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PURIFICATION AND SOME PROPERTIES OF FIVE ANTHRAQUINONE-SPECIFIC GLUCOSYLTRANSFERASES FROM *CINCHONA SUCCIRUBRA* CELL SUSPENSION CULTURE

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Key Word Index—*Cinchona succirubra*; Rubiaceae; glucosyltransferases; purification; FPLC; anthraquinones; plant cell culture.

Abstract—Five anthraquinone-specific glucosyltransferases were partially purified from *Cinchona succirubra* cell suspension culture by fractional precipitation with ammonium sulphate, gel filtration and chromatofocusing on a fast protein liquid chromatography system. Five, distinct glucosylating activities were resolved with apparent pI values of 5.3, 4.8, 4.5, 4.3 and 4.1. They accepted emodin, anthrapurpurin, quinizarin, 2,6-dihydroxy anthraquinone and 1,8-dihydroxy anthraquinone as the best substrates, respectively. These enzymes exhibited similar characteristics as to pH optimum (pH 7) in histidine/HCl buffer, M_r 50 000, had no cation requirement and were inhibited by various SH-group reagents. The K_m value of the respective anthraquinones for either of the five enzymes was 10 μM. The physiological role of these novel enzymes is discussed in relation to the biosynthesis of anthraquinone glucosides in this tissue.

INTRODUCTION

Many species of the Rubiaceae are known to synthesize anthraquinones (AQs) both *in vivo* [1] and *in vitro*. Among the latter, *Morinda citrifolia* [2–5], *Galium mullugo* [6–8], *Rubia cordifolia* [9–11] and *Cinchona* spp. [12–17] are well documented. Except for the early enzymatic steps catalysed by *o*-succinyl benzoate CoA synthase [18] and 1,4-dihydroxy-2-naphthoate CoA synthase [19], little is known of the enzymes involved in the later steps of biosynthesis of AQ glucosides.

Very recently, we reported on the production of AQ glucosides in *Cinchona succirubra* (Rubiaceae) cell culture, and the presence of a glucosyltransferase (GT) activity in this tissue [20]. This report describes, for the first time, the isolation and some properties of five distinct GTs (E.C. 2.4.1-) which catalysed the transfer of the glucosyl moiety from UDP-glucose to the hydroxyl group(s) of AQs.

RESULTS AND DISCUSSION

Growth of Cinchona *cell culture*

In order to study the glucosylation of AQs, it was essential to determine the peak of GT activity in relation to culture growth and the production of AQs by *C. succirubra* cells. Figure 1 shows that the enzymatic glucosylation of AQs paralleled cell growth and was concomitant with maximum protein content as well as AQ production in *Cinchona* cells.

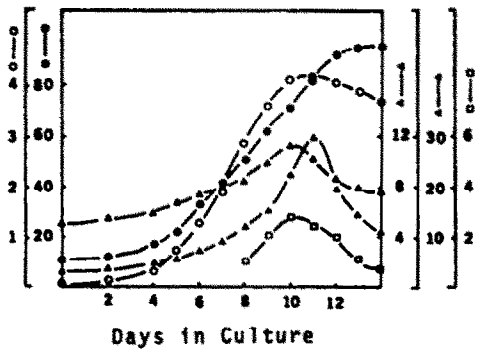

Fig. 1. Changes with time in fresh weight (g/l) (●—●); dry weight (g/l) (○—○); protein content (mg/l) (△—△); total anthraquinone content (μmol/l) (▲—▲) and glucosyltransferase activity (pkat/mg protein) (□—□) of *C. succirubra* suspension culture.

Purification of GT activity

The GT activity of *C. succirubra* was purified by fractional precipitation with ammonium sulphate, gel filtration on Superose 12 HR 10/30 column (Fig. 2) followed by chromatofocusing on Mono P HR 5/20 column (Fig. 3). The latter purification step resulted in the resolution of five peaks of GT activity with apparent pI values of 5.3, 4.8, 4.5, 4.3 and 4.1. These exhibited distinct specificity towards different substituted AQs. The combined purification steps resulted in an increase in specific

Abbreviations: AQ, anthraquinone; DTE, dithioerythritol; FPLC, fast protein liquid chromatography; GT, glucosyltransferase; 2-ME, 2-mercaptoethanol; UDP-glucose, uridine diphospho-glucose.

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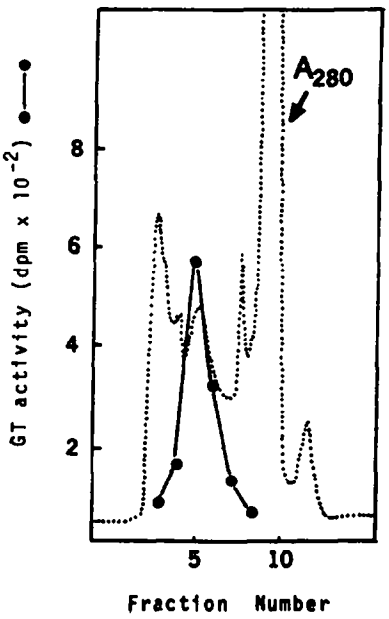

Fig. 2. Elution profile of glucosyltransferase activity after gel filtration on Superose 12 HR 10/30 column using emodin as substrate.

activity of 202-, 337-, 228-, 417- and 823- fold for peaks I, II, III, IV and V, respectively, as compared with that of the crude extract (Table 1).

Substrate specificity

The different activity peaks (Fig. 3) accepted preferentially different substrates, so that peak I accepted emodin (1,6,8-trihydroxy-3-methyl-AQ); peak II, anthrapurpurin (1,2,7-trihydroxy-AQ); peak III, quinizarin (1,4-dihydroxy-AQ); whereas peaks IV and V accepted 2,6-dihydroxy anthraquinone and 1,8-dihydroxy anthraquinone as the best substrates, respectively (Table 2). Due to the difficulty in the separation of AQ glucosides [20, 21], the individual reaction products could not be identified. However, based on the substitution pattern of the substrates used (Table 2), it seems that these GTs may be attacking positions C-1 and C-2 of AQs, or their equivalents. Furthermore, all five enzymes accepted quercetin, as well as 3,7,3′,4′-tetramethylquercetin as substrates to various extent (Table 2). This may be due to the fact that positions C-5 and C-7 of a flavone are structurally equivalent to positions C-1 and C-2 of an AQ, respectively (Fig. 4). It is interesting to note that the least reactive 5-hydroxyl of quercetin could be subjected to enzymatic glucosylation, despite the fact that it is chelated with the carbonyl group, as is evident from the reactivity of 3,7,3′,4′-tetramethylquercetin (Table 2). Other phenolic compounds tested were poor glucosyl acceptors.

Effect of pH

The pH optimum for the glucosylation reaction, as determined in different buffers, was found to be pH 7. Maximum GT activity was observed in histidine/HCl buffer, and a change of one pH unit resulted in a loss of 60% of enzyme activity.

Linearity of the glucosylation reaction

At optimum pH and 10 μM of substrate, the glucosylation of AQs was linear with time up to 60 min and with protein concentration up to 50 μg.

Enzyme stability

In the absence of SH-group portectors, the partially purified enzymes lost more than 50 % of their activity within 48 hr. However, the addition of 14 mM 2-ME to

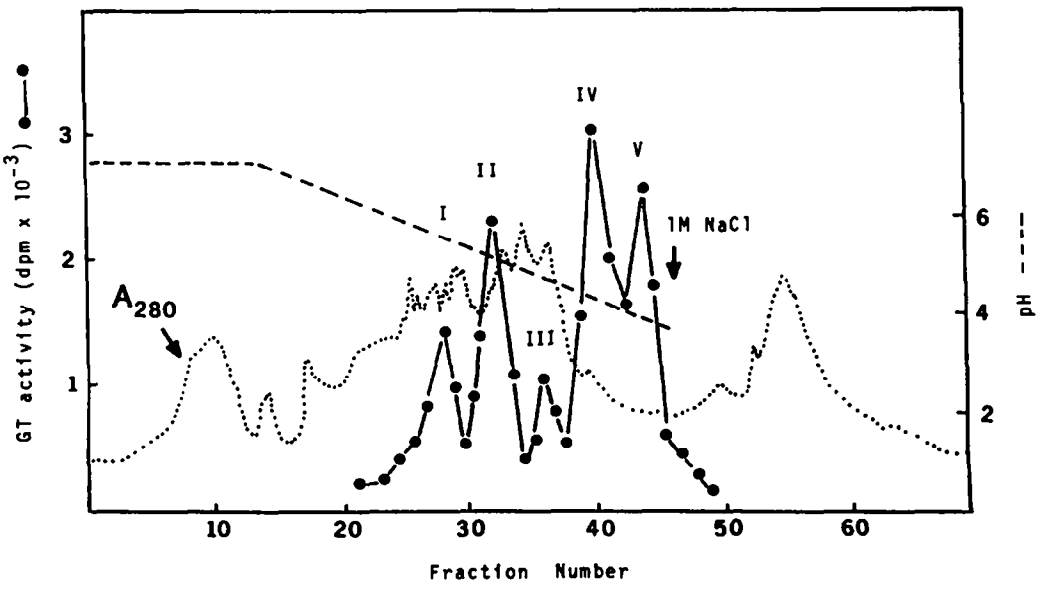

Fig. 3. Elution profile of glucosyltransferase activity after chromatofocusing on Mono P HR 5/20 column using emodin as substrate.

Table 1. Purification of *C. succirubra* glucosyltransferases

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
Crude extract*	101	1.07	—	100
Ammonium sulphate (35–80%)†	92.7	1.21	1.1	100
Gel filtration‡	7.4	12.0	11.2	82
Chromatofocusing§				
peak I	0.138	216	202	28
peak II	0.102	361	337	34
peak III	0.087	244	228	20
peak IV	0.078	446	417	32
peak V	0.054	881	823	44

*Dowex 1 × 2.
†Desalted on Sephadex G-25.
‡On Superose 12 HR 10/30 column.
§On Mono P HR 5/20 colomn.

Table 2. Substrate specificity of *C. succirubra* glucosyltransferases

Substrate	Relative activity (%)*				
	I	II	III	IV	V
Alizarin (1,2-diOH-AQ)	44	50	46	44	59
Quinalizarin (1,2,5,8-tetra-OH-AQ)	34	22	14	21	55
Chrysophanol (1,8-diOH-3Me-AQ)	41	50	86	85	80
Emodin (1,6,8-triOH-3Me-AQ)	100	80	87	87	67
2,6-diOH-AQ	73	49	35	100	54
Purpurin (1,2,4-triOH-AQ)	32	29	25	30	48
1,8-diOH-AQ	39	42	85	87	100
Quinizarin (1,4-diOH-AQ)	33	50	100	77	64
Anthrarufin (1,5-diOH-AQ)	11	28	43	32	34
Anthrapurpurin (1,2,7-triOH-AQ)	48	100	43	44	54
3,7,3',4'-Tetra-OMe-quercetin	29	72	82	22	71
Apigenin	35	32	29	34	27
Kaempferol	58	37	48	42	36
Quercetin	60	52	77	76	71
p-Coumaric acid	35	30	32	29	30
Caffeic acid	40	45	35	37	41

*100% activity represents 216, 361, 244, 446, and 881 pkat/mg protein for peaks I, II, III, IV and V, respectively using the standard enzyme assay.

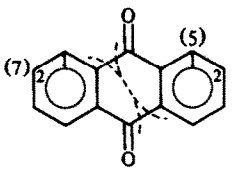

Fig. 4. Formula of an anthraquinone ring system showing structural similarity of positions C-1 and C-2 to those of C-5 and C-7 in a flavonoid ring.

either of the enzymes resulted in 50% loss of activity after two weeks. The partially purified GTs were stored in 25 mM histidine/HCl buffer pH 7.0 containing 10% glycerol and 10 mM DTE at −20° and were fairly stable for two months.

Effect of divalent cations and SH-group reagents

The effect of divalent cations and SH-group inhibitors on the glucosylation reaction was studied (Table 3). The fact that EDTA did not inhibit the enzyme activity seems to indicate that the glucosylation of AQs has no requirement for divalent cations. However, low concentrations of Co^{2+}, Cu^{2+} and Zn^{2+} inhibited the glucosylation reaction by 50–75%. Furthermore, GT activity was inhibited by various SH-group reagents, but the addition of DTE resulted in 100% recovery from inhibition (Table 3).

Other properties

The five GTs had an apparent M_r of 50000 and an apparent K_m value for their respective AQ substrates of

Table 3. Effect of divalent cations and SH-group reagents on *C. succirubra* glucosyltransferase activity *

Additions	Concentration (mM)	Relative activity (%)
None	—	100
Mg^{2+}	1	110
Mg^{2+}	10	105
Mn^{2+}	1	105
Mn^{2+}	10	55
Ca^{2+}	1	110
Co^{2+}	1	40
Cu^{2+}	1	50
Zn^{2+}	1	23
EDTA	1	130
EDTA	10	125
DTE	10	163
Iodoacetamide	1	40
Iodoacetate	1	70
N-Ethylmaleimide	1	8
Phenyl mercuriacetate	1	20
Phenyl mercuriacetate + DTE	1, 10	116
p-Chloromercuribenzoate	1	53
p-Chloromercuribenzoate + DTE	1, 10	131

* 100% activity represents 216 pkat/mg of peak I assayed using emodin as substrate.

10 μM. The properties of these enzymes appear to be similar to those of other reported phenol GTs with respect to pH optimum, M_r, requirements for SH-groups and inhibition by divalent cations [22, 23].

It is interesting to note that the activity of peaks III, IV and V following chromatofocusing was recovered only when 1 ml fractions were collected into 0.25 ml of existing 0.2 M Tris–HCl buffer pH 8.0. This is probably due to the fact that these enzymes have low pI values and are therefore not stable at that pH.

These results demonstrate the presence, in *Cinchona* cell cultures, of several enzymes that catalyse the glucosylation of hydroxy-AQs. These enzymes expressed preference for different compounds indicating specificity towards their phenolic substrates. The fact that the best substrates for these GTs were emodin, anthrapurpurin, quinizarin, 1,8-dihydroxy-AQ and 2,6-dihydroxy-AQ is consistent with the accumulation of their glucosides in *C. succirubra* cell suspension cultures [20].

EXPERIMENTAL

Culture conditions. Cinchona succirubra cells were grown in the control medium as was described in [20]. Culture growth was determined by measuring cell fresh and dry wt, protein content, as well as monitoring the pH and conductivity of the medium [20].

Estimation of AQ content. This was calculated from the absorbance values of cell extracts at 435 nm, using an extinction coefficient of 5500 [20].

Buffers. The following buffers were used: A, 0.2 M Tris–HCl pH 7.6 containing 14 mM 2-ME, 5 mM EDTA and 10 mM diethylammonium diethyldithiocarbamate; B, 25 mM Tris–HCl pH 7.2 containing 14 mM 2-ME and 10% glycerol; C, Polybuffer (74): water (1:10, v/v) pH 4.0 containing 14 mM 2-ME and 10% glycerol. Buffers B and C were passed through a 0.22 μm Millipore filter and degassed before use.

Protein extraction. Unless otherwise stated, all procedures were carried out at 4°. Filtered cells (*ca* 10 g) were frozen in liquid N_2, mixed with Polyclar AT (1:5, w/w) and ground to a fine powder. The latter was homogenized with buffer A (1:4, w/v) and the homogenate was filtered through nylon mesh. The filtrate was centrifuged at 12000 *g* for 10 min. The supernatant was stirred with Dowex 1 × 2 (20%, w/v) which had previously been equilibrated with the same buffer, then filtered through glass wool. The filtrate was fractionated with solid $(NH_4)_2SO_4$, and the protein fraction that precipitated between 35 and 80% salt saturation was collected by centrifugation.

Enzyme purification. The GT activity was purified using the Pharmacia Fast Protein Chromatography (FPLC) system. The protein pellet was suspended in the minimal amount of buffer B and chromatographed on a Superose 12 HR 10/30 column, which had previously been equilibrated with the same buffer. The column was developed with buffer B at a flow rate of 0.5 ml/min (2.5 MPa) and 1 ml fractions were collected and assayed for GT activity using emodin as substrate. The active fractions were pooled and subjected to chromatofocusing on a Mono P HR 5/20 column which had previously been equilibrated with buffer B. Elution of the bound proteins was performed with 40 ml of buffer C, at a flow rate of 1.0 ml/min (2.0 MPa), which generated a linear gradient of pH 7–4. Fractions (1 ml) were collected into 0.25 ml of 0.2 M Tris buffer pH 8.0, containing 14 mM 2-ME, and fractions were assayed for GT activity using emodin as substrate.

GT assay. The assay mixture consisted of 10 μM of the AQ substrate (in 10 μl of 50% DMSO), 3 μM of [U-^{14}C]UDP glucose (0.05 μCi), 25 mM Tris–HCl buffer pH 7.5 and 14 mM 2-ME in a total volume of 100 μl. The reaction was started by the addition of protein, and the mixture was incubated for 30 min at 30°. The reaction was stopped by the addition of 10 μl of 6N HCl, and the products were extracted with 250 μl of EtOAc. An aliquot of the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid.

M_r *determination.* An estimate of the M_r of the partially purified enzyme preparation was obtained by determining its elution vol. on gel filtration using M_r standards.

Determination of pI. The pI values of the enzymes were determined from their elution patterns following chromatofocusing on a Mono P column HR 5/20 [24].

Protein determination. Protein was determined according to the method of Bradford [25] using the Bio-Rad protein reagent and bovine serum albumin as standard.

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