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Purification and characterization of gentisic acid glucosyltransferase from the cultured cells of *Catharanthus roseus*

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Abstract

A 41 kDa glucosyltransferase was isolated from the cultured cells of *Catharanthus roseus*. The enzyme specifically catalyzed the monoglucosylation of phenolic compounds, such as hydroxybenzoic acid and hydroxycoumarins. The enzyme activity was optimal at pH 8.0 and 37 °C and was strongly inhibited by divalent cations, such as Mn^{2+}, Co^{2+}, Zn^{2+} and Fe^{2+}. Gentisic acid was the best substrate with a K_m value of 9 μM. The enzyme glucosylates regioselectively 5-hydroxyl group of 2,5-dihydroxybenzoic acid (gentisic acid) by the transfer of glucose from UDP-glucose (UDPG).

Keywords: Glucosyltransferase; Gentisic acid; *Catharanthus roseus*

1. Introduction

Glucosyltransferases are widely distributed in plant tissues and has been implicated in a number of biological processes of plant growth. For example, glucosylation reactions by glucosyltransferase relating to detoxification of exogenous toxic phenolic hydroxyl group [1] and accumulation of secondary metabolites including phytoalexins against exogenous stress for plant [2–5] have been reported.

We recently found that the cultured cells of *Catharanthus roseus* were capable of hydroxylating the 5-position of 2-hydroxybenzoic acid (salicylic acid) to give 2,5-dihydroxybenzoic acid (gentisic acid) and then regioselectively glucosylating the newly-introduced hydroxyl group [6]. On continuing the studies on glucosylation of phenolic compounds, we have found a new glucosyltransferase responsible for regioselective glucosylation of gentisic acid and report here the purification and characterization of the glucosyltransferase from *C. roseus*.

2. Experimental

2.1. Materials

Suspension cell cultures of *C. roseus* were grown at 25 °C for 4 weeks in SH medium according to the procedure described previously [6]. REACTIVE GREEN 19 agarose-gel and UDP-glucose (UDPG) were obtained from Sigma. Sephadex G-25 and G-200 were obtained from Pharmacia Co. Ltd. DEAE Toyopearl was obtained from Tosoh Co. Ltd. CENTRICUT U-20 was obtained from Kurabo Co. Ltd.

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2.2. Purification of glucosyltransferase from C. roseus

All procedures for enzyme purification were performed at 4 °C. Frozen cells (40 g fresh weight) of *C. roseus* were pulverized in liquid nitrogen using a mortar and pestle. The homogenate was suspended in 20 ml of buffer A (50 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid (HEPES) buffer (pH 7.5) containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM ascorbic acid, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Tissue slurry was removed by centrifugation (18,000 × *g* for 20 min at 4 °C) and the supernatant was loaded onto a column of Sephadex G-25 column (2.5 cm × 15 cm). The desalted fractions were purified with a RE-ACTIVE GREEN 19 agarose-gel affinity column (1.5 cm × 16 cm). The column was washed with three bed volume of buffer A and was eluted with a linear UDPG gradient (0–4 mM UDPG) and were concentrated with a CENTRICUT U-20. The active fractions from the affinity column were applied to a Sephadex G-200 column (2.5 cm × 40 cm). The column was eluted with the buffer B (50 mM HEPES buffer (pH 7.5) containing 10% glycerol) at a flow rate of 0.1 ml/min and the active fractions were concentrated with a CENTRICUT U-20. The concentrated enzyme fraction was loaded onto a DEAE Toyopearl 650 M anion-exchange column (1 cm × 5 cm) equilibrated with buffer B. The column was washed with three bed volume of buffer B and was eluted with a linear HEPES gradient (0.05–1.0 M HEPES buffer B). Active fractions were pooled and used for experiment.

Samples from each purification steps were analyzed by SDS-PAGE [7]. Protein bands were visualized with silver staining procedure. Protein concentration was quantified by protein assay (Bio-Rad) using bovine serum albumin (BSA) as standard according to Bradford [8]. The molecular mass of the denatured enzyme was estimated by SDS-PAGE and that of the native enzyme was estimated by gel filtration through a TSK G3000SW HPLC column using BSA, ovalbumin, chymotrypsinogen A, trypsin inhibitor and ribonuclease A as marker proteins under the following condition; flow rate 1 ml/min of 50 mM HEPES buffer (pH 7.5) containing 0.3 M NaCl; monitor by UV adsorption at 280 nm.

2.3. Products identification

The glucosylcoumarins (such as 7-*O*-glucosylcoumarin, 7-*O*-glucosyl-6-hydroxycoumarin (esculin) and 7-*O*-glucosyl-6-methoxycoumarin (scopolin)) and 5-*O*-glucosyl-2-hydroxybenzoic acid were analyzed and isolated by HPLC on Puresil C18 (Waters) under the following conditions. For glucosylcoumarins: flow rate 0.5 ml/min of 25% methanol solution; detection by UV adsorption at 320 nm. For 5-*O*-glucosyl-2-hydroxybenzoic acid: flow rate 0.75 ml/min of 15% acetonitrile solution; detection by UV adsorption at 290 nm. The glucosides isolated were identified by comparison of HPLC, NMR (JEOL GSX500) and FAB-MS (JEOL SX102A) with authentic glucosides [6,9].

2.4. Assay of glucosyltransferase activity

The standard reaction mixture (1 ml) consisted of 10 mM of substrate, 20 mM of UDPG and enzyme fractions in buffer B. After gentle shaking at 37 °C for 24 h, the reaction was stopped by the addition of 50 μl of 1 M HCl. The supernatant separated by centrifugation was analyzed using HPLC as described in Section 2.3. The glucosyltransferase activity of the enzyme fractions was estimated by measuring the amount of glucosides produced in the reaction mixtures on the basis of the peak area of their HPLC by use of the corresponding standard curves prepared with authentic samples [6,9]. One unit of enzyme activity was defined as the amount of the enzyme that catalyzes the formation of 1 μmol of glucoside per minute.

Glucosyltransferase properties were determined with the standard reaction mixture as follows. Optimum pH was determined by the enzyme reaction in pH range between 6.0 and 9.0 using 50 mM HEPES buffer. Effect of metal ions was examined in the enzyme reaction in the presence of 5 mM metal ions in buffer B. Thermal stability was determined by use of the enzyme preparation treated at various temperature from 20 to 60 °C.

3. Results and discussion

3.1. Purification of the glucosyltransferase

A glucosyltransferase was extracted as soluble enzyme from *C. roseus* suspention cells with buffer

Table 1
Purification of glucosyltransferase from the cultured cells of *C. roseus*

Steps	Total protein (mg)[a]	Total activity ($\times 10^{-3}$ U)[b]	Specific activity ($\times 10^{-3}$ U/mg)	Fold
Crude extract	197	366.8	2	1
REACTIVE GREEN 19	1.37	35.7	26	13
Sephadex G-200	0.19	27.2	143	72
DEAE Toyopearl	0.02	7.1	355	178

[a] Total protein was estimated by protein assay (Bio-Rad) with BSA as standard.
[b] One unit was defined as the amount of enzyme which catalyzes the formation of 1 μmol of 5-*O*-glucosyl-2-hydroxybenzoic acid per minute from gentisic acid.

A. The glucosyltransferase was purified by three chromatographic steps (Table 1). Crude enzyme extract was loaded onto a REACTIVE GREEN 19 agarose-gel column. The green dye column which is able to bind UDP-sugar-dependent enzymes was used to eliminate many of the UDP-sugar-non-dependent enzymes [10] (Fig. 1). This step allowed the protein sample to be purified approximately 13-fold and to be concentrated for gel filtration chromatography. Sephadex G-200 gel filtration chromatography was achieved to eliminate proteins with low molecular mass. This step provided a 72-fold increase in the purification of the glucosyltransferase. After this step, glucosyltransferase active fraction was identified to contain three protein bands with molecular mass of 38, 41 and 43 kDa by SDS-PAGE (Fig. 2). The appeared 38 and 43 kDa protein bands were considered to be fragments of other protein with high molecular mass. Further purification was achieved on a DEAE Toyopearl 650 M anion-exchange column. The glucosyltransferase was successfully released with HEPES linear gradient. This final step provided a 178-fold increase in the purification of the glucosyltransferase. After the final purification step, glucosyltransferase protein appeared as a dominant single peptide on a silver-stained gel (Fig. 2).

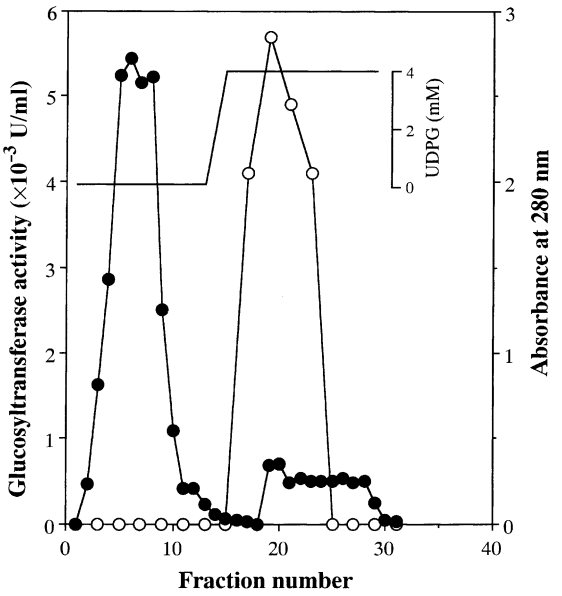


Fig. 1. Elution profiles of glucosyltransferase from the cultured cells of *C. roseus* by REACTIVE GREEN 19 column: (○) glucosyltransferase activity; (●) protein concentration.

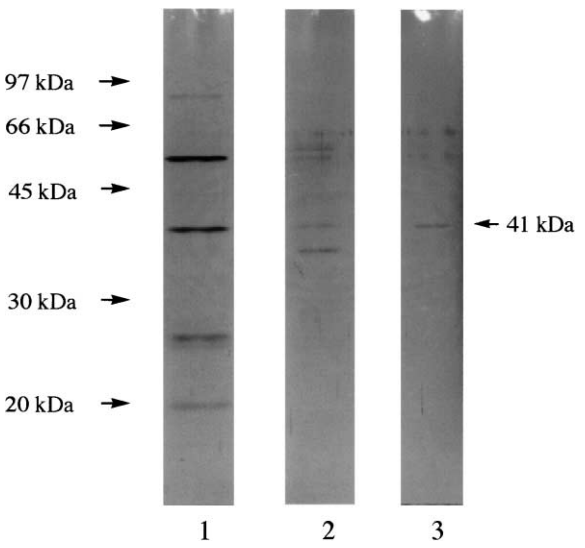


Fig. 2. SDS-PAGE of purified glucosyltransferase from *C. roseus*: lane 1, REACTIVE GREEN 19 affinity; lane 2, Sephadex G-200; lane 3, DEAE Toyopearl.

The molecular mass of glucosyltransferase was estimated by SDS-PAGE to be 41 kDa. The molecular mass of the native glucosyltransferase was estimated by gel filtration through a TSK G3000SW HPLC column to be 41 kDa. These results showed the enzyme to be a monomer protein.

3.2. Properties of glucosyltransferase

The optimum pH of the glucosyltransferase on the enzymatic activity was 8.0. The enzyme was stable below 35 °C, but the activity was found to decrease rapidly over 40 °C.

It was reported that many plant glucosyltransferases require metal ions to enhance the activity [4,11–13]. So the effect of metal ions, such as Mn^{2+}, Mg^{2+} and Ca^{2+} on the enzyme activity with the glucosyltransferase was examined. It was found that divalent metal ions, such as Mg^{2+} and Ca^{2+} slightly inhibited the enzyme activity, while the other divalent metal ions, Mn^{2+}, Co^{2+}, Zn^{2+} and Fe^{2+} strongly inhibited the activity of glucosyltransferase, as shown in Table 2. These results suggested that the glucosyltransferase from *C. roseus* does not require metal ion as cofactor for its activity and is different from many other plant glucosyltransferases.

3.3. Substrate specificity

The substrate specificity of the glucosyltransferase was examined with gentisic acid (**1**), 2-, 3- and 4-hydroxybenzoic acids (**2-4**), hydroxycoumarins (**9-13**) and 2-, 3- and 4-hydroxybenzylalcohols (**19-21**) by incubating with glucosyltransferase (4.8 μg) under the standard reaction conditions. Gentisic acid (**1**) was the best substrate, followed by hydroxycoumarins (Table 3). On the other hand, the salicylic acids (**2-4**) and salicyl alcohols (**19-21**) were not glucosylated by the glucosyltransferase. Interestingly, only 5-hydroxyl group of gentisic acid (**1**) was regioselectively glucosylated to give 5-*O*-glucosyl-2-hydroxybenzoic acid (**5**); 2-*O*-glucosylated form and glucosyl ester form were not detected by HPLC analysis. In addition, only 7-hydroxyl groups of hydroxycoumarins, such as 7-hydroxycoumarin (**9**), 6,7-dihydroxycoumarin (esculetin, **10**) and 6-methoxy-7-hydroxycoumarin (scopoletin, **11**) were glucosylated to give 7-*O*-glucosylated forms, but no 6-*O*-glucosylated form of **10** was found. The glucosylated forms of 3-hydroxycoumarin (**12**) and 4-hydroxycoumarin (**13**) were not detected by HPLC analysis. These results showed that the glucosyltransferase was specific for the glucosylation of 5-hydroxyl group of gentisic acid and 7-hydroxyl group of hydroxycoumarins.

Table 2
Effect of metal ions on the glucosyltransferase activity[a]

Metal	Relative activity (%)
None	100
Mg^{2+}	64
Mn^{2+}	0
Ca^{2+}	36
Co^{2+}	0
Zn^{2+}	0
Fe^{2+}	0

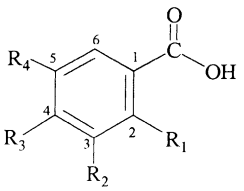
[a] The enzyme reactions were carried out in the standard reaction mixture with 5 mM metal ions.

Table 3
Enzyme activities of the purified glucosyltransferase toward phenolic compounds

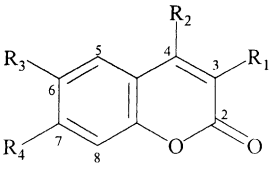
Substrate	Product	Conversion (%)	Relative activity (%)
1	**5**	29.6	100
2	**6**	0	0
3	**7**	0	0
4	**8**	0	0
9	**14**	0.35	1.2
10	**15**	0.13	0.4
11	**16**	0.05	0.2
12	**17**	0	0
12	**17**	0	0
13	**18**	0	0
19	**22**	0	0
20	**23**	0	0
21	**24**	0	0

Table 4
Kinetic parameters in the glucosylation of **1** and **9-11** with the glucosyltransferase from the cultured cells of *C. roseus*

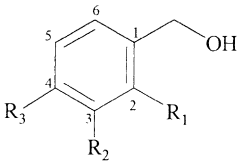
Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($(s\,\mu M)^{-1}$)
1	9	1056	117
9	25	308	12
10	58	185	3
11	20	75	4



1: R_1=R_4=OH, R_2=R_3=H
2: R_1=OH, R_2=R_3=R_4=H
3: R_1=R_3=R_4=H, R_2=OH
4: R_1=R_2=R_4=H, R_3=OH
5: R_1=OH,R_2=R_3=H, R_4=OGlc
6: R_1=OGlc, R_2=R_3=R_4=H
7: R_1=R_3=R_4=H, R_2=OGlc
8: R_1=R_2=R_4=H, R_3=OGlc



9: R_1=R_2=R_3=H, R_4=OH
10: R_1=R_2=H, R_3=R_4=OH
11: R_1=R_2=H, R_3=OCH_3, R_4=OH
12: R_1=OH, R_2=R_3=R_4=H
13: R_1=R_3=R_4=H, R_2=OH
14: R_1=R_2=R_3=H, R_4=OGlc
15: R_1=R_2=H, R_3=OH, R_4=OGlc
16: R_1=R_2=H, R_3=OCH_3, R_4=OGlc
17: R_1=OGlc, R_2=R_3=R_4=H
18: R_1=R_3=R_4=H, R_2=OGlc



19: R_1=OH, R_2=R_3=H
20: R_1=R_3=H, R_2=OH
21: R_1=R_2=H, R_3=OH
22: R_1=OGlc, R_2=R_3=H
23: R_1=R_3=H, R_2=OGlc
24: R_1=R_2=H, R_3=OGlc

Kinetic parameters of glucosyltransferase were determined with substrates as shown in Table 4 from Lineweaver–Burk plots. The K_m value for gentisic acid (**1**) was determined to be 9 μM in the presence of 90 mM UDPG and was lowest among substrates tested (Table 4). The K_m value for UDPG in the reaction with 10 mM gentisic acid (**1**) was 43 μM. On the other hand, turn over (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of the glucosylation reaction of **1** was the highest among substrates examined. These results suggested that the glucosyltransferase was specific for the glucosylation of gentisic acid (**1**).

Thus, 41 kDa glucosyltransferase which catalyzes the glucosylation of gentisic acid (**1**) was isolated from the cultured cells of *C. roseus*. The enzyme exhibited potent glucosyltransferase activity and specifically catalyzed the monoglucosylation of the 5-hydroxyl group of gentisic acid (**1**) with UDPG as glucose donor from the result of kinetic examination. To our knowledge, Edwards reported that a glucosyltransferase isolated from *Nicotiana tabacum* converted salicylic acid (**2**) into its 2-*O*-glucoside [14]. Also, Lee and Raskin reported that the enzyme induced by pathogen in tobacco cells also catalyzed glucosylation of salicylic acid (**2**) to give its 2-*O*-glucoside and glucosyl salicylate [12]. Since salicylic acids (**2-4**) were not directly glucosylated by the 41 kDa glucosyltransferase (Table 3), the 41 kDa glucosyltransferase is clearly different from these reported enzymes with respect to substrate specificity. Though it is well known that gentisic acid (**1**) is biosynthesized from 3-hydroxybenzoic acid (**3**) [15], it was found that *C. roseus* cells hydroxylated the 5-position of salicylic acid (**2**) to give gentisic acid (**1**) and then glucosylated the introduced hydroxyl group [6]. It is interesting to clarify the role of enzymes in the metabolic processes of salicylic acid, but further investigations are necessary.

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