   S   A   T
960     ACT GAA AAG ATT GAT AAA CTA ATT GAC GAG AAA CTG AGC GCC ACT
          G   L   V   L   K   *
1005    GGT TTA GTT CTA AAA TGA AATGCGCAAGATCACATCCTTTGCTCAGCTATTTG
1058    AAAGTTTATTGGTTGAAATTTCCTGTATTAAATAATTAAGGGTTATGTGTCTCTCTCTC
1117    TATCATTATGTCATGTTATATCTTATGTCTATCATTATGTCTATCATTATGTCATGTTC
1176    TATATTTGAGATTTAAACTATCATGTTCGTATGTCTATTAATAATCAAAAAAAAAAAAA
1235    A
```

Figure 31. Nucleotide and deduced amino acid sequence of pFST4'.

base-pair cDNA insert with a long ORF starting at nucleotide 67 and terminating at nucleotide 1029. Two other ATG codons were present at nucleotides 97 and 106 of the ORF. None of the three in-frame methionine codons in the 5' region of the cDNA was surrounded by the AACAATGGC consensus sequence which is commonly associated with ATG translation initiation codons in plant genes (Luetcke et al 1987). A 213-base pair non-coding sequence separates the stop codon from the 14 nucleotide polyadenylic acid tail at the 3' end. The longest ORF encodes a putative protein of 320 amino acids with a calculated molecular weight of 37,212 D. The deduced polypeptide contains 48 basic and 44 acidic residues and is made of 28.8% charged and 36.3% apolar amino acids.

D.3.4 pFST3 and pFST4' sequence alignment

The nucleotide and the amino acid sequences of pFST3 and pFST4' were compared using the FASTA software (Pearson and Lipman 1988). The search revealed nucleotide and amino acid identities of 74% in 1141 nucleotides overlap and 69% in 311 amino acids overlap, respectively (Figs. 32, 33). It is interesting to note that the alignment matches the ATG of pFST3 with the second ATG of pFST4'. Furthermore, no sequence identity at the nucleotide level is observed between the two clones for the nucleotides 5' to the ATG of pFST3. These results suggest that the second ATG codon might be used for initiation of translation of the 4'-ST mRNA. Another possibility will be that the cDNA clone of the 3-ST is not full length. The very high similarities observed in the amino acid sequences of pFST3 and pFST4' make it difficult to deduce sequences which might be functionally important. However, there are few regions which exhibit distinct variability. The regions covering the amino acids 16 to 39, 110 to 132, and 260 to 267 of pFST3 represent most of the divergences found. The structural similarities between *F. chloraefolia* 3- and 4'-STs were further revealed by comparing their hydropathy profiles (Fig. 34).

Figure 32. Comparison between pFST3 and pFST4' nucleotide sequences. The alignment was made using the FASTA software. (:) indicates an identical nucleotide. Dashes represent gaps introduced for maximal alignment.

```
                          10        20        30        40
pFST3                GTATCATTTCGATCAATTATGGAAGATATTATCAAAACACTTCCACAA
                                  :::: ::: :: :::::::: ::::::::::
pFST4' ATGGAAACTACAAAAACCCAGTTTGAGTCCATGGCAGAAATGATCAAAAAACTTCCACAA
      60        70        80        90        100       110

       50        60        70        80        90        100
pFST3  CACACTTGTAGCTTTCTGAAACACAGGTTCACCTTGTACAAGTATAAAGACGCCTGGAAT
       :: :::::::: :   :::::  :::: :::: :: :: :::::: :::::   :::  :
pFST4' CATACTTGTAGTTCGTTGAAAGGCAGGATCACGTTATATAAGTATCAAGACTTTTGGGGT
     120       130       140       150       160       170

      110       120       130       140       150       160
pFST3  CATCAGGAGTTTCTTGAAGGACGAATTTTATCTGAACAAAAGTTCAAGGCTCATCCCAAT
       : :::: :     :::::::: :::: :  :: :::::  :::::::::  ::  ::
pFST4' CTTCAGAACAACATTGAAGGAGCAATTCTTGCTCAACAAAGTTTCAAGGCTCGACCAGAT
     180       190       200       210       220       230

      170       180       190       200       210       220
pFST3  GATGTATTTCTCGCTAGTTATCCCAAAAGTGGCACAACATGGCTAAAAG-CTTGGATT-T
       ::::::::::    :::::::::::::::::::::::::::::::: : ::::: :: :
pFST4' GATGTATTTCTATGCAGTTATCCCAAAAGTGGCACAACATGGCTAAAGGCCTTGGCTTAT
     240       250       260       270       280       290

         230       240       250       260       270       280
pFST3  G-CATCATAACGCGAGAAAAGTTTGATGATTCCACAAGTCCTTTGCTCACAACCATGCCT
       : :::  :::: :::::::::::::::::: : ::: ::::: :::::::: : ::: :::
pFST4' GCCATTGTAACCCGAGAAAAGTTTGATGAATTCACGAGTCCGTTGCTCACGAACATTCCT
     300       310       320       330       340       350

         290       300       310       320       330       340
pFST3  CATGATTGCATTCCTCTCCTAGAGAAAGACCTTGAAAAAATTCAAGAAAACCAAAGGAAC
       ::: ::::::::::   : : :::::::::::: :::::::    :::::::::  :::
pFST4' CATAATTGCATTCCCTACATTGAGAAAGACCTTAAAAAAATAGTTGAAAACCAAAATAAC
     360       370       380       390       400       410

         350       360       370       380       390       400
pFST3  TCGCTCTACACACCCATCTCCACACACTTTCATTACAAATCCCTACCGGAGTCGGCCCGA
       ::   :: :::::::::  : ::::::: : :  ::: :    :::::  : ::    : :
pFST4' TCATGCTTCACACCCATGGCTACACACATGCCCTACCATGTTCTACCAAAATCCATTCTA
     420       430       440       450       460       470

         410       420       430       440       450       460
pFST3  ACATCAAACTGCAAGATAGTTTACATATACCGGAACATGAAAGATGTCATTGTTTCTTAT
       : : :::::::::::: :::::::::: :: :: :: :::::::::: ::::::: :
pFST4' GCTTTAAACTGCAAGATGGTTTACATATATCGCAATATAAAAGATGTCATAGTTTCTTTT
     480       490       500       510       520       530
```

```
          470        480        490        500        510        520
pFST3   TACCATTTCCTGAGACAGATAGTTAAACTATCTGTGGAAGAGGCCCCATTTGAGGAGGCT
        ::::::::   :::: : :::   :::::: :  :::::::: :::::::::::::::::
pFST4'  TACCATTTTGGGAGAGAAATAACAAAACTACCCTTGGAAGATGCCCCATTTGAGGAGGCA
      540        550        560        570        580        590

          530        540        550        560        570        580
pFST3   TTTGATGAGTTTTGTCAGGGTATTTCAAGTTGTGGACCATATTGGGAACACATCAAGGGA
        ::::: ::::: : ::: :: :::::    :  :::::::::::::: ::   :   ::::
pFST4'  TTTGACGAGTTCTATCATGGCATTTCCCAATTCGGACCATATTGGGATCATCTATTGGGA
      600        610        620        630        640        650

          590        600        610        620        630        640
pFST3   TACTGGAAAGCAAGCTTGGAGAAGCCGGAGATATTTCTTTTCTTGAAATACGAAGACATG
        :: :::::::::::::::::: : ::: ::: :: :: : :::::::::::: :::::  ::
pFST4'  TATTGGAAAGCAAGCTTGGAAAGGCCAGAGGTAATTTTATTCTTGAAATATGAAGATGTG
      660        670        680        690        700        710

          650        660        670        680        690        700
pFST3   AAAAAGGATCCGGTACCAAGTGTGAAGAAACTTGCGGATTTCATTGGGCATCCCTTTACA
        ::::::::::    :   : ::: :::: ::::::::: ::::::::: :::: ::::::
pFST4'  AAAAAGGATCCTACAAGTAATGTAAAGAGACTTGCGGAGTTCATTGGGTATCCATTTACA
      720        730        740        750        760        770

          710        720        730        740        750        760
pFST3   CCCAAAGAAGAGGAAGCGGGTGTGATAGAAGATATTGTAAAGTTATGTAGTTTTGAGAAA
           :::::::: :::  ::::::::::::   ::  ::::::: :::::::: :::::
pFST4'  TTTGAAGAAGAGAAAGAAGGTGTGATAGAAAGCATCATAAAGTTGTGTAGTTTCGAGAAT
      780        790        800        810        820        830

          770        780        790        800        810        820
pFST3   TTGAGCAGCTTAGAAGTAAACAAAAGTGGCATGCATCGTCCCGAAGAAGCTCATTCCATT
        ::::::: ::::::::::::::::::::::      :  :  :  ::: :  :: : : :::
pFST4'  TTGAGCAACTTAGAAGTAAACAAAAGTGG------TAATTCAAAAGGATTTCTTCCAATT
      840        850        860                870        880        890

          830        840        850        860        870        880
pFST3   GAAAACCGGCTTTACTTCAGGAAGGGTAAGGATGGAGATTGGAAGAACTACTTCACTGAT
        ::::: :: ::::::::::::::: :::::: ::::::::::::::::::::::::: :::
pFST4'  GAAAATCGACTTTACTTCAGGAAGGCTAAGGACGGAGATTGGAAGAACTACTTCACGGAT
           900        910        920        930        940        95C

          890        900        910        920        930        940
pFST3   GAGATGACTCAGAAAATAGACAAACTGATCGACGAGAAACTGGGTGCCACTGGTTTAGTT
        :: :::::: : :: :: :: ::::: :: :::::::::::: : ::::::::::::::
pFST4'  GAAATCACTGAAAAGATTGATAAACTAATTGACGAGAAACTGAGCGCCACTGGTTTAGTT
           960        970        980        990       1000       1010

          950        960        970        980        990       1000
pFST3   CTAAAATGAAAGGCTTCAAGATGCTATCTAGTTGAGAGCAAAACTTGATTTCAAACTATC
        :::::::::: ::  ::::::   :::   :      ::   : ::   :::: ::: : :
pFST4'  CTAAAATGAAATGC-GCAAGATCACATCCTTT-----GCTCAGCT--ATTTGAAAGTTTA
          1020       1030       1040            1050       1060
```

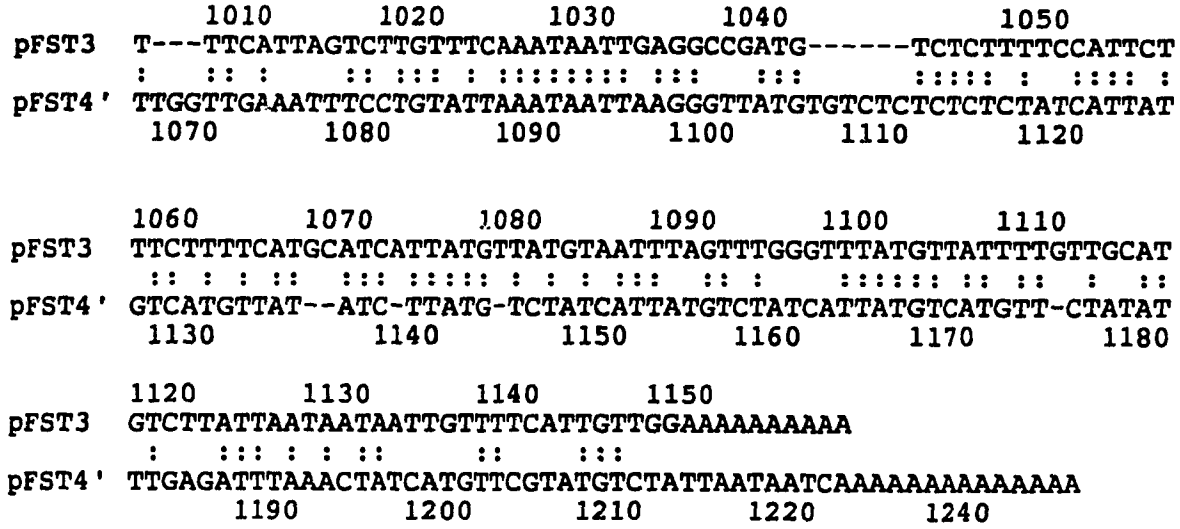


Figure 32. Comparison between pFST3 and pFST4' nucleotide sequences. The alignment was made using the FASTA software. (:) indicates an identical nucleotide. Dashes represent gaps introduced for maximal alignment.

```
                          10        20        30        40        50
pFST3                MEDIIKTLPQHTCSFLKHRFTLYKYKDAWNHQEFLEGRILSEQKFKAHPN
                     :...:.:::::::: :: :.:::::.: :. :. .:: ::..:.:::.:.
pFST4' METTKTQFESMAEMIKKLPQHTCSSLKGRITLYKYQDFWGLQNNIEGAILAQQSFKARPD
                    10        20        30        40        50        60

                   60        70        80        90        100
pFST3  DVFLASYPKSGTTWLKAWIC-IITREKFDDSTSPLLTTMPHDCIPLLEKDLEKIQENQRN
       :::: ::::::::::::   . :.:::::::. :::::::..::.::: .:::::.:: :::.:
pFST4' DVFLCSYPKSGTTWLKALAYAIVTREKFDEFTSPLLTNIPHNCIPYIEKDLKKIVENQNN
                    70        80        90       100       110       120

     110       120       130       140       150       160
pFST3  SLYTPISTHFHYKSLPESARTSNCKIVYIYRNMKDVIVSYYHFLRQIVKLSVEEAPFEEA
       : .::..::..:. ::.:  . :::.:::::::.:::::::::: :.:.::..:.:::::::
pFST4' SCFTPMATHMPYHVLPKSILALNCKMVYIYRNIKDVIVSFYHFGREITKLPLEDAPFEEA
                   130       140       150       160       170       180

     170       180       190       200       210       220
pFST3  FDEFC-GISSCGPYWEHIKGYWKASLEKPEIFLFLKYEDMKKDPVPSVKKLADFIGHPFT
       :::::. :::  :::::.:. :::::::::.::..:::::::.::::...::.:.:::.:::
pFST4' FDEFYHGISQFGPYWDHLLGYWKASLERPEVILFLKYEDVKKDPTSNVKRLAEFIGYPFT
                   190       200       210       220       230       240

      230       240       250       260       270       280
pFST3  PKEEEAGVIEDIVKLCSFRKLSSLEVNKSGMHRPEEAHSIENRLYFRKGKDGDWKNYFTD
        .::..::::.:.::::: .::.:::::::  ....  .:::::::::.::::::::::
pFST4' FEEEKEGVIESIIKLCSFENLSNLEVNKSG--NSKGFLPIENRLYFRKAKDGDWKNYFTD
                   250       260       270       280       290

      290       300       310
pFST3  EMTQKIDKLIDEKLGATGLVLK
       :::.:::::::::::.:::::::
pFST4' EMTEKIDKLIDEKLSATGLVLK
      300       310       320
```

Figure 33. Comparison of the deduced amino acid sequence of pFST3 and pFST4'. The alignment was made using the FASTA software. (:) indicates the presence of the same residue while (.) represents a conserved residue. Dashes represent gaps introduced for maximal alignment.

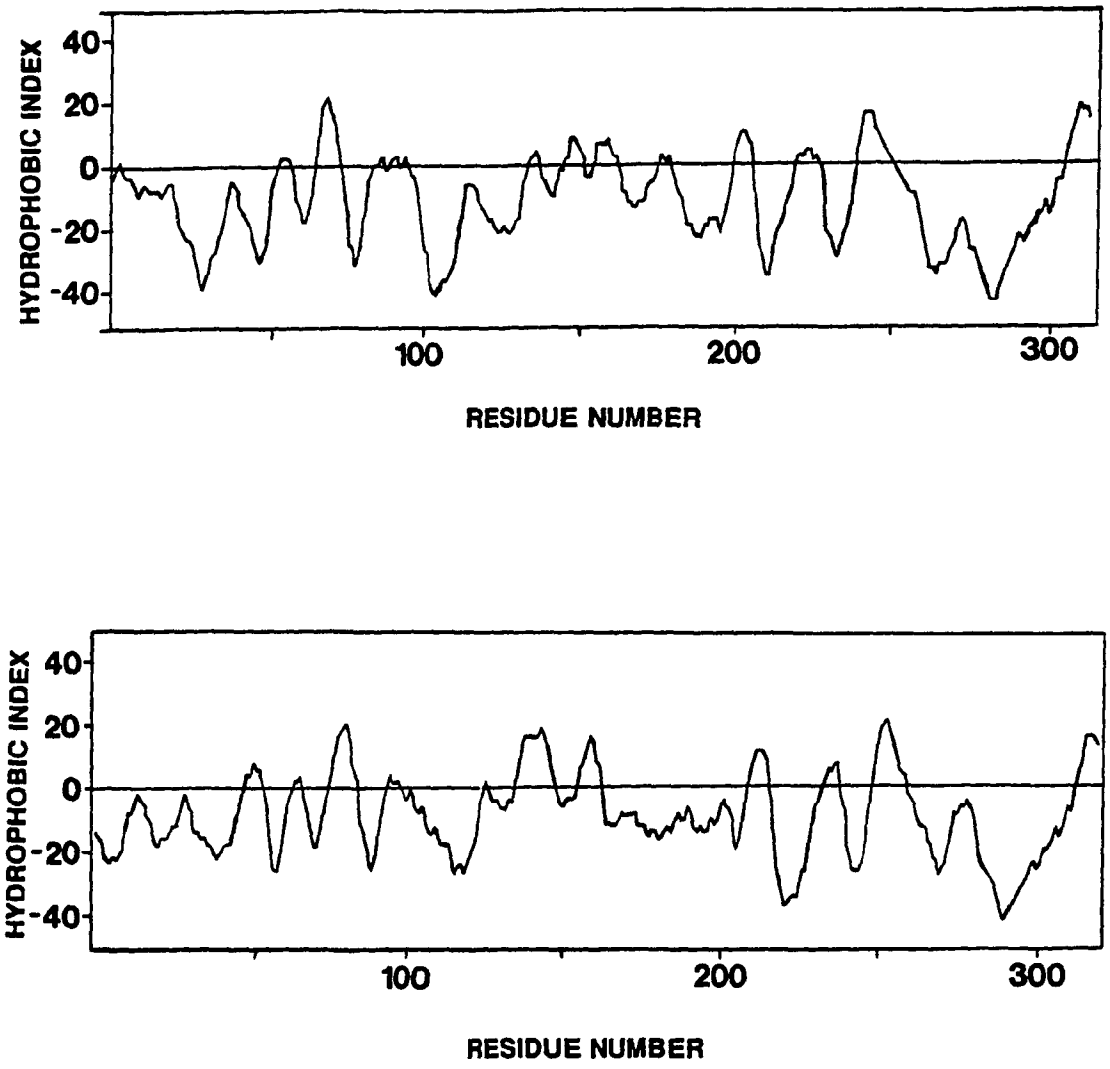


Figure 34. Hydropathy profile of pFST3 (A) and pFST4' (B). Each value was calculated as the average hydropathic index of a sequence of nine amino acids and plotted to the middle residue of each sequence. Positive and negative values indicate hydrophobic and hydrophilic regions of the protein, respectively (Kyte and Doolittle 1982).

D.3.5 Homology to other proteins

The sequence of pFST3 was used to search the Genbank, European Molecular and Biological Laboratory, and National Biomedical Research Foundation data bases. 28.8% identity in 246 amino acid overlap was found with the protein sequence of hydroxysteroid ST from rat liver, 29.9% identity in 284 amino acid overlap with the sequence of estrone ST from bovine placenta, and 30.7% identity in 192 amino acid overlap with the senescence marker protein 2 (SMP 2) of rat liver whose enzymatic function is not known (Table 18). Multialignment of the four ST sequences and of SMP 2 reveals similarities which extend throughout the polypeptides with four well conserved regions (Fig. 35).

TABLE 18

Identity of pFST3 derived amino acid sequence with other related sequences[1]

Sequences compared[2]	Amino acids in overlapping reading frame	Shared amino acids in overlapping reading frame	% Identity in overlapping reading frame
pFST3 + pFST4'	311	216	69.2
pFST3 + HSST	264	76	28.8
pFST3 + EST	284	105	29.9
pFST3 + SMP2	192	59	30.7
HSST + SMP2	285	207	72.6

[1] Optimal alignment and computing performed with the FASTA software

[2] HSST = Hydroysteroid ST
EST = Oestrogen ST
SMP2 = Senescence marker protein 2

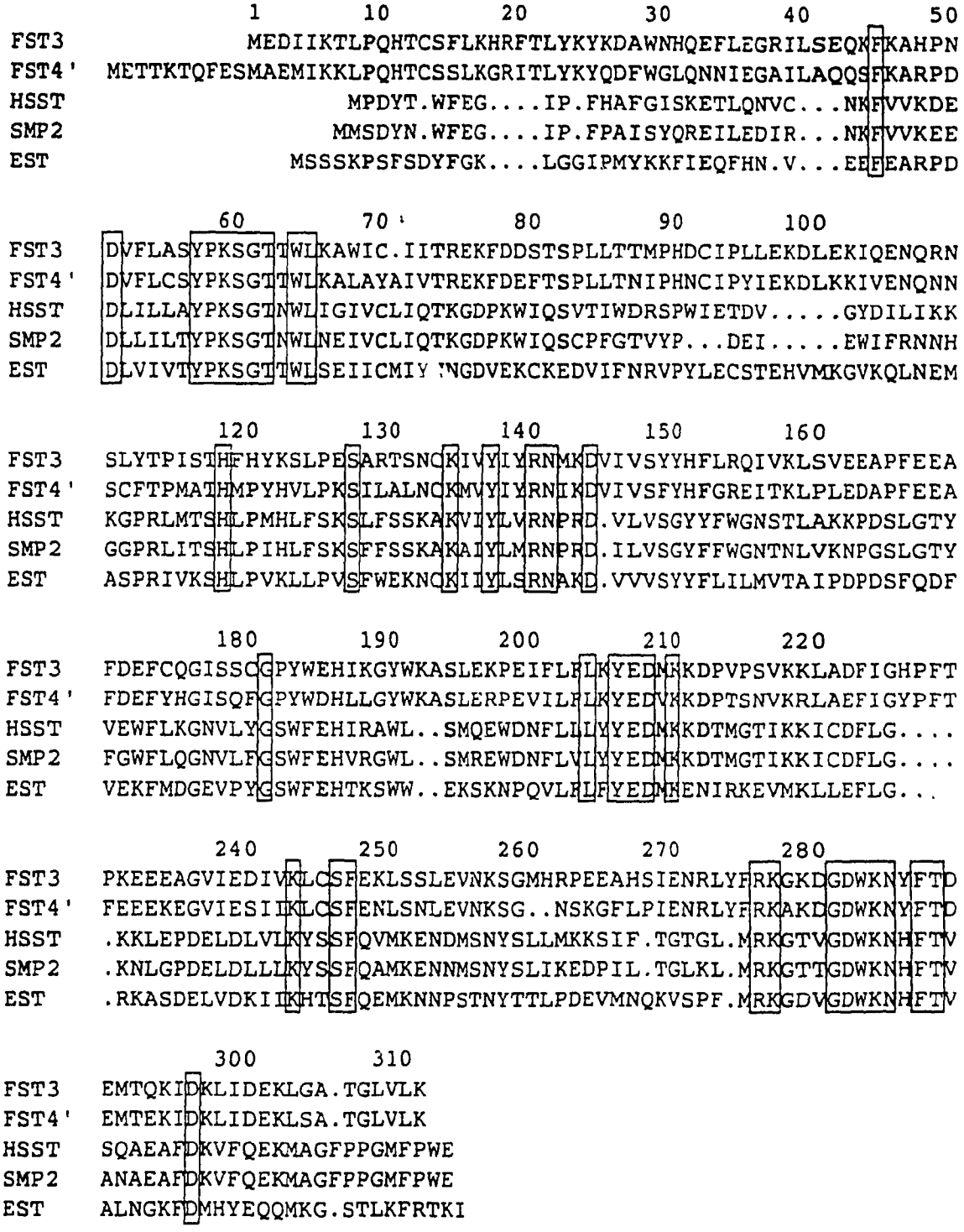


Figure 35. Amino acid alignment of F. chloraefolia pFST3 and pFST4', rat liver hydroysteroid ST (HSST), rat liver senescence marker protein 2 (SMP2), and bovine placenta oestrogen ST (EST). Boxes represent residues present in all five sequences. Bold residues are present in four sequences. Amino acid (standard one-letter code) are numbered for the 3-ST.

E. DISCUSSION

E.1 Phytochemical and biosynthetic studies

We have demonstrated the presence, in *F. bidentis*, of the 3-O-glucosides of kaempferol, 6-methoxykaempferol and patuletin, as well as several flavonol sulfate esters belonging to quercetin and isorhamnetin. Except for quercetin 3,7- disulfate, all other sulfated flavonols have previously been described (Barron et al. 1988). The fact that this compound has not been reported in the previous studies conducted with this plant is quite surprising, considering the fact that it represents about 70% of the total flavonol sulfates present in the seedlings. On the other hand, 6-methoxykaempferol 3- glucoside has been reported as a new compound in *F. brownii* (Al-Khubaizi et al. 1978); whereas patuletin 3-glucoside was reported in *F. linearis* and *F. trinervia* as well as other Compositae (Wagner et al. 1971). These two glucosides, as well as quercetin 3,7-disulfate are being reported here for the first time in *F. bidentis.*

An interesting feature of the flavonoid pattern in *F. bidentis* is that a given aglycone is either glucosylated or sulfated. In *F. chloraefolia* on the other hand, the same aglycone can be glucosylated or sulfated (e.g. patuletin). The co-occurrence in *F. chloraefolia* of patuletin 3-glucoside and patuletin 3-sulfate raises an interesting question as to the competition of the different transferase reactions for the same aglycone in this species. Furthermore, the quantitative differences observed in the different organs of *F. bidentis* seedlings, as compared with mature shoots, represent another interesting feature of this species. The predominance of the sulfate esters in the seedlings, especially quercetin 3,7- disulfate which represented about 70% of total flavonoids as compared with 9% in mature shoots, raises the question as to the significance of the variety of sulfated flavonols in the growth and development of this species.

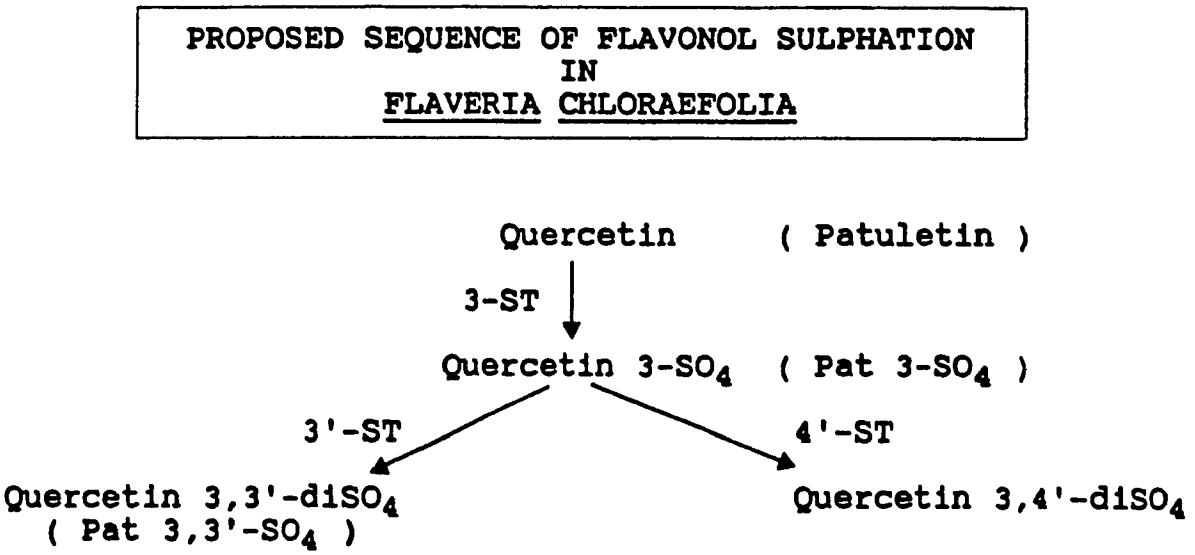
The biosynthesis of *F. bidentis* flavonoids from [^{3}H]cinnamate and [^{35}S]sulfate showed that the cinnamate label was incorporated predominantly into flavonol

glucosides, whereas that of sulfate, into flavonol sulfate esters which increased with increasing level of sulfation. The lack of incorporation of [^{3}H]cinnamate into sulfated flavonols may be due to a low endogenous level of 3'- phosphoadenosine 5'-phosphosulfate (PAPS), the sulfate donor.

E.2 Enzymology of the sulfation reaction.

Three distinct, flavonol-specific STs were highly purified from shoot tips of *F. chloraefolia* and exhibited strict specificity for positions 3 of various flavonol acceptors, and of 3' and 4' of flavonol 3-sulfate. Enzymic sulfation seems to be a later step in flavonoid biosynthesis, since a number of methylated flavonols were good sulfate acceptors for the 3-ST. Furthermore, the availability of flavonol substrates with different substitution patterns permitted the assignment of position specificities of the 3'- and 4'-STs without ambiguity. In view of the strict position specificity of these three STs, we propose the following designations for these enzymes: PAPS:flavonol 3-ST, PAPS:flavonol 3-sulfate 3'-ST, and PAPS:flavonol 3-sulfate 4'- ST.

These novel STs constitute the enzyme complement involved in the sequential sulfation of flavonols in this tissue, and represent the first report of a family of position-specific flavonol STs in plants. Based on the substrate specificity of the three enzymes characterized from *F. chloraefolia*, we propose that the biosynthesis of polysulfated flavonols in this species follows the pathway depicted in Scheme 2. The results of the *in vitro* study of the sulfation reaction correlates well with the metabolites which accumulate in this plant (Table 3)(Barron et al. 1988). It is interesting to note that patuletin 3,4'-disulfate has not been reported to accumulate in *F. chloraefolia*. This fact may be explained by the higher affinity of the 3'-ST for patuletin 3-sulfate when compared with the 4'-ST (Table 10). Furthermore, the absence of a 7-ST in chloraefolia is in agreement with the absence of flavonoids sulfated at this position in this species.

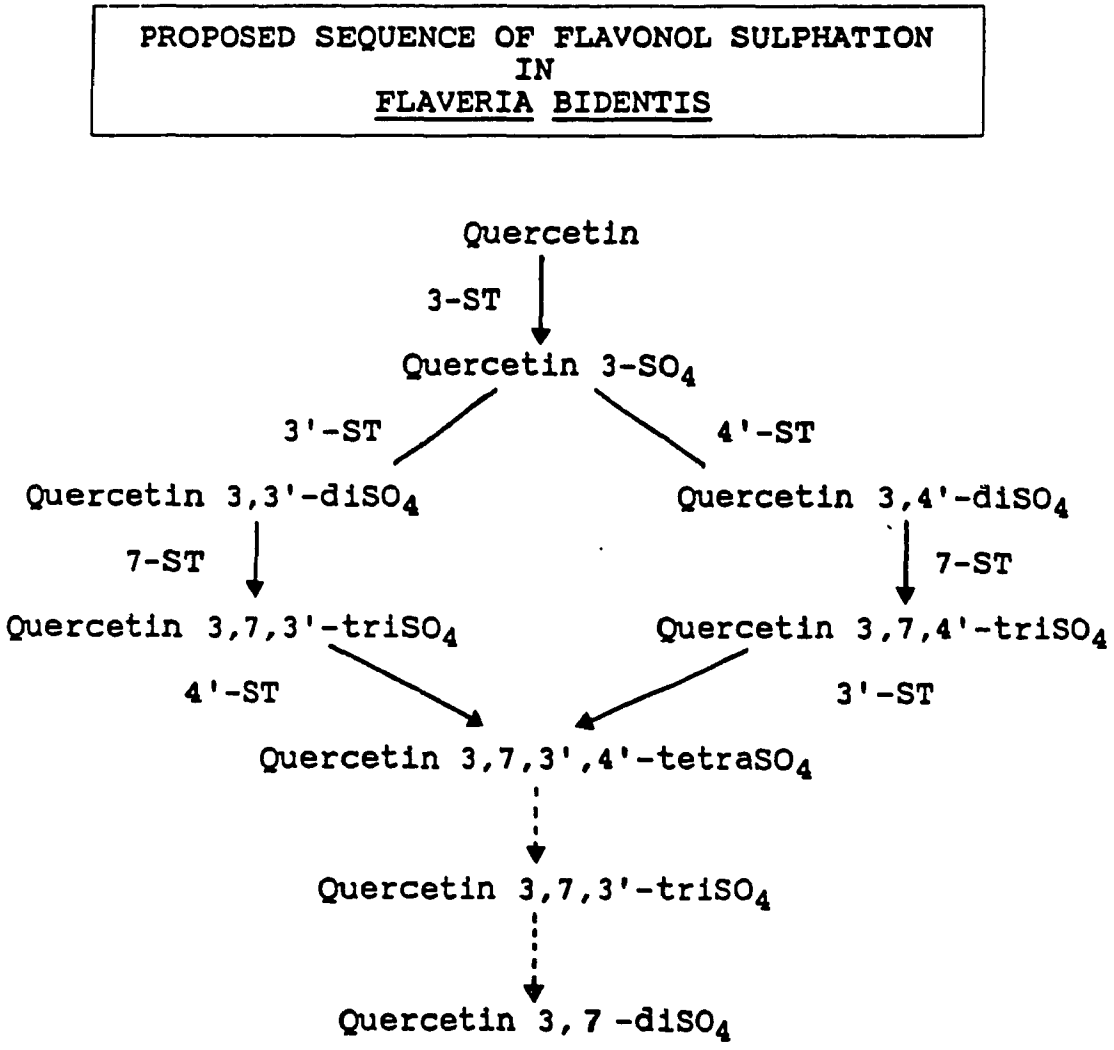


Scheme 2. Proposed model for the formation of polysulfated flavonols in Flaveria chloraefolia

Although the purification procedure reported here was efficient in separating the three enzyme activities, it did not yield homogeneous proteins. A different protocol had to be developed to purify the 3-ST to apparent homogeneity.

The study of enzymatic sulfation of flavonoids in *F. bidentis* led to the identification of two 7-ST isoenzymes in this species. The presence of two 7-ST isoenzymes is responsible for the accumulation, in *F. bidentis*, of polysulfated flavonoids bearing a sulfate group at position 7. The presence of isoenzymes of the 7-ST contrasts with the results obtained with the *F. chloraefolia* STs. Polymorphisms have been reported for other enzymes of the phenylpropanoid and flavonoid pathways. Further studies as to their regulation and tissue distribution will be required to assign the role of the 7-ST I and II in *F. bidentis*. In view of the strict position specificity of the 7-ST, we propose the following designation for this enzyme: PAPS:flavonol 3,3'-/3,4'- disulfate 7-ST.

As compared with *F. chloraefolia*, the biosynthesis of polysulfated flavonols in *F. bidentis* represents a more complex situation. The fact that both 7-ST isoenzymes of *F. bidentis* accept only quercetin 3,3'- or 3,4'-disulfates suggests that sulfation at position 7 is the third step in the formation of quercetin 3,7,3',4'-tetrasulfate in this species. Therefore, we would like to propose, as a working model, the pathway depicted in Scheme 3 for the formation of polysulfated flavonols in *F. bidentis*. The first two steps in this pathway are probably identical to those already observed in *F. chloraefolia*, even though quercetin 3,3'-disulfate has yet to be isolated from *F. bidentis*. Two pieces of evidence support this hypothesis: (a) quercetin 3-sulfate is the only monosulfate formed when a crude extract of *F. bidentis* is incubated with quercetin, and (b) the 7-STs accept only the 3,3'- or 3,4'-disulfates of quercetin to give rise to 3,7,3'- and 3,7,4'-trisulfates known to accumulate in this species (Cabrera and Juliani 1979; Cabrera et al. 1985).



Scheme 3. Proposed model for the formation of polysulfated flavonols in Flaveria bidentis

Finally, one or both of the trisulfates may be further sulfated to give rise to quercetin 3,7,3',4'-tetrasulfate. However, the presence of a ring B-specific 3'- or 4'-STs that accepts only trisulfated substrates remains to be demonstrated. Since the 7-ST does not accept either quercetin, its 3-, or 3'-monosulfate derivatives, one should exclude its involvment in the biosynthesis of quercetin 3,7-disulfate, despite the fact that the latter accumulates in large quantity in *F. bidentis.* Although we do not have yet any evidence for the catabolic desulfation of sulfated flavonoids in plants, it is conceivable that flavonoid-specific aryl sulfatase(s) may exist in *Flaveria spp.*, in a manner similar to microorganisms and animal tissues (for a review, see Dodgson et al. 1982). Hence, the action of sulfatase on quercetin tri- and/or tetrasulfate may explain the accumulation of the 3,7- disulfate in *F. bidentis.*

Flaveria STs are quite distinct from the much studied sulfating enzymes of mammalian tissues, which exhibited broad specificity toward major chemical classes of substrates (e.g. phenols, steroids, and bile acids) but none for specific functional groups or positions. The high position and substrate specificities of the flavonol STs, as compared to animal STs, suggest a more specialized role of the former enzymes, other than their involvment in detoxification of reactive hydroxyl groups. In addition, the involvment of position-specific STs in the sequential sulfation of steroid- and bile salt disulfates has yet to be demonstrated.

The high affinity of the *Flaveria* STs for both the sulfate donor and the flavonol acceptors suggests that both metabolites are present in low concentration inside the cells. Furthermore, the inhibition of enzyme activity at flavonol substrate concentration above Km suggests a very tight control of flavonol sulfate biosynthesis. Similar substrate inhibition patterns were obtained with the hydroxysteroid ST from human liver (Falany et al. 1989) and for the flavonoid *O*-methyltransferases of *Chrysosplenium americanum* (Ibrahim et al. 1987). It is interesting to note that the sulfation reaction by *Flaveria* STs was catalyzed in the presence of PAPS but not APS, which supports earlier

proposals (Harborne 1977) that the former compound is the physiological sulfate donor. This is in agreement with the recently reported STs of cress (Glendening and Poulton 1988) and *Brassica juncea* (Jain et al. 1990) which utilized PAPS in the sulfation of desulfobenzylglucosinolate to its sulfated derivative.

The flavonol 3-ST from *F. chloraefolia* has been purified to apparent electrophoretic homogeneity by a simple five step chromatographic procedure. The conclusion that the 34,500D band is the 3-ST is based on several lines of evidence. The 34,500D band co-fractionated with the activity peak in the final Mono Q column (Fig. 21). In addition, the enzyme activity in the gel filtration column correlated with the activity eluted from the native-PAGE (Fig. 23). Both gel filtration and SDS-PAGE were consistent with a monomeric molecule of about 34,500D. The observed molecular weight falls within the range observed for animal STs (28,000 to 38,000D), but is in contrast with the presence of dimeric or multimeric forms reported for the majority of animal STs (Table 2). Furthermore, the molecular weight of the 3-ST is distinctly lower than the 44,000D reported for plant desulphobenzylglucosinolate STs (Glendening and Poulton 1988; Jain et al. 1990).

The availability of a purified 3-ST permitted the development of the necessary tools for the isolation of a cDNA clone of the gene encoding it. Segments of its primary amino acid sequence were determined, and monospecific polyclonal anti-3-ST antibodies were produced in rabbits. The antibodies were found to recognize a protein band which co-migrated with the 3-ST protein (Fig. 25). Partially purified 3'- and 4'-STs were also recognized by the anti-3-ST antibodies, suggesting sequence similarities between the three enzymes (Fig. 26). This result was not unexpected in view of the similar catalytic functions and chromatographic behavior of the three enzymes. However, antibodies raised against phenol ST from human liver (Falany et al. 1990) did not recognize *Flaveria* 3-ST (C.N. Falany, pers. comm.)

E.3 Molecular cloning of *F. chloraefolia* 3- and 4'-STs

In the present study cDNA clones coding for the flavonol 3- and 4'-STs were isolated from a cDNA library produced from poly(A)$^+$ mRNAs isolated from shoot tips of *F. chloraefolia.*

Several direct and indirect evidences confirm the authenticity of the pFST3 and pFST4' as coding for the 3- and 4'-ST respectively, these are: (1) The matches between the deduced and directly determined amino acid sequences of the 3-ST (Fig. 30), (2) The recognition by the anti-3-ST antibodies of a protein expressed in bacteria harboring pFST3 or pFST4' plasmids but not in bacteria harboring a control plasmid (Figs. 28, 29) and, (3) Finally, and probably the most significant evidence is the position and substrate specificity of the ST activities expressed in *E. coli* harboring the pFST3 and pFST4' plasmids (Figs. 28 and 29).

The low frequency of cDNA clones coding for the 3-ST (6), compared to the 4'-ST (40), is quite surprising when we consider that the 3-ST is catalysing the first step in the sequential formation of polysulfated flavonoids. Furthermore, we were not able to isolate a cDNA clone coding for the 3'-ST. This low representation might be due to the high instability of the 3- and 3'-ST mRNAs, or to the low expression of their respective genes at the time of mRNA extraction. In order to isolate a 3'-ST cDNA clone, the screening of a larger number of clones from the cDNA library will be required.

The complete nucleotide sequence of pFST3 and pFST4' has been determined. The deduced amino acid sequence of pFST3 and pFST4' revealed an overall positional identity of 69.2% in 311 amino acid residues between the two proteins. In addition to the high sequence similarity, the even distribution of the amino acid identities throughout the protein sequences renders difficult the identification of domains which might be necessary for catalysis or determinants for the specificity of the two enzymes. The homology between the two STs suggests that early in the evolutionary process,

gene duplication followed by sequence divergence between the two loci may have occurred to the point where they no longer function as duplicate loci but rather as complementary genes. The sequence similarities between the 3- and 4'-STs provide an explanation for the difficulty encountered in the separation of the two proteins by chromatographic methods, and the cross-reactivity of the anti-3-ST antibodies with the 4'-ST.

The two STs of *F. chloraefolia* also share sequence similarities with other STs characterized from animal tissues (Table 18). 28.9 and 30.7 identity is observed with the hydroxysteroid ST cDNA clone isolated from rat liver and the estrogen ST cDNA clone isolated from bovine placenta. Furthermore, 30.7% identity is observed between the 3-ST cDNA clone and the senescence marker protein 2 (SMP2) isolated from rat liver. The catalytic function of SMP-2 was unknown when the cloning of its cDNA was reported (Chatterjee et al. 1987). Recently however, Ogura et al. (1990) reported the isolation of a cDNA clone coding for a hydroxysteroid ST which has 74% sequence identity with the SMP-2 deduced amino acid sequence. This result, together with the similarities observed with the deduced amino acid sequences of the flavonol STs, strongly suggest that SMP-2 is coding for a ST.

The common evolutionary origin of plant and animal STs becomes more evident when we analyze their aligned sequences (Fig. 35). In addition to their similar length, the presence of identical amino acid residues is distributed throughout the sequences with four well conserved regions. The stability of the amino acid sequence in these four regions through divergent evolution suggests that they play an important role in the function of these proteins. We can speculate on the role of these regions as to their requirement for the appropriate protein folding to create the catalytic domain. One or more of these conserved regions might be involved in the binding of the co-substrate PAPS.

E.4 Perspectives for future work

The availability of antibodies as well as ST cDNA clones provide the basic tools for new experimental strategies in order to define the role of the sulfate conjugation with flavonoids in plant tissues. These include:

1) The localization of the ST enzymes at the tissue and subcellular level using immunocytochemical techniques (Ibrahim 1990), in relation to the compartmentation of final metabolites.

2) The study of the environmental and developmental factors governing ST gene expression.

3) The isolation of genomic clones and analysis of *cis*-acting gene regulatory signals such as promoters, enhancers and silencers by the introduction of dissected gene constructs in transgenic plants.

4) The analysis of protein structure-function relationship by the construction of chimaeric genes using segments of pFST3 and pFST4'.

5) The mapping of the amino acids which are critical for ST activity by site directed mutagenesis.

6) *In vivo* manipulation of plants to create new sulfate metabolic sink through the expression of the 3-ST gene into flavonol producing plants which accumulate sulfur-containing deleterious metabolites, e.g. glucosinolates in the Brassicaceae family. The latter strategy implies that the newly expressed sulfated metabolites neither have any deleterious effect on plant growth, nor affect the nutritive quality of the seed meal/fodder produced by such transgenic plants. It also implies that overexpression of the flavonol ST enzymes can compete with the glucosinolate ST for the sulfate donor PAPS . The recent work of Lefebvre (1990) demonstrates the feasibility of this approach in creating an artificial sulfur sink by introducing a foreign gene coding for a product rich in the amino acid cysteine, thus reducing the sulfur pool of the plant and, consequently, reducing glucosinolate biosynthesis.

The production of transgenic *Brassica napus* harboring the flavonol 3-ST gene with the goal of creating a variety low in glucosinolates is presently in progress.

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