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Purification and Properties of Flavonol-Ring B Glucosyltransferase from *Chrysosplenium americanum*[1]

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ABSTRACT

A novel glucosyltransferase which catalyzed the transfer of glucose from UDP-glucose to positions 2′ and 5′ of partially methylated flavonols was isolated from the shoots of *Chrysosplenium americanum* Schwein ex Hooker. It was purified 225-fold by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, hydroxyapatite, and polybuffer ion exchanger. This glucosyltransferase appeared to be a single polypeptide with an apparent molecular weight of 42,000 daltons, pH optimum of 7.5 to 8.0, and an isoelectric point of 5.1. It had low but similar K_m values for the 2′ and 5′ positions of flavonol substrates and the cosubstrate UDP-glucose and was inhibited by both reaction products, the glucosides formed, and UDP.

Glucosyltransferase activity was independent of divalent cations, was not inhibited by EDTA, but showed requirement for SH groups. The differential effect on enzyme activity of metal ions, especially cupric ion, and various SH group reagents seemed to indicate the involvement of two active sites in the glucosylation reaction; the site specific for 2′ activity being more susceptible than that of the 5′ activity. The substrate specificity expressed by this glucosyltransferase and the requirement of at least two *para*-oriented B-ring substituents (at 2′ and 5′) for activity support this view.

The transfer of the glucosyl moiety from sugar nucleotides to flavonoid acceptors has been widely studied (12) and is considered to be a terminal step in flavonoid biosynthesis (9, 10). Whereas glucosyltransferases were believed to possess a broad substrate specificity, recent reports tend to indicate their specificity towards the different classes of flavonoids, *e.g.* flavones, isoflavones, flavonols, and anthocyanidins (18, 22, 23). Position specificity has also been reported for the 7-*O*-glucosyltransferases of flavones, isoflavones, and flavonols (18, 23, 24), as well as the much investigated 3-*O*-glucosyltransferases of flavonols and anthocyanidins (21–24). No glucosyltransferase specific for ring B of flavonoids has, so far, been reported except for the detection of a weak glucosylating activity for positions 5, 7, and 3′ of the flavonol quercetin by nonpurified extracts of *Zea mays* (7).

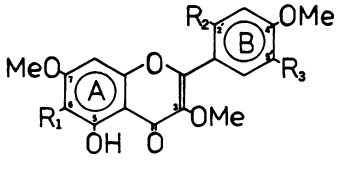
Chrysosplenium americanum, a Saxifragaceae, contains two 2′-*O*-D-glucosides of partially methylated 2′-hydroxyquercetin and four 5′-*O*-D-glucosides of partially methylated 6-hydroxy- and 6,2′-dihydroxyquercetin (Fig. 1; Ref. 4). Our current interest in the enzymic synthesis of partially methylated flavonols (4–6, 15) prompted us to study the glucosyltransferase(s) involved in their biosynthesis. In view of the position specificity exhibited by a number of *O*-methyltransferases isolated from this plant (6), it was interesting to find out whether glucosylation of the 2′ and 5′ positions was catalyzed by one or two distinct enzymes. We wish to report in this paper on the purification and some properties of a novel enzyme which catalyzed the transfer of glucose from UDP-glucose to positions 2′ and 5′ of partially methylated flavonols in *C. americanum*.

MATERIALS AND METHODS

Plant Material. *Chrysosplenium americanum* Schwein ex Hooker (Saxifragaceae) was collected from Sutton junction, Eastern Townships, Province of Québec, and was maintained in the greenhouse under natural light at 18 to 22°C.

Chemicals. UDP-[U-^{14}C]glucose (334 mCi/mmol) was obtained from New England Nuclear; UDP, UDPG, *N*-ethylmaleimide, *p*-chloromercuribenzoate, and Dowex chelating resin were purchased from Sigma. Sephadex G-25, G-100, DEAE-Sephadex A-25, polybuffer ion exchanger PBE-94, polybuffer PB-74, and the reference proteins used for mol wt determination were from Pharmacia Fine Chemicals. Hydroxyapatite (Bio-gel HT) was from Bio-Rad, and DEAE-cellulose (DE-52) was from Whatman Chemical Separation Ltd, England. Flavonoid substrates and reference compounds were from our laboratory collection and from those previously isolated and identified (4). Imidazole was recrystallized from ethylacetate before use.

Enzyme Extraction and Purification. Unless stated otherwise, all procedures were carried out at 2 to 4°C. About 15 g of shoot tips were frozen in liquid N_2, mixed with Polyclar AT (1:5, w/w), and ground to a fine powder. The mixture was homogenized with 0.2 M Tris-HCl buffer (pH 7.6; 1:4, w/v), containing 14 mM 2-mercaptoethanol, 5 mM EDTA, and 10 mM diethylammonium diethyldithiocarbamate (buffer A). The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 15,000*g* for 15 min. The supernatant was stirred for 20 min with Dowex 1×2 (20%, w/v) which had previously been equilibrated with buffer A, then filtered through glass wool. The filtrate was frac-



Compound	R_1	R_2	R_3	Glucose
A	H	OH	OH	R_2
B	H	OH	OMe	R_2
C	OH	H	OH	R_3
D	OMe	H	OH	R_3
E	OMe	OH	OH	R_3
F	OMe	OMe	OH	R_3

FIG. 1. Structural formulae of the partially methylated flavonol aglycones (A–F) used as substrates for the glucosyltransferase assay; their 2′ (R_2) and 5′ (R_3) glucosides occur naturally in *C. americanum*. Note that whenever the 2′ position is substituted then numbering of the *o*-dihydroxy grouping becomes 4′,5′- instead of 3′,4′-, since the 3′- and 5′-positions are identical.

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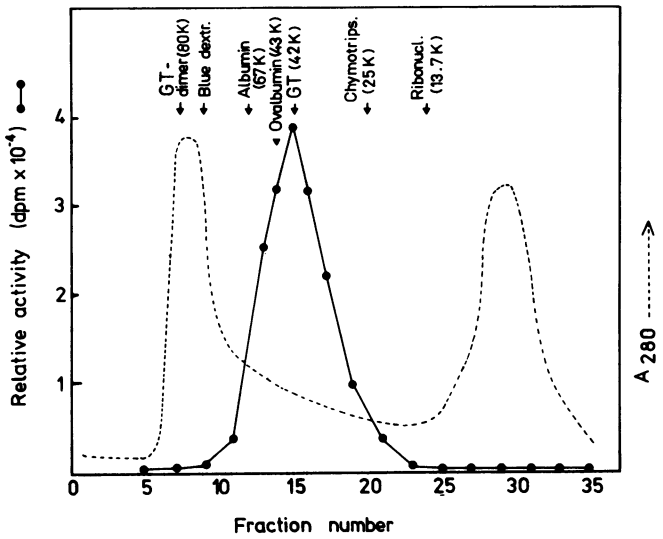

FIG. 2. Elution profile of glucosyltransferase activity from Sephadex G-100 column using compound F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) as substrate. The column was preequilibrated and the protein eluted with 20 mM Tris-HCl buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol. The mol wt of the enzyme was determined with a similar column which had previously been calibrated with the indicated reference proteins.

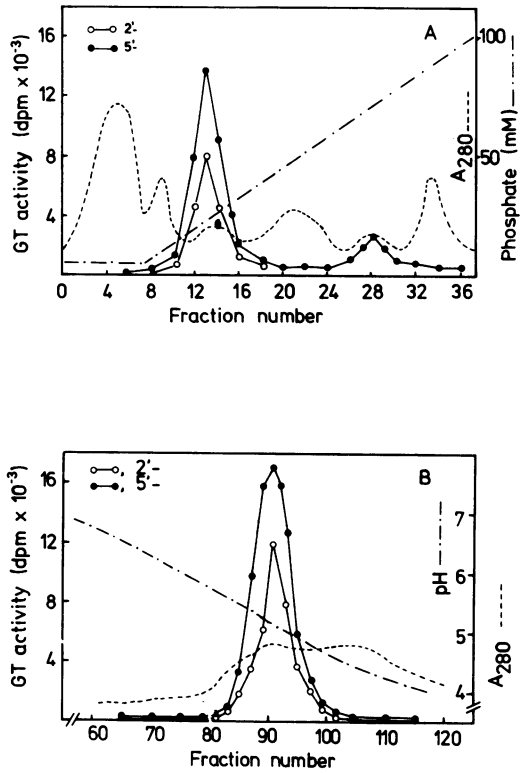

FIG. 3. A, Elution profile of glucosyltransferase activity from hydroxyapatite column. Fractions from Sephadex G-100 with high enzyme activity were pooled and applied to a column previously equilibrated with 10 mM phosphate buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol. The protein was eluted with a linear gradient (10–200 mM) phosphate in the same buffer, and 2.5-ml fractions were assayed for enzyme activity using substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) for 2' and 5' activities, respectively. B, Chromatofocusing of glucosyltransferase activity on polybuffer ion exchanger (PBE-94). The enzyme protein was applied to a column preequilibrated with 25 mM imidazole buffer (pH 7.4) containing 14 mM 2-mercaptoethanol and 10% glycerol. Elution was carried out using polybuffer-HCl (pH 4.0) which generated a linear gradient between pH 7 and 4. Fractions were assayed against substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,-7,2',4'-pentamethoxyflavone) for 2' and 5' activities, respectively.

tionated with solid ammonium sulfate, and the protein fraction which precipitated between 35 and 70% salt saturation was collected by centrifugation and resuspended in the minimal amount of 20 mM Tris-HCl (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol (buffer B). The latter was chromatographed on a Sephadex G-100 column which was previously equilibrated with the same buffer, and 2.5-ml fractions were collected and assayed for enzyme activity. Fractions with glucosyltransferase activity were pooled and applied to a hydroxyapatite column which was previously equilibrated with 10 mM phosphate buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol (buffer C). After washing the column with buffer C, the protein was eluted using a linear gradient (10–150 mM) of K-phosphate in buffer C, and 2.5-ml fractions were collected for assay of enzyme activity.

For chromatofocusing, the enzyme was extracted and partially purified in the usual manner, except that 25 mM imidazole-HCl buffer (pH 7.4) containing 10% glycerol and 14 mM 2-mercaptoethanol (buffer D) was used for elution of the protein from the Sephadex G-100 column. The pooled fractions containing glucosyltransferase activity were applied to a column containing polybuffer ion exchanger (PBE-94) which had previously been equilibrated with buffer D. Elution was carried out using polybuffer-HCl (pH 4.0) which had been diluted according to manufacturer's specifications and generated a linear gradient between pH 7 and 4. The component proteins were thus eluted at their apparent isoelectric points (pI).

Chromatography on DEAE-cellulose was carried out using a linear gradient (0–200 mM) of KCl in buffer C. For chromatography on DEAE-Sephadex A-25 (16), the column was preequilibrated with buffer B and the protein was eluted with 0.5 M KCl in the same buffer. The soluble protein content was determined by the Bio-Rad method (2).

Enzyme Assay and Product Identification. The standard assay mixture consisted of 15 μM of the flavonoid substrate (in 10 μl of 50% dimethyl sulfoxide), 1.5 μM of UDP-[U-^{14}C]glucose (containing 25 nCi), 20 mM Tris-HCl buffer (pH 7.6), and 14 μM 2-mercaptoethanol in a total volume of 130 μl. The reaction was started by the addition of protein, and the mixture was incubated for 30 min at 30°C. The reaction was stopped by adding 20 μl of 6 N HCl, and the reaction products were extracted with 400 μl of ethylacetate. An aliquot of the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid.

For product identification, the ethylacetate extracts of several assays were pooled and evaporated to near dryness, then chromatographed on commercial Polyamid-6 (Machery and Nagel) TLC plates in solvent systems: A, butanone:water (15:85); and B, toluene:ethylformate:ethanol:water (60:20:19:1). Identity of the glucosylated products was confirmed by cochromatography with reference compounds (4), visualization in UV light (366 nm), and by autoradiography.

Mol Wt Determination. An estimate of the mol wt of the purified enzyme was obtained by determining its elution volume from a Sephadex G-100 column which had previously been calibrated with proteins of known mol wt.

Electrophoresis. Gel electrophoresis of the chromatofocused enzyme was performed using 10% acrylamide according to the method of Weber and Osborne (25), except that the running buffer contained 5 mM 2-mercaptoethanol and 2 mM EDTA. In the absence of SDS, electrophoresis was carried out at 6°C. For the

Table I. *Purification of Chrysosplenium O-Glucosyltransferase*

The standard enzyme assay was used as described in "Materials and Methods." The substrate used was 5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone (Fig. 1, F).

Purification Step	Protein	Specific Activity	Purification	Recovery
	mg	*pkat/mg*	*-fold*	%
Crude extract	14.0	0.36		100
Dowex 1×2	10.5	0.47	1.3	99
Ammonium sulfate[a]	3.6	1.44	4.0	103
Sephadex G-100	0.26	18.0	50	96
Hydroxyapatite	0.07	44.8	124	62
Polybuffer ion exchanger	0.007	81.2	225	11

[a] 35 to 70% saturation.

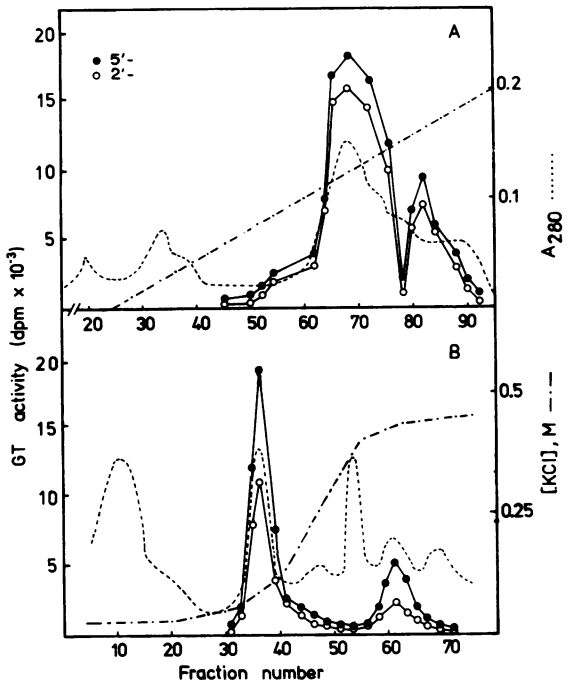

FIG. 4. A, Elution profile of glucosyltransferase activity from DEAE-cellulose column. The enzyme activity from Sephadex G-100 was applied to a column preequilibrated with 10 mM phosphate buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol. The protein was eluted with a linear gradient (0–200 mM) KCl in the same buffer, and fractions were assayed using substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) for 2' and 5' activities, respectively. B, Elution profile of glucosyltransferase activity from DEAE-Sephadex A-25 column. The enzyme activity from Sephadex G-100 was applied to a column preequilibrated with 20 mM Tris-HCl buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and 10% glycerol. The protein was eluted with 0.5 M KCl in the same buffer, and fractions were assayed against substrates B and F.

determination of enzyme activity, the gels were sliced into 2-mm sections and were disintegrated in 200 µl of Tris-HCl buffer (pH 7.6) containing 14 mM 2-mercaptoethanol and incubated overnight at 4°C. The eluates were assayed for enzyme activity using the 2' and 5' hydroxylated substrates, B and F, respectively (Fig. 1).

Determination of SH and Disulfide Groups. The enzyme preparation was made free of 2-mercaptoethanol by passing through a small column of Sephadex G-75 (19). SH-groups were determined before and after reduction with sodium borohydride in 8 M urea using the potassium salt of 5,5'-dithio-bis-2-nitrobenzoic acid as thiol-disulfide exchanger (3). The latter was prepared by the method of Wilson *et al.* (26). The amount of SH groups was calculated using an extinction coefficient of 12,000 M^{-1} cm^{-1} for nitrobenzoate anions.

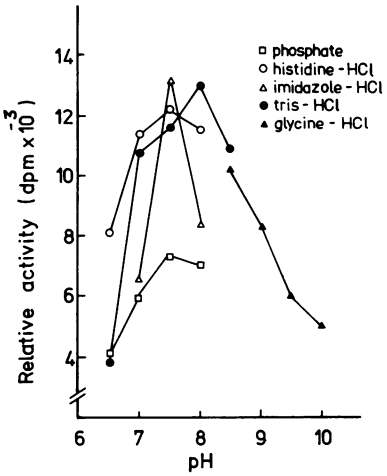

FIG. 5. pH Optima of the 5' glucosylating activity using substrate F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone). Twenty µl of the enzyme protein was added to 100 µl of 0.2 M buffer adjusted to the indicated pH and assayed for activity. Similar curves were obtained for the 2' activity.

Table II. *Effect of Divalent Cations and SH Group Inhibitors on Chrysosplenium Glucosyltransferase Activity*

The enzyme source was Sephadex G-100 fraction. Substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) were used for the 2'- and 5'-glucosylating activities, which amounted to 11,500 and 12,000 dpm/assay of the control (= 100%), respectively.

Additions	Concn.	Relative Activity	
		2'-	5'-
	mM	%	
Divalent Cations			
Mg^{2+}	0	100	100
	1	100	100
	10	50	60
Mn^{2+}	1	85	100
	10	20	60
Ca^{2+}	1	90	100
	10	42	75
Cu^{2+}	1	60	70
Zn^{2+}	1	24	22
Fe^{2+}	1	10	16
EDTA	5	130	120
Phosphate buffer[a]	100	35	58
SH Group Reagents			
Iodoacetamide	10	5	40
Iodoacetate	10	16	16
N-Ethylmaleimide	1	76	60
+Dithioerythritol	10	100	100
Phenylmercuriacetate	1	48	85
+Dithioerythritol	10	100	100
p-Chloromercuribenzoate	4	20	10
+Dithioerythritol	40	85	82

[a] Compared with 100 mM Tris-HCl buffer as control.

RESULTS

Enzyme Purification. Crude enzyme preparations of *C. americanum* catalyzed the glucosylation of the 2' and 5' positions of the

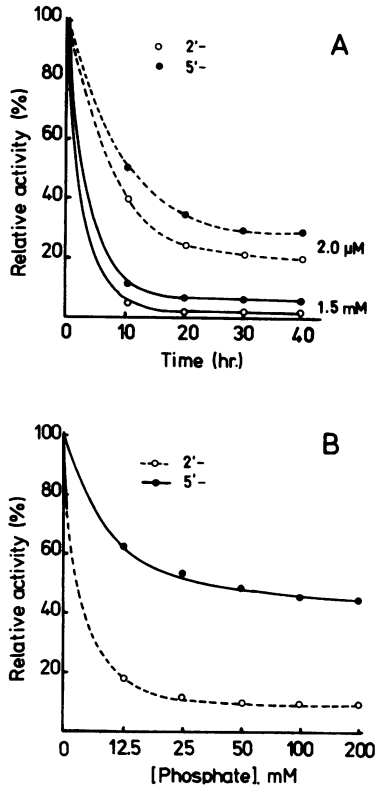

FIG. 6. A, Effect of cupric ion on 2′ and 5′ glucosylating activity. $CuSO_4$ solution at a final concentration of 2 μM and 1.5 mM was added to the enzyme preparation in 0.1 M Tris-HCl buffer (pH 7.6) and the activity was determined over a period of 40 h using substrates B (5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone) and F (5,5′-dihydroxy-3,6,7,2′,4′-pentamethoxyflavone), respectively. B, Inhibition of 2′ and 5′ glucosylating activity by phosphate ion. Twenty μl of the enzyme was added to 100 μl of phosphate buffer (pH 7.6) which had previously been passed through Dowex chelating column and then assayed with substrates B and F, respectively.

partially methylated flavonols B and F, respectively (Fig. 1) in presence of UDP-glucose as the glucosyl donor. The enzyme was purified by fractional precipitation with ammonium sulfate and successive chromatography on Sephadex G-100 (Fig. 2), hydroxyapatite (Fig. 3A), and polybuffer ion exchanger (Fig. 3B) columns. The enzyme activity was eluted at pI 5.1 from the latter column. The combined purification steps resulted in an increase in specific activity of 225-fold as compared with that of the crude preparation (Table I) when using either substrate B (referred to as 2′ activity) or F (5′ activity). Both activities, however, were recovered within the same protein peak, even when the Sephadex G-100 active fracion was further chromatographed on either DEAE-cellulose (Fig. 4A) or DEAE-Sephadex A-25 (Fig. 4B) columns. Furthermore, another peak with low glucosyltransferase activity was observed on these columns as well as that of hydroxyapatite (Fig. 3A), where the 2′ activity was generally lower than that of 5′ activity in the presence of phosphate buffer.

Effect of pH. The pH optimum for 2′ and 5′ activities, as determined in different buffers, was found to be 7.5 to 8.0 (Fig. 5). Maximum glucosyltransferase activity was observed in imidazole-HCl, Tris-HCl, and histidine-HCl, buffers with a pronounced sharp peak in the former buffer. There was a rapid drop in enzyme activity below pH 7.0 or above pH 8.5. In phosphate buffer, the 5′ activity was about 50% of that observed in Tris-HCl (Fig. 5) and it was markedly lower in case of the 2′ activity (not shown).

Linearity of Reaction. At optimum pH, the reaction rate for both 2′ and 5′ positions was linear for at least 50 min and was proportional to the amount of protein up to 30 μg/ml of the Sephadex G-100 enzyme preparation.

Table III. *Substrate Specificity of Chrysosplenium Glucosyltransferase*

The standard enzyme assay was used with the purified enzyme as described in "Materials and Methods." Identity of the reaction products was verified by co-chromatography with reference compounds and autoradiography (Fig. 7). There was no activity against any of the phenylpropanoid compounds, flavones, dihydroflavonols, or any of the glucosides tested.

Flavonoid Substrate[a]	Substitution		Relative Activity[b]	
	Hydroxy-	Methoxy-	2′-	5′-
			%	
B	5,2′-	3,7,4′,5′-	100	
F	5,5′-	3,6,7,2′,4′-		100
A	5,2′,5′-	3,7,4′-	80	20
E	5,2′,5′-	3,6,7,4′-	20	80
C	5,6,5′-	3,7,4′-		30
D	5,5′-	3,6,7,4′-		30
	3,5,5′-	7,4′-		14
	3,5,7,4′-	5′-		12

[a] Structural formulae of substrates A to F are shown in Figure 1. Substrate concentration for all compounds was 20 μM, except for E (40 μM).

[b] 2′- and 5′-Glucosylating activities (100%) amounted to 11,000 and 12,000 dpm/assay, respectively.

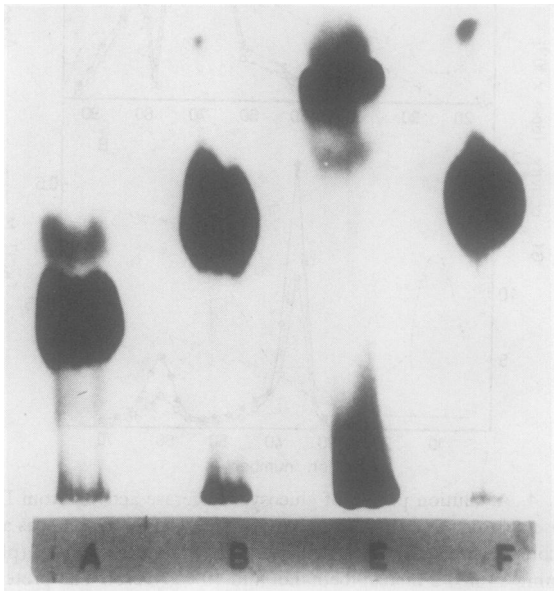

FIG. 7. Photograph of an autoradiogram of the chromatographed reaction products of glucosyltransferase activity against compounds A, B, E, and F (structures shown in Fig. 1) in butanone:water (15:85, v/v). The major products shown cochromatographed with the natural glucosides of these substrates.

Enzyme Stability. In the absence of SH group protectors, the partially purified enzyme lost more than 70% of its activity within 24 h. Storage of the enzyme in presence of 14 mM 2-mercaptoethanol resulted in 50% loss of the 2′ activity after 1 week, whereas that of the 5′ was fairly stable for several weeks. Both activities were lost within 1 week in the presence of phosphate buffer. The enzyme was stored in 20 mM Tris-HCl buffer (pH 7.6) containing 10% glycerol and 10 mM dithioerythritol, under N_2 at −20°C and was stable for more than 2 months.

Effect of Divalent Cations and Inhibitors. Mg^{2+}, Mn^{2+}, or Ca^{2+} at 1 mM concentration had no effect on glucosylation of either 2′

Table IV. *Kinetic Properties of Chrysosplenium Glucosyltransferase*

Properties	Glucosyltransferase Activity[a]	
	2'-	5'-
K_m Substrate (μM)	B,8.5	F,9.1
	A,11	E,20
V_{max} (nkat/mg)[b]	2.44	4.40
K_m UDPGlucose (μM)[b]	44.4	40.8
K_i Glucoside (μM)[c]	25	45
K_i UDP (μM)	510	780

[a] See Figure 1 for structural formulae of substrates A, B, E, and F.
[b] For substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone), respectively.
[c] For 2'- and 5'-*O*-glucosides of substrates B and F, respectively.

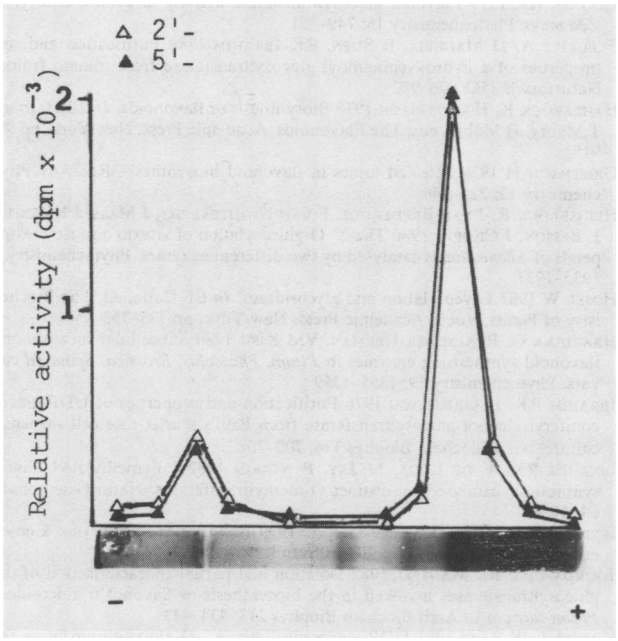

FIG. 8. Glucosyltransferase activity after acrylamide gel electrophoresis. The purified enzyme preparation was layered on 10% polyacrylamide gels and electrophoresed in a buffer containing 5 mM 2-mercaptoethanol and 2 mM EDTA at 6°C. The gels were sliced into 2-mm sections, disintegrated in Tris-HCl buffer (pH 7.6) containing 14 mM 2-mercaptoethanol, and assayed for enzyme activity against substrates B (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) for 2' and 5' activities, respectively.

or 5' positions (Table II). The fact that 5 mM EDTA did not inhibit enzyme activity seems to indicate that glucosylation of either position had no requirement for divalent cations. However, there was strong inhibitory effect of these ions at higher concentration (10 mM), especially that of Mn^{2+} and Ca^{2+} on the 2' activity. Other cations, such as Cu^{2+}, Fe^{2+}, and Zn^{2+} strongly inhibited glucosylation at 1 mM concentration (Table II).

Both 2' and 5' glucosyltransferase activities were inhibited by various SH group reagents. *N*-Ethylmaleimide, phenylmercuriacetate, and *p*-chloromercuribenzoate were inhibitory at lower concentration (1 mM), whereas iodoacetate and iodoacetamide were effective at higher concentration (Table II). It is interesting to note that the neutral inhibitors iodoacetamide and phenylmercuriacetate strongly inhibited the 2' activity as compared with that of 5'. The addition of 10 and 40 mM dithioerythritol resulted in 50% and 100% recovery from inhibition, respectively (Table II).

The effect of phosphate buffer on glucosyltransferase activity (Table II; Fig. 5) was further studied in view of the reported occurrence of trace metal contaminants in ionic buffers, especially Cu^{2+} (1) and the inhibitory effect of enzyme activity by the latter ion. The addition of a very low concentration of Cu^{2+} (2 μM) to the enzyme protein in presence of Tris-HCl buffer resulted in a rapid drop of activity during the first 10 h, which was followed by a slower rate of inactivation for another 10 to 20 h (Fig. 6A). Higher concentration of Cu^{2+} (1.5 mM) resulted in almost complete elimination of either 2' or 5' activities. Reactivation of the enzyme could be achieved by incubation with 10 mM dithioerythritol at 4°C for several days. These results seem to indicate an oxidative effect by Cu^{2+} ions of the free SH groups resulting in disulfide linkages which can be reduced in presence of dithioerythritol. In another experiment, the phosphate buffer was purified from metal ion contaminants by passing through Dowex chelating resin. However, partial inhibition of the enzyme activity was still observed at 25 mM of the purified buffer and amounted to 50% and 90% inhibition of 5' and 2' activities, respectively (Fig. 6B). Determination of the free SH groups before and after reduction with sodium borohydride showed the presence of 8.9 groups/mol of the enzyme. On the other hand, the enzyme preparation in phosphate buffer, which retained 40% of the original activity, was found to contain 6.6 SH groups/mol of the enzyme, thus indicating the oxidation of at least two SH groups which resulted in 60% loss of enzyme activity.

Substrate Specificity. Of the various substituted flavonols tested for their glucose acceptor ability, the best substrates were compounds A and B for 2' activity, and compounds E and F for 5' activity (Fig. 1; Table III). It is interesting to note that compounds A and E which have 2',5'-dihydroxy positions available for glucosylation gave predominantly their naturally occurring 2' and 5' glucosylated derivatives, respectively. The reaction products were identified by cochromatography with reference compounds (4) and autoradiography (Fig. 7). Unlike substrates A, B, E, and F which possess trisubstituted B-ring, disubstituted flavonols such as compounds C and D were poor glucosyl acceptors, as were other flavonol aglycones and phenylpropanoid compounds (Table III). Regardless of the nature of substitution on the flavonoid ring system, flavonol glucosides were not accepted for further glucosylation.

Kinetic Data and Product Inhibition. The apparent K_m values of the purified enzyme for substrates B and F were similar (8.5 and 9.1 μM) for the 2' and 5' activities, respectively; those for substrates A and E were also of the same order of magnitude (Table IV). The apparent K_m values for the cosubstrate UDP-glucose were 44.4 and 40.8 μM for both activities, respectively. Glucosyltransferase activity was strongly inhibited by both products of the reaction, the flavonol glucoside and UDP (Table IV). The K_i values for the former were 25 and 45 μM, and for the latter were 510 and 780 μM for the 2' and 5' activities, respectively.

Mol Wt. An estimation of the mol wt of this glucosyltransferase was found to be approximately 42,000 D. The second peak of activity which was eluted from DEAE-cellulose or DEAE-Sephadex A-25 columns (Fig. 4, A and B) had an apparent mol wt of 80,000 D.

DISCUSSION

To our knowledge, this is the first reported instance where a glucosyltransferase specific to ring B of partially methylated flavonols has been purified and characterized. It catalyzed the transfer of glucose from UDP-glucose to both 2' and 5' hydroxyl groups of the naturally occurring flavonol aglycones of *C. americanum* (4). This soluble enzyme had an apparent mol wt of 42,000 D and an isoelectric point of 5.1. Its general properties were similar to those of other reported flavonoid glucosyltransferases with respect to pH optimum, mol wt, requirement for SH groups, inhibition by divalent cations and by either of the reaction products (8, 13, 14, 17, 18, 20, 24).

Enzyme activity was strongly inhibited by Cu^{2+} ions (Fig. 6A) as well as in the presence of phosphate buffer (Fig. 6B). Inhibition by the latter was shown to be possibly due to oxidation of SH groups which resulted in partial loss of enzyme activity. A similar result was reported for the isoflavone 7-*O*-glucosyltransferase from chick pea (18). Glucosyltransferase activity of *C. americanum* exhibited no requirement for divalent cations which compared well with some reported glucosyltransferases (12, 14) but is in contrast with that of C-glycoflavones (3). The substrate specificity expressed by this enzyme and the low K_m values for its flavonoid substrates and the cosubstrate UDP-glucose indicate high affinity as compared with other flavonoid glucosyltransferases (17, 21, 22).

The fact that both 2′ and 5′ glucosylating activities eluted in a single peak from a number of chromatographic columns seemed to indicate the presence of one enzyme that catalyzed the glucosylation of both positions. This view was supported by the fact that, when substrates B and F were mixed at near saturating concentration, no additive effect was observed. Instead, 60% reduction of glucosylating activity was obtained as compared with either of the controls. The similarity of the K_m values for the substrates specific for 2′ (8.6 μM) and 5′ (9.0 μM) and those of the cosubstrate UDP-glucose (44 and 41 μM), respectively tend to substantiate the above concept. Further evidence for the presence of one enzyme acting on both 2′ and 5′ positions was obtained from polyacrylamide gel electrophoresis of the purified preparation in the presence and absence of SDS. Both 2′ and 5′ glucosylating activities were found in one polypeptide band (Fig. 8).

However, this should not exclude the possibility of the existence of two enzymes with similar chromatographic and electrophoretic properties that may attack each position. The presence of another peak with low glucosylating activity suggests the existence of a dimeric form of the enzyme which had an apparent mol wt of 80,000 D.

The differential effects towards 2′ and 5′ activities of divalent cations, SH group protectors and their inhibitors, as well as the inhibition by the reaction products suggest the involvement of two active sites in the glucosylation reaction. The results presented here (Table II) seem to indicate that the site for 2′ activity is more susceptible to the above mentioned factors than that of the 5′ activity.

The data on substrate specificity show that substrates with *O*-disubstituted B-ring were poor glucosyl acceptors (Table III, C and D) as compared with those trisubstituted compounds having two *para*-oriented substituents. It seems plausible, therefore, that at least two *para*-oriented substituents are required for substrate binding as shown with compounds A, B, E, and F (Table III). This is in agreement with the fact that, in *C. americanum*, the natural glucosides of compounds C and D occur as minor constituents (4). This may raise the question as to whether efficient glucosylation of these two substrates requires some specific factor(s) or is mediated by another glucosyltransferase.

The physiological role of this enzyme is to catalyze the 2′ glucosylation of two partially methylated flavonols A and B, and the 5′ glucosylation of those of E and F. Current work in this laboratory (6, 8–10) has demonstrated the stepwise *O*-methylation of flavonol aglycones to their mono-, di-, and trimethyl derivates in *C. americanum*. These results, together with the present work, strongly indicate that glucosylation is a later step in the biosynthesis of partially methylated flavonols and is in agreement with earlier proposals (9, 12).

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