Title:

Purification and characterization of UDP-glucose: hydroxycoumarin

7-*O*-glucosyltransferase, with broad substrate specificity from

tobacco cultured cells

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Abstract

The enzyme UDP-glucose: hydroxycoumarin 7-O-glucosyltransferase (CGTase), which catalyzes the formation of scopolin from scopoletin, was purified approximately 1,200-fold from a culture of 2,4-D-treated tobacco cells (*Nicotiana tabacum* L. cv. Bright Yellow T-13) with a yield of 7%. Purification to apparent homogeneity, as judged by SDS-PAGE, was achieved by sequential anion-exchange chromatography, hydroxyapatite chromatography, gel filtration, a second round of anion-exchange chromatography, and affinity chromatography on UDP-glucuronic acid agarose. The purified enzyme had a pH optimum of 7.5, an isoelectric point (pI) of 5.0, and a molecular mass of 49 kDa. The enzyme did not require metal cofactors for activity. Its activity was inhibited by Zn^{2+}, Co^{2+} and Cu^{2+} ions, as well as by SH-blocking reagents. The Km values for UDP-glucose, scopoletin and esculetin were 43 μM, 150 μM and 25 μM, respectively. A study of the initial rate of the reaction suggested that the reaction proceeded via a sequential mechanism. The purified enzyme preferred hydroxycoumarins as substrates but also exhibited significant activity with flavonoids. A database search using the amino terminus amino acid sequence of CGTase revealed strong homology to the amino acid sequences of other glucosyltransferases in plants.

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1. Introduction

In higher plants, secondary metabolites are often converted to their

glycoconjugates, which are then accumulated and compartmentalized in

vacuoles. Such glucosylation reactions are catalyzed by glucosyltransferases

(GTases). Several roles have been postulated for the glucosylation of various

low-molecular -mass compounds in plants, as follows: 1) Glucosylation

allows solubilization of the compounds in water [1]; 2) it acts to detoxify

harmful metabolites or environmental compounds, such as herbicides [2];

and 3) it can regulate the action of functional compounds, such as the plant

hormones, IAA and cytokinins [3,4]. Although various GTases have been

purified from a large number of plant species, the enzymes have been

purified to homogeneity in only a few cases [1,3,5-7]. By contrast, the

cloning of genes for plant GTases has been quite successful. The first such

gene was isolated from maize in an analysis of the *bronze* locus that encodes

UDP- glucose: flavonol glucosyltransferase (UFGT). Subsequently, related

genes for enzymes that mainly catalyzed the glucosylation of flavonols in the biosynthesis of anthocyanins were cloned from many plant species using the gene for UFGT from maize as probe (for review, [8]). Other genes for GTase-related enzymes that catalyze the glucosylation of a variety of low-molecular-mass compounds have been isolated from plants: the gene for an IAA glucosyltransferase from maize [3]; the gene for a solanidine glucosyltransferase from potato [9]; and some genes for GTase-homologous enzymes that are expressed during the ripening of fruit [10], in response to wounding or in response to treatment with salicylic acid [11]. Much further work is needed to clarify the relationships between the structures and functions of GTases and to understand their roles in plants.

Coumarins are common secondary metabolites that have been found in many botanical families [12]. They are reported to function in the protection of plants, having, for example, antimicrobial activity, the ability to deter feeding by insects, an autoinhibitory effect on germination, and a shielding effect against ultraviolet irradiation. Most coumarins in higher plants exist as glucoconjugates, and appear to be accumulated in vacuoles [12-14].

Scopolin, a 7-O-glucoconjugate of scopoletin, which is a coumarin derivative, is accumulated in tobacco cultured cells (strain T-13). The conversion of scopoletin to scopolin is catalyzed by a scopoletin glucosyltransferase (SGTase). The level of scopolin in cells increases with the activation of SGTase when T-13 cells are treated with 2,4-D [15]. In this report, the purification and characterization of a UDP-glucose:

hydroxycoumarin 7-*O*-glucosyltransferase (CGTase) that converts scopoletin to scopolin from tobacco cells that have been treated with 2,4-D is described. The substrate specificity of the purified enzyme differs from that of the previously described SGTase.

2. Materials and methods

2.1 *Culture of plant cells.*

  Cells of T-13 habituated callus of *Nicotiana tabacum* L. cv. Bright Yellow [15] were used in this study. They were maintained on hormone-free MS-agar medium as described previously [14]. At the late logarithmic phase of cell growth, 2,4-D was added to a final concentration of 1 mg liter^{-1}, and cells were incubated for an additional 24 hours. They were collected by vacuum aspiration on Miracloth™ (Calbiochem) and stored at -80 ^{o}C prior to use.

2.2 *Extraction and purification of CGTase*

    Frozen cells (1,000 g fresh weight) were extracted with 1.5 liter of buffer A (50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol) that contained 0.5 mM PMSF and 3% (w/v) polyvinylpolypyrrolidone. All subsequent operations were carried out at 4 ^{o}C unless otherwise stated. The homogenate was filtered through two layers of cotton cloth and centrifuged for 10 min at 5,000 x g to remove all cell debris. The supernatant was loaded onto a column of DEAE-cellulose (7 cm i.d. x 10 cm; Wako Pure

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Chemical Industries) equilibrated with buffer A. The column was washed with 150 ml of buffer A, then the absorbed protein was eluted with 300 ml of buffer A that contained 0.3 M NaCl. The eluent was dialyzed against buffer B (20 mM sodium phosphate, pH 7.5, 5 mM 2-mercaptoethanol), then loaded onto a hydroxyapatite column (2.1 cm i.d. x 12 cm; Macro-Prep Ceramic Hydroxyapatite type I, 40 µm; Bio-Rad) equilibrated with buffer B. After washing with 100 ml of buffer B, the column was eluted with a linear gradient of sodium phosphate buffer (500 ml, 20 to 200 mM sodium phosphate buffer, pH 7.5) at a flow rate of 40 ml h^{-1}. Fractions with strong GTase activity were fractionated by the addition of solid ammonium sulfate. The precipitate at 35% to 65% saturation was dissolved in a minimal volume of buffer A, then was applied to a Sephadex G-100 column (2.1 cm i.d. x 55 cm; Pharmacia) equilibrated with buffer A. The column was eluted with 150 ml of buffer A at a flow rate of 10 ml h^{-1}, and the fractions with enzymatic activity were are loaded onto a DEAE-Sepharose CL-6B column (1.7 cm i.d. x 10 cm; Pharmacia) equilibrated with buffer A. The column was washed with 50 ml of buffer A and was eluted with a linear NaCl gradient (300 ml, 0 to 200 mM NaCl in buffer A). Active fractions were pooled, concentrated, and desalted by ultrafiltration with Centricon-10 (Amicon).

The enzyme preparation was applied to a UDP-glucuronic acid agarose column (2 ml; Sigma) equilibrated with buffer A. The column was washed with 7 ml of buffer A and then adsorbed proteins were eluted with 5 ml of

buffer A that contained 2 mM UDP-glucose. Active fractions were pooled

and stored at 4 ^{o}C until use.

2.3 *Enzyme activity assay and determination of kinetic parameters.*

GTase activity, with scopoletin as the substrate was assayed as described

previously [15] for identification of active fractions after column

chromatography. For kinetic studies, HPLC was used for the detection of

scopolin as product to achieve greater sensitivity [14]. The standard

reaction mixture (200 µl) contained the enzyme preparation (50-100 ng

protein), 200 µM scopoletin, 200 µM UDP-glucose, 50 mM Tris-HCl (pH 7.5),

5 mM 2-mercaptoethanol, and 0.01% (w/v) bovine serum albumin. Each

reaction was initiated by addition of the enzyme, and incubated at 30 ^{o}C for

60 min. Reactions were terminated by adding 20 µl of 20% (w/v)

trichloroacetic acid. Excess scopoletin was removed by chloroform

extraction. The upper layer was saved and subjected to analysis by HPLC

using 4-methylumbelliferone as the internal standard. Under the standard

conditions, the rate of the reaction was constant for up to 90 min.

For determinations of Km values, the concentrations of scopoletin and

UDP-glucose were varied between 50 and 500 µM and between 10 and 250

µM, respectively. For other compounds, the concentration of UDP-glucose

was fixed at 500 µM. The Km values were calculated as described [16]. The

molecular mass of CGTase was estimated by SDS-PAGE on a 10%

polyacrylamide gel. The isoelectric point was determined by electrofocusing

on an acrylamide gel with Pharmalyte (pH range: 4-6.5; Pharmacia) as the polybuffer. For determination of the optimum temperature, the effect of the temperature on incubation of the reaction mixture was examined between 4 and 70 ºC, after 30 min of incubation. For checking the effect of sulfhydryl reagents, enzyme was mixed with reaction buffer with or without sulfhydryl-blocking reagents, p-mercuribenzoic acid (PCMB) and $HgCl_2$ (final 1 mM). Then the substrate mixture with or without 2-mercaptethanol (10 mM) were added to the enzyme for starting the reaction. Protein was quantitated by Protein Assay (Bio-Rad) with bovine serum albumin as the standard.

2.4 *Substrate specificity of CGTase*

Each reaction mixture (10 µl) contained CGTase (0.1 pkat) purified by affinity chromatography, 0.5 mM UDP- [U-^{14}C] glucose (740 MBq mmole^{-1}; American Radiolabeled Chemicals), 0.5 mM substrate, 0.01% BSA, 5 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.5). After incubation for 90 min, an aliquot was spotted on a silica-gel plate (Kieselguhr 60 F254; Merck), and the plate developed with a mixture of ethyl acetate, methanol and water (20:4:3, v/v) that had been acidified with one drop of acetic acid. The plate was exposed to an imaging plate (Fuji Film Co.) for autoradiography. The intensity of each radioactive spot was estimated with a Storm 860 system (Molecular Dynamics).

2.5 *Amino acid sequence of CGTase*

The band of purified CGTase, after SDS-PAGE on a 10% polyacrylamide gel, was blotted onto a PVDF membrane (Bio-Rad) using a semi-dry protein blotter (Sartorius). The amino-terminal amino acid sequence was determined with a peptide sequencer (model 477A; Perkin-Elmer).

2.6 *Reagents*

Glucosides used as standards were kindly donated by Dr. S. Tanaka (Tokyo University of Agriculture) and Dr K. Yoshitama (Kumamoto University). All other chemicals and solvents were obtained from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

3. Results and discussion

3.1 *Purification of the CGTase*

In general, GTases that use secondary metabolite as substrates are minor constituent proteins in plant cells [17]. In this study, we purified a GTase from tobacco cells (fresh weight, 1,000 grams) that had been cultured for 24 hours after addition of 2,4-D to the medium, since, as shown previously, 2,4-D increases the activity that glucosylates scopoletin 10-fold in cultured tobacco cells [15]. After chromatography on hydroxyapatite, the major peak of activity was collected. The enzyme was further purified by gel filtration, by a second round of anion-exchange chromatography and by affinity chromatography. SDS-PAGE revealed that the protein had been

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purified to apparent homogeneity (Fig. 1). The purification resulted in an approximately 1,200-fold purification of the protein with 7% recovery (Table 1). Among the various purification steps, affinity chromatography on the UDP-glucuronic acid agarose was particularly effective. The specific activity of the enzyme was increased approximately 30-fold during this step. There was some activity that was not adsorbed to the column, we used the adsorbed enzyme for further experiments. It seems very likely that GTases other than the purified enzyme are able to catalyze the glucosylation of scopoletin. We also tested other affinity resins, such as UDP-hexanolamine agarose (Sigma), UDP-agarose (Sigma), Affigel Blue (Pharmacia) and Hg-UDP-glucose (synthesized as described [18] using UDP-glucose as the substrate) coupled with SH-Sepharose 4B (Pharmacia). These are not effective on this activity. In the purification, we also detected a minor peak of activity after chromatography on hydroxyapatite (data not shown). It corresponded to less than 10% of the total activity. Further investigations are required to clarify the existence and activities of related enzymes.

3.2 *Physical properties*

The enzyme eluted from the column of Sephadex G-100 in an elution volume that corresponded to that of proteins with a molecular mass of 47 to 50 kDa. SDS-PAGE also indicated that the CGTase had a molecular mass of 49 kDa, suggesting that the enzyme was a monomer. Isoelectric focusing indicated that the CGTase had a pI of 5.0. Other plant GTases associated

with secondary metabolism also have molecular masses of 40-62 kDa [17], consist of a single subunit or are dimers of subunits of 20-32 kDa, and have a pI near 5.0 [5, 7, 19-21]. Thus, the physical characteristics of the CGTase were similar to those of other plant GTases.

3.3 *Substrate specificity*

We examined the substrate specificity of the purified CGTase using various phenolic compounds as glucose acceptors (Table 2). Strong glucosylation activity was detected with hydroxycoumarins, namely, scopoletin (a putative major substrate in this tobacco cells), esculetin, umbelliferone and 4-methyl umbelliferone. The Km values for UDP-glucose, scopoletin, and esculetin were 43 μM, 150 μM, and 25 μM, respectively. Strong activity was also found with flavonols, kaempferol and quercetin, and a flavone, baicalein (Table 2). Glucosyltransferase activity was also detected with other phenylpropanoid compounds, isoflavone and hydroxycinnamates as substrates, but negligible activity was detected when hydroxybenzoates were tested as substrates. These reactions were performed using homogeneously purified CGTase. Thus, the enzyme had a broad substrate specificity and reacted with several kinds of phenylpropanoids. In this tobacco cells, scopoletin and scopolin are the major phenolic compound, and none of esculetin or its glucoside were found. Some of hydroxycinnamates or flavonols were found in T-13 cells, though the contents were less than scopolin. It could be postulated that this enzyme

would work as a multiple phenylpropanoid glucosylation enzyme, especially scopoletin, in tobacco cells. Although the physiological roll of this enzyme is unclear, it may be ascribed to changing the storage site of certain phenolic compounds for further utilization or for avoidance of their toxic effect. In *Stevia*, two steviol GTases react with numerous natural substrates, steviols and steviosides, but have much stronger affinity for flavonols [7]. In livingstone daisy, betanidin GTases also react with the several hydroxygroups of flavonols and anthocyanidins [22]. It is likely that individual plant GTases are able to catalyze multiple reactions in the metabolism of phenolic compounds. From its substrate specificity, the purified CGTase seems to be different from two other GTases previously characterized from tobacco, namely, a scopoletin glucosyltransferase (SGTase; [15]) and a dihydrocoumarin glucosyltransferase [23], since neither of these two enzymes was able to utilize flavonols as acceptors of the sugar moiety. Furthermore, SGTase was reported to show highest activity with scopoletin, and less activity for esculetin [15]. The reported Km values of dihydrocoumarin glucosyltransferase for esculetin and scopoletin were 95 µM and 1.43 mM, i.e. much higher than the enzyme that we reported here. However, all three enzymes might have overlapping roles and might all function in converting scopoletin to scopolin in tobacco cells.

3.4 *Other properties*

The CGTase was activated by sulfhydryl compounds, such as 10 mM of 2-mercaptoethanol (141 % of the activity of control). SH-blocking reagents, namely, $HgCl_2$ and 4-chloromercuribenzoic acid (PCMB), inhibited the activity dramatically at the concentration of 1 mM (0 and 1 % of the activity of the control, respectively). This inhibition was reversed by the addition of 10 mM of 2-mercaptoethanol (49 %for $HgCl_2$, and 26 % for PCMB), suggesting that the purified CGTase had an SH-group at or near the catalytic center.

We also examined the effects of metal ions on the CGTase activity. At 1 mM, Mn^{2+}, Ca^{2+} and Mg^{2+} each slightly enhanced the activity of the CGTase (133, 114, and 112 % of the activity of the control, respectively), while the activity was inhibited by Zn^{2+}, Co^{2+} and Cu^{2+} (4, 13, and 45 % of the activity of the control, respectively) at the same concentration. The pH optimum for the reaction, examined in Tris-HCl (pH 7.0 to 9.0) and sodium phosphate buffer (pH 5.5 to 8.0), was 7.5. The optimum temperature was 45 $^{\circ}$C in Tris-HCl buffer (pH 7.5). In this respect, the enzyme was similar to other plant GTases that are involved in the metabolism of secondary products [19, 20]. Studies of initial rates of reaction, using double-reciprocal plots for the two-substrate reaction for scopoletin, showed that the reaction fit the criteria for a sequential bireactant mechanism rather than a ping-pong bireactant mechanism, that the slope of the plots were decreased when the concentration of scopoletin was increased (Fig. 2) [16]. It was reported in several cases the GTase for phenylpropanoid are reacted with ordered bi-bi

mechanisms, where UDP-glucose was the first substrate to bind to the enzyme [19, 24]. When the reaction of CGTase was inhibited by UDP, the result was no conflict with an ordered bi-bi mechanism (data not shown). Though the inhibition of the reaction with scopolin was not possible in this assay, it was unclear, the reaction of CGTase may fit for the criteria for an ordered bireactant mechanism as like as the reported enzymes.

3.5 *Amino-terminal amino acid sequence of the purified CGTase*

The amino acid sequence of the amino terminus of the purified enzyme was determined with an automated protein sequencer. The amino terminus of the enzyme was not blocked, and 27 amino-terminal amino acids were identified (Fig. 3A). A homology search of databases using the NCBI "blastp" program revealed that the putative amino acid sequences of some plant cDNAs showed a significant similarity to the amino-terminal sequence of the CGTase (Fig. 3B). The products of these cDNAs are considered to be GTases since all of them possess the consensus sequence that is deduced to be the UDP-binding site of GTases at their carboxyl termini [25].

A large number of GTases from mammalian sources have been reported and analyzed in terms of their structure and function [26]. By contrast, plant GTases have not been examined in detail. In tobacco, several GTase-like enzymes that are involved in secondary metabolism [15, 23, 27], inducible by salicylic acid (IS5a and IS10a, [11], or inducible by jasmonate (JIGT,

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[28]) have been reported so far. However, the physiological roles of these GTases in tobacco remain to be clarified. In this report, we purified and characterized a CGTase, which is different from SGTase. CGTase is very similar in the N-terminus sequence to the product of IS5a and IS10a, but it may differ from them because they prefer flavonols as the substrates when they are expressed in *E. coli*, mammalian, and plant cells. (D. Horvath, personal communication). It was also reported that the products of homologues genes glucosylate several kinds of phenylpropanoids, but flavonoids were not considered as possible substrates [29]. Scopoletin is known as one of the phytoalexins that can be induced by fungal infection or by treatment with plant hormones in tobacco. In tobacco cells, scopoletin usually accumulates as its glucoconjugate form, scopolin [14, 15, 30]. The purification of the present CGTase should help in future to shed light on the process of formation and the accumulation of scopolin and /or other phenylpropanoids in tobacco cells.

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Table 1. Purification of CGTase from tobacco cells.

purification step	protein	total activity	specific activity	purity	recovery
	mg	nkat	pkat / mg protein	-fold	%
Crude enzyme	1106	1.60	1.45	1	100
DEAE-cellulose	599.2	1.84	3.07	2.1	115
Hydroxyapatite	89.8	0.710	7.91	5.5	44.4
Sephadex G-100	20.6	0.472	22.9	15.9	29.5
DEAE-Sepharose	4.6	0.285	62.0	43.1	17.8
Affinity column	0.067	0.113	1690	1176	7.1

Enzyme purification was carried out as described in " Materials and Methods".

Table 2 Substrate specificity of CGTase, as determined with various phenolic compounds as possible acceptors of glucose.

Substrate	Relative activity [a]
hydroxycoumarins	
scopoletin	100
esculetin	363
4-methylumbelliferone	135
umbelliferone	60
4-coumaric acid	50
hydroxycinnamates	
caffeic acid	44
ferulic acid	18
hydroxybenzoates	
4-hydroxybenzoic acid	9
salicylic acid	5
flavonoids	
kaempferol	294
quercetin	77
baicalein	240
formononetin	43

Reactions were carried out as described in "Materials and Methods" with UDP-[^{14}C-U] glucose as the donor of glucose.

[a] Percent of the activity observed with scopoletin as the substrate.

Legends for figures

Figure 1. Analysis by SDS-PAGE of each step in the purification of CGTase. A) Five micrograms of protein from each fraction, namely, the crude extract (lane 1); fractions after chromatography on DEAE-cellulose (lane 2), hydroxyapatite (lane 3) and Sephadex G-100 (lane 4); after ammonium sulfate precipitation (lane 5); and after chromatography on DEAE-Sepharose CL-6B (lane 6), were fractionated and stained with coomassie brilliant blue R-250. In the case of the active fraction after affinity chromatography on UDP-glucuronic acid agarose (lane 7), only 0.5 μg of protein was subjected to SDS-PAGE. The molecular mass of the purified CGTase is indicated. Standard proteins were loaded in lane M. B) The active fraction after UDP-glucuronic acid agarose (0.5 μg) was fractionated and stained with silver.

Figure 2. Double-reciprocal plots of initial rates of reactions catalyzed by the purified CGTase. Analysis was performed as described by Cleland (1963).

Figure 3. The amino-terminal amino acid sequence of CGTase. A) The amino-terminal amino acid sequence of the CGTase. B) Comparison of amino acid sequences deduced from nucleotide sequences of four genes for GTases with the amino acid sequence determined for the CGTase: tobacco

salicylic acid-inducible glucosyltransferases (IS5a and IS10a; Gene Bank

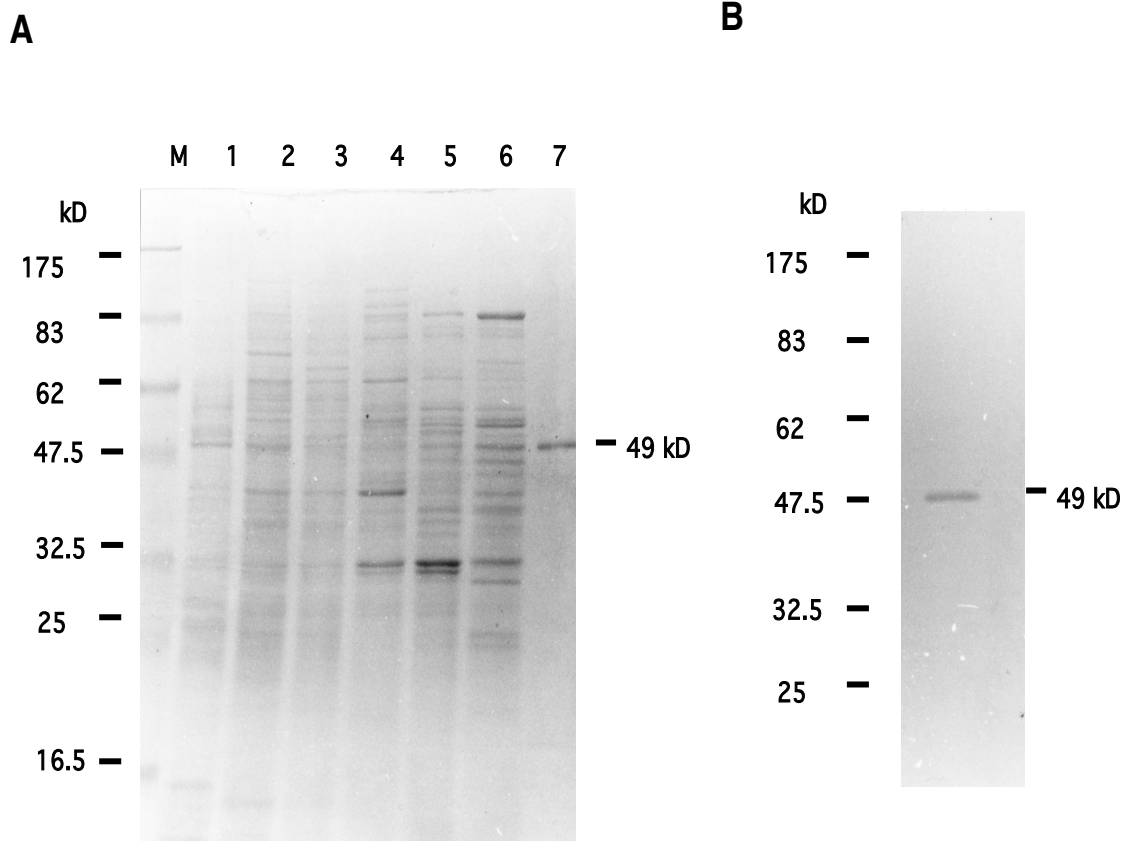accession no.U32643 and U32644); *Scutellaria* flavonoid

7-O-glucosyltransferase (SB7GT; AB031274) tomato wounding-inducible

gene (twi1; X85138); *Dorotheanthus* betanidin-5-O-glucosyltransferase

(DB5GT; Y18871); *Vigna* flavonoid glucosyltransferase (VMFGT;

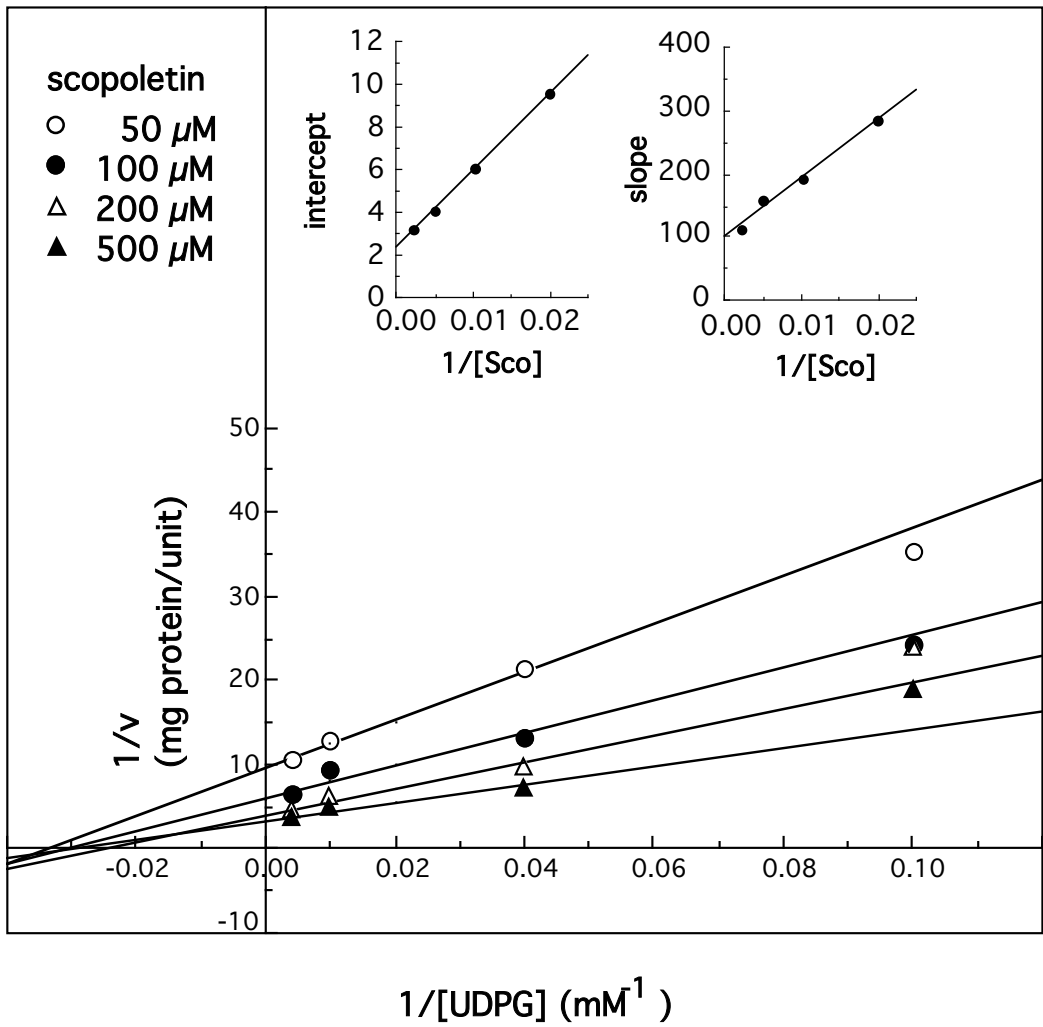AB012114); and potato solanidine glucosyltransferase (SolGT; U82367).

Numbers indicate positions relative to the putative initial methionine (as

deduced from the four genes) or the amino terminus (CGTase). Shadowed

residues at specific positions are identical. The analysis was performed with

Genetyx ver. 8.0 (Software Development Co., Tokyo).

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A

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N'-    Glu-Gln-Leu-His-Ile-Phe-Phe-Phe-Pro-Val-   10
       (Gly)

       Met-Ala-His-Gly-His-Met-Ile-Pro-Thr-Leu-   20


       Asp-XXX-Ala-Lys-Leu-Ile-Ala-                27
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B

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CGTase   1   EQLHIFFFPVMAHGHMIPTLDXAKLIA--   27
IS5a     2   GQLHIFFFPVMAHGHMIPTLDMAKLFASR   30
IS10a    2   GQLHFFFFPVMAHGHMIPTLDMAKLVASR   30
SBGT     2   GQLHIVLVPMIAHGHMIPMLDMAKLFSSR   30
twi1     1   ---HFFFFPDDAQGHMIPTLDMANVVACR   26
DB5GT    8   PDLHVVFFPFLAHGHMIPSLDIAKLFAAR   36
VMFGT    3   TPLKIYFLPFFAQGHQIPMVQLARLIASR   31
SolGT    9   EILHVLFLPFLSAGHFIPLVNAARLFASR   37
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Figure 3. The amino-terminal amino acid sequence of CGTase.

A) The amino-terminal amino acid sequence of the CGTase. B) Comparison of amino acid sequences deduced from nucleotide sequences of four genes for GTases with the amino acid sequence determined for the CGTase: tobacco salicylic acid-inducible glucosyltransferases (IS5a and IS10a; Gene Bank accession no.U32643 and U32644); *Scutellaria* flavonoid 7-O-glucosyltransferase (SB7GT; AB031274) tomato wounding-inducible gene (twi1; X85138); *Dorotheanthus* betanidin-5-O-glucosyltransferase (DB5GT; Y18871); *Vigna* flavonoid glucosyltransferase (VMFGT; AB012114); and potato solanidine glucosyltransferase (SolGT; U82367). Numbers indicate positions relative to the putative initial methionine (as deduced from the four genes) or the amino terminus (CGTase). Shadowed residues at specific positions are identical. The analysis was performed with Genetyx ver. 8.0 (Software Development Co., Tokyo).