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ENZYMOLOGY OF UDP-GLUCOSE:SINAPIC ACID GLUCOSYLTRANSFERASE FROM *BRASSICA NAPUS*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Brassica napus*; Cruciferae; UDP-glucose:sinapic acid glucosyltransferase; sinapine; enzymology

Abstract—UDP-glucose:sinapic acid glucosyltransferase (SGT; EC 2.4.1.120) was purified from 60-h-old seedlings of *Brassica napus*. The purified SGT appears to be a cytosolic monomeric polypeptide with a M_r of 42 kDa and a pI of 5. Kinetic analysis suggested that the catalytic mechanism used by SGT best fits a "random bi–bi" model, with a $K_{m\ (UDP\text{-}glucose)}$ of 2.4 mM and $K_{m\ (sinapic\ acid)}$ of 0.16 mM. SGT also catalyzes the reverse reaction *in vitro*, using UDP and sinapoylglucose to form UDP-glucose. No cofactors are required for enzyme activity, but reducing agents and glycerol are required to stabilize the activity. The enzyme is strongly inhibited by p-OH-mercuribenzoic acid, UDP, TDP, Zn^{++}, Cu^{++} and Hg^{++}.

INTRODUCTION

Sinapine (O-sinapoyl choline) is known to be a remarkable chemotaxonomic character in many crucifers, especially in *Brassica* and closely-related species [1]. In edible oilseed *Brassica* (canola) it accumulates in the mature seeds to levels that limit the utilization of the protein-rich post-crushing meal as an animal feed supplement [2]. Among the enzymes required in sinapine biosynthesis and metabolism, UDPG:sinapic acid glucosyl transferase (SGT), which catalyzes the formation of 1-O-sinapoyl-β-D-glucose from sinapic acid and UDP-glucose (UDPG), is notable because it is required at two points. Its product, sinapoylglucose, is an essential substrate both for sinapine formation in the seed, and for sinapoylmalate formation in the leaves of the plant [3].

SGT activity has been demonstrated previously in leaf tissue of *B. oleracea* [4], and in seeds and young seedlings of *Raphanus sativus* [1], and it has been partially purified from seedlings of *R. sativus* [1, 5, 6]. Outside the Brassicaceae family, SGT activity has also been detected in extracts of cultured cells of *Daucus carota*, where its product is believed to serve as an intermediate in the synthesis of a sinapoyl anthocyanin derivative accumulated by the carrot cells [7]. However, as a class, glucosyltransferases are not abundant proteins in plant tissues, and only a small number of nucleotide sugar-dependent glucosyltransferases have been purified to varying degrees [8–12].

Because of its participation in two different pathways that operate at different stages of plant development, and in different tissues, the mechanisms which regulate SGT expression are of interest. As a key enzyme in sinapine biosynthesis, SGT is also a potential target for genetic engineering approaches to creation of an agronomically-desirable low-sinapine *Brassica* genotype. Here, we report the purification and characterization of SGT from seedlings of *B. napus* (canola).

RESULTS AND DISCUSSION

Inducibility of SGT

SGT activity in *B. napus* changes dramatically during the growth cycle of the plant [13]. The underlying regulatory mechanisms are unknown, but could include both developmental and environmental cues. One possible control factor might be changes in the size of the intracellular pool of sinapate, arising from

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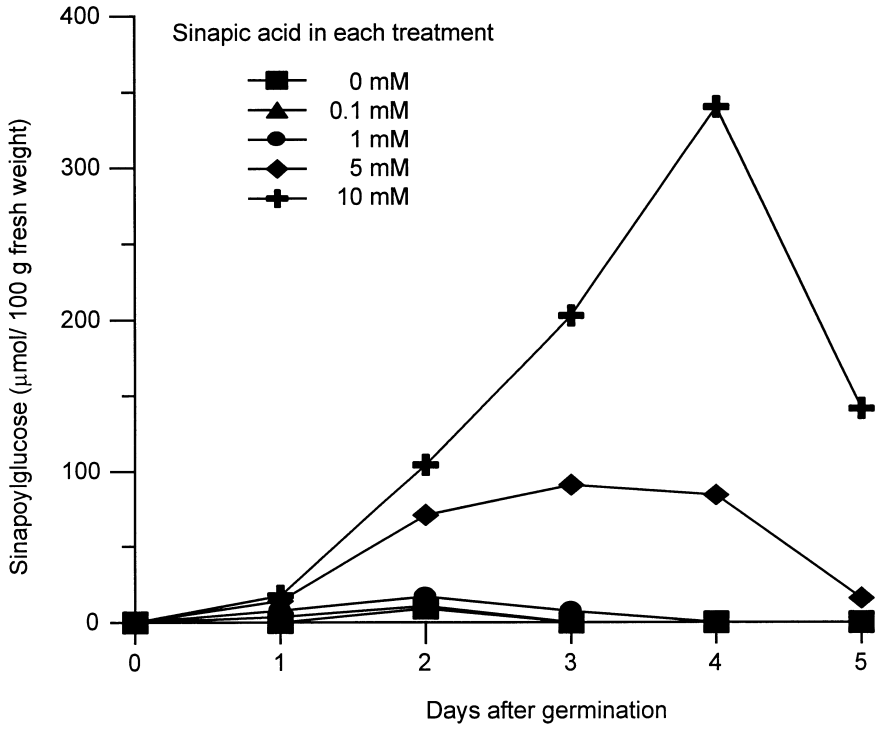


Fig. 1. Dynamics of sinapoylglucose accumulation in young seedlings of *B. napus* cv. Westar grown in medium containing various concentrations of sinapic acid. The data presented are the means of three duplicates.

either *de novo* synthesis in the developing seed, or from sinapine hydrolysis during seed germination. A potential environmental cue is the perception of increased UV exposure in aerial tissues, since sinapoyl esters appear to provide at least part of the UV-B screen in crucifer leaves [14]. It is also possible that sinapoyl ester synthesis forms part of an overall stress response in these plants. To test these ideas, young *B. napus* seedlings were exposed to three different regimes: (1) an exogenous supply of sinapic acid, (2) visible light/UV-B irradiation, or (3) heat-shock stress. The results showed that exogenous supply of a high (10 mM) concentration of sinapic acid could induce a modest increase (1.8-fold) in SGT activity in the seedlings (data not shown). Under these conditions, the level of sinapoylglucose accumulated in the tissue was enhanced, and the peak of accumulation was delayed by 24–48 hours (Figure 1).

The concentration of sinapine in *B. napus* cv. Westar seeds is $\sim 40\,\mu$mol/g dry wt., but two days after germination, $\sim 4\,\mu$mol sinapine/g fresh wt. and $\sim 0.4\,\mu$mol sinapate/g fr. wt. were detected in young seedlings. The level of SGT activity extractable from 2-day-old *B. napus* seedlings (~ 500 pkat/g fresh tissue) should thus be sufficient to re-esterify any endogenous free sinapic acid released from sinapine during germination, and also seems adequate to esterify exogenously-supplied sinapic acid. The relatively weak induction of SGT activity observed in seedlings fed sinapic acid is in contrast to the strong induction (5 to 25-fold) reported for other glucosyltransferases by exogenous supply of their substrates [15–19]. Environmental stresses such as UV-B irradiation or temperature shifts are also known to induce higher glucosyltransferase activities in plant tissues [20], but in *B. napus* seedlings neither the expression pattern nor the absolute level of SGT activity were affected by the tested UV or heat stress regimes (data not shown).

Subcellular localization of SGT

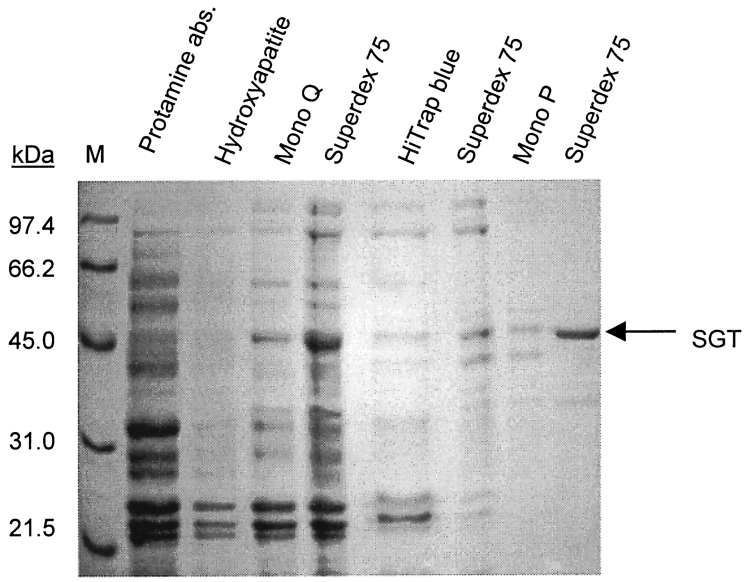
Most glucosyltransferases involved in glycosylation of plant secondary products appear to be freely soluble, and some have been confirmed to be located in the cytosol [21–25]. Fractionation of protein preparations from *B. napus* seedlings by centrifugation in Percoll gradients provided no evidence for association of SGT activity with any organellar or membrane fraction, and use of non-ionic detergents did not enhance the recovery of the enzyme [data not shown]. The apparent cytosolic location for seedling SGT is consistent with an earlier proposal that the product of the SGT reaction, sinapoylglucose, must be transported from the cytosol to the vacuole to support subsequent sinapoylmalate biosynthesis [26, 27].

Purification of SGT

Protein extracts of two-day-old *B. napus* seedlings contained large amounts of cotyledonary storage pro-

Table 1. SGT purification schedule and protein profile of each step on SDS-PAGE

Purification step	Total Activity (pkat)	Specific Activity (pkat/mg protein)	Total protein (mg)	Enriched (fold)
Crude extract	250,427	37	6760	1.0
Protamine absorption	239,733	37	6445	1.0
Differential surface binding (Macro-prep Hydroxyapatite)	151,749	45	3368	1.2
Anion exchange (Prep-Mono Q)	102,368	115	894	3.1
Gel filtration (Superdex 75)	62,450	220	284	5.9
Affinity binding (HiTrap blue dye)	31,503	272	116	7.4
Gel filtration (Superdex 75)	22,874	401	57	10.8
Chromatofocusing (Mono P)	12,515	544	23	14.7
Gel filtration (Superdex 75)	2,500	5000	0.5	135.1
Recovery (%)	1		0.007	



teins that complicated the isolation of SGT. After extensive chromatography, SGT was purified 135-fold with an overall yield of 1% of the initial activity (Table 1). This low yield reflects loss of much of the SGT activity during the time-consuming purification process. No isoforms of SGT were detected in the seedling extracts in any of the chromatographic procedures (Figure 2). Attempts to recover SGT activity from native PAGE gels were unsuccessful. After the final chromatography step, the protein profile of the most active fraction on stained 10% SDS-PAGE gels contained a single major species.

Physico-chemical characterization of SGT

Molecular mass. Based on calibrated Superose 12 gel filtration, native SGT has a M_r of 42 ± 1 kDa. If the major polypeptide in the most highly purified enzyme fraction on SDS-PAGE gels represents SGT, the migration of this putative SGT in a calibrated SDS-PAGE system generates a calculated M_r of about 42 kDa for the denatured enzyme. SGT is thus apparently a monomeric polypeptide.

pI, pH stability and pH optimum. The peak of SGT activity recovered during chromatofocussing was detected at pH 5.1. The stability of SGT at this pH was markedly reduced compared to its stability at pH 6.3 or 7.0, with a loss of 60% of the activity within one hour. The optimum reaction pH was found to be pH 6.0 when MES buffer was used. No activity could be detected below pH 4.5 or above pH 8.5.

Thermal stability and optimum temperature. SGT lost $>90\%$ of its original activity after 24 h at 25°, but $<10\%$ after two days at 4°. After one month at this temperature, $<10\%$ of the original activity remained, but little activity was lost at $-20°$ over the same period. The optimum assay temperature was 32°, and no activity could be detected at incubation temperatures $>50°$.

Effect of metal ions, reducing agents and inhibitors.

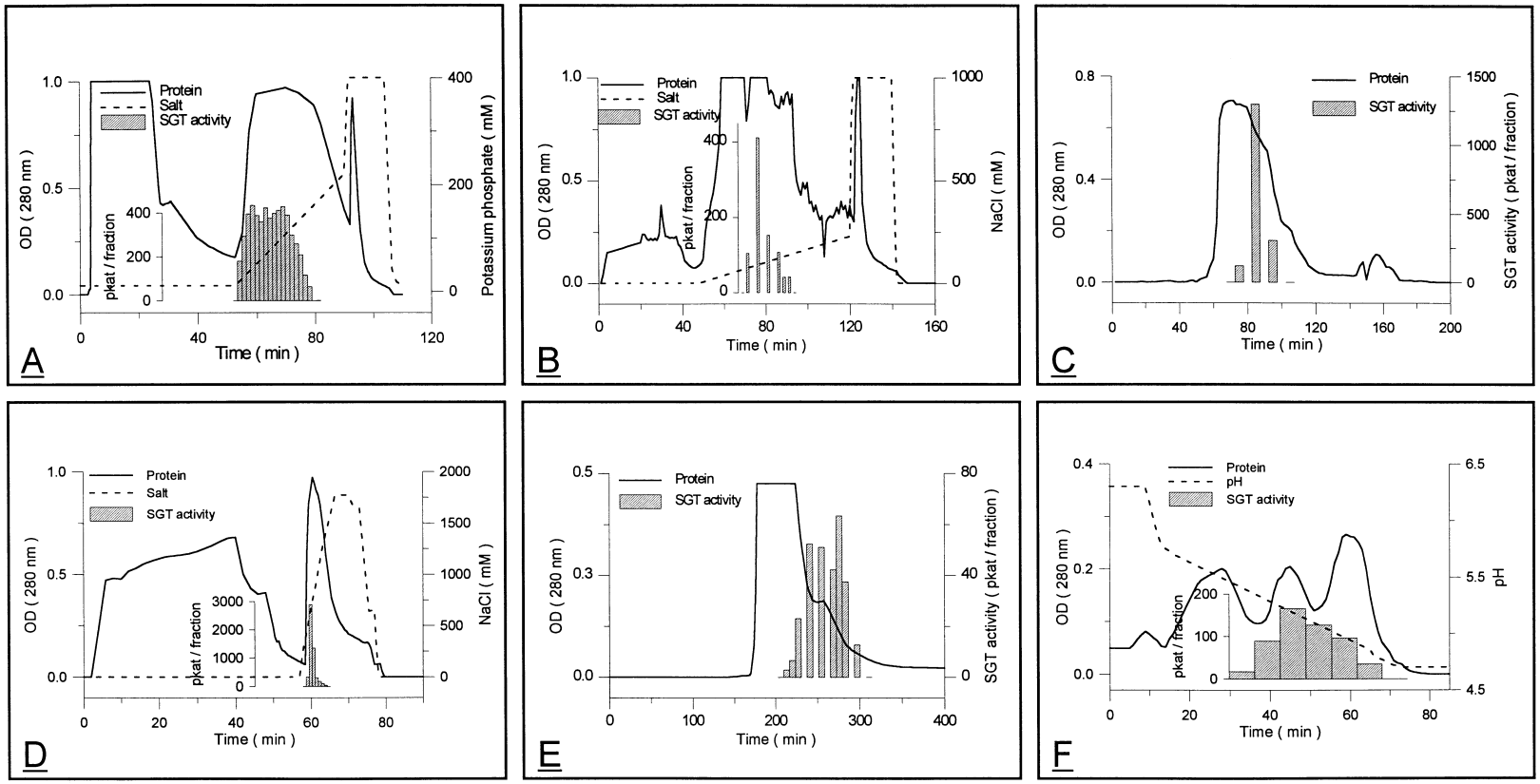


Fig. 2. Chromatograms of systematic purification of SGT from seedlings of *B. napus* cv. Westar by column chromatography. (A) Differential surface binding by Macro-prep hydroxyapatite; (B) anion exchange on Prep-Mono Q; (C) first gel filtration on Superdex 75; (D) affinity binding on HiTrap blue dye; (E)second gel filtration on Superdex 75; (F) chromatofocusing on Mono P.

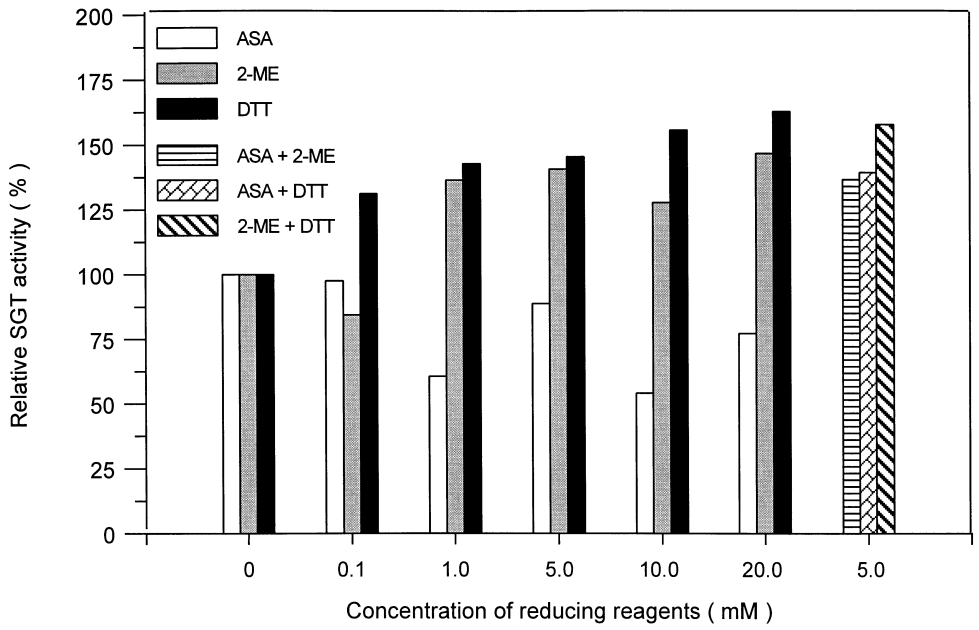


Fig. 3. Effects of reducing reagents on SGT activity. Partially purified SGT from seedlings of *B. napus* cv. Westar was incubated with various reducing reagents at 4° for 1 h, then mixed with the substrates. After incubation at 37° for 30 min, the SGT activity of each sample was determined by the HPLC method.

Thiol reducing agents such as β-mercaptoethanol (2-ME) or dithiothreitol (DTT) were required to maintain SGT activity after extraction. The activity was completely lost after three days storage at −20° if there was no reducing agent in the buffer. Ascorbic acid, on the other hand, was somewhat inhibitory (Figure 3), although this could be countered by addition of 2-ME or DTT. This pattern differs from the behaviour of UDPG:cyanidin 3-O-glucosyltransferase from carrot cell cultures, whose activity was stimulated >10-fold by ascorbate [28]. This stimulatory effect was not observed with thiol reducing agents.

No metal ion was absolutely required as a co-factor for SGT activity (Table 2), but Mg^{++} and Mn^{++} showed a modest stimulatory effect, and this could be eliminated by addition of EDTA. SGT was inhibited by several divalent cations, of which Zn^{++} was the most effective. The inhibition by Zn^{++} ions could be overcome by EDTA, but the inhibition by Cu^{++} and Hg^{++} could not. This sensitivity of SGT to metal ions capable of forming stable mercaptides is consistent with the enzyme's apparent requirement for one or more free thiol groups. Among the substrate analogues and various known -SH group and reactive serine group inhibitors tested, only p-hydroxymercuribenzoate showed a significant inhibitory effect.

Substrate specificity. Different nucleotide-activated glucose compounds (ADPG, CDPG, TDPG and UDPG) were tested for their ability to serve as sugar donors in the SGT reaction. Of these, only UDPG and TDPG were accepted by the enzyme (Table 3). SGT accepts a broader range of glucose acceptors as second substrate, but among those tested, sinapic acid was used most efficiently. A strong inhibitory effect was observed when SGT was incubated with UDP or TDP (Table 4). UDP-mannose, UDP-xylose and UDP-galactose were less inhibitory and glucose itself had no effect.

Reversibility. SGT has the capability to catalyze both the forward and reverse reactions *in vitro*. Adding UDP or TDP to a reaction mixture in which formation of sinapoylglucose is being catalyzed, immediately drove the reaction backward (Figure 4-I). When the reaction solution included only sinapoylglucose and enzyme, but no UDP, the reverse reaction did not proceed until UDP was added (Figure 4-II). This result demonstrates that the SGT preparation was not contaminated by any esterases capable of hydrolyzing sinapoylglucose, and that it is only in the presence of U/TDP that the glucose moiety of sinapoylglucose can be transferred to an acceptor substrate. This reversibility of UDPG-dependent glucosyltransferases has also been observed in enzymes from several other plant species [1, 29–33]. The energy-rich nature of the glycosidic linkage between glucose and carboxylic acid/phenolic functional groups may mean that such glycosides, in addition to their other roles, provide a source of readily mobilized hexose.

Kinetic parameters. The pattern of dependence of the SGT reaction rate on both substrate concentrations was consistent with Michaelis–Menten kinetic behaviour (Figure 5). For the purified enzyme, the calculated $K_{m[UDPG]}$ was 0.24 mM, the $K_{m[sinapic\ acid]}$ was 0.16 mM and the V_{max} was 10.6 pkat. Analysis of

Table 2. Metal ion effects on SGT activity. Partially purified SGT from seedlings of *B. napus* cv. Westar was incubated with various metal ion solutions at 4° for 2 h, then mixed with substrates. After reaction at 37° for 1 h, the SGT activity of each treatment was determined by the HPLC method*

Sample	SGT activity (pkat) Mean	SD ($n=3$)	Relative activity (%)
Control	2.02	0.05	100
Control + EDTA	2.03	0.18	100
Sodium chloride	2.00	0.20	99
NaCl + EDTA	2.05	0.23	102
Potassium chloride	1.90	0.10	94
KCl + EDTA	1.92	0.17	95
Calcium chloride	1.95	0.00	96
$CaCl_2$ + EDTA	1.40	0.02	69
Cobaltous chloride	0.94	0.07	47
$CoCl_2$ + EDTA	1.62	0.11	80
Cupric chloride	0	0	0
$CuCl_2$ + EDTA	0	0	0
Ferrous sulphate	0.93	0.03	46
$FeSO_4$ + EDTA	1.09	0.10	54
Mercuric chloride	0	0	0
$HgCl_2$ + EDTA	0	0	0
Magnesium chloride	2.22	0.36	110
$MgCl_2$ + EDTA	1.99	0.15	99
Manganese chloride	2.44	0.12	121
$MnCl_2$ + EDTA	1.84	0.08	91
Zinc sulphate	0	0	0
$ZnSO_4$ + EDTA	1.10	0.13	55

* The pH of various metal ion solutions was adjusted to pH 6.0. The final concentration of metal ion was 0.93 mM and EDTA was 7.4 mM in the reaction solutions.

Table 3. The specificity of SGT for sugar donor and acceptor. Partially purified SGT from seedlings of *B. napus* cv. Westar was used. For the sugar donor test, sinapic acid was used as the sugar acceptor, and the formation of the product was determined by HPLC. For the sugar acceptor test, UDP-[β-D-glucose (U-^{14}C)] was used as the sugar donor, and the formation of the product was determined by the radioactivity assay

Sugar donor	SGT relative activity (%)	Sugar acceptor	SGT relative activity (%)
UDP-Glucose	100	Sinapic acid	100
TDP-Glucose	96	Ferulic acid	77
ADP-Glucose	0	5-OH-Ferulic acid	39
CDP-Glucose	0	Cinnamic acid	24
GDP-Glucose	0	*p*-Coumaric acid	21
		Caffeic acid	14
UDP-Galactose	0	Syringic acid	10
UDP-Mannose	0		
UDP-Xylose	0		
Glucose	0		

the kinetic parameters suggested that the *B. napus* SGT catalytic mechanism best fits a "random bi–bi" model. This differs from the conclusion reached earlier for the enzyme from *R. sativus*, which was reported to use an "ordered bi–bi" mechanism [6], but the basis of this apparent difference is not clear. Inhibition of the *B. napus* enzyme by UDPG analogues suggests that one binding site does display structural specificity for the nucleotide moiety of the sugar donor substrate, but chromatography matrices bearing UDPglucose or UDPglucuronic acid ligands both failed to bind SGT under the conditions tested (data not shown). On the

Table 4. Effects of UDPG analogues on SGT activity. Partially purified SGT from seedlings of *B. napus* cv. Westar was incubated with each analogue at 4° for 1 h and then mixed with substrates. After reaction at 37° for 30 min, the SGT activity of each sample was determined by the HPLC method*

Analogues	SGT activity (pkat) Mean	SD ($n=3$)	Relative activity (%)
Control	7.77	0.56	100
β-D-Glucose	8.02	0.35	103
UDP-Galactose	7.01	0.77	90
UDP-Xylose	6.43	0.23	83
UDP-Mannose	3.05	0.12	39
UDP	1.01	0.04	13
TDP	0.78	0.08	10

* The concentration of UDPG analogue for inhibitory effect test was 2 mM.

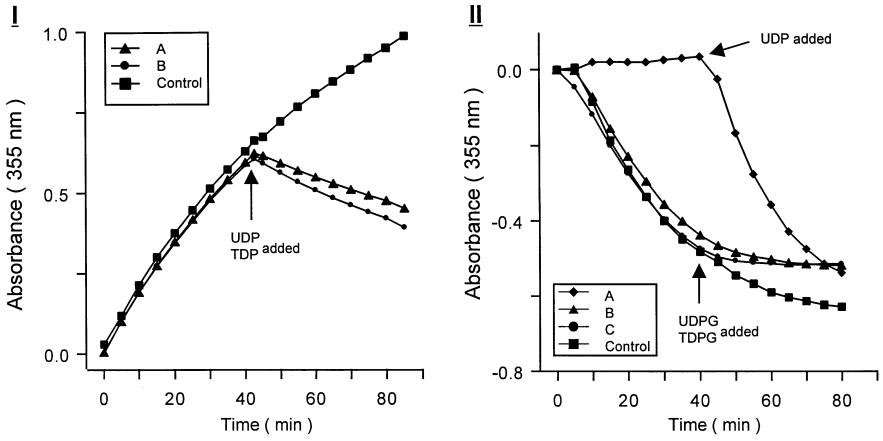


Fig. 4. Reversibility of the SGT reaction. (I) The enzymatic reaction was initiated in cuvettes containing SinA and UDPG. After addition of SGT, the absorbance at 355 nm was recorded at 5 min intervals for 40 min. At that point, TDP was added to cuvette A, or UDP to cuvette B, or buffer for the control, and the absorbance was monitored for another 40 min. (II) Four cuvettes were charged with incubation mixtures containing SinG and UDP. After addition of SGT, the absorbance at 355 nm was recorded at 5 min intervals for 40 min. Then, UDP was added to cuvette A, or TDPG to cuvette B, or UDPG to cuvette C, or buffer to the control, and the absorbance was monitored for another 40 min at 5 min intervals.

other hand, SGT was effectively bound on an immobilized sinapic acid matrix, from which it could then be eluted by free sinapic acid, but not by UDPG. While this pattern seems inconsistent with an "ordered bi–bi" model in which SGT would first bind UDPG and then sinapic acid, the overall mechanism may be more complex than such simple models suggest.

EXPERIMENTAL

Chemicals and plant material

UDP-[-β-D-glucose-(U-^{14}C)] was obtained from New England Nuclear; the protease inhibitors, from Boehringer Mannheim, and Macro-Prep ceramic hydroxyapatite matrix, from Bio-Rad Laboratories. The remaining chromatographic matrices were obtained from Pharmacia, and the Microsep centrifugal concentrators were obtained from Filtron Technology Corp. All other chemicals were obtained from Sigma Chemical Company. *Brassica napus* cv. Westar seeds were provided by Agriculture and Agri-food Canada, Saskatoon, Saskatchewan.

Preparation of crude enzyme

Fresh seedlings (~5 g) were ground to a fine powder in a mortar with liquid nitrogen and extracted with 5 ml extraction buffer (100 mM Tris/HCl, pH 6.5 with 4% (w/v) of insoluble polyvinylpolypyrrolidone (PVPP), 10 mM 2-ME and 5% (v/v) glycerol) at 4° for 1 h. The extract was centrifuged at 14,000 × g for 20 min. The supernatant was desalted on a PD-10

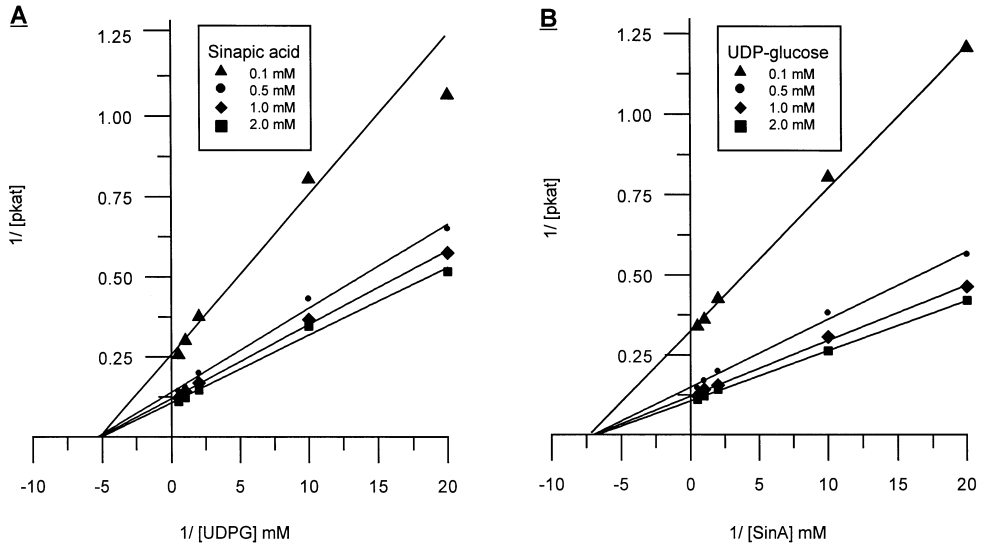


Fig. 5. Lineweaver-Burk plots of SGT reaction velocity at four different concentrations of the second substrate. (A) Determination of K_m for UDPG; (B) determination of K_m for sinapic acid. Partially purified SGT from seedlings of *B. napus* cv. Westar was used, and all data are the means of three measurements.

column equilibrated with the extraction buffer minus PVPP. The eluate was used as the crude enzyme solution.

Determination of protein concentration

Protein concentrations were measured using the dye-binding method [34], with bovine serum albumin as the standard.

SGT activity assay—Multi-well plate method

A simple semi-quantitative assay was developed for screening SGT activity during chromatography procedures. Each well of a flat-bottom 96-well plate was loaded with 25–50 μl enzyme solution and 25 μl standard substrate mixture (1.5 mM sinapic acid, 1.5 mM UDPG, 30 mM 2-ME, 30% glycerol and 240 mM MES, pH 6.0). After incubation for 30–60 min at 37°, the reaction was stopped by addition of 200 μl CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer, pH 11. These basic conditions result in a differential bathochromic shift in the absorption spectra of sinapic acid and sinapoylglucose, which allows measurement of the concentration of sinapoylglucose (yellow color developed) at 405 nm in a multi-well plate reader. For quantitative analysis, a standard curve was generated using the $OD_{405\,nm}$ and the corresponding concentration of sinapoylglucose standard (0, 0.25, 0.50 and 1.25 μmol/well).

3.1.5.. *SGT activity assay—HPLC method* In a 1.5 ml HPLC sample vial, 25–50 μl protein extract was incubated with 25 μl standard substrate mixture at 37° for 15–30 min. The reaction was stopped by adding 250 μl acetonitrile, then made up to a final volume of 1 ml with water. For HPLC analysis, 100 μl of this solution was injected onto a Hypersil C_8 reverse phase column (4.6 × 250 mm, MOS 5 μ, Alltech Chromatography Inc.). A linear gradient elution started from 30% to 50% solvent B (2% HOAc in acetonitrile) in solvent A (2% HOAc) in 2 min and held at 50% B for 0.5 min, then increased to 80% B over 0.5 min. The gradient was held at 80% B for 0.5 min and decreased to 30% B over 0.5 min, then held at 30% B for 2.5 min. The flow rate was 1.2 ml/min and eluate was monitored at 330 nm.

SGT activity assay—Radioisotope method

A radioactive substrate mix was prepared at a ratio of 1 μl UDP-[β-D-glucose (U-^{14}C)] (0.025 μCi, 125 pmoles UDPG per μl) to 24 μl standard substrate mixture. The enzymatic reaction started by incubating 10–50 μl enzyme solution and 25 μl radioactive substrate mix at 30° for 30 min. The reaction was stopped by adding 10 μl 6 N HCl. [^{14}C] labeled sinapoylglucose in the reaction was separated from the substrates by extracting with 1 ml EtOAc. The organic phase (0.7 ml) was transferred to a liquid scintillation vial and counted for radioactivity.

Induction of SGT by sinapic acid

B. napus cv. Westar seeds (0.1 g) were germinated under cool white fluorescent light at 23° in a 5 cm Petri dish with two layers of Whatman No. 1 filter paper wetted with a solution containing 0, 0.1, 1, 5 or 10 mM sinapic acid in a standard MS macronutrient solution (pH 6.5). The filter paper was moistened daily with fresh solution. Seedlings were harvested after 1, 2, 3, 4 or 5 days, rinsed with distilled water and patted dry on filter paper. The seedlings were frozen with liquid

nitrogen and stored at -70° before extracted for SGT activity measurement by the HPLC method.

Purification of SGT

The following procedures were used for large scale purification. All buffers contained 5% glycerol and a reducing reagent (2-ME or DTT) at the concentration indicated, and all procedures were performed at 4° except where noted. The FPLC was used for all column chromatography except size exclusion, which was performed on a ConSep 1000 LC system (Millipore Corp.).

Step 1. Protein extraction: Freshly harvested 65-h-old seedlings (800 g) were flash-frozen in liquid nitrogen and ground to a fine powder with a coffee mill containing a small amount of sea sand. The powder was extracted for 30 min with 1500 ml 100 mM potassium phosphate buffer (KPi, pH 7.0), including 5% glycerol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 15 mM 2-ME and 4% insoluble PVPP. The homogenate was filtered through 2 layers of Miracloth, centrifuged (15,000 × g; 30 min) and the supernatant re-filtered.

Step 2. Protamine sulphate (PS) precipitation: A 2% stock of PS was added to the supernatant to a final concentration of PS at 0.1%. The mixture was stirred for 30 min, and centrifuged (20,000 × g, 20 min). The supernatant was filtered (Miracloth) and dialyzed in an 8 kDa-cut-off dialysis tube against 2 L 10 mM KPi buffer pH 7.0, including 15 mM 2-ME. After 6 h, the buffer was renewed and dialysis was continued overnight. The dialyzed protein extract was then centrifuged (20,000 × g; 15 min).

Step 3. Hydroxyapatite: The supernatant from Step 2 was divided into 10 portions (150 ml each) and each portion was loaded individually at flow rate 4 ml/min on a Macro-prep hydroxyapatite column (HTP, 16 × 200 mm), which was equilibrated with a 10 mM KPi loading buffer, pH 7 (including 15 mM 2-ME). The unbound proteins were removed by washing with 250 ml loading buffer, and SGT was eluted with a linear increase of the concentration of KPi from 10 to 200 mM in 320 ml at a flow rate 8 ml/min. The fractions (12 ml) were collected and assayed for SGT activity by the multi-well plate method. The SGT-active fractions were pooled and dialyzed against Mono Q loading buffer (20 mM 2-ME and 20 mM Bis-Tris, pH 6.5) overnight. The buffer was renewed and dialysis continued for another day. The dialyzed HTP fractions were concentrated in dialysis tubes by water absorption with polyethylene glycol powder (MW 8,000). The dialyzed and concentrated HTP fractions were centrifuged (20,000 × g, 20 min).

Step 4. Anion exchange: The supernatant from Step 3 was divided into 8 portions (25 ml each). One portion at a time was loaded at a flow rate 2 ml/min onto an anion exchange column (Prep-Q HR, 10 × 100 mm), which was equilibrated with the Mono Q loading buffer. The unbound protein was removed by washing with 100 ml loading buffer, and SGT was eluted at a flow rate 2 ml/min with a linear gradient of NaCl (2 M NaCl in loading buffer) from 0 to 200 mM in a 160 ml volume. The fractions (5 ml) were collected and assayed for SGT activity by the multi-well plate method. The SGT-active fractions were pooled and precipitated by ammonium sulfate at 80% of saturation for 3 h. After centrifugation (20,000 × g, 30 min), the supernatant was discarded. The pellet was stored at -20° until used in the next step.

Step 5. The first size exclusion: The protein pellet obtained in Step 4 was dissolved in Superdex 75 loading buffer (20 mM Bis-Tris, pH 6.5, with 2 mM DTT and 150 mM NaCl), and divided into seven portions (2 ml each). Each portion was loaded at a flow rate 0.5 ml/min onto a Superdex 75 gel filtration column (10 × 1020 mm) equilibrated with Superdex 75 loading buffer. The SGT was eluted with 100 ml of the same buffer at 0.5 ml/min. The fractions (5 ml each) were collected and assayed for SGT activity by the multi-well plate method. The SGT-active fractions were pooled and used directly in the next step.

Step 6. Affinity binding: The pooled gel filtration fractions were divided into two portions (50 ml). Each portion was loaded at a flow rate 0.5 ml/min onto a blue dye affinity matrix column (HiTrip Blue, 16 × 25 mm) equilibrated with HiTrip Blue loading buffer (20 mM Bis-Tris/HCl, pH 6.5 and 2 mM DTT). Unbound proteins were removed with 60 ml loading buffer, and SGT was eluted at a flow rate 5 ml/min with a linear gradient of NaCl from 0 to 2 M in loading buffer, over a 50 ml volume. The fractions (4 ml) were collected and assayed for SGT activity by HPLC. The SGT-active fractions were pooled and precipitated by ammonium sulfate at 70% of saturation for 3 h. After centrifugation (20,000 × g, 30 min), the supernatant was discarded, and the pellet was stored at -20°.

Step 7. The second size exclusion: The protein pellet from Step 6 was dissolved in Superdex 75 loading buffer and divided into 3 portions (2 ml each). The second size exclusion was carried out on the Superdex 75 column using the conditions described in Step 5 except that the flow rate was 0.17 ml/min. Fractions (1 ml) were collected and assayed for SGT activity by HPLC. The SGT-active fractions were pooled and precipitated with ammonium sulfate (80% of saturation) for 3 h. After centrifugation (15,000 × g, 1.5 h), the pellet was dissolved in a Mono P loading buffer (25 mM 2-methyl-piperazine, pH 6.3 and 2 mM DTT), and desalted on a PD-10 column equilibrated with the loading buffer.

Step 8. Chromatofocusing: The desalted protein solution from Step 7 was divided into 3 portions (2 ml each). One portion at a time was loaded at a flow rate 0.5 ml/min onto a chromatofocusing column (Mono P HR, 5 × 200 mm) equilibrated with the Mono P loading buffer. The column was eluted with 42 ml Polybuffer 74 (10% Polybuffer 74, pH 4.5 and 2 mM DTT), and SGT was separated by a linear gradient of pH from 4.7 to 5.7, which was generated on the col-

umn by Polybuffer 74. In order to neutralize the acidity of the elution buffer, the fractions (1.5 ml) were collected in 2 ml microcentrifuge tubes containing 0.2 ml 250 mM Bis-Tris, pH 6.5 and assayed for SGT activity by HPLC. The SGT-active fractions were pooled and concentrated to a 2 ml volume using a 10 kDa-cut-off Microsep centrifugal concentrator.

Step 9. The third size exclusion: The concentrated protein solution from Step 8 was divided into 3 portions (0.7 ml each) and subjected to a third size exclusion on the Superdex 75 gel filtration column. The gel filtration conditions were the same as described in Step 7. The SGT activity in each fraction (1 ml) was detected by the radioassay method. The active fractions were concentrated using a Microsep centrifugal concentrator and stored at -20°.

A partially purified SGT preparation from Step 7 was used for characterization of the enzymatic properties, and activity was assayed by the HPLC method, unless otherwise indicated.

Determination of molecular weight

The molecular weight of native SGT (from Step 8) was determined by FPLC on a Superose 12 HR gel filtration column (10×30 mm), which was calibrated by a set of native low molecular weight reference proteins (Boehringer Mannheim). The buffer system was the same as described in Step 7, and the flow rate was 0.2 ml/min.

The molecular weight of denatured SGT was determined by 10% discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [35]. The gel was stained (Coomassie Blue R-250) and protein mobility assessed in comparison with a Bio-Rad low molecular weight protein mix.

Determination of SGT pH stability and pH optimum

The buffer in the SGT preparation was changed to 20 mM Tris/HCl (pH 7.0), 25 mM 2-methyl-piperazine (pH 6.3) or 10% Polybuffer 74 (pH 5.1) using PD-10 columns. All buffers contained 5% glycerol and 2 mM DTT. The protein solutions from each treatment were stored at -20° and sampled 1, 3, 6 and 12 days later. The treatment with 10% Polybuffer 74 was also sampled 0.5, 1, 2, 3 and 4 h after incubation on ice.

In order to investigate the optimum pH for SGT activity, 400 mM Tris/HCl buffers at pH 6.0–9.5, 400 mM MES/NaOH buffers at pH 5.4–6.5, and 400 mM NaAc/HAc buffers at pH 3.5–5.5 were employed. The SGT preparation (40 μl) was incubated with 25 μl 50 mM 2-ME in 50% glycerol and 25 μl test buffer at 4° for 2 h. The mixture was then incubated with 5 μl 10 mM sinapic acid in MeOH and 5 μl 10 mM UDPG in distilled water at 37° for 30 min.

Determination of SGT thermal stability and temperature optimum

The SGT preparation was stored at 23°, 4° and -20°, and sampled 2, 4, 8, 24 h, and 2, 4, 7 and 30 days later.

To establish the optimum temperature for SGT activity, the SGT preparation (50 μl) was first pre-incubated at 4°, 23°, 32°, 37°, 42°, 50° or 65° in a 1.5 ml HPLC sample vials for 15 min, and then mixed with 25 μl substrate mix. The reaction was incubated at the same temperature as pre-incubation for another 30 min.

Requirement for metal ions

Various metal ions, including NaCl, KCl, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$ and $ZnSO_4$, were tested for their effects on SGT activity. The SGT preparation (50 μl) was pre-incubated with 50 μl 2.5 mM metal ion mix, including 25 mM 2-ME, 25% glycerol and 250 mM MES, pH 6.0 at 4° for 1 h. Where the reversibility was to be tested, 25 μl 40 mM EDTA (made in 250 mM MES, pH 6.0) was added, otherwise 25 μl MES buffer. After incubation at 4° for 1 h, 10 μl SGT substrate mix (5 mM sinapic acid, 5 mM UDPG and 50% MeOH) were added and incubated at 37° for 1.5 h.

Requirement for reducing reagents

Reducing reagents (ascorbic acid, 2-ME or DTT) were tested at concentrations ranging from 0.1 to 20 mM when they were used individually, or at 5 mM each when they were applied in combination. The SGT source was the crude extract from 65-h-old seedlings, except that no reducing reagent was included in the extraction buffer. The desalted seedling extract (50 μl) was first pre-incubated at 4° for 1 h with 25 μl 50% glycerol plus 25 μl reducing reagent solution prepared in 0.4 M MES (pH 6.0). The mixture was then incubated with 25 μl substrate mix (2.5 mM sinapic acid, 2.5 mM UDPG and 5% MeOH) at 37° for 30 min.

Determination of SGT substrate specificity

In order to investigate specificity for the sugar donor, 50 μl SGT preparation was incubated with 80 μl substrate mix, including 0.4 M MES, pH 6.0, 15 mM 2-ME, 15% glycerol, 3% MeOH, 0.8 mM sinapic acid and 0.8 mM sugar donor (ADPG, CDPG, GDPG, TDPG, UDPG, UDP-galactose, UDP-mannose, UDP-xylose or β-D-glucose) at 37° for 15 min.

To test SGT specificity for the sugar acceptor, the radioisotope method was used. SGT preparation (50 μl) was incubated with 12 μl 2.6 mM glucose acceptor (cinnamic acid, *p*-coumaric acid, caffeic acid, 5-hydroxyferulic acid, ferulic acid, sinapic acid or syringic acid in 20% MeOH in 0.4 M MES; pH 6.0) and

13 μl radioactive substrate mix (minus sinapic acid) at 30° for 60 min. SGT activity was determined as described earlier.

Determination of the inhibitory effect of UDPG analogues

UDP-galactose, UDP-mannose, UDP-xylose, UDP, TDP and β-D-glucose were used as UDPG analogues to test for their inhibitory effect on SGT activity. The SGT preparation (50 μl) was pre-incubated with 50 μl 5 mM UDPG analogue mix, containing 25 mM 2-ME and 25% glycerol and 0.4 M MES, pH 6.0 in a HPLC sample vial at 4° for 1 h. The mixture was then incubated with 25 μl substrate mix (2.5 mM sinapic acid, 2.5 mM UDPG and 5% MeOH) at 37° for 30 min.

Determination of effects of other inhibitors

N-ethylmaleimide, Na iodoacetate, *p*-hydroxymercuribenzoic acid, phenylmethanesulfonylfluoride, 3,5-dihydroxybenzoic acid and 4-hydroxy-3,5-dimethoxybenzaldehyde azine, were tested for their ability to inhibit SGT activity. The SGT preparation (50 μl) was pre-incubated with 75 μl 1.67 mM inhibitor mix, including 16.7 mM 2-ME, 16.7% glycerol, 2% MeOH and 0.4 M MES, pH 6.0 at 4° for 2 h. The mixture was then incubated with 25 μl substrate mix (2.5 mM sinapic acid, 2.5 mM UDPG and 5% MeOH) at 37° for 15 min.

Determination of SGT reversibility—From the forward reaction

The enzymatic reaction was carried out in a 1.5-ml cuvette (A, B or C) containing 240 μl 50 mM 2-ME in 50% glycerol, 15 μl 44 mM sinapic acid in 20% MeOH, 15 μl 44 mM UDPG and 780 μl 150 mM MES (pH 6.0). The reaction was started by adding 150 μl SGT preparation to cuvettes A and B (the sample cuvettes), and 150 μl heat-denatured SGT preparation was added to cuvette C (the reference cuvette). The $OD_{355\,nm}$ of the reaction solution was recorded at five minute intervals. After 40 min reaction, 15 μl 100 mM TDP was added to cuvette A, 15 μl 100 mM UDP was added to cuvette B and 15 μl distilled water was added to cuvette C. These additions were designed to reverse the reaction direction in cuvettes A and B, as follows: SinA + TDPG ← SinG + TDP, or SinA + UDPG ← SinG + UDP. The decrease in $OD_{355\,nm}$ was monitored for another 40 min at five minute intervals.

From the reverse reaction

To each of four 1.5-ml cuvettes (A, B, C and D), 240 μl 50 mM 2-ME in 50% glycerol, 2 μl 44 mM sinapoylglucose, 6 μl 100 mM UDP (except to the cuvette A) and 802 μl 150 mM MES (pH 6.0), were added. The reaction was started by adding 150 μl SGT preparation to cuvettes A, B and C, and 150 μl heat-denatured SGT preparation to cuvette D. The $OD_{355\,nm}$ of the reaction was recorded at five minute intervals. After 40 min reaction, 15 μl 100 mM UDP was added to cuvette A, 15 μl 100 mM TDPG was added to cuvette B, 15 μl 100 mM UDPG was added to cuvette C and 15 μl distilled water was added to the cuvette D. The reverse reaction (SinA + UDPG ← SinG + UDP) was monitored in cuvette A, while forward reactions (SinA + TDPG → SinG + TDP, or SinA + UDPG → SinG + UDP) were monitored in cuvettes B and C. The $OD_{355\,nm}$ was monitored for another 40 min at 5 min intervals.

Determination of SGT substrate kinetics

The apparent K_m values of the enzyme for its substrates were determined by varying one substrate (sinapic acid or UDPG) from 0.1 to 2.0 mM at a fixed concentration of the second substrate. The SGT preparation (50 μl) was incubated with 50 μl 0.2 M MES buffer, pH 6 (including 25 mM 2-ME and 25% glycerol), 5 μl sinapic acid in 100% MeOH (2.2, 11, 22 or 44 mM) and 5 μl UDPG (2.2, 11, 22 or 44 mM) at 37° for 15 min. The kinetic parameters and reaction mechanism were analyzed using the EZ-Fit program (Dr. Frank W. Perrella, Glenolden Laboratory, E.I. DuPont de Nemours & Co.).

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