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FAST PROTEIN AFFINITY CHROMATOGRAPHY OF TWO FLAVONOID GLUCOSYLTRANSFERASES

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SUMMARY

Chrysosplenium americanum contains a glucosyltransferase which attacks the 2′- or 5′-positions of partially methylated flavonols. The two enzyme activities were not resolved on either Superose 12 (gel filtration), Mono Q (ion exchange) or Mono P (chromatofocusing) columns, suggesting similar chromatographic properties. The combined use of the affinity columns UDP-glucuronic acid agarose and Reactive Brown 10-agarose, coupled with a fast protein liquid chromatography system allowed the separation, for the first time, of the two flavonol-ring B-specific glucosyltransferases.

INTRODUCTION

Chrysosplenium americanum (Saxifragaceae) accumulates a variety of 2′- and 5′-O-glucosides of partially methylated flavonols[1]. Recently a novel flavonol-ring B-specific O-glucosyltransferase (GT) was partially purified from this tissue. This enzyme catalyzed the transfer of the glucosyl moeiety of UDP-glucose to the 2′- or 5′-positions of partially methylated flavonols[2] (Fig. 1). In view of the high substrate and position specificity of the O-methyltransferases in this tissue[3] and of the GTs in general for their flavonoid acceptors[4,5], it was considered of interest to determine whether glucosylation of the 2′- and 5′-positions was catalyzed by one or two distinct enzymes. Previous work on *Chrysosplenium* GT indicated the close similarity in both chromatographic[2] and kinetic properties[6] of the 2′- and 5′-glucosylating activities.

We report in this paper on the separation of the 2′- and 5′-glucosylating activities by a combination of UDP-glucuronic acid agarose and dye ligand affinity chromatography, using a fast protein liquid chromatography (FPLC) system.

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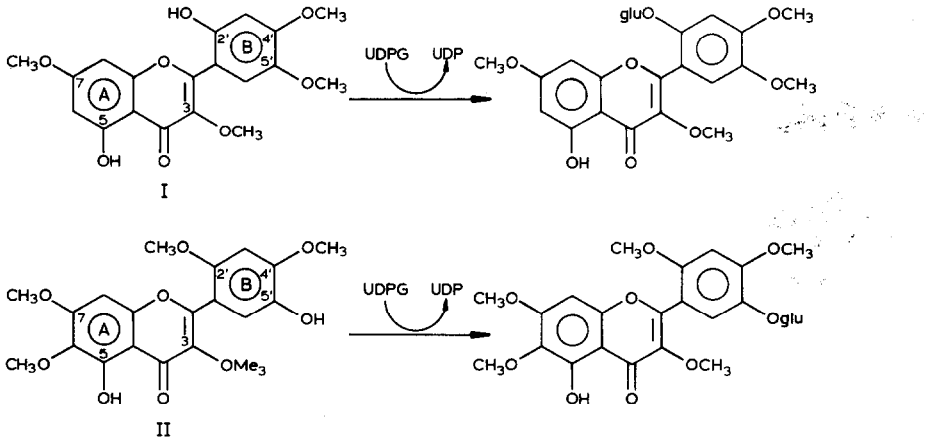


Fig. 1. O-Glucosylation of the 2'- and 5'-positions of the partially methylated flavonols I (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and II (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone), respectively, of *C. americanum*.

EXPERIMENTAL

Plant material

Chrysosplenium americanum Schwein ex Hooker (Saxifragaceae) was collected from Sutton Junction (Province of Quebec, Canada), and was maintained under controlled conditions of light, temperature and humidity.

Materials

Uridine 5'-diphospho-[U-^{14}C]glucose (278 Ci/mol) was obtained from Amersham (Arlington Heights, IL, U.S.A.); UDP-glucuronic acid-agarose and Reactive Brown 10 cross-linked agarose from Sigma (St. Louis, MO, U.S.A.). Sephadex G-25, Superose 12 (prep grade), prepacked Superose 12 HR 10/30, Mono P HR 5/20 and Mono Q HR 5/5 columns as well as the FPLC system (see below) were from Pharmacia (Uppsala, Sweden). Dowex 1-X2 was from Bio-Rad Labs. (Richmond, CA, U.S.A.) and all other chemicals and solvents were of analytical grade reagents.

Buffers

The following buffers were used: (A) 25 m*M* imidazole (pH 7.2) containing 14 m*M* 2-mercaptoethanol (2-ME) and 10% glycerol; (B) Polybuffer (74)–water (1:10, v/v) (pH 4.0) containing 14 m*M* 2-ME and 10% glycerol; (C) 20 m*M* Tris–HCl (pH 7.8) containing 14 m*M* 2-ME and 10% glycerol; (D) 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME (E) same as buffer D containing 10% glycerol; and (F) 0.2 *M* potassium chloride in 25 m*M* imidazole (pH 8.0) containing 14 m*M* 2-ME and 10% glycerol.

Enzyme source

Unless stated otherwise, all procedures were carried out at 4°C. Protein was extracted as previously described[2]. The protein extract was fractionated with solid

ammonium sulfate and the protein fraction that precipitated between 35 and 70% salt saturation was collected by centrifugation.

Chromatofocusing

The 35–70% ammonium sulfate pellet was suspended in the minimal amount of buffer A and subjected to gel filtration on Superose 12 HR 10/30 column (300 × 10 mm I.D.) which had previously been equilibrated with the same buffer. The column was developed with buffer A at a flow-rate of 0.5 ml/min (2.5 MPa) and 1-ml fractions were collected and assayed for the 2′- and 5′-glucosylating activity. The active fractions were pooled and subjected to chromatofocusing on Mono P HR 5/20 column (200 × 5 mm I.D.) which had previously been equilibrated with buffer A. The bound proteins were eluted with 50 ml of buffer B, which generated a linear gradient between pH 7.0 and 4.0. The flow-rate was 0.5 ml/min (3.0 MPa) and 1-ml fractions were collected and assayed for GT activity.

Ion-exchange chromatography

The 35–70% ammonium sulfate pellet was suspended in the minimal amount of buffer C and was desalted on a Sephadex G-25 column (160 × 15 mm I.D.) which had previously been equilibrated with the same buffer. The desalted protein was subsequently subjected to ion-exchange chromatography on Mono Q HR 5/5 column (50 × 5 mm I.D.) which had previously been equilibrated with buffer C. The column was washed with five bed volumes of the same buffer. The bound proteins were eluted using a linear salt gradient of 0–0.5 *M* potassium chloride in buffer C. The flow-rate was 1 ml/min (3.0 MPa) and 1-ml fractions were collected and assayed for GT activity.

Affinity chromatography

UDP-glucuronic acid agarose. The 35–70% ammonium sulfate pellet was suspended in the minimal amount of buffer D and was desalted on a Sephadex G-25 column (160 × 15 mm I.D.) which had previously been equilibrated with the same buffer. The desalted protein was subsequently chromatographed on a UDP-glucuronic acid agarose column (70 × 10 mm I.D.) which had previously been equilibrated with buffer D. The column was washed with five bed volumes of the same buffer and the bound proteins were eluted with a linear gradient using 0–100% of buffer F in buffer E, at a flow-rate of 0.3 ml/min. Fractions of 1 ml were collected and assayed for GT activity. The active fractions were pooled and subjected to gel filtration on Superose 12 (prep grade) HR 16/50 column (500 × 16 mm I.D.) which was packed using the FPLC system as per the manufacturers instructions. The protein was loaded onto the column which had previously been equilibrated with buffer D. The column was developed using the same buffer at a flow-rate of 0.5 ml/min (0.25 MPa) and 3-ml fractions were collected and assayed for GT activity.

Reactive brown 10 agarose. The active fractions were pooled and subjected to dye ligand affinity chromatography on Reactive Brown 10 agarose C 10/10 column (100 × 10 mm I.D.), which was packed using the FPLC system as per the manufacturers instructions. The protein was loaded onto the column which had previously been equilibrated with buffer D. The column was washed with five bed volumes of the same buffer. The bound proteins were eluted using a linear gradient of 0–100%

buffer F in buffer E. The flow-rate was 0.2 ml/min (0.1 MPa) and 0.5-ml fractions were collected and assayed for GT activity.

Fast protein liquid chromatography system

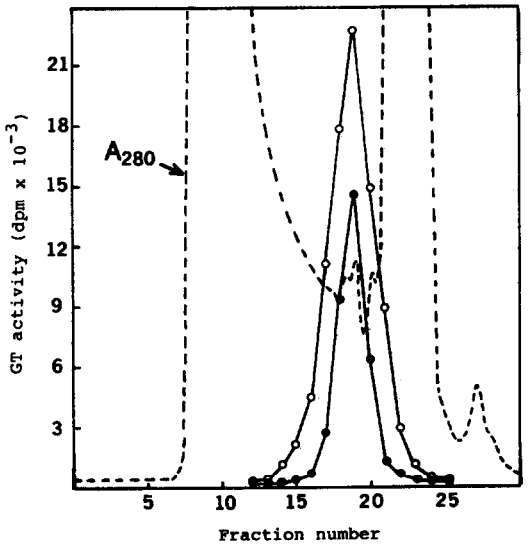
The FPLC system consisted of two P-500 pumps, GP-250 gradient programmer, V-7 injection valve, 10-ml superloop, UV monitor with HR flow cell, REC-482 chart recorder and pH monitor with a flow-through electrode. All buffers were filtered through 0.22-μm Millipore filter and degased under vacuum before use.

Glucosyltransferase assay and product identification

The standard enzyme assay was performed as described previously[2] using UDP-[U-^{14}C]glucose as the glucosyl donor. The substrates used were I (5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone) and II (5,5′-dihydroxy-3,6,7,2′,4′-pentamethoxyflavone) for the 2′- and 5′-glucosylating activities, respectively (Fig. 1). The reaction products were identified by co-chromatography with their corresponding 2′- and 5′-glucosides[1] on Polyamid-6 MN using benzene–methyl ethyl ketone–methanol (8:1:1) as the solvent system, and autoradiographed on X-ray film.

Protein determination

Protein was determined according to the method of Bradford[7] using the Bio-Rad reagent and bovine serum albumin as standard.



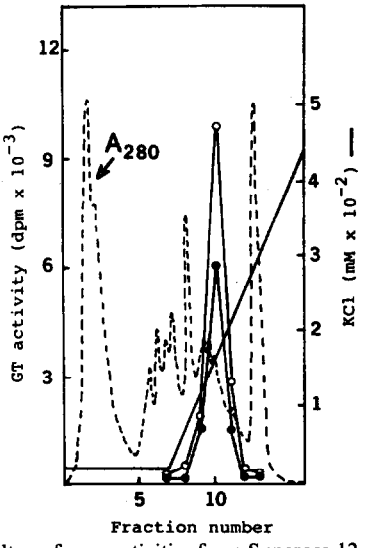


Fig. 2. Elution profile of the 2′- (○—○) and 5′- (●—●) glucosyltransferase activities from Superose 12 column using compounds I and II as substrates, respectively. The column was pre-equilibrated and developed with 25 m*M* imidazole (pH 7.2) containing 14 m*M* 2-ME and 10% glycerol and 1-ml fractions were collected, and assayed for GT activity.

Fig. 3. Elution profile of the 2′- (○—○) and 5′- (●—●) glucosyltransferase activities from Mono Q column using compounds I and II as substrates, respectively. The desalted enzyme was applied to a column pre-equilibrated with 20 m*M* Tris–HCl (pH 7.8) containing 14 m*M* 2-ME and 10% glycerol. The bound proteins were eluted using a linear salt gradient (0–0.5 *M* potassium chloride) in the same buffer and 1-ml fractions were collected, and assayed for GT activity.

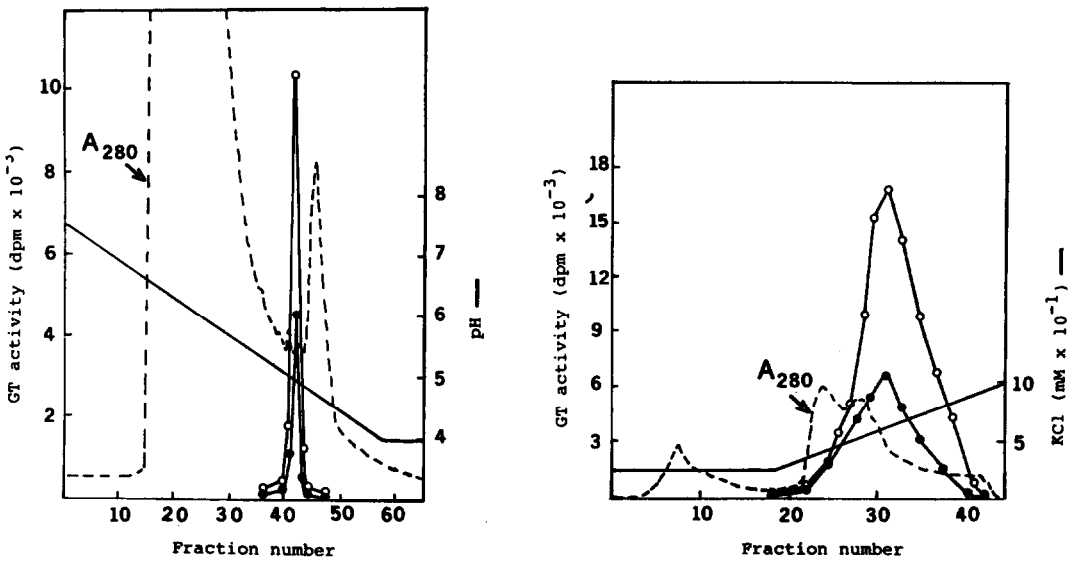


Fig. 4. Elution profile of the 2'- (○—○) and 5'- (●—●) glucosyltransferase activities from a Mono P column using compounds I and II as substrates, respectively. The active fractions from gel filtration on Superose 12 were pooled and applied to a column pre-equilibrated with 25 m*M* imidazole (pH 7.2) containing 14 m*M* 2-ME and 10% glycerol. Elution of the bound proteins was carried out using Polybuffer (74)–water (1:10, v/v) (pH 4.0) containing 14 m*M* 2-ME and 10% glycerol, which generated a linear gradient between pH 7.0 and 4.0. Fractions of 1 ml were collected, and assayed for GT activity.

Fig. 5. Elution profile of the 2'- (○—○) and 5'- (●—●) glucosyltransferase activities from a UDP-glucuronic acid agarose column using compounds I and II as substrates, respectively. The desalted protein was loaded onto a column which had previously been equilibrated with 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME. The column was washed with the same buffer and the bound proteins were eluted using a linear gradient of 0–100% 25 m*M* imidazole (pH 8.0) (containing 0.2 *M* potassium chloride, 14 m*M* 2-ME and 10% glycerol) in 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME and 10% glycerol. Fractions of 1 ml were collected, and assayed for GT activity.

RESULTS AND DISCUSSION

Previous work in this laboratory[2] indicated that both the 2'- and 5'-glucosyltransferase activities were not separated by conventional chromatography on gel filtration, ion exchange, hydroxyapatite columns or by chromatofocusing. Similar results were obtained when Superose 12 (Fig. 2). Mono Q (Fig. 3) or Mono P (Fig. 4) columns were used with an FPLC system. These results seemed to indicate that both the 2'- and 5'-glucosylation reactions were catalyzed by one enzyme. On the other hand, both glucosylations may be mediated by two distinct enzymes with similar chromatographic properties.

Unlike the coniferyl alcohol GT[8], the kinetic mechanism of *Chrysosplenium* GT indicated that UDP binds the free enzyme[6]. This kinetic property allowed the binding of the enzyme to the UDP-glucuronic acid agarose affinity support, although it did not bind to UDP-agarose. Both the 2'- and 5'-GT activities were eluted at approximately 60 m*M* potassium chloride (Fig. 5), which resulted in about ten-fold purification (Table I). Since the enzyme was eluted from this affinity column using

TABLE I

PURIFICATION OF 2'- AND 5'-GLUCOSYLTRANSFERASE ACTIVITIES FROM *C. AMERICANUM*

Purification step	*Total protein (mg)*	*Specific activity (pkat/mg)*		*Purification (-fold)*	
		2'-	*5'-*	*2'-*	*5'-*
Crude extract*	9.45	0.028	0.012	–	–
Ammonium sulfate** (35–70% saturation)	6.0	0.038	0.018	1.35	1.5
UDP-glucuronic acid agarose	1.69	0.28	0.12	9.8	10.0
Gel filtration***	0.1	4.86	1.8	173	150
Brown 10 agarose	<0.001	34.5	15.1	1230	1200

* After treatment with Dowex 1-X2.
** Desalted on Sephadex G-25.
*** On Superose 12 (prep grade) column.

a buffer of high pH and ionic strength, therefore, the active fractions were pooled and desalted on a preparative Superose 12 column (Fig. 6). The latter step was necessary for desalting the enzyme protein and changing the pH of the buffer, in order to allow binding of the enzyme to the dye ligand column. Elution from the latter was performed using a linear pH salt gradient, which resulted in the separation of the 2'- and 5'-enzyme activities at pH values of 7.8 and 7.3, respectively (Fig. 7). The combined purification steps described above resulted in an increase in specific activity of

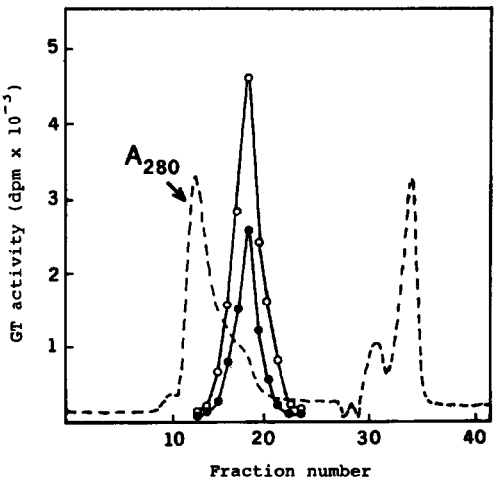


Fig. 6. Elution profile of the 2'- (○—○) and 5'- (●—●) glucosyltransferase activities from Superose 12 (prep grade) column using compounds I and II as substrates, respectively. The active fractions from UDP-glucuronic acid agarose column were pooled and applied to a preparative Superose 12 column which had previously been equilibrated with 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME. The column was developed with the same buffer and 3-ml fractions were collected, and assayed for GT activity.

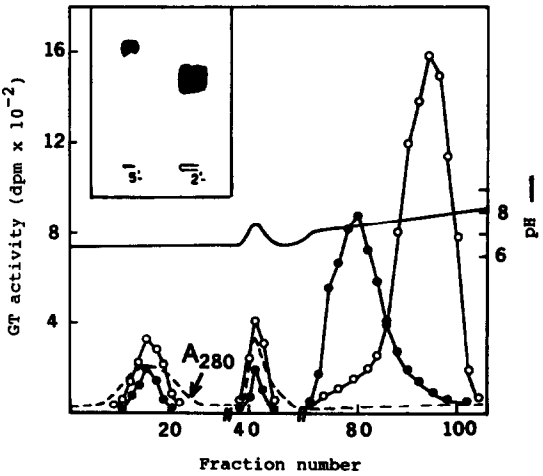


Fig. 7. Elution profile of the 2′- (○—○) and 5′- (●—●) glucosyltransferase activities from Reactive Brown 10 agarose column using compounds I and II as substrates, respectively. The active fractions from gel filtration on Superose 12 (prep grade) were pooled and applied to the dye ligand column which had previously been equilibrated with 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME. The column was washed with the same buffer and the bound proteins were eluted using a linear gradient of 0–100% 25 m*M* imidazole (pH 8.0) (containing 0.2 *M* potassium chloride, 14 m*M* 2-ME and 10% glycerol) in 25 m*M* imidazole (pH 6.4) containing 14 m*M* 2-ME and 10% glycerol. Fractions of 0.5 ml were collected, and assayed for GT activity. Insert, autoradiogram of the chromatographed enzyme reaction products on Polyamid-6 MN using benzene–methyl ketone–methanol (8:1:1).

1230-fold and 1200-fold for the 2′- and 5′-GTs, respectively, as compared with the crude extract (Table I). Each of the purified fractions gave a single product when assayed with its respective substrate as shown by autoradiography (Fig. 7, insert).

Affinity chromatography has become a widely used tool for enzyme purification[9–11]. Unlike other commonly employed fractionation methods, affinity chromatography makes use of the highly specific binding sites of enzymes, thus allowing their separation according to their ability to bind to a particular ligand. It is interesting to note that, of the several dye ligands used, the brown agarose was the only support which allowed binding and separation of the two GT activities reported here, suggesting selectivity of the enzymes for the chemical nature of this ligand. Therefore, this technique proves to be a powerful tool for the separation of enzymes with similar or closely related chromatographic properties. The low concentration of protein as well as the prolonged exposure of the enzymes to drastic changes of pH and ionic strength resulted in poor recovery of enzyme activities. However, the combined use of dye ligand chromatography and a FPLC system made it possible to separate, for the first time, two flavonol-ring B-specific GTs from *C. americanum*. Unequivocal evidence for the involvement of two distinct enzymes in the glucosylation of the 2′- and 5′-positions of polymethylated flavonols in *C. americanum* should be obtained from further work using immunological techniques. To our knowledge, this is the first reported instance of the use of fast protein affinity chromatography in enzyme purification.

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