

# Glucose Polyester Biosynthesis. Purification and Characterization of a Glucose Acyltransferase<sup>1</sup>

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Glandular trichomes of the wild tomato species *Lycopersicon pennellii* secrete 2,3,4-*O*-tri-acyl-glucose (-Glc), which contributes to insect resistance. A Glc acyltransferase catalyzes the formation of diacyl-Glc by disproportionating two equivalents of 1-*O*-acyl- $\beta$ -Glc, a high-energy molecule formed by a UDP-Glc dependent reaction. The acyltransferase was purified 4,900-fold from *L. pennellii* leaves by polyethylene glycol fractionation, diethylaminoethyl chromatography, concanavalin A affinity chromatography, and chromatofocusing. The acyltransferase possesses an isoelectric point of 4.8, a relative molecular mass around 110 kD, and is composed of 34- and 24-kD polypeptides as a heterotetramer. The 34- and 24-kD proteins were partially sequenced. The purified enzyme catalyzes both the disproportionation of 1-*O*-acyl- $\beta$ -Glcs to generate 1,2-di-*O*-acyl- $\beta$ -Glc and anomeric acyl exchange between 1-*O*-acyl- $\beta$ -Glc and Glc.

Glandular trichomes of the wild tomato species *Lycopersicon pennellii* secrete sugar polyesters, a trait not possessed by cultivated tomato species. Sugar polyesters, also known as acylsugars, contribute to resistance to potato aphid (Goffreda et al., 1988; Goffreda and Mutschler, 1989), leaf-miner (Hawthorne et al., 1992), green peach aphids (Rodriguez et al., 1993), silver leaf whitefly (Liedl et al., 1995), tomato fruitworm, and beet armyworm (Juvik et al., 1994). Acylsugars are also produced by other Solanaceae, including tobacco, potato, petunia, and *Datura* (Severson et al., 1985; King et al., 1986, 1987a, 1987b; King and Calhoun, 1988; Shinozaki et al., 1991; Son et al., 1994), and exhibit feeding-deterrent or antibiotic-resistant activities against insects, bacteria, and fungi (Cutler et al., 1986; Holley et al., 1987; Neal et al., 1989, 1990). Knowledge of the biosynthesis of acylsugars will provide information for manipulation of acylsugar production and thus for improvement of insect resistance in tomato and other crops.

*L. pennellii* accessions produce both acyl-Glc and acyl-Suc. The amount of the total acylsugars and the ratio

between acyl-Glcs and acyl-Sucs vary among different accessions (Shapiro et al., 1994). Acyl-Glcs are complexes of 2,3,4-tri-*O*-acyl-Glcs (Burke et al., 1987). The acyl substituents are branched- and straight-chain, short (C<sub>4</sub>–C<sub>5</sub>)- to medium (C<sub>10</sub>–C<sub>12</sub>)-chain-length fatty acids (Burke et al., 1987; Walters and Steffens, 1990). The acyl-Sucs are complexes of 3',3,4-*O*-tri-acyl- and 3',3,4,6-*O*-tetra-acyl-Sucs (J.C. Steffens, unpublished data).

Acyl-Glc biosynthesis is a combination of the synthesis of branched amino acids to provide branched fatty acid primers, extension reactions for synthesis of medium-chain-length fatty acids, de novo fatty acid biosynthesis, UDP-Glc-dependent activation of the fatty acids, and regiospecific transfer of activated fatty acids to Glc hydroxyls (Walters and Steffens, 1990; Ghangas and Steffens, 1993, 1995).

Stable isotope-labeling studies show that branched-chain amino acids are either incorporated into triacyl-Glc as short-chain acyl substituents, or are elongated in 2-carbon increments to 10:0 and 12:0 and then incorporated into triacyl-Glc (Walters and Steffens, 1990). Similar incorporation of branched amino acids into acyl-Sucs was also reported in tobacco (Kandra et al., 1990).

In contrast to the well-known thioester-dependent (acyl-carrier protein and CoA) mechanisms of fatty acid activation, in acyl-Glc biosynthesis fatty acids are activated by UDP-Glc:fatty acid glucosyltransferase to form a high-energy 1-*O*-acyl- $\beta$ -Glc intermediate. This acyl alkyl acetal serves as the acyl donor molecule in the subsequent transacylations (Fig. 1). Two UDP-Glc:fatty acid glucosyltransferases with differential specificity toward short- and medium-chain fatty acids have been identified and purified (Kuai et al., 1997).

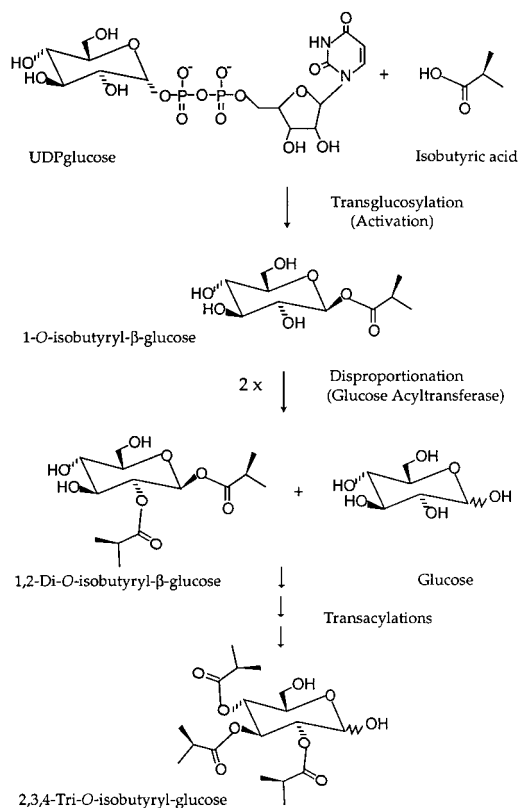
The early steps in Glc transacylation are characterized by two distinct acylation reactions (Ghangas and Steffens, 1995). The first is disproportionation between two equivalents of the 1-*O*-acyl- $\beta$ -Glc, yielding diacyl-Glc and free Glc (Fig. 1). The second reaction is anomeric acyl exchange, in which the anomeric acyl moiety of 1-*O*-acyl- $\beta$ -Glc is transferred to  $\beta$ -Glc. In previous work it was not clear whether disproportionation and anomeric exchange activities are inherent in a single acyltransferase, or whether they reside on separate polypeptides (Ghangas and Steffens, 1995). The steps between diacyl-Glc and triacyl-Glc are currently unclear, although it is possible that 1-*O*-acyl- $\beta$ -Glcs serve as acyl donors for further acylations to form triacyl-Glcs. Anomeric acyl exchange activity may contribute to the synthesis of triacyl-Glcs by transferring the anomeric acyl group

<sup>1</sup> This work was supported by the Cornell Center for Advanced Technology in Biotechnology, which is sponsored by the New York State Science and Technology Foundation and by industrial partners, and by Hatch Project no. 149,417.

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**Figure 1.** Acyl-Glc biosynthetic pathway. Fatty acids are activated via UDP-Glc-dependent transglucosylation to form the high-energy acyl donor molecule 1-*O*-acyl-β-Glc. Two equivalents of the activated 1-*O*-acyl-β-Glc intermediate then undergo enzyme-catalyzed disproportionation to form 1,2-substituted diacyl-Glc and Glc. In this figure, isobutyrate represents the acyl group; however, branched and straight short ( $C_4$ – $C_5$ ) to medium ( $C_{10}$ – $C_{12}$ ) chain fatty acids are utilized in acyl-Glc biosynthesis and are activated by chain-length-specific UDP-Glc:fatty acid transglucosylases (Kuai et al., 1997).

of a 1,2,3,4-tetra-*O*-acyl-β-Glc intermediate to a free Glc (Ghangas and Steffens, 1993, 1995).

The purpose of this study was to reveal the mechanism for this acylation pathway by purifying and characterizing the Glc acyltransferase that catalyzes the formation of diacyl-Glc.

## MATERIALS AND METHODS

### Routine Activity Assay

Disproportionation activity was detected by measuring the formation of diacyl-Glc from 1-*O*-[1- $^{14}$ C-isobutyryl]-β-Glc. Reactions were performed in a 15-μL solution containing 50 mM HEPES-NaOH, pH 7.5, 1 mM 1-*O*-[1- $^{14}$ C-isobutyryl]-β-D-Glc ( $10^5$  cpm), and an appropriate amount of protein obtained at different purification steps (approximately 30 μg–50 ng). All reactions were carried out at 37°C for 30 min unless otherwise specified. Reaction solution (5 μL) was loaded on a TLC plate and developed in chloroform:methanol:water (75:22:3, v/v). Autoradiography was then performed on the TLC plates (Ghangas and Steffens, 1993, 1995). 1-*O*-Isobutyryl-β-D-Glc and 1-*O*-[1- $^{14}$ C-

isobutyryl]-β-Glc were synthesized as previously described (Ghangas and Steffens, 1995).

Anomeric acyl exchange activity was monitored by measuring the transfer of the isobutyryl group from 1-*O*-isobutyryl-β-D-Glc to [U- $^{14}$ C]Glc (American Radiolabelled Chemicals, St. Louis). Reactions were performed in a 15-μL solution containing 50 mM HEPES-NaOH, pH 7.5, 2 mM 1-*O*-isobutyryl-β-D-Glc, 40 μM [U- $^{14}$ C]Glc ( $2 \times 10^5$  cpm), and an appropriate amount of proteins obtained at different purification steps (approximately 30 μg–50 ng). TLC monitoring was the same as with disproportionation (Ghangas and Steffens, 1993, 1995).

### Purification

Purification of Glc acyltransferase consisted of extraction, PEG fractionation, DEAE chromatography, concanavalin A (ConA) chromatography, and HPLC chromatofocusing (Mono P 5/20 column, Pharmacia). The first three steps were performed at 4°C, while the last two steps were conducted at room temperature. Gel filtration was performed to estimate the relative molecular mass of the enzyme at 4°C.

*L. pennellii* LA 1376 leaves (500 g) were homogenized with 750 mL of ice-cold buffer A (100 mM HEPES-NaOH, pH 7.5, 250 mM Suc, 10 mM DTT, 1% [w/v] acid-washed polyvinylpyrrolidone, and 1 mg/mL diethyldithiocarbamate), and filtered through cheesecloth. The homogenate was centrifuged for 20 min at 20,000g. PEG 3350 was added to the supernatant to a concentration of 0.13 g/mL. The sample was then centrifuged for 20 min at 20,000g. PEG was added to the supernatant to reach a concentration of 0.22 g/mL, and the sample was again centrifuged for 20 min at 20,000g. The pellet was resuspended in 100 mL of buffer B (50 mM HEPES-NaOH, pH 7.5, 10% [v/v] glycerol, and 1 mM DTT).

Resuspended sample was loaded onto a 1- × 25-cm DEAE-Sepharose column equilibrated in buffer B at 19.6 mL/h and washed overnight in 200 mL of buffer B. A gradient of 5 bed volumes was performed with 0 to 0.25 M KCl in buffer B. Fractions (3 mL) were collected and active fractions were pooled after activity assay.

KCl was added to the pooled DEAE fractions to reach a final concentration of approximately 0.2 M KCl. The sample was loaded onto a 0.7- × 13-cm ConA column equilibrated in buffer C (50 mM HEPES-NaOH, pH 7.5, 10% [v/v] glycerol, and 0.2 M KCl) at 19.2 mL/h. The column was washed with 4 bed volumes of buffer C and 2 bed volumes of 50 mM α-D-methylglucoside in buffer C. A gradient elution of 10 bed volumes was then performed with 50 mM to 1 M α-D-methylglucoside in buffer C. Fractions (2 mL) were collected. Active fractions were pooled and concentrated to about 1 mL using an ultrafiltration cell (YM10 membrane, Amicon) at 4°C. The sample was then dialyzed against buffer D at 4°C (25 mM piperazine-HCl, pH 5.5) overnight.

A Mono P column was used for HPLC chromatofocusing. The column (0.5 × 20 cm) was equilibrated with buffer D at a flow rate of 0.8 mL/min. After the sample was injected, the column was eluted with 32 mL of 1:10 (v/v) diluted

polybuffer 74 brought to pH 4.0 with HCl. The column was then washed with 1 M KCl in buffer D. Fractions were collected following the peak of  $A_{280}$ . After the pH gradient was measured, fractions were buffered by adding one-tenth volume of 0.5 M HEPES-NaOH, pH 7.5, and one-tenth volume of glycerol.

Gel filtration was used to determine the native molecular mass of the Glc acyltransferase. The active fraction (1.5 mL) from the DEAE purification step was loaded onto a 2 × 65-cm Sephacryl 200 column (Pharmacia). The column was eluted with buffer C at 25.1 mL/h.  $\beta$ -Amylase, alcohol dehydrogenase, BSA, carbonic anhydrase, and Cyt *c* were used as protein standards.

### Electrophoresis and Protein Sequencing

SDS-PAGE (10%) and silver staining (Schoenle et al., 1984) were routinely used for protein separation and identification. Native PAGE was performed as reported previously (Lewinsohn et al., 1992). The native gel was cut into a series of thin slices, and proteins were eluted (Lewinsohn et al., 1992). The proteins were either used for activity assay or loaded on SDS-PAGE.

After the Mono P step, 100 pmol of protein was transferred to a PVDF membrane (Bio-Rad) in 25 mM Tris, 192 mM Gly, and 10% (v/v) methanol at 250 mA for 2 to 4 h at 4°C. The membrane was stained with Ponceau dye and the 34- and 24-kD bands were cut out and destained. Tryptic peptides derived from the 34-kD band were separated by HPLC and sequenced (Harvard Microchemicals, Cambridge, MA). The N-terminal sequence of the 24-kD protein was determined at the Cornell University sequencing facility (Ithaca, NY).

### Characterization

For determination of the optimum pH and temperature for the disproportionation activity, ConA-purified (1,200-fold) enzyme was used. Citrate-phosphate (50 mM, pH 4.0 or 5.0), MES-HCl (pH 6.0 or 6.5), HEPES-NaOH (pH 7.0, 7.5, or 8.0), Gly-NaOH at pH 8.5 or 9.0, and Gly-NaOH at pH 10.0 were used in the pH study. Other conditions were the same as the routine assay. Disproportionation activities were tested in the temperature range from 0°C to 90°C.

The effect of HgCl<sub>2</sub> and *p*-hydroxymercuribenzoate (*p*-HMB) on disproportionation activity were examined using enzymes obtained from the Mono P step (purified about

4,900-fold). *p*-HMB was dissolved in 1 mM NaOH at 5 mM, and HgCl<sub>2</sub> was dissolved in water at 10 mM. The stock solutions were diluted to various concentrations with 50 mM Tris-HCl, pH 7.5. *p*-HMB or HgCl<sub>2</sub> was preincubated with the enzyme at 37°C for 90 min. Routine activity assays were then performed after the pre-incubations.

GC-MS was performed to analyze the disproportionation product. The enzymatic reaction proceeded for 3 h at room temperature in a 30- $\mu$ L solution containing 4 mM 1-*O*-isobutyryl- $\beta$ -Glc and ConA-purified enzyme (purified about 1,200-fold). The reaction was stopped by vortexing with 970  $\mu$ L of chloroform:methanol (3.8:1, v/v) and the sample was dried under N<sub>2</sub>. The sample was then acetylated overnight at room temperature with 60  $\mu$ L of pyridine and 30  $\mu$ L of acetic anhydride, and then dried under N<sub>2</sub> at 40°C. The acetylated sample was resuspended in 30  $\mu$ L of methylene chloride, and 1  $\mu$ L was injected onto an HP5 column in a GC-MS (model 5890, Hewlett-Packard). Temperature programming was 80°C for 1 min, 200°C at 12°C/min, and 260°C at 5°C/min, and the temperature was held at 260°C for 15 min. NH<sub>3</sub> and isobutane chemical ionization-MS (CI-MS) were performed.

## RESULTS

### Purification of Glc Acyltransferase

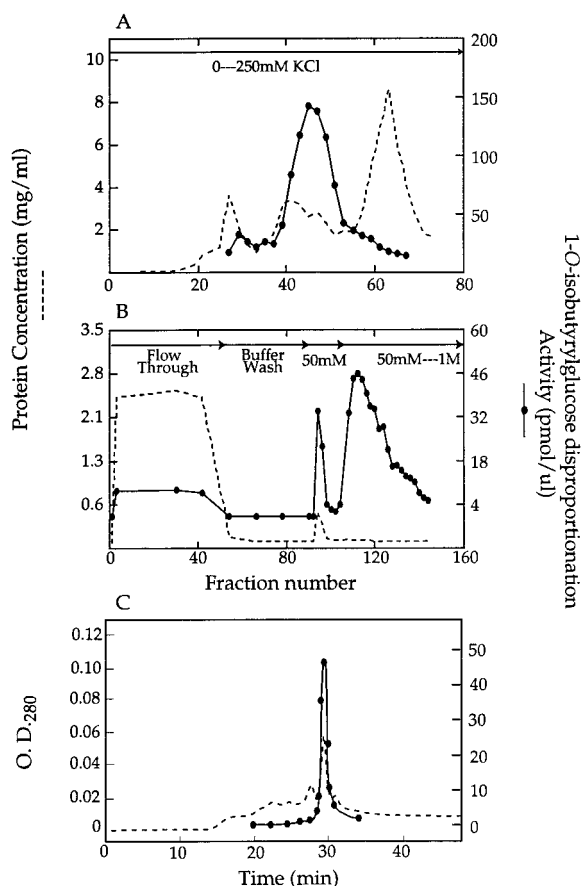
Disproportionation activity (1-*O*-acyl- $\beta$ -Glc:1-*O*-acyl- $\beta$ -Glc acyltransferase) was monitored throughout the purification. Approximately a 5-fold purification was obtained by PEG fractionation (0.13–0.22 g/mL) (Table I). Because of the low specific activity in the original extract, the uncertainty in the measured radioactivity of the product was relatively high. Thus, the 108% yield was most likely caused by variability in measurement rather than being an indication of the existence of inhibitors.

DEAE chromatography separated the activity from the bulk of the protein and resulted in an approximately 5-fold increase in specific activity (Fig. 2A). In addition to the protein represented in Figure 2A, one-fifth of the loaded protein did not bind to the column.

ConA-affinity chromatography proved to be the most effective purification step for the Glc acyltransferase. Disproportionation activity was separated not only from the majority of nonglycosylated proteins in the flow-through, but also from the majority of the glycoproteins that eluted with 50 mM  $\alpha$ -methylglucoside (Fig. 2B). The presence of the

**Table I.** Acyltransferase purification: disproportionation and acyl exchange activities

Purification step	Disproportionation Activity (1- <i>O</i> -Isobutyryl- $\beta$ -Glc:1- <i>O</i> -Isobutyryl- $\beta$ -Glc Acyltransferase)					Acyl Exchange Activity (1- <i>O</i> -Isobutyryl- $\beta$ -Glc:Glc Acyltransferase)
	Total protein mg	Specific activity nmol min <sup>-1</sup> $\mu$ g <sup>-1</sup>	Total activity nmol min <sup>-1</sup>	Yield %	Purification -fold	Purification -fold
Extraction	3,354	$4.3 \times 10^{-4}$	1,442			
PEG precipitation	707	$2.2 \times 10^{-3}$	1,555	108	5	3.5
DEAE-Sepharose	118	$9.8 \times 10^{-3}$	1,156	80.2	23	23
Con A-Sepharose	0.44	0.55	242	16.8	1,279	1,054
Mono-P	0.035	2.1	73.5	5.1	4,884	4,850



**Figure 2.** Purification of 1-*O*-isobutyryl- $\beta$ -Glc:1-*O*-isobutyryl- $\beta$ -Glc acyltransferase activity. A, DEAE-Sepharose chromatography. B, ConA-affinity chromatography. C, Chromatofocusing (Mono P). Protein was estimated using the Bradford method in A and B and by  $A_{280}$  in C.

minor activity peak eluting with 50 mM  $\alpha$ -methylglucoside may be due to natural glycosylation variants or changes in glycosylation status during purification. The 50 mM to 1 M  $\alpha$ -methylglucoside gradient elution yielded two-thirds of the total activity recovered from ConA chromatography. In addition, specific activity in the 50 mM to 1 M gradient elution was significantly higher than either the flow-through or the 50 mM  $\alpha$ -D-methylglucoside elution step (Fig. 2B). Therefore, only the 50 mM to 1 M fractions were pooled and concentrated. This step yielded a 55-fold increase in specific activity, resulting in an approximately 1,200-fold overall purification (Table I).

Final purification was achieved by chromatofocusing on a Mono P column. In addition to the protein shown in Figure 2C, a significant amount of protein eluted with 1 M KCl following the pH gradient. The enzyme is relatively unstable at its pI (pH 4.8). Therefore, fractions were buffered to pH 7.5 immediately after elution. The enzyme was purified approximately 4,900-fold after the chromatofocusing step (Table I).

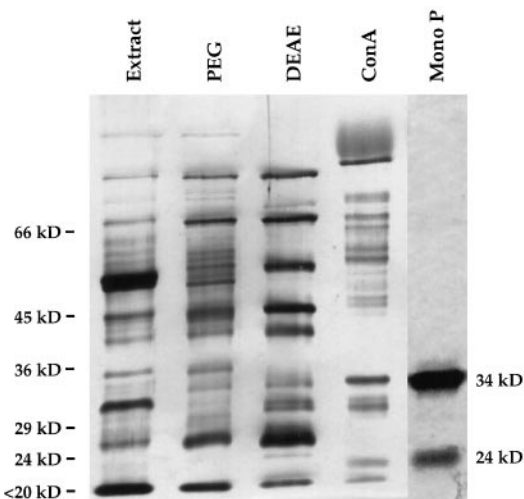
The purification resulted in enrichment of two SDS-PAGE silver-stained bands with estimated molecular masses of 34 and 24 kD (Fig. 3). These two bands were also

enriched in the ConA sample, but were not evident in any previous steps (Fig. 3). To determine the correlation between disproportionation activity and these two bands, proteins from ConA and Mono P columns containing the same amount of disproportionation activity were loaded on a SDS-PAGE column. Intensities of the 34- and 24-kD bands were similar in these two samples (not shown), suggesting that they are likely candidates for the enzyme.

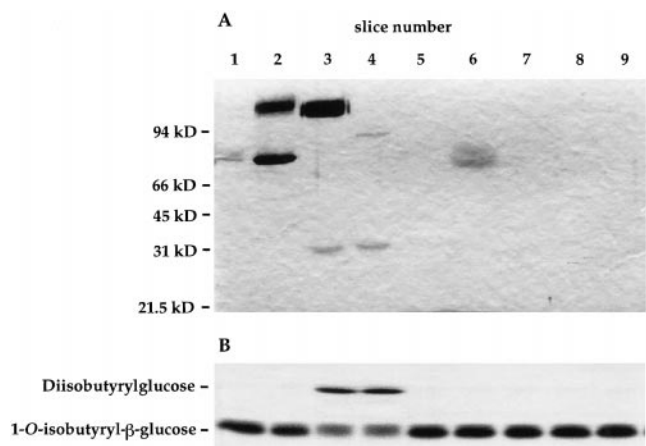
To confirm the identity of the proteins responsible for activity, native PAGE was performed using a ConA-purified enzyme preparation. The gel was cut into nine equal slices from top to bottom, and the protein eluted from each gel slice was used for activity assays and SDS-PAGE. Only protein samples from slices 3 and 4 possessed disproportionation activities. The common bands in slices 3 and 4 were the 34- and 24-kD polypeptides (Fig. 4A) (The 24-kD protein was too faint to reproduce, but could be seen in the original gel.) The 34- and 24-kD band intensities in slices 3 and 4 were similar, which is in agreement with the activities exhibited by these two samples (Fig. 4B).

Gel filtration on Sephacryl 200 showed that the acyltransferase relative molecular mass was approximately 110 kD (Fig. 5). This indicates that the active enzyme may be a heterotetramer consisting of two pairs of the 34- and 24-kD proteins. The 34-kD protein was digested with trypsin. Seven tryptic peptides were purified by HPLC and sequenced. The N terminus of the 24-kD protein was also sequenced. Results are shown in Table II. No significant sequence similarities were found in GenBank.

Acyl exchange activities were also assayed after each purification step and compared with the disproportionation activity (Table I). In each step, similar degrees of purification were obtained for both disproportionation and acyl exchange activity. Furthermore, the Mono P sample (the purified acyltransferase) still catalyzed the acyl exchange reaction. Therefore, we conclude that the anomeric



**Figure 3.** SDS-PAGE analysis of each purification step. Equal amounts of protein (approximately 10  $\mu$ g) were loaded on the gel for the extract, PEG, DEAE, and ConA steps. Approximately 5  $\mu$ g of Mono P-purified protein was loaded. The gel was silver-stained.



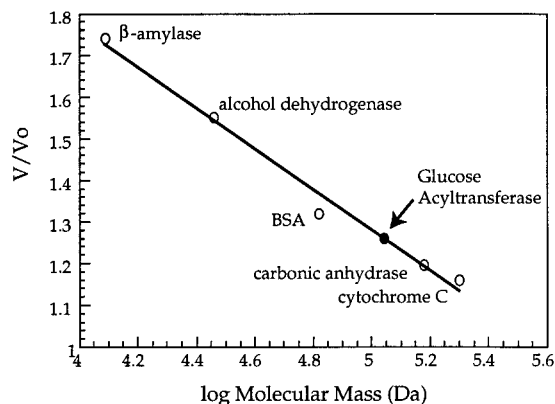
**Figure 4.** A, Silver-stained SDS-PAGE of recovered native PAGE samples. Native PAGE was performed for ConA-purified acyltransferase. The gel was cut into nine equal slices from top to bottom, and the protein eluted from each gel slice was used for activity assays and SDS-PAGE. The numbers indicate the slices. The 34- and 24-kD (too faint to reproduce in this figure) polypeptides were common to slice 3 and 4. B, TLC autoradiography of disproportionation activity for proteins eluted from each slice of the native PAGE.

acyl exchange activity and the disproportionation activity are properties of the same enzyme.

#### Characteristics of the Disproportionation Enzyme

Disproportionation activity was tested between pH 4.0 and 10.0. The formation of diacyl-Glc was relatively independent of pH between 4.0 and 8.5 (Fig. 6A). Activity was also insensitive to temperature between 20°C and 60°C (Fig. 6B).

The effects of the the sulfhydryl group (-SH)-modifying reagents  $\text{Hg}^{2+}$  and *p*-HMB are shown in Figure 6C. At a concentration of 0.25 mM,  $\text{Hg}^{2+}$  decreased the disproportionation activity by 50% and inhibition reached 100% at 2 mM  $\text{Hg}^{2+}$ , suggesting involvement of sulfhydryl groups in



**Figure 5.** Gel filtration determination of relative molecular mass for the Glc acyltransferase. β-Amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), carbonic anhydrase (29 kD), and Cyt *c* (12.4 kD) were used as protein standards. The relative molecular mass of the acyltransferase (indicated by the arrow) is about 110 kD.

**Table II.** Partial amino acid sequences from the 34- and 24-kD subunits

PT, Peptides from trypsin digestion.

Tryptic peptide sequences from the 34-kD subunit

PT15: S I D F N G R

PT21: G Y I Q G N A L T D R

PT24: Y A N H M G L I S D K

PT25: I Y D G I E V G D R P

PT40: L E L N S Y S W

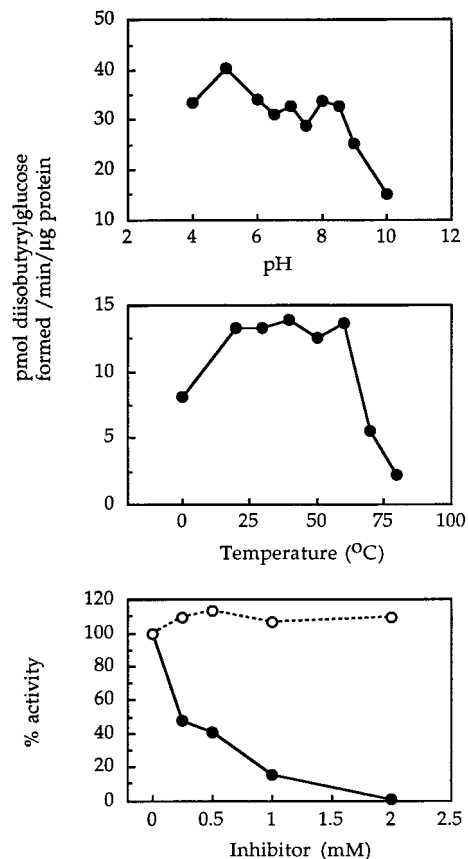
PT50: E H F I V E T L P G F H

PT60: A N - N G N Y I D V D P N N I L (L)(L) N D

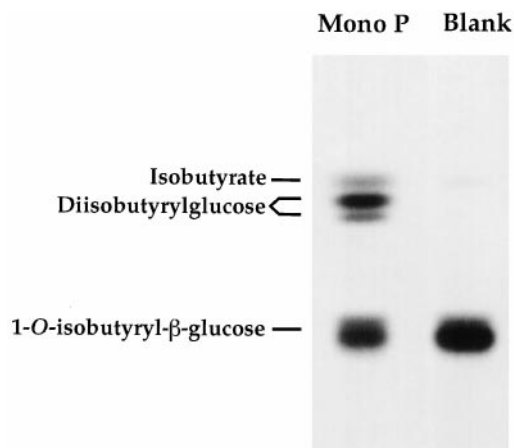
N-terminal sequence of the 24-kD subunit

N 24: G Q S V F P I A G P

maintaining the acyltransferase in an active conformation. However, no inhibition could be observed up to 2 mM *p*-HMB. This may involve the relative valence of  $\text{Hg}^{2+}$  and *p*-HMB and the stability of the resulting -SH complex. It is also possible that the location of reactive SH groups in the protein structure differ in their accessibility to these reagents.



**Figure 6.** Characteristics of the Glc acyltransferase. Approximately 0.5 μg of ConA-purified enzyme was used in each reaction. Top, pH optima for disproportionation activity. Middle, Temperature optima for disproportionation activity. Bottom, Effects of *p*-HMB and  $\text{Hg}^{2+}$  on the disproportionation of 1-O-isobutyryl-β-Glc. *p*-HMB (○) or  $\text{HgCl}_2$  (●) was incubated with the enzyme at 37°C for 90 min prior to the activity assay.



**Figure 7.** TLC autoradiograph of the disproportionation reaction. Disproportionation of 1-*O*-[1-<sup>14</sup>C-isobutyryl]- $\beta$ -Glc by acyltransferase purified 4,900-fold. Blank, No added enzyme.

### The Glc Acyltransferase Is Responsible for Converting the 1-*O*-Acyl- $\beta$ -Glc to Diacyl-Glc

The formation of diacyl-Glcs was previously established by comparing the  $R_F$  values of the products of *in vitro* acyl transfer with those of the diacyl-Glcs generated by ammoniolysis of natural triacyl-Glcs (Ghangas and Steffens, 1993, 1995). TLC of the disproportionation reaction using 4,900-fold Mono P-purified enzyme is shown in Figure 7. GC-MS analysis confirmed the formation of diacyl-Glcs in this reaction (Fig. 8). The reaction mixtures were acetylated prior to GC-MS. The primary product of disproportionation, diisobutyryl-Glc, eluted at 19.99 min (Fig. 8A). The  $\text{NH}_3$  CI-MS of this product is shown in Figure 8B. The ion at  $m/z$  464.6 is the  $[\text{M} + \text{NH}_4]^+$  adduct of di-isobutyryl-Glc triacetate.  $m/z$  359.3 represents loss of  $(\text{CH}_3)_2\text{CHCOONH}_4$  from  $[\text{M} + \text{NH}_4]^+$ , indicating the presence of an isobutyryl moiety at the anomeric position of Glc.

Unreacted substrate 1-*O*-isobutyryl- $\beta$ -Glc tetraacetate eluted at 18.16 min, and  $\alpha$ - and  $\beta$ -Glc pentaacetate resulting from disproportionation eluted at 16.40 and 16.54 min, respectively. A small amount of nonanomerically substituted mono-isobutyryl-Glc resulting from disproportionation of the diisobutyryl product eluted at the 18.30 and 18.37 min. In  $\text{NH}_3$  CI-MS, these monoacyl-Glcs generate  $[\text{M}-59]^+$  (loss of  $\text{CH}_3\text{COONH}_4$  from  $[\text{M} + \text{NH}_4]^+$ , adduct of monoisobutyryl-Glc tetraacetate), but not  $[\text{M}-87]^+$  (loss of  $[\text{CH}_3]_2\text{CHCOONH}_4$  from  $[\text{M} + \text{NH}_4]^+$ ), indicating that the isobutyryl moiety is not at the anomeric position of the Glc. A minor shoulder at 20.07 min may represent a second diisobutyryl-Glc product that is acylated at different hydroxyls; however, the amount is too low for identification.

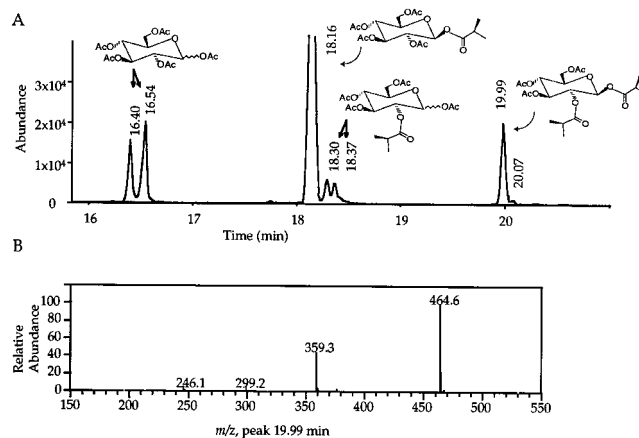
### DISCUSSION

We purified the Glc transferase that catalyzes the disproportionation of two equivalents of 1-*O*-acyl- $\beta$ -Glc to form diacyl-Glc and free Glc. The enzyme appears to be a heterotetramer composed of 34- and 24-kD subunits. The status of the acyltransferase as a glycoprotein suggests the

possibility that the acyltransferase is associated with a secretory vesicle pathway through which polyacylsugars are synthesized, packaged, and secreted from glandular trichomes (D. Maiti and J.C. Steffens, unpublished data).

Previously, it was unclear whether disproportionation activity and anomeric acyl exchange activity (transacylation between 1-*O*-acyl- $\beta$ -Glc and Glc) reside on the same or separate polypeptides (Ghangas and Steffens, 1995). Because acyl exchange and disproportionation activities copurify through a 4,900-fold enrichment process, we conclude that disproportionation and the acyl exchange are inherent activities of the same enzyme.

The formation of diacyl-Glc in the disproportionation reaction was confirmed by GC-MS analysis. <sup>1</sup>H-NMR studies indicate that the primary product is 1,2-di-*O*-acyl- $\beta$ -Glc (J.C. Steffens, unpublished data). Thus, the Glc acyltransferase primarily catalyzes a regiospecific acyltransfer reaction. A small amount of another diacyl-Glc besides 1,2-diacyl-Glc appears when HEPES (Fig. 7) or Tris is used in the reaction buffer. It is currently not clear whether the formation of the other diacyl-Glc is an enzymatic reaction or the result of a nonenzymatic intramolecular acyl migration. 1,2-di-*O*-acyl- $\beta$ -Glcs also serve as acyl donors to monoacyl-Glcs or Glcs to form nonanomerically substituted monoacyl-Glc (the 18.30- and 18.37-min peaks in the GC-MS analysis), as proposed previously (Ghangas and Steffens, 1993, 1995).



**Figure 8.** GC-MS analysis of the disproportionation reaction. Disproportionation of 1-*O*-isobutyryl- $\beta$ -Glc was catalyzed by acyltransferase purified 1,200-fold from *L. pennellii* leaves. The reaction products were acetylated prior to analysis. A, GC separation of the substrate (1-*O*-isobutyryl- $\beta$ -Glc) and products of disproportionation. The 16.40- and 16.54-min peaks are  $\alpha$ - and  $\beta$ -Glc pentaacetates, respectively; the 18.16-min peak is the 1-*O*-isobutyryl- $\beta$ -Glc tetraacetate; and the 18.30- and 18.37-min peaks are nonanomerically substituted mono-isobutyryl-Glc tetraacetates. The peak at 19.99 min is the primary product of the disproportionation reaction, 1,2-di-*O*-isobutyryl- $\beta$ -Glc. The minor 20.07-min shoulder may be another diisobutyryl-Glc, but the amount is too low to be identified. B,  $\text{NH}_3$  CI-MS of the 1,2-di-*O*-isobutyryl- $\beta$ -Glc product (19.99 min) of 1-*O*-isobutyryl- $\beta$ -Glc disproportionation.  $m/z$  464.6 is the  $[\text{M} + \text{NH}_4]^+$  adduct of di-*O*-isobutyryl-Glc triacetate.  $m/z$  359.3 represents the loss of  $(\text{CH}_3)_2\text{CHCOONH}_4$  from  $[\text{M} + \text{NH}_4]^+$ .

UDP-Glc-dependent acyl activation and 1-*O*-acyl- $\beta$ -Glc-mediated transacylation are involved in the synthesis of other secondary metabolites such as gallotannins, sinapate esters, and chlorogenic acid (3-*O*-caffeoylquinic acid). In gallotannin synthesis, gallic acid is activated by UDP-Glc to form  $\beta$ -glucogallin (1-*O*-galloyl- $\beta$ -Glc), which serves as a galloyl donor for the synthesis of 1,2,3,4,6-pentagalloyl-Glc (Gross and Denzel, 1991). At least four different galloyltransferases have been tentatively identified in the synthesis of 1,2,3,4,6-pentagalloyl-Glc, each with a different regiospecificity for transacylation (Denzel et al., 1988; Cammann et al., 1989; Gross et al., 1990; Hagenah and Gross, 1993). Sinapate esters such as sinapoyl malate, sinapoyl choline, and 1,2-di-sinapoyl-Glc, which have potential roles in plant UV resistance (Chapple et al., 1994), are synthesized through an analogous series of transacylations, each of which utilizes 1-*O*-sinapoyl- $\beta$ -Glc (Dahlbender and Strack, 1986; Grawe et al., 1992). It is interesting that disinapoyl-Glc is diacylated at the 1 and 2 positions, similar to the diacyl-Glc formed in the synthesis of Glc polyesters. Chlorogenic acid, a ubiquitous polyphenol in plants, is synthesized using caffeoyl-Glc as the acyl donor (Villegas and Kojima, 1986; Moriguchi et al., 1988) as well as by an acyl-CoA-dependent route (Zenk, 1977). The similarity between the *L. pennellii* Glc acyltransferase and other transferases underlines the mechanistic conservation of phenolic and fatty acid transacylation reactions in secondary metabolism.

UDP-Glc-dependent acyl activation and 1-*O*-acyl- $\beta$ -Glc-mediated transacylation are also critical steps in the conjugation of the plant growth hormone IAA. The conjugates of IAA serve functions such as transport, protection against peroxidative attack, storage, and regulation of IAA. Ester-linked conjugates such as IAA-*myo*-inositol are formed through transacylation from 1-*O*-indol-3-ylacetyl- $\beta$ -D-Glc to *myo*-inositol (Kesy and Bandurski, 1990; Szerszen et al., 1994; Bandurski et al., 1995). Although not a C<sub>6</sub> sugar alcohol, inositol is a cyclic hexahydric alcohol. Thus, this transacylation also shares similarities to the acyl exchange reaction catalyzed by the *L. pennellii* Glc acyltransferase. It will be interesting to compare these two enzymes when the indole-3-ylacetyltransferase is purified.

In the disproportionation of 1-*O*-isobutyryl- $\beta$ -Glc catalyzed by the 4,900-fold-purified Glc acyltransferase, GC-MS did not indicate significant formation of tri- and tetraacyl-Glcs. Thus, this acyltransferase does not appear to be responsible for higher-order acylation in vitro. By analogy to gallotannin biosynthesis, other regiospecific acyltransferases may be responsible for higher-order acylations using 1-*O*-acyl- $\beta$ -Glc as the acyl donor. However, it is also possible that other acyl transfer mechanisms, such as one utilizing a fatty acyl CoA, are responsible for further acylation of diacyl-Glc and that the combined pathways contribute to the synthesis of 2,3,4-triacyl-Glc. Further studies will identify these reactions and their corresponding enzymes to determine the entire pathway utilized for the synthesis of sugar polyesters.

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