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ISOLATION AND CHARACTERIZATION OF UDP-GLUCOSYLTRANSFERASE IN THE CULTURED CELLS OF *NICOTIANA TABACUM*

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Abstract - A glucosyltransferase, catalyzing the transfer of glucose from UDP-glucose to hydroxycoumarins, was isolated from the cultured cells of *Nicotiana tabacum*. The glucosyltransferase was purified to electrophoretic homogeneity by a procedure involving Sephadex G-25, Agarose-gel affinity, and Sephadex G-200 columns; the enzyme has molecular mass of 61 kDa and pH optimum at 8.0 and the activity was inhibited by divalent metal ions such as Co^{2+} and Zn^{2+}. The enzyme glucosylated preferentially 7-hydroxyl group of hydroxycoumarins.

INTRODUCTION

The ability of glucosyltransferases to convert phenolic compounds into glucosides is important for preparation of pharmacologically useful compounds. A number of studies on biotransformation of the phenolic compounds such as phenols,[1-3] coumarins[4,5] and fravonoids[6,7] by plant cell cultures have been reported. We recently reported regioselective glucosylation of exogenous benzyl alcohols by the cultured cells of *Nicotiana tabacum*.[8,9] On the other hand, it was found that administration of hydroxycoumarins to the cultured cells of *N. tabacum* caused the secretion of intracellular phytoarexin, scopoletin, into the cultured medium and the ladder fragmentation of genomic DNA in the cells, and also that the exogenous hydroxycoumarins were transformed into their corresponding glucosides as a result of detoxification of toxic substrates.[10]

In this paper, we describe the purification and the characterization of a UDP-glucosyltransferase (UDPGTase) participating in the detoxyfication reaction of harmful phenolic compounds in the cultured cells of *N. tabacum*.

EXPERIMENTAL

Materials

Suspension cell cultures of *N. tabacum* were grown at 25 ℃ for 3 weeks in Murashige-Skoog (MS) medium according to the procedure described previously.[9] REACTIVE GREEN 19 Agarose-gel and UDP-glucose were obtained from Sigma Co. Ltd. Sephadex G-25 and Sephadex G-200 were obtained from Pharmacia Co. Ltd.

Isolation and purification of the UDPGTase from *N. tabacum*

The cultured cells (110 g) of *N. tabacum* were pulverized in liquid nitrogen using a mortar and pestle, and resuspended in 50 mL of 50 mM *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid (HEPES) buffer (pH 7.5) containing 5 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 15% glycerol (the standard buffer). The tissue slurry was removed by centrifugation (18,000×g, for 20 min at 4 ℃) and the supernatant was desalted by Sephadex G-25 column. The active fractions were layered onto a REACTIVE GREEN 19 Agarose-gel affinity column. After washing the column with the standard buffer, the active fractions were eluted from the column with the standard buffer containing 4 mM UDP-glucose and were concentrated with a CENTRICUT U-20. Active fractions from the affinity column were applied to the Sephadex G-200 column at a flow rate of 0.1 mL/min with the standard buffer. The enzyme fractions from each purification steps were analyzed by SDS-PAGE.[11] Protein bands were visualized with silver staining procedure. Protein concentration was quantified by protein assay (Bio-Rad) using bovine serum albumin (BSA) as standard.

Products identification

The glucosylcoumarins were analyzed and isolated using HPLC on Waters Puresil C18 column under the following conditions: flow rate 0.5 mL/min of 25% methanol solution; monitor by UV adsorption at 320 nm. The isolated glucosides (**6**-**9**) were identified by direct comparison of HPLC and the spectral data of NMR (JEOL GSX500) and FAB-MS (JEOL SX102A) analyses with authentic samples.[10]

Assay of UDPGTase activity

The standard reaction mixture (1 mL) was consisted of substrate (10 μmol), UDP-glucose (20 μmol), and enzyme preparations in the standard buffer. After gentle shaking in the dark at 37 ℃ 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 above. The UDPGTase 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.[10] One unit of enzyme activity was defined as the amount of the enzyme that catalyzes the formation of 1 μmol of glucoside per min.

UDPGTase 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 the standard buffer. Effect of metal ions was examined in the enzyme reaction under the presence of 5 mM metal ions in the standard buffer without EDTA. pH stability was determined by use of the enzyme preparations stored in the standard buffer at various pH from 6.0 to 9.0 for 6 h at 4 ℃. Thermal stability was determined by use of the enzyme preparations treated at various temperature from 20 ℃ to 60 ℃ for 2 h.

RESULT AND DISCUSSION

A glucosyltransferase was extracted as soluble enzyme with HEPES-NaOH buffer from the cultured cells of *N. tabacum*. The purification step developed is shown in Table 1. The crude enzyme fraction was layered onto a REACTIVE GREEN 19 Agarose-gel column in order to eliminate many of the UDPsugar-non-dependent enzymes.[12] A further purification was achieved on the Sephadex G-200 gel filtration column. This final step provided a 33-fold increase in the purification of the UDPGTase. After the final purification step, UDPGTase appeared as a dominant single protein band in its SDS-PAGE analysis. The molecular mass of UDPGTase was estimated to be 61 kDa by the SDS-PAGE. The native form of the purified UDPGTase was estimated to be 60 kDa based on gel filtration of Sephadex G-200 column. These results suggested native UDPGTase to be a monomer structure. The optimum pH of the

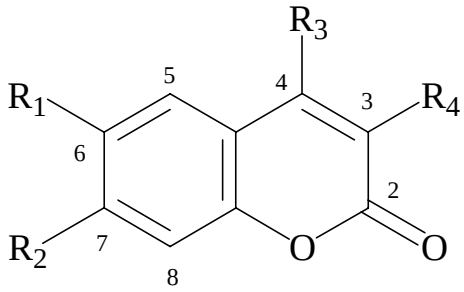
Table. 1 Purification of UDPGtase from the cultured cells of *N. tabacum*

Steps	Total protein [a] (mg)	Total activity [b] ($x10^{-3}$U)	Specific activity ($x10^{-3}$U/mg)	Purification (Fold)
Crude extract	310	30	0.1	1
REACTIVE GREEN 19	13	21	1.7	17
Sephadex G-200	0.8	2.6	3.3	33

[a] Total protein was estimated by protein assay (Bio-Rad) with BSA as a standard.

[b] One unit was defined as the formation of 1 μmol / min of 7-O-glucosylcoumarin from 7-hydroxycoumarin.

UDPGTase on the enzyme activity was 8.0. The enzyme was stable at below 35 ℃, but the activity was found to decrease rapidly at over 40 ℃.



1: R_1=R_3=R_4=H, R_2=OH
2: R_1=OCH_3, R_2=OH, R_3=R_4=H
3: R_1=R_2=OH, R_3=R_4=H
4: R_1=R_2=R_4=H, R_3=OH
5: R_1=R_2=R_3=H, R_4=OH
6: R_1=R_3=R_4=H, R_2=OGlc
7: R_1=OCH3, R_2=OGlc, R_3=R_4=H
8: R_1=OH, R_2=OGlc, R_3=R_4=H
9: R_1=R_2=R_4=H, R_3=OGlc

The substrate specificity of the UDPGTase was examined with hydroxycoumarins and salicyl alcohols. 7-Hydroxycoumarin (**1**) was the best substrate, producing 7-*O*-β-D-glucopyranosyl-coumarin (**6**).[10] The conversion of scopoletin (**2**) into scopolin (**7**)[10] was slightly lower than that of **1**, and the conversion of 6,7-dihydroxycoumarin (**3**) and 4-hydroxycoumarin (**4**) into 7-*O*-β-D-glucopyranosyl-6-hydroxycoumarin (**8**)[10] and 4-*O*-β-D-glucopyranosylcoumarin (**9**),[10] respectively, resulted in low yield. On the other hand, 3-hydroxycoumarin (**5**) was not glucosylated with the UDPGTase (Table 2). Interestingly, only 7-hydroxy group of 6,7-dihydroxycoumarin (**3**) was glucosylated to give 7-*O*-β-D-glucopyranosyl-6-hydroxycoumarin (**8**); 6-*O*-glucosylated and 6,7-diglucosylated forms were not detected in spite of careful analysis with HPLC. The orientations of the glucosidic linkage of all the products (**6**-**9**) were β, defined by the coupling constant for H-1' in their ^{1}H NMR spectra. When the *o*-, *m*- and *p*-hydroxybenzyl alcohols were used as substrates, no glucosides were obtained. These observations showed that the UDPGTase is specific for the glucosylation of 7-hydroxy group of coumarins.

Table. 2 Enzyme activities of the purified UDPGTase toward various hydroxycoumarins

Substrates	Products	Conversion(%)	Relative activity
1	**6**	7.6	100
2	**7**	5.6	73
3	**8**	1.3	17
4	**9**	0.7	9
5	——	0	0

Kinetic parameters of UDPGTase were determined from Lineweaver-Burk plots. The Km value for 7-

Table 3. Kinetic parameters in the glucosylation reaction with the UDPGTase from the cultured cells of *N. tabacum*[a]

Substrates	Km (μM)	kcat (s^{-1})	kcat / Km ($\mu M^{-1}s^{-1}$)
1	17	93	5.5
2	47	82	1.8
3	30	116	3.9
4	18	19	1.1

[a] The kinetic parameters were measured with 90 mM UDPG and 10 mM hydroxycoumarins.

hydroxycoumarin (**1**) was determined to be 17 μM in the reaction with a fixed concentration of 90 mM UDP-glucose, and those of the other hydroxycoumarins were also shown in Table 3. The Km value for UDP-glucose in the reaction with 10 mM 7-hydroxycoumarin (**1**) was 70 μM. On the other hand, turn over (kcat) of the glucosylation reaction of **1** was highest among hydroxycoumarins examined. The catalytic efficiency (kcat/Km) for glucosylation of hydroxycoumarins was highest for the glucosylation of **1** among the hydroxycoumarins. These results confirmed that the UDPGTase is specific for the 7-hydroxyl group of hydroxycoumarins.

It was reported that many plant UDP-glucosyltransferases require metal ions for maximal activity.[5,13-15] So, the effect of metal ions on the UDPGTase activity was examined. It was found that divalent metal ions, such as Mg^{2+} and Ca^{2+}, at 1 mM concentration did not affect on the enzyme activity, while Co^{2+} and Zn^{2+} strongly inhibited the activity of UDPGTase, as shown in Table 4. These results suggested that the UDPGTase does not require metal ion cofactor for activation.

Thus, 61 kDa UDPGTase which catalyzes the glucosylation of hydroxycoumarins was isolated from the cultured cells of *N. tabacum*. The UDPGTase specifically catalyzed the monoglucosylation of 7-hydroxyl group of coumarins in the presence of UDP-glucose as the glucosyl donor. To our knowledge, several glucosyltransferases have been isolated

Table. 4 Effect of metals ion on the UDPGTase activity [a]

Metal ion	Relative activity (%)
Control	100
K^{+}	91
Na^{+}	88
Mg^{2+}	100
Mn^{2+}	58
Ca^{2+}	100
Co^{2+}	6
Zn^{2+}	3
Fe^{2+}	62

[a] The enzyme reactions were carried out in the standard reaction mixture with 5 mM metal ions in the standard buffer solution without EDTA.

from *N. tabacum*; a 49 kDa glucosyltransferase isolated by Taguchi et al. catalyzed glucosylation of a broad range of substrates and required metal ions as cofactor.[15] The 61 kDa UDPGTase was different from the reported 49 kDa glucosyltransferase with respect to the molecular mass, substrate specificity, and requirement of metal ions. In our previous report, it was reported that 7-hyroxycoumarin is high cytotoxic in the cells of *N. tabacum*, but is detoxified by its glucosylation. Although the UDPGTase isolated may concern with the detoxification of toxic phenolics, further investigations will be necessary to complete.

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REFERENCES

1. Y. Orihara, T. Furuya, N. Hashimoto, and Y. deguchi, *Phytochemistry*, 1992, **31**, 827.
2. H. Mizukami, T. Terao, A. Amano, and H. Ohashi, *Plant Cell Physiol.*, 1986, **27**, 645.
3. C. Langebartels and H. Harms, *Z. Pflanzenphysiol*., 1984, **113**, 201.
4. T. Tabata, Y. Umetani, M. Ooya, and S. Tanaka, *Phytochemistry*, 1988, **27**, 809.
5. M. Kalinowska, *Phytochemistry*, 1994, **36**, 617.
6. G. A. Barber, *Biochemistry*, 1962, **1**, 463.
7. R. L. Durren and C. A. Mcintosh, *Phytochemistry*, 1999, **52**, 793.
8. Y. S. Lee, D. I. Ito, K. Koya, S. Izumi, and T. Hirata, *Chem. Lett*., **1996**, 719.
9. T. Hirata, K. Koya, K. J. Sarfo, K. Shimoda, D. I. Ito, S. Izumi, S. Ohta, and Y. S. Lee, *J. Mol Cat. **B**: Enzymatic*, 1999, **6**, 67.
10. T. Hirata, K. Shimoda, T. Fujino, and S. Ohta, *J. Mol Cat. **B**: Enzymatic*, 2000, **10**, 477.
11. U. K. Laemmie, *Nature*, 1970, **227**, 680.
12. M. Bar-Peled, E. Lewinsohn, R. Fruhr, and J. Gressel, *J. Biol. Chem*., 1991, **266**, 20953.
13. S. Hasegawa, C. G. Suhayda, W. Hsu, and G. H. Robertson, *Phytochemistry*, 1997, **46**, 33.
14. H. Lee and I. Raskin, *J. Biol. Chem*., 1999, **274**, 36637.
15. G. Taguchi, H. Imura, Y. Maeda, R. Kodaira, N. Hayashida, M. Shimosaka, and M. Okasaki, *Plant Science*, 2000, **157**, 105.