

Characterization and Properties of Different Glucosyltransferases Isolated from Suspension-Cultured Cells of *Daucus carota*

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Particulate enzymes (14,000 g pellet) from suspension-cultured carrot cells (*Daucus carota* L.) incorporated glucose from UDP-glucose and GDP-glucose into ethanol-insoluble products which were characterized as glucans or glucoprotein. Based on the test system to assay glucansynthetases I and II four different enzymatic activities could be distinguished on the basis of their substrate and divalent cation requirements, the influence of active substances such as nucleotides, nucleotide sugars, cellobiose, and *in vivo* inhibitors of cell wall glucan synthesis, their distribution in linear sucrose gradient and the nature of their products. The enzymatic activities which incorporated glucose from UDP-glucose or GDP-glucose at low substrate concentrations (10^{-6} M) were both localized in membranes of a density of $1.129 \text{ g} \cdot \text{cm}^{-3}$ (Golgi membranes) and synthesized a β -1,4-glucan chain. Both showed similar properties in most of the characterization experiments. The glucosyltransferase that catalysed the formation of a β -1,3-glucan from UDP-glucose (0.48 mM) was found in membranes which accumulated at a density of $1.170 \text{ g} \cdot \text{cm}^{-3}$ (plasma membrane) and differed in its properties from the Golgi-localized glucosyltransferase activities in many aspects. A soluble glucosyltransferase (175,000 \times g supernatant) which was also active at low concentrations of UDP-glucose (10^{-6} M) but showed enhanced activity under conditions where the other glucosyltransferases were inactive incorporated glucose into a proteinase-sensitive product. In linear sucrose gradients this enzyme migrated to different gradient densities depending on conditions.

Introduction

Although cellulose synthesis with cell-free preparations has not yet been achieved, the synthesis of β -glucans by particulate fractions is possible without any problem, as has been demonstrated on many objects including bacteria, fungi, monocotyledonous and dicotyledonous plants. But a comparison of the assay conditions as well as the treatment of the *in vitro* synthesized products used to assay the involved enzymes by different groups reveals a number of differences. Because a detailed characterization of the enzymes is often not given, the question is justified whether the same enzymes are assayed in all cases

since, for example, β -1,4-linkages occur in such quite different substances as xyloglucans, glucomannans, cellulose, mixed linked glucans, glycoproteins or glycolipids. Two β -glucansynthetases are widely used as marker enzymes to identify membrane fractions. These enzymes also serve as a basis for investigations of the *in vitro* synthesis of cell wall components (β -glucans) and are known as glucansynthetase I and II [1]. The assay conditions are based on work of van der Woude *et al.* [2], Raymond *et al.* [3] and Ray [1]. Depending on the substrate concentration and the availability of MgCl_2 , β -1,3 or β -1,4 linked glucans are synthesized. With UDP-glucose the glucansynthetase I, which is used as marker for Golgi membranes, mainly produces β -1,4-glucans and may be involved in xyloglucan synthesis as proposed by Ray [4] but contradicted by Hayashi and Matsuda [5], or may serve as a precursor of "cellulose synthase" [6]. A GDP-glucose: 1,4- β -D-glucan- 4- β -D-glucosyltransferase (EC 2.4.1.29) was reported to act in glucomannan synthesis [7–9].

The glucansynthetase II (UDPG) used as the marker for plasmamembranes mainly produces β -1,3-glucans [1]. Callose (β -1,3-glucan) is deposited rapidly on wounds or infections by pathogens [10, 11]. In order to get an insight into the control mecha-

Abbreviations: DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β -aminoethyl ether)N,N'-tetraacetic acid; GT IG, glucosyltransferase I, assayed with GDPG; GI IU, glucosyltransferase I, assayed with UDPG; GT IIU, glucosyltransferase II, assayed with UDPG; GT IsU, glucosyltransferase I soluble, assayed with UDPG; IDPase, inosine diphosphatase; PNP, *para*-nitrophenyl; TFA, trifluoroacetic acid.

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nisms of cell wall formation the properties of enzymes which are thought to be involved in the formation of cell wall components must be investigated in detail. In this paper, we will report on the detailed characterization of four glucosyltransferase activities which can be measured in crude homogenates and particulate fractions of suspension-cultured carrot cells based on the assay conditions described by Ray [1] including UDP-glucose and GDP-glucose as substrate. The effects of temperature, pH, divalent ions, nucleotides, nucleotide sugars and several inhibitors and activators on the enzyme activities were determined as well as the localization of the enzymes by linear sucrose density gradients. The products of the different glucosyltransferases synthesized by particulate and gradient fractions were also characterized.

Materials and Methods

Chemicals

Guanosine diphosphate glucose, [glucose- $^{14}\text{C}(\text{U})$] (8.87 G Bq·mmol $^{-1}$) was purchased from New England Nuclear (Dreieich, FRG) and uridine diphospho-D-[U- ^{14}C]glucose ammonium salt (10.8 G Bq·mmol $^{-1}$) from Amersham Buchler (Braunschweig, FRG). Unlabeled UDP-glucose was from Boehringer (Mannheim, FRG), unlabeled GDP-glucose, PNP-phosphate, PNP- α -mannopyranoside and PNP- β -mannopyranoside were from Sigma (München, FRG). Endo- β -1,4-glucanase (*Streptomyces*), endo- β -1,3-glucanase (*Rhizopus* Q 1031) and exo- β -1,3-glucanase (*Sporotrichum dimorphosporum*) were kindly donated by Prof. E. T. Reese (Food Science Laboratory, U.S. Army Research and Development Command, Natick, USA). TH-echinocandin B and TH-echinocandin B-(2-aminoethyl)-ether were generously provided by Dr. M. Dreyfuss (Sandoz Ltd., Basle, Switzerland), Papulacandin B by Prof. Dr. J. Nüesch (Ciba-Geigy, Basle, Switzerland), 2,6 dichlorobenzonitrile by Dr. J. Konze (Bayer AG, Leverkusen, FRG).

Plant material

Suspended cells of an anthocyan-containing variety of carrot (*Daucus carota*) were grown at 26 °C in the dark as previously described [12] on a modified Murashige-Skoog medium [13] and subcultivated every 7 days. Cells from the log phase (4–6d) were used for enzyme preparations.

Preparation of enzyme fractions

Cells were collected by filtration under suction. Usually, frozen cells were ground with a mortar and pestle under liquid nitrogen to a fine powder. The cell powder was transferred to homogenization buffer (Tris-Mes 100 mM, KCl 5 mM, sucrose 350 mM, pH 7.6) and carefully thawed at room temperature. 1.5 ml buffer was used per g cells (fresh weight). After thawing the homogenate was filtered through Miracloth (Calbiochem, Pösel, Frankfurt) and pre-centrifuged for 10 min at $1,200 \times g$. The supernatant was used for further centrifugation steps. The $1,200$ to $14,000 \times g$ pellet (40 min centrifugation time) was resuspended in Tris-Mes 100 mM, KCl 5 mM, sucrose 250 mM, pH 7.2, and used as the enzyme source for glucosyltransferase I with UDPG or GDPG as substrate and glucosyltransferase II. For GT I soluble a resuspended, desalted ammonium sulphate precipitate between 30 and 35% saturation of a $175,000 \times g$ supernatant was used as the enzyme source.

For membrane-separation experiments fresh cell material was used. The cells were homogenized in the presence of buffer (Tris-Mes 100 mM, KCl 5 mM, sucrose 350 mM, pH 7.6; 1.5 ml per g fresh weight) in the cold with a motor-driven (900 rev.min $^{-1}$; 12 strokes) Potter homogenizer with a Teflon pestle (Braun, Melsungen, FRG), filtrated through Miracloth and centrifuged at $1200 \times g$ for 10 min.

The supernatant was adjusted to 11% (w:w) sucrose and layered onto a linear sucrose gradient (32 ml) of 13% to 38% (w/w) sucrose in Tris-Mes 10 mM, KCl 5 mM, pH 7.2. A 1.5 ml cushion of 50% (w/w) sucrose was placed at the bottom of the tube. After centrifugation for 3 or 15 h at 4 °C in a Kontron TGA 65 (SW27 rotor) at 26,000 rev.min $^{-1}$ the gradient was fractionated into 1.4 ml fractions whose density was determined by refractometry. The protein concentrations of the different fractions were determined according to Bradford [14].

Enzyme assays

The activities of the different glucosyltransferases (GT IU, GT IG, GT IIU, GT IsU) were determined according to Ray [1] and Langebartels *et al.* [12] with various modifications. 20 μl of a reaction mixture containing ions, substrate and buffer substance were combined with 10 μl of either aqua dest. or stock solutions of active substances. The reaction was started by adding 50 μl of the enzyme fraction.

Table I. Composition of the reaction mixture. Values are final concentrations in the assay.

Composition of the reaction mixture	GT IU	GT IG	GT I soluble U	GT IIU
Tris-acetate pH 8	16.8 mM	16.8 mM	16.8 mM	16.8 mM
Substrate labeled*	UDPGlc 1 μ M	GDPGlc 1 μ M	UDPGlc 1 μ M	UDPGlc 1 μ M
Ions	MgSO ₄ 60 mM	MgSO ₄ 60 mM	ZnCl ₂ 1 mM	CaCl ₂ 1 mM
Substrate unlabeled	—	—	—	UDPGlc 0.48 mM
Test conditions				
Incubation time	up to 5 min	30 sec	up to 5 min	up to 10 min
Temperature	25 °C	25 °C	40 °C	25 °C

* UDP-glucose: 10.8 G Bq/mmol.

GDP-glucose: 8.87 G Bq/mmol.

Final concentrations and test conditions in the standard assay were as shown in Table I. In some cases, GT IIU was measured in presence of cellobiose (50 mM). The reaction was stopped by adding 0.5 ml TCA (5%). 50 μ l of a boiled supernatant (1,500 \times g; 10 min) was added to each sample to support precipitation and the reaction tubes were kept in boiling water for 1 min. After that, 1.5 ml 98% ethanol were added to each of the samples, which were stored for at least 2 h at 4 °C. The incorporation of labeled sugar into a 70% ethanol-insoluble product was measured. To remove unreacted substrate the samples were washed 4 times with 70% ethanol by centrifugation (5 min, 2,500 \times g) and resuspension of the sedimented material. The pellet of the last centrifugation step was resuspended in 2 ml of absolute ethanol and transferred quantitatively to 8 ml of a toluene-PPO-POPOP scintillant for counting.

The activity of hydrolases were determined using various paranitrophenyl derivatives as described by Hopp *et al.* [15] using PNP- α -mannopyranoside for α -mannosidase, PNP- β -mannopyranoside for β -mannosidase and PNP-phosphate for acid phosphatase. 0.4 ml of 0.1 M sodium citrate buffer (pH 4.5) containing 10 mM PNP-derivatives as substrate were preincubated for 5 min at 25 °C. The reaction was started by adding 0.1 ml of enzyme fractions. After incubation for 30 min (acid phosphatase, α -mannosidase) or 120 min (β -mannosidase) the reaction was terminated by adding 0.8 ml 0.2 M borate buffer (pH 9.8). The absorbance of para-nitrophenol was measured at 405 nm. Samples treated with borate buffer before adding the enzyme were used as controls.

Characterization of the products

Solubility: 70% ethanol-insoluble products were resuspended in water, KOH (24%), SDS (1%) or DMSO (100%) and incubated for 24 h at room temperature under a drop of toluene. After that, the samples were sedimented by centrifugation and the sediments were washed again with 70% ethanol before the radioactivity was determined.

Stability against acid: Products were heated for 4 h at 100 °C in TFA (2 N). TFA was evaporated, the samples resolved in aqua dest. and used for chromatography.

Enzymatic hydrolysis: *In vitro* synthesized products were washed as described for the standard assay procedure but without adding boiled material to support precipitation. The sediments of the last washing were resuspended in 0.5 ml buffer (Tris-Mes 10 mM, KCl 5 mM) or buffered enzyme solution, covered with 50 μ l toluene to prevent microbial contamination and incubated in test tubes with a marble on the top. Proteinase K (0.5 mg \cdot ml⁻¹), trypsin (0.5 mg \cdot ml⁻¹) and α -amylase (0.05 mg \cdot ml⁻¹) were incubated at pH 7 and 21 °C, the different glucanases (0.2 mg \cdot ml⁻¹) at pH 5 and 40 °C for 24 h. The incubation was terminated by adding 1.5 ml 98% ethanol and the samples were boiled for 1 min. Non hydrolyzed products were collected by centrifugation (3 washings in 70% ethanol) and resuspended in 98% ethanol, after which the radioactivity was determined as described above. The supernatants of the first washing containing the hydrolysed fragments were air-dried and redissolved in aqua dest. including sugar references for chromatography.

Paper chromatography

Samples of acid and enzymatic hydrolysis were separated by descending chromatography on Whatman 3MM paper (50 cm) for 28 h using isopropanol/acetic acid/water (29:4:9 v/v/v) as solvent. The standards were cochromatographed with the labeled fragments and detected with aniline phthalate (developed for 5 min at 100 °C). The chromatogram was cut into 25 segments of 2 cm each and the radioactivity was determined as described above in 6 ml scintillation cocktail.

Results

NDP-glucose-glucosyltransferases in carrots

Based on the test conditions for UDP-glucose- β -D-glucan β -D-glucosyltransferases (EC 2.4.1.12 and EC 2.4.1.34, *i.e.*, glucansynthetase I and II) according to Ray [1] four different enzymatic activities could be measured in crude homogenates and particulate fractions of suspension cultured carrot cells. With reference to the glucansynthetases I and II, the activities were called glucosyltransferase I with UDP-glucose (GT IU) or GDP-glucose (GT IG) (EC 2.4.1.29) when micromolar concentrations of substrate were used and glucosyltransferase II (GT IIU) for mil-

limolar substrate concentrations. In none of the cases tested could a glucosyltransferase be measured using millimolar concentrations of GDP-glucose. In membrane-free supernatants ($175,000 \times g$; 60 min) a UDP-glucose using glucosyltransferase was detectable which was called GT I soluble (GT IsU). The properties of this enzyme are quite different from the membrane-integral GT IU, GT IG, and GT IIU. The membrane-integral glucosyltransferases already sedimented nearly completely at $14,000 \times g$ (40 min). In the $30,000 \times g$ and $60,000 \times g$ pellet traces of GT IG and GT IU were present but no GT IIU activity. For this reason the $14,000 \times g$ pellet was used as the enzyme source to characterize the membrane-bound glucosyltransferases. The GT IsU could be precipitated by ammonium sulfate (between 30%–35% saturation) from the $175,000 \times g$ supernatant. The resuspended, desalted (Sephadex G-25) protein was used to characterize the "soluble" glucosyltransferase (GT IsU).

Localization of glucosyltransferases

The distribution pattern of the glucosyltransferases in a linear sucrose density gradient is shown in Fig. 1a. Three distinct areas of glucosyltransferase

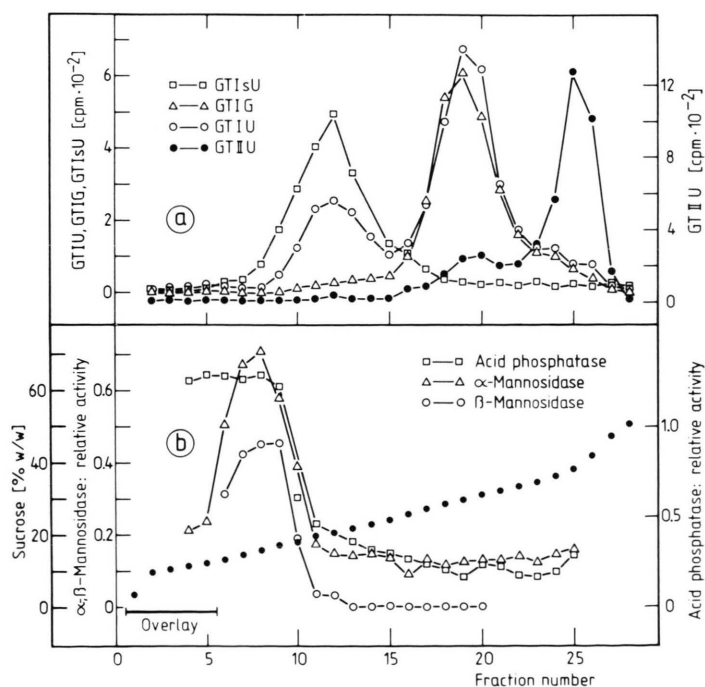


Fig. 1. (a-b) Isopycnic distribution of the relative activities of different glucosyltransferases (a) and possible marker enzymes of vacuoles (b) in a linear sucrose gradient (32 ml, 13–38% w/w; 1.5 ml cushion 50% w/w; 15 h; $80,000 \times g_{av}$) from $1,200 \times g$ supernatant of suspension cultured carrot homogenates. Fractions of 1.4 ml were collected. The activities of the glucosyltransferases are expressed as the incorporation of radioactive labeled sugar into ethanol insoluble glucan assayed according to Table I. Final concentration of UDP- $[^{14}C]$ glucose was 2 μ M, that of GDP- $[^{14}C]$ glucose 1 μ M. GT IIU was assayed in presence of cellobiose 50 mM.

activity can be distinguished from one another. The glucosyltransferase II accumulated at the beginning of the 50% sucrose cushion with a peak at 39.5% sucrose ($1.17 \text{ g} \cdot \text{cm}^{-3}$). In addition there was a small shoulder around 30% ($1.129 \text{ g} \cdot \text{cm}^{-3}$) sucrose. In linear gradients from 15–45% sucrose the maximum of its activity was at 40% sucrose ($1.179 \text{ g} \cdot \text{cm}^{-3}$). At 30% sucrose the only peak of GT I GDPG activity banded. Using UDPG as substrate (GT IU) there was also a maximum at 30% sucrose, but a second peak of GT IU activity banded around 20% sucrose ($1.08 \text{ g} \cdot \text{cm}^{-3}$). This second peak of GT IU corresponded with the profile of the GT IsU (tested at 40 °C in presence of ZnCl_2 1 mM). The GT IsU, however, showed no activity at 30% sucrose where the main peak of GT IU was found. Experiments with ammonium sulphate precipitated protein showed that GT IsU also functions under the test conditions of GT IU. On the other hand, the membrane-bound GT IU did not function under the test conditions of GT IsU.

In gradients (13–38% sucrose) which were only run for 3 hours at $80,000 \times g_{\text{av}}$ the maximum of GT IsU and the second peak of GT IU already appeared at 13 to 15% sucrose although the GT IG and the maximum of GT IU were found at 30% sucrose as in the gradient running for 15 hours. In this case, the activity of GT IsU was also found in the overlay fractions. It seems that the GT IsU slowly migrated from the overlay to higher densities with increasing centrifugation time.

Marker enzymes of plasmamembrane (K^+ -ATPase, EC 3.6.1.3) measured as described previously [16, 17] and Golgi membranes (latent IDPase, EC 3.6.1.6) assayed according to Ray *et al.* [18] in the presence of digitonin (0.015%) banded around the GT IIU (at 38%) or around the GT IU and GT IG (at 33%) although the maximum fractions did not coincide (data not shown). Under the conditions used the cytochrome *c*-reductase (EC 1.6.99.3) used as an ER marker and measured according to Hodges and Leonard [16] showed a broad distribution with increasing activity and highest values at 38% sucrose as also described by Wienecke *et al.* [19] using carrots in the presence of MgCl_2 (3 mM).

To elucidate the nature of the material which accumulated around 20% sucrose and showed the activity of the so-called GT IsU, possible marker enzymes for vacuoles were assayed (Fig. 1b). Acid phosphatase, α -mannosidase, and β -mannosidase all

were measurable in fractions of the gradient. Acid phosphatase showed high activities in the overlay and the first fractions of the gradient (up to 17% sucrose). The α -mannosidase and β -mannosidase also had a maximum at 16 to 17% sucrose whereas the maximum of GT IsU activity could be measured at 20% sucrose.

Activity and stability

The incorporation of glucose into 70% ethanol insoluble product by the four glucosyltransferases was quite different. Depending on the experiment, the values measured from crude homogenate per min and mg protein were as follows: GT IU 1–2 pmol, GT IG 30–50 pmol, GT IIU 1–2 nmol, GT IsU 0.5–1 pmol. All enzymes could be stored at –70 °C for several weeks without any marked loss of activity. High concentrations of protein were advantageous for storing. Interestingly, the enzymes – including the membrane-bound ones – remained active after lyophilization of the enzyme fractions ($14,000 \times g$ pellet and NH_4SO_4 precipitated protein) and resuspension.

Properties of the glucosyltransferases

Effect of temperature and pH: There was no great difference among the membrane-bound glucosyltransferases with respect to incubation temperature and pH. The best activities were obtained at a pH of 7.5 and 25 °C. The GT IIU showed a very sharp pH optimum curve whereas the rise and fall of the GT IU and GT IG curves were not so steep. At 30 °C and at pH 8 the activity of GT II was markedly diminished, but the GT IU and GT IG were only slightly reduced. The optimum for GT IsU was between pH 7 to 7.5. The incorporation of glucose increased continuously up to 40 °C. Heat-denatured protein (1 min, 100 °C) showed no activity.

Effect of ions: All four glucosyltransferases were dependent on divalent cations as shown by their inhibition by EDTA and EGTA, but the nature of the ions required differed (Fig. 2). GT IU and GT IG did not work in the absence of exogenously applied ions. Both enzymatic activities were stimulated by Mg^{2+} and Mn^{2+} ($> 0.1 \text{ mM}$). At concentrations higher than 10 mM the activities decreased again with MgCl_2 and MnCl_2 but remained high with MgSO_4 (up to 120 mM). Mg^{2+} or Mn^{2+} were essential for these activities. At high salt concentrations the Cl^- anion

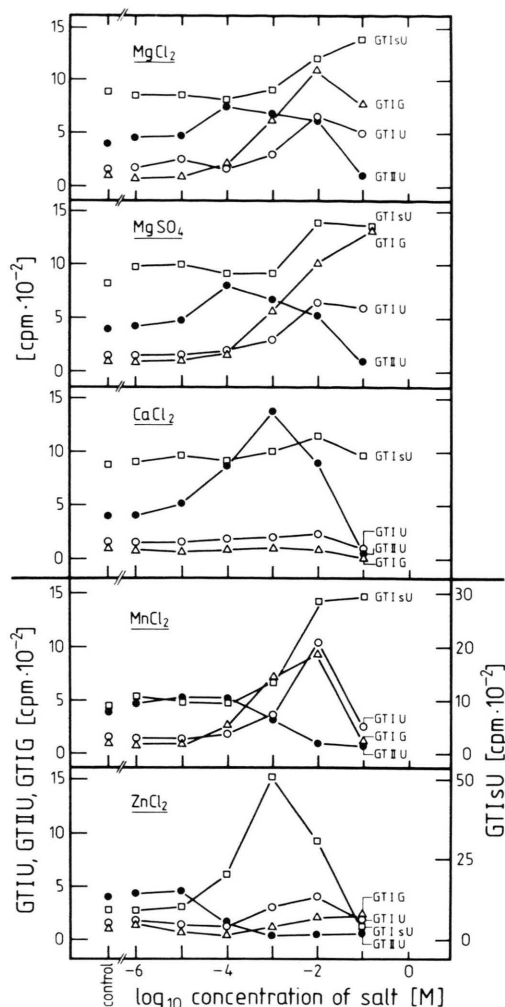


Fig. 2. Effects of divalent cations on the activity of the glucosyltransferases. The test conditions were as given in Table I but without ions in the reaction mixture. The concentration of ions in the assay was adjusted by adding stock solutions of the different salts. The content of protein in the assay and the time of incubation were 67 μ g, 3 min (GT IU); 18 μ g, 30 sec (GT IG); 31 μ g, 5 min (GT IIU); 43 μ g, 3 min (GT IsU). Incorporation of radioactive labeled glucose into ethanol insoluble glucans was measured.

had an inhibiting effect. GT IIU and GT IsU were active without the addition of exogenous ions. GT IIU was stimulated (80–100%) by low concentrations of Mg^{2+} (0.1 mM) and inhibited at concentrations higher than 10 mM. $CaCl_2$ (1 mM), however, caused a much higher activation of GT IIU (237%), but also inactivated the enzyme with increasing concentration to a total loss of activity at 100 mM $CaCl_2$.

The GT IsU was stimulated to various extents by all the ions used. The best activation was obtained with $ZnCl_2$ (1 mM), but $MnCl_2$ (10 mM) and $LaCl_3$ (1 mM) were also potent activators. In none of the cases could KCl replace the divalent salts. Incubation in the presence of a mixture of two salts which stimulated the glucosyltransferases when applied alone did not cause an additional stimulation of any of the four glucosyltransferases.

Effect of cellobiose: Only GT IIU was stimulated by cellobiose in concentrations higher than 1 mM. At 50 mM cellobiose the stimulation was about 2-fold. GT IU, GT IG, and GT IsU remained unaffected. Stimulation of GT IIU was obtained both in the absence and presence of Ca^{2+} (see also Fig. 3).

Substrate requirement: The app. K_m for GT IIU without exogenously applied ions in the assay was determined to be 1.52×10^{-3} M. The value was lowered by $CaCl_2$ (1 mM) to 0.6×10^{-3} M and $CaCl_2$ (1 mM)/cellobiose (50 mM) to 0.37×10^{-3} M. In addition, the app. V_{max} increased from 9.6 to 15.8 nmol incorporated glucose \cdot min $^{-1}$ \cdot mg $^{-1}$ protein. For GT IG an app. K_m of 1.3×10^{-6} M was measured. Depending on the range of the substrate concentrations used different results were obtained for GT IU. At concentrations of 1 to 21×10^{-6} M UDPG an app. K_m of 29.7×10^{-6} M was determined. The affinity to the substrate decreased and the app. V_{max} increased at higher concentrations of UDPG (app. K_m of 85×10^{-6} M at 2 to 152×10^{-6} M UDPG). This behaviour may be due to an activation of the enzyme by the substrate as also described by Delmer [20] for a glucansynthase in cotton or by the activation of another glucosyltransferase with increasing levels of substrate. The GT IsU showed the highest affinity to the substrate with an app. K_m of 0.55×10^{-6} M.

Effect of nucleotides (Fig. 4): In none of the cases examined could a significant stimulation of the glucosyltransferases by ATP, ADP, GTP, GDP, UTP and UDP (tested from 10^{-7} to 10^{-2} M) be found. On the contrary, all of the nucleotides showed an inhibitory effect at higher concentrations which were above the concentration of substrate used and therefore seemed to be non-specific. ADP and ATP had the least influence. All of the enzymes remained active up to 0.1 mM nucleotide concentration. GT IIU and GT IsU were best inhibited by UDP and UTP but only slightly affected by GDP and GTP. GT IU and GT IG were most inhibited by GDP and GTP, whereby GT IG was most sensitive.

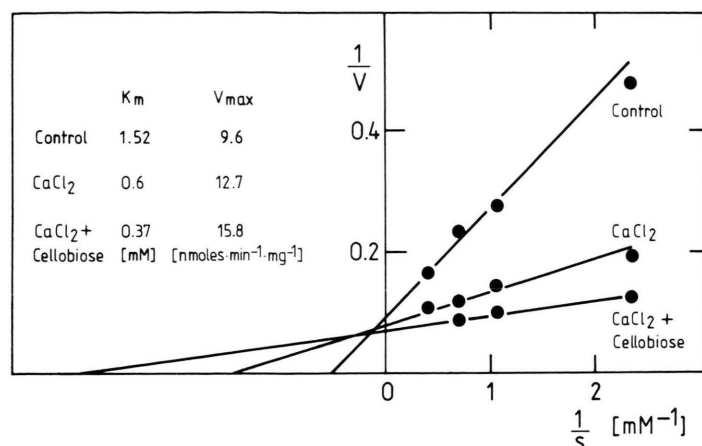


Fig. 3. Kinetics of ¹⁴C-labeled polysaccharide formation from high concentrations of UDP-[¹⁴C] glucose (GT IIU) without and in the presence of CaCl_2 (1 mM) or CaCl_2 (1 mM) and cellobiose (50 mM). The incubation was performed with 50 μg protein ($14,000 \times g$ sediment) for 5 min at 25 °C. Velocity (V) is given as nmol incorporated ¹⁴C-glucose per min and mg protein. Substrate concentration (S) is given in mmol per litre.

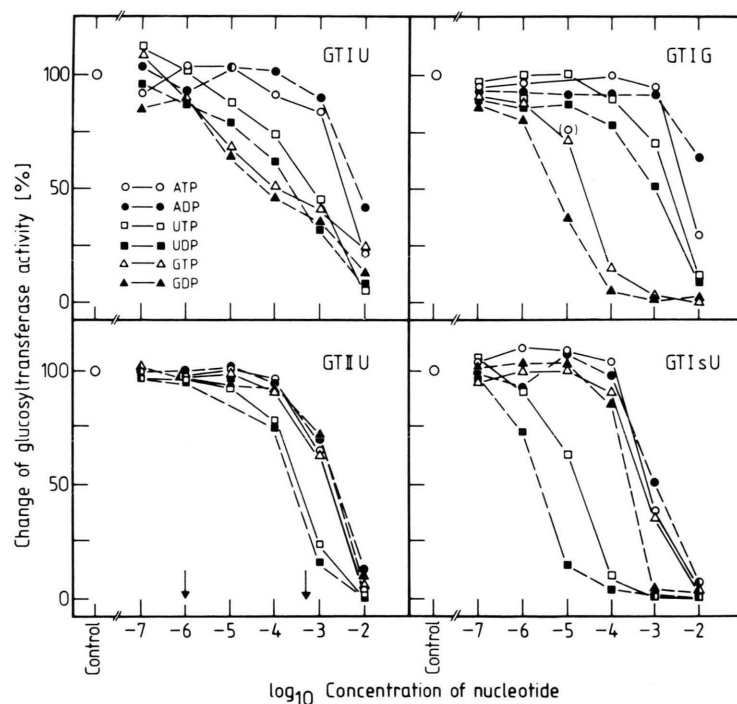


Fig. 4. Influence of different nucleotides on the activity of different glucosyltransferases. The activity is given in % referred to the control. The arrows indicate the concentration of substrate in the assay, 1 μM in case of GT IU, GT IG and GT IsU, 0.48 mM for GT IIU.

Additionally, GT IU was inhibited by UDP and UTP while GT IG showed less inhibition. In each case the nucleotide diphosphates had a stronger inhibitory effect than the nucleotide triphosphates.

Effect of other sugar-nucleotides: In order to examine whether the glucosyltransferases are involved in the synthesis of heteroglycans such as xyloglucan or glucomannan, other unlabeled sugar nucleotides

(UDP-galactose, UDP-xylose, UDP-N-acetylglucosamin, GDP-mannose) were added to the assay. At 1 μM UDP-xylose or UDP-galactose the incorporation of glucose by GT IsU was about 30 to 40% of the control. In the assay for GT IIU (0.48 mM UDPG) UDP-xylose (0.48 mM) caused a decrease of 40% and UDP-galactose (0.48 mM) a decrease of 25%. The inhibition increased with increasing concentra-

tions of UDP-xylose or UDP-galactose. GT IG and GT IU (concentration of labeled substrate $1\ \mu\text{M}$) showed no change in the incorporation of glucose when UDP-xylose ($1\ \mu\text{M}$) or UDP-galactose ($1\ \mu\text{M}$) were added. UDP-N-acetylglucosamin had no influence on GT IG, GT IU, GT IIU and GT IsU either when applied at the same concentrations as the substrate. Only GDP-mannose ($> 1\ \mu\text{M}$) increased the incorporation of glucose by GT IU and GT IG by elongating the period of the linear incorporation of glucose. GT IG in particular may play a role in synthesis of glucomannans (Ingold and Seitz, submitted).

Competition between UDPG and GDPG for glucosyltransferase I: The activity of GT I with $2\ \mu\text{M}$ UDPG was decreased in the presence of unlabeled $1\ \mu\text{M}$ GDPG by 33% and with $50\ \mu\text{M}$ GDPG by 57%. The same data were obtained with a substrate concentration of $32\ \mu\text{M}$ UDPG. With $77\ \mu\text{M}$ UDPG the inhibition at $1\ \mu\text{M}$ GDPG was 16% and at $50\ \mu\text{M}$ GDPG 36%. The activity of GT I with GDPG ($1\ \mu\text{M}$) was not affected when unlabeled $1\ \mu\text{M}$ UDPG was added. $10\ \mu\text{M}$ of UDPG caused a decrease of 12% and $50\ \mu\text{M}$ UDPG an inhibition of 19%. When the

concentration of labeled GDPG was raised to $6\ \mu\text{M}$ no inhibition by $50\ \mu\text{M}$ UDPG occurred. These data indicated that the inhibition in both cases is partial and competitive by a mechanism where two or more substrates are involved.

Effect of inhibitors: Different active substances which are known to influence the synthesis of cell wall components in *in vivo* systems were used in the *in vitro* assays described above. The protein contents were $60\ \mu\text{g}$ per assay for GT IU, GT IG and GT IIU and $21\ \mu\text{g}$ for GT IsU. Coumarin ($10\text{--}500\ \mu\text{M}$) [21, 22] and 2,6-DCB ($1\text{--}100\ \mu\text{M}$) [23, 24], known inhibitors of *in vivo* cellulose synthesis, were ineffective. Papulacandin B and echinocandin are inhibitors of β -glucansynthesis in yeast spheroplasts [25]. In the carrot system the glucosyltransferase II with UDPG was stimulated by tetrahydro-echinocandin B ($100\ \mu\text{g}\cdot\text{ml}^{-1}$) and its water soluble derivative tetrahydro-echinocandin B-(2-aminoethyl)-ether ($100\ \mu\text{g}\cdot\text{ml}^{-1}$) to 245% and 206% (Fig. 5) compared to the control (100%) while papulacandin B had a slightly inhibitory effect (10–20%) in the range of 10^{-6} to 10^{-4} M. Calcofluor white, a cellulose dye [26], which binds to the synthesized glucan chains *in vivo* and thus prevents crystallization but not glucansynthesis [27], totally inactivated GT IU, GT IG and GT IIU at 0.1% (w/w). GT IsU lost about 55% of its activity at 0.1% calcofluor white.

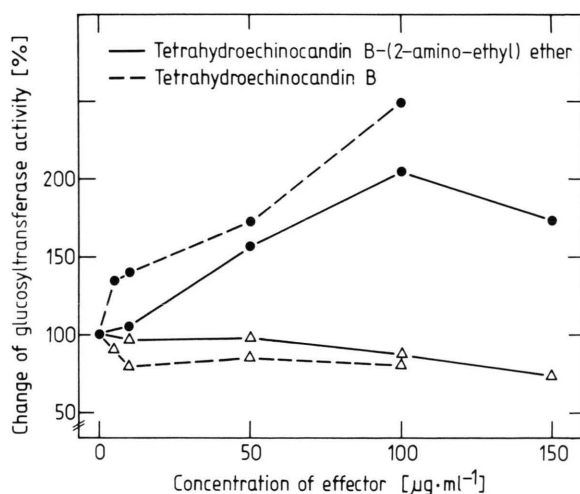


Fig. 5. Effects of tetrahydro-echinocandin B and tetrahydro-echinocandin B-(2-aminoethyl)ether on glucosyltransferase I GDP-glucose and glucosyltransferase II UDP-glucose. The protein concentration in the assay was $30\ \mu\text{g}$ ($14,000 \times \text{g sediment}$). Enzymes were incubated for 1 min (GT IG) or 10 min (GT IIU). The enzyme fractions were preincubated (10 min, 4°C) with the active substances. TH-echinocandin B was presolved in DMSO (final concentration in the assay was 2%) and its derivative in aqua bidest.

Characterization of the products

The *in vitro* products (70% ethanol insoluble) of all four glucosyltransferases were partly soluble in water. The solubility was enhanced by KOH. Only small amounts of 24% KOH insoluble products were found: GT IU 12%, GT IG 15%, GT IIU 2% and GT IsU 2%. Ninety percent of the product of GT IIU was soluble in 100% DMSO, whereas the products of the other glucosyltransferases were DMSO insoluble. The solubility of the GT IsU product in water was increased by SDS.

In order to determine their stability in acid the products were incubated with TFA (2 N, 4 h, 100°C), the whole sample chromatographed after evaporating the remaining TFA and the amount of radioactivity at the origin and at the glucose reference measured. 13% (GT IU), 47% (GT IG), 13% (GT IIU) and 0% (GT IsU) of the total recovered radioactivity remained at the origin. 75%, 48%, 87% and 99% of the radioactivity co-migrated with the

Table II. Enzymatic hydrolysis of the ethanol-insoluble products of glucosyltransferases GT IU, GT IG, GT IsU and GT IIU. Samples were treated with different hydrolases as described in Material and Methods. After incubation the samples were washed with 70% ethanol and the radioactivity in the remaining sediments was determined. Values are given as release of radioactivity in % from products by treatment with hydrolases as compared with the control (treated with buffer). The membrane sediment at $14,000 \times g$ (a) and the peak fractions of each glucosyltransferase of a linear sucrose gradient (see Fig. 1) (b) were used as source of enzymes. In case of GT IsU an ammonium sulphate precipitate (30–35% saturation) of a $175,000 \times g$ supernatant (a) was used instead of the $14,000 \times g$ sediment.

	GT I UDPGlc		GT I GDPGlc		GT II UDPGlc		GT IsU UDPGlc	
	a	b	a	b	a	b	a	b
	30% w/w		30% w/w		38% w/w		20% w/w	
α -Amylase	11	n.d.	0	n.d.	4	n.d.	0	n.d.
Trypsin	0	0	0	0	0	5	98	89
Proteinase K	0	10	n.d.	2	5	0	99	97
Exo- β -1,3-glucanase	14	17	3	25	84	93	0	0
Endo- β -1,3-glucanase	20	14	1	18	0	37	55	86
Endo- β -1,4-glucanase	71	93	84	98	79	60	32	14

n.d. = not determined.

glucose reference. The release of incorporated [^{14}C]glucose from ethanol insoluble products by various degrading enzymes was assayed. The products synthesized by unpurified enzyme fractions ($14,000 \times g$ sediment; ammonium sulfate 30–35% precipitate) were compared with the products of partly purified enzyme fractions of linear sucrose density gradient (Table II). The results showed that the products were not contaminated with starch. Proteinases were able to digest the product of GT IsU. The endo- β -1,3-glucanase preparation also attacked the product of GT IsU. The products of GT IU and GT IG were best degraded by the endo- β -1,4-glucanase and that of GT IIU by the exo- β -1,3-glucanase although the endo- β -1,4-glucanase preparation also was able to digest this product to some extent.

The products synthesized by the enzymes of sucrose gradient fractions seemed to be more homogeneous, as indicated by the enhanced percentage of release by the optimal hydrolase. The fragments of hydrolysis were separated by paper chromatography and identified by the co-chromatography of references. Although all glucanases of Table II released radioactivity from the products of GT IU and GT IG in varying amounts only glucose, cellobiose and some material at the origin were detected by paper chromatography. No laminaribiose as subunit of a β -1,3-glucan could be detected by this method. The products of GT IU and GT IG were β -1,4-glucans but the occurrence of some β -1,3-linkages is not absolutely excluded. The profile of the GT IIU product treated with exo-1,3-glucanase

showed only one peak of radioactivity which comigrated with glucose. When this product was incubated with endo- β -1,4-glucanase in addition to glucose, a peak of radioactivity at laminaribiose and in the area of oligoglucans could be seen. No radioactivity was detected on the spot of co-chromatographed cellobiose. These results revealed the product of GT IIU to be a β -1,3-glucan and that the used endo- β -1,4-glucanase preparation also contained β -1,3-glucanase activity. The product of GT IsU showed the properties of a glucoprotein whose side glucosyl chains must be very short.

Discussion

In cell-free systems, besides some β -1,3-linkages, mainly β -1,4-glucans are synthesized at micromolar concentrations of UDPG [28, 29]. When GDPG is used as a substrate the only linkage that is found is β -1,4 [9]. On the other hand, when millimolar concentrations of UDPG are applied a β -1,3-glucan chain is the predominant product and only small portions of β -1,4-linkages occur [3, 29]. These facts are indications of several glucosyltransferases working with different activities in the same assay. The use of purified membrane fractions by density fractionation or phase partition, for example, will lower the possibility that several glucosyltransferases are assayed in the same test. But probably different enzymes that are involved in the synthesis of cell wall components are located in the same membrane. An extended characterization of the *in vitro* active glucosyltrans-

ferases will help find the specific assay conditions which would make it possible to assay certain enzymes selectively.

Under the assay conditions for glucansynthases I and II, which are widely used as marker enzymes for Golgi and plasma membranes, four different glucosyltransferases (GT) were measurable in cell-free extracts of suspension-cultured carrot cells. In addition to the two enzymes mentioned above two further enzymatic activities were found at low concentrations (10^{-6} M) of substrate, one of which used GDP-glucose while the other one used UDP-glucose. The latter one was also found in particulate fractions and high speed supernatants and was called soluble (GT IsU).

In this system the separation of GT I with UDP-glucose or GDP-glucose from GT IIU in linear sucrose gradients was very good although a small shoulder of GT IIU appeared at densities where GT IU and GT IG accumulated (30%). But there was no activity of the glucosyltransferases working at low concentrations of substrate in the plasma membrane as reported for *Saprolegnia monoica* [30]. In sugar cane [31] the glucansynthases I and II were found in Golgi membranes as well as in the plasma membrane. And in *Lolium multiflorum* both were distributed throughout the endomembrane system but were also found in the plasmamembrane [32]. As the glucansynthases I and II are reported to be usable marker enzymes in many objects [33], the failure to separate the enzymes in density gradients may be typical for gramineae as suggested by Robinson and Glas [31].

In carrots UDP-glucose as well as GDP-glucose could serve as glucosyl donor to catalyse the synthesis of β -1,4-glucan chains at low substrate concentrations. Both enzymatic activities were located in membranes of the same density ($1.13 \text{ g} \cdot \text{cm}^{-3}$) which was also found by Ray *et al.* [18] using pea seedlings. The authors reported that the polysaccharide synthase particles which form β -1,4-glucan from UDP-glucose and GDP-glucose had a density of about $1.15 \text{ g} \cdot \text{cm}^{-3}$, which corresponds to Golgi membranes. These results indicate that the GT IU and the GT IG are not directly involved in the formation of cellulose, which in higher plants is thought to occur at the cell surface. Their function might be the formation of the glucan moiety in xyloglucan (UDPG) as suggested by Ray [4] and glucomannan (GDPG) as suggested by Elbein [7] and Villemez [8]. Although some differ-

ences existed the properties of GT IU and GT IG from carrots were quite similar and were not distinct enough to prove that two different enzymes are responsible for the incorporation of glucose from UDPG and GDPG. In addition GDP-mannose enhanced the incorporation of glucose from both sugar nucleotides, which UDP-xylose or other sugar nucleotides did not. The stimulation by GDP-mannose indicates the cooperation of different enzymes using these substrates (Ingold and Seitz, submitted).

On the contrary the inhibition of GT IU by GDPG and of GT IG by UDPG supports the idea that only one enzyme exists which can use both substrates with different affinities. On the other hand Henry and Stone [29] reported that in *Lolium multiflorum* only a 1,4- β -glucan was synthesized with GDPG, but no 1,3:1,4- β -glucan, in contrast to UDPG. This indicates the existence of two different enzymes. But mixed linked glucans are typical for monocotyledonous plants and we found no hint that 1,3:1,4- β -glucans were synthesized in our system.

Products which were synthesized by enzymes separated by linear sucrose gradients were better digestable by the corresponding glucanases than if particulate membrane fractions were used, indicating that fewer side reactions occurred. Under the assay conditions of GT IU a glucosyltransferase was active which, depending on the time of centrifugation, migrated into the gradient to different densities and which was also found in $175,000 \times g$ supernatants (GT IsU). This migration effect was taken as one criterion by Nagahashi and Baker [34] to show the solubility of a β -glucosidase which was thought to be associated to vacuoles.

Marker enzymes of vacuoles — α -mannosidase [35] and acid phosphatase [36] — accumulated at a density of $1.065 \text{ g} \cdot \text{cm}^{-3}$ (16% sucrose). The β -mannosidase which was discussed by Gross [37] and Hopp *et al.* [15] as a putative marker which may be bound or integrated into the tonoplast showed the same distribution as the α -mannosidase. Although in a 15-h run the GT IsU banded at densities which were cited in the literature for tonoplast (1.08 – $1.10 \text{ g} \cdot \text{cm}^{-3}$) we think that the GT IsU is more likely to be a soluble or detached enzyme than a vacuolar one. The properties of this enzyme were quite different from the membrane-bound ones. In contrast to them it was stimulated by ZnCl_2 and high temperature and it synthesized an ethanol-insoluble product which was protease sensitive.

Only the β -1,3-glucan synthesizing GT IIU which accumulated around 39% sucrose ($1.17 \text{ g} \cdot \text{cm}^{-3}$) was affected by cellobiose, a component which was reported to stimulate glucan synthesis [20, 29]. Cellobiose increased both the affinity to the substrate and the maximal velocity in the presence and absence of Ca^{2+} . Calcium also stimulated the GT IIU and might be involved in the regulation of β -1,3-glucan synthesis as shown by Köhle *et al.* [11] for the soy bean. The catalysis of β -1,3-glucan from particulate fractions of carrot was also affected by tetrahydro-echinocandin B and its water-soluble derivative tetrahydro-echinocandin B-(2-aminoethyl)-ether, which are described as causing the lysis of growing cells of *Candida albicans* [38]. Sawistowska-Schröder *et al.* [39] showed that echinocandin inhibited a β -1,3-glucan synthase assayed from particulate enzyme fractions of *Candida albicans*. Whereas Heininger [40] found that the activity of a UDP-glucose:1,3- β -glucan synthase in potato tubers was not affected, in carrots both active substances stimulated the *in vitro* activity of GT IIU.

The involvement of nucleotides in the regulation of glucosyltransferases as shown for *Acetobacter* [41] and certain fungi [42, 43] was not supported because

only inhibitory effects were obtained by the different nucleotides.

The four glucosyltransferases were all dependent on divalent cations as shown by their inhibition by chelating agents. By making use of the different requirements of the enzymes for divalent cations and their reaction to various active substances and assay conditions the selectivity of the assays could be enhanced even when total-membrane fractions were used as source of the enzymes.

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