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# Characterization of two glucan synthases from shoot of *Dendrocalamus latiflorus*

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**Abstract.** Two glucan synthases from shoot of *Dendrocalamus latiflorus* were characterized from plasma membrane preparations partially purified by two-phase partitioning method. One of the glucan synthases reacted at high substrate condition (1 mM UDP-glucose) has a pH optimum of 7.2 and reaction temperature optimum of 30-32°C. The other glucan synthase reacted at low substrate condition (3 $\mu$M UDP-glucose) has a pH optimum of 6.5 and temperature optimum of 15-20°C. Cellobiose and magnesium chloride are necessary for the enzyme activity and show significant effect of activation. Calcium chloride can not replace magnesium chloride in the reaction. Under the high substrate concentration, the kinetic constants for the substrate, UDP-glucose, $Mg^{2+}$ and cellobiose are 0.82 mM, 0.53 mM, and 2.1 mM, respectively. Under the low substrate condition, the kinetic constants for the $Mg^{2+}$ and cellobiose are 0.62 mM and 3.1 mM, respectively. Solubility of the enzyme products in 24% KOH was found different between the two glucan synthases where the product from high substrate glucan synthase was soluble, and the product from low substrate glucan sysnthase was not. Digitonin and CHAPS both at specific concentration showed significant effect of activation on the glucan synthase activity. Periodate oxidation and methylation analysis were used to show that both glucan synthase products are 1-3 glycosidic linkage. Localization study by sucrose density gradient centrifugation revealed that the enzyme activities of both glucan synthases coincided with the plasma membrane fraction even though high substrate glucan synthase showed more than one peak. Thus it would seem that these two glucan synthases are functionally different enzymes.

**Key words:** *Dendrocalamus latiflorus*; Glucan synthase; Two-phase partitioning.

## Introduction

$\beta$-glucan synthases which use UDP-glucose as substrate have been found associated with the plasma membrane and the Golgi apparatus (Ray *et al.*, 1969). It is still an open question as to which way these enzymes are localized within the membrane systems. It now seems fairly well-accepted that there are two UDP -glucose: $\beta$-glucan synthase activities in plant cells. One of these, cellulose synthase, is active in intact cells; callose synthase is latent in intact cells and rapidly activated in response to a variety of perturbations. So far in higher plants, almost all attempts to obtain *in vitro* synthesis of cellulose have resulted in the synthesis of callose, ((1-3)-$\beta$-glucan), using radioactive UDP-glucose as the substrate with membrane preparations derived from higher plants (Delmer, 1977). It also has been found that the product synthesized by the *in vitro* preparation is also dependent upon the concentration of UDP-glucose. High level of UDP-glucose, in mM concentration, give predominantly $\beta$-(1-3) linkages (Peaud-lenoel and Axelos, 1970; Franz and Heiniger, 1981). In cereals, like the cell walls from suspension -cultured ryegrass endosperm cells contain 49% 1,3: 1,4 -$\beta$-glucans (Smith and Stone, 1973). These mixed-lin-

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kage $\beta$-glucans have also been found in several other monocots (see refs in Henry and Stone, 1982), so may be different glucan synthases in addition to the above mentioned are also involved in cell wall biosynthesis.

We are interested in the glucan synthase system in bamboo comparing with those of other system. Bamboo offers a large quantity of material for the membrane preparation and the glucan synthase activity is reasonably high in a preliminary experiment. In this communication we describe the properties of two glucan synthases isolated from the shoot of *Dendrocalamus latiflorus*.

## Materials and Methods

*Chemicals*

All chemicals were purchased from Sigma Chemical Co. BCA protein reagent was from Pierce Chemical Co. UDP-($^{14}$C)-glucose (300 mCi/mmole) was from Amersham International.

*Plant Materials*

The fresh shoot of *Dendrocalamus latiflorus* was purchased from the local market. After bringing to the lab, the vagina (bract) were removed and the fresh shoot was used for the isolation of plasma membrane.

*Tissue Homogenization*

Bamboo shoot 100 g were homogenized with 200 ml of homogenization buffer (250 mM sucrose, 70 mM Tris-HCl, pH 7.2, 10 mM Bis-Tris-Propane, 3 mM dithiothreitol (DTT), 0.1% bolvine serum albumin (BSA), 0.5% polyvinylpyrrolidon (PVP). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 10,000 g for 15 min. The supernatant was centrifuged at 100,000 g for 30 min again and the resulting crude membrane pellet was resuspended in 250 mM sucrose, 5 mM $KPO_4$, pH 7.8 at a concentration about 15 mg/ml.

*Plasma Membrane Preparation*

Two-phase partitioning was used for the preparation of plasma membrane. A 2 ml sample of the resuspended crude membrane pellet was added to a premade, 8.0 g, two-phase system. The two phase system was prepared according to Kjellbom and Larsson (1984), and consisted of 7.75% dextran T500, 7.75% PEG 3350, 312.5 mM sucrose, 3.75 mM KCl, 6.25 mM $KPO_4$, pH 7.8. The two-phase system was then accelerated by centrifugation at 2400 g for 10 min. The upper phase was collected and washed by vigorously mixing with a fresh lower phase and then was centrifuged again at 2400 g for 10 min. The final upper phases were diluted 10 folds with homogenization buffer and centrifuged at 120,000 g for 30 min. The plasma membrane pellets were resuspended in 0.46 M sucrose, 5 mM MES, pH 7.5 and stored at small vials in liquid nitrogen.

*Enzyme Assay*

Two glucan synthase activities were detected in the membrane preparation. A preliminary experiment was carried out to determine the optimal condition for the assay. Basically the assay condition can be separated as high substrate and low substrate condition. When enzymes were assayed for high substrate glucan synthesis, the complete mixture (total volume 200 $\mu$l) contained 20 mM TES, pH 7.2, 5 mM $MgCl_2$, 10 mM cellobiose, 1 mM UDP-($^{14}$C)-glucose (500 cpm/nmole), and enzyme preparation 1-4 $\mu$g. The mixtures were incubated at 32°C for 30 min. Reactions were terminated by addition of 3 ml of 66% ethanol. About 10 mg $\alpha$-cellulose was added to the reaction vessel as a carrier to faciliate precipitation of ethanol-insoluble glucan. The sample was stored at -20°C for 30 min (to overnight) and then filtered through a Whatman GF/C glass fiber filter under suction. The filter was washed 3 times with 66% ethanol and dried. Radioactivity was determined by liquid scintillation counting in 5 ml of Beckman Ready-Solv cocktail using a Kontron BETAmatic liquid scintillation system. When enzyme were assayed for low substrate glucan synthesis, the complete mixture (total volume 200 $\mu$l) contained 20 mM MES, pH 6.5, 3 $\mu$M UDP-($^{14}$C)-glucose (200 cpm/pmole), 5 mM $MgCl_2$, 10 mM cellobiose, 0.003% digitonin and enzyme preparation 1-4 $\mu$g. The mixtures were incubated at 20°C for 30 min. Rest of the procedure is same as that for high substrate glucan synthesis. Enzyme activity was expressed as nmole or pmole of glucose incorporated into glucan per mg protein per min.

The activities of UDP-glucose sterol glucosyltransferase and latent IDPase were used as the marker enzymes for plasmalemma and Golgi, respectively. The assay method was described by Chanson *et al.* (1984). $Mg^{2+}$-$K^+$-ATPase activity (Hodges and Leonard, 1974) was also used as the marker enzyme for plasmalemma

in the experiment of two phase partitioning.

*Protein Solubilization and Assay*

Plasma membrane was solubilized in 0.1% Triton but the final concentration of Triton was adjusted to < 0.01%. Protein concentration was determined by the method of Smith *et al.* (1985) using the Pierce Chemial Co. prepared BCA reagent and BSA was used as the standard.

To solubilize the glucan synthase from the membrane system, enzyme preparation was mixed with different concentration of digitonin or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS). The mixture was incubated on ice for 30 min with occational vortexing. Afterwards the mixture was assayed for glucan synthase activity under high or low substrate conditions as that mentioned under "enzyme assay".

*Periodate Oxidation and Paper Chromatography*

Periodate oxidation was performed by a method described previously (Heiniger and Delmer, 1977). Separation of monosaccharides was achieved by descending irrigation of a 50 cm long of Whatman No. 1 paper with n-butanol-pyridine-water (6:4:3) for about 20 hours. Reducing sugars were identified by the method of Trevelyan *et al.* (1950).

*Methylation Analysis of Reaction Product*

Glucan product was prepared with a scale-up assay with 20 fold as that used in standard assay and the incubation was allowed to proceed 12 hours. Samples were then washed with water and ethanol, and dried in vacuo. Methylation was conducted by the procedure of Hakomori (1964) as modified by Harris *et al.* (1984). Methylated polysaccharide was isolated by overnight dialysis against distilled water. The methylated polysaccharide was hydrolyzed using 2 N trifluoroacetic acid and reduced acetylated as described (Harris *et al.*, 1984). Partially methylated alditols were separated in HP 5890A gas chromatography equipped with FID (300°C). A OV 17 column, 25 m × 0.2 mm, was used, and the temperature elevated from 160°C (2 min) to 240°C (10 min), with a rate of 2°C/min. Identities of the alditols were confirmed by comparison of their relative mobilities with alditols derived from cellulose ($\beta$-1, 4-linkage) and lichenin (mixture of $\beta$-1, 3 and $\beta$-1, 4-linkages).

*Homogenization and Linear Sucrose Gradients*

Bamboo shoot 10 gm were chopped at 4°C with razor blade in the presence of 6 ml 50 mM Hepes-KOH, pH 7.0, 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT. Homogenate was filtered through nylon and centrifuged at 1000 g for 10 min to remove cell-wall debris and nuclei. Filtered homogenate was centrifuged again at 6000 g for 10 min to sediment mitochondria. Five ml of supernatant was layered onto 25 ml linear 20-55% (W/W) sucrose gradients made up in 50 mM Hepes-KOH, pH 7.0, 0.1 mM $MgCl_2$, 1 mM DTT in 2.5 × 8.8 cm Ultraclear tubes. The gradients were centrifuged 3 h at 130,000 g in SW 28 rotor (Beckman L8-M ultracentrifuge). Each gradient was divided into 28 fractions (about 1.25 ml each).

## Results and Discussion

*Plasma Membrane Preparation*

Table 1 shows the purity of plasma membrane after the isolation by two-phase partitioning technique. $Mg^{2+}$-$K^+$-ATPase is used as the marker enzyme for plasma membrane. The enzyme activity of upper and lower phase is about 30% of the total ATPase activity. This indicated inactivation of this enzyme happened during the process of purification. The low recovery also happened to glucan synthase. However the specific activity of glucan synthase has been highly enriched in the upper phase fraction. The upper phase could not be further purified by detergent treatment. So the upper phase was considered as the plasma membrane fraction and used for the following experiments.

The specific activity of glucan synthase under high substrate condition (HGS) was 35.6 nmole/min · mg by measuring the activity under suboptimal condition. The value could be much improved and reached 110 nmole/min • mg protein, if the optimal assay condition was used.

*Component of Reaction Mixtures*

Before the properties of glucan synthase were studied, the linearity of enzyme quantity and activity of HGS has been checked. Results showed that the linearity could be obtained for the protein up to at least 50 $\mu$g in each assay. In addition, the linearity of reaction time and product increment were agreed each other well.

From the literature, the activity of glucan synthase

**Table 1.** *Plasma membrane enrichment by two-phase partitioning.*
Experiment started with 200 g fresh shoot of *Dendrocalamus latiflorus*.

| Fraction | Total volumol (ml) | Total protein (mg) | Mg-K-ATPase | | Glucans synthase | |
|---|---|---|---|---|---|---|
| | | | Total activity (nmol Pi • $min^{-1}$) | Specific activity (nmol Pi • $min^{-1}$ • $mg^{-1}$) | Total activity (nmol Glu • $min^{-1}$) | Specific activity (nmol Glu • $min^{-1}$ • $mg^{-1}$) |
| Microsome | 11 | 213 | 10811 | 50.7 | 2440 | 11.4 |
| Upper phase | 8 | 22.6 | 1848 | 81.7 | 807 | 35.6 |
| Lower phase | 65 | 41.4 | 1586 | 38.3 | 100 | 2.41 |

is influenced by chemicals like: $MgCl_2$, $CaCl_2$, cellobiose, glycerol, CHAPS and Triton. In order to obtain the optimal assay condition for glucan synthesis, the chemicals were checked. The results showed: $CaCl_2$ could not replace $MgCl_2$ in the reaction mixture. $CaCl_2$ reduced the product to 40% when compared with $MgCl_2$. $MgCl_2$ was very much needed for glucan synthesis. The product reduced to 20% if $MgCl_2$ were omitted from the reaction mixture. Cellobiose was needed too. The product reduced to 40% or less if cellobiose were not included. Triton at 1% final concentration inhibited the glucan synthesis up to 95%, and at 0.1% final concentration indeed could increase the product for glucan synthesis.

*PH Optimal*

The result of pH optimal is presented in Figure 1. These experiments showed that there are two different pH curves for the glucan synthesis. Both curves were narrow in the optimal pH ranges. pH 6.5 is optimal for the glucan synthase under low substrate concentration (LGS), and pH 7.2 is optimal for the HGS.

*Optimal Reaction Temperature for Glucan Synthesis*

Temperatures 10-45°C were tested for the glucan synthesis. Under the high substrate condition, 30-32°C is the best temperature for glucan synthesis. Under the low substrate condition, 15-20°C is the optimal temperature (Fig. 2).

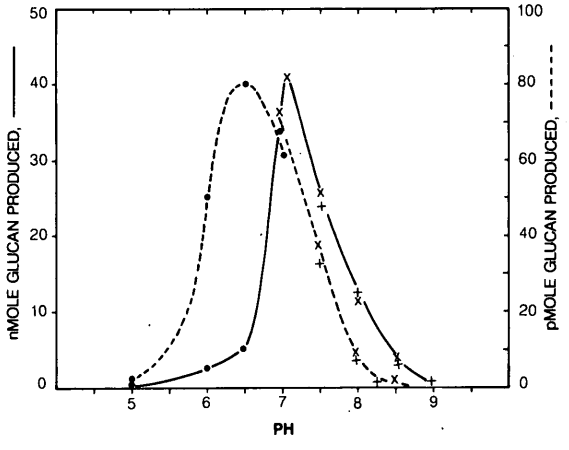


Fig. 1. pH dependence of β-glucan synthesis from 1 mM UDP -glucose (——), and 3 μM UDP-glucose (----).

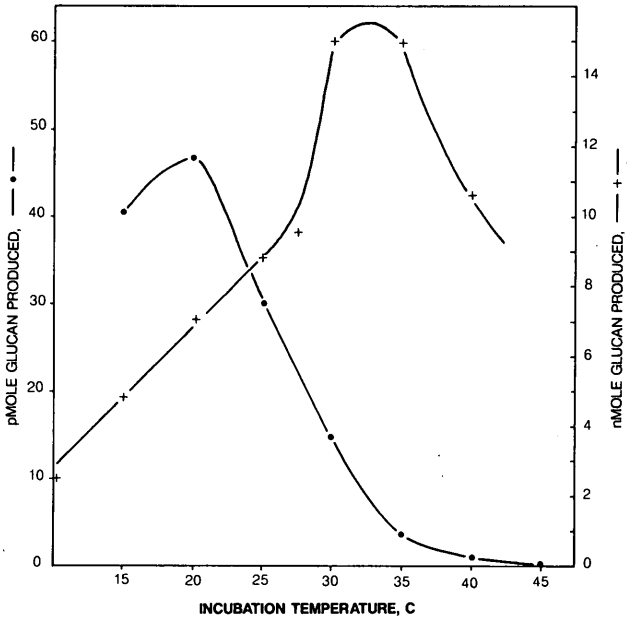


Fig. 2. Effect of incubation temperature (10-45°C) upon the incorporation of radioactivity from 1 mM (+) or 3 μm (●) UDP-($^{14}$C) glucose into glucan.

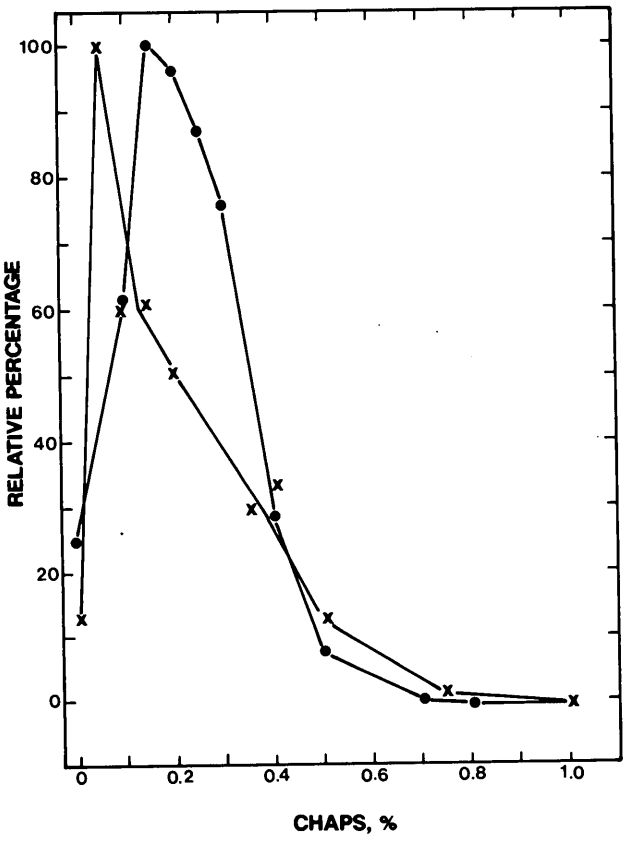


Fig. 3. Glucan synthase activity under high (×) and low (●) substrate assay condition after the membrane was solubilized with CHAPS in different concentrations.

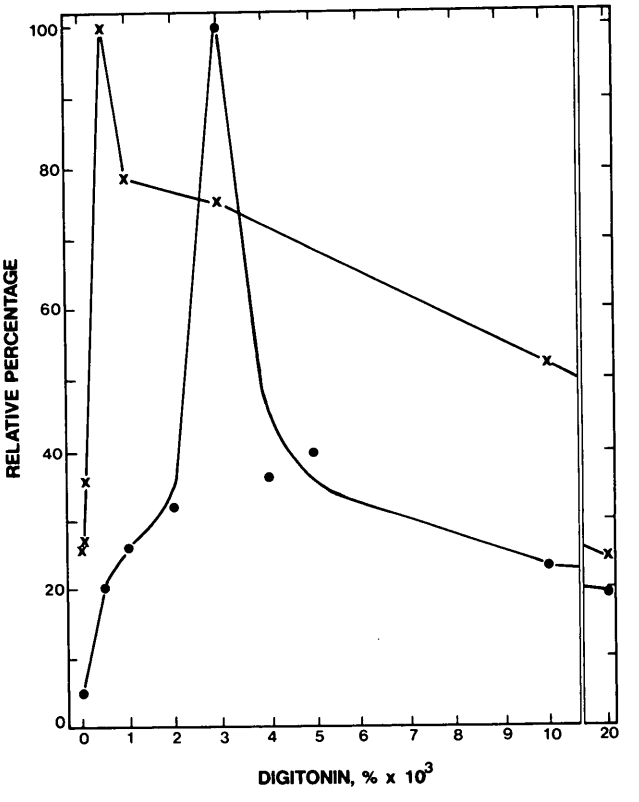


Fig. 4. Glucan synthase activity under high (×) and low (●) substrate assay condition after the membrane was solubilized with digitonin in different concentrations.

*Solubility of the Products in Ethanol, KOH and Acetic-nitric Reagent*

Since callose, the β-1, 3-linkage, and cellulose, β-1, 4-linkage, are different in their solubility in KOH and acetic-nitric reagent (Updegraff, 1969), we were interested in knowing the property of the products of HGS and LGS. Table 2 showed the results of solubility study. The radioactivity in the precipitates of 66% ethanol was considered as 100% in both the high substrate and low substrate concentration. The major difference was in the solubility in 24% KOH. The product from low substrate glucan synthesis was not dissolved in 24% KOH. On the contrary, the product from the high substrate glucan synthesis was highly dissolved in 24% KOH. Both products were almost completely dissolved in acetic-nitric reagent. These results indicated that the glucans synthesized from HGS and LGS may be different in chemical structure.

**Table 2.** *Solubility of reaction product from glucan synthase under the condition of high UDP-glucose and low UDP-glucose concentrations*

The reaction conditions see "Materials and Methods"

| | Reaction product insoluble in | | |
|---|---|---|---|
| | 66% Alcohol | 24% KOH | Acetic-nitric reagent |
| High UDP-glucose | 100[1] | 39 | 1 |
| Low UDP-glucose | 100[1] | 94 | 2 |

[1]The product quantity in cpm was considered as 100%.

*Solubilization of Glucan Synthase in Detergents*

After the dissociation of plasma membrane by the detergent, CHAPS and digitonin at different concentration, the glucan synthase activity was then assayed under high substrate condition and low substrate condition. Under both assay conditions, CHAPS activated

glucan synthase for several folds (Fig. 3). Under high substrate condition, the optimal CHAPS concentration is 0.05%, which is compared with 0.15% of the low substrate condition (Fig. 3). HGS was also activated by digitonin and showed an optimal final reaction concentration of 0.0005% (Fig. 4), while the LGS was activated at a final concentration of 0.003% (Fig. 4).

*Kinetic Study*

Under different substrate, UDP-($^{14}$C)-glucose, concentrations, the reaction rates were measured. The Km for UDP-glucose was obtained from a double reciprocal plot. Km for the high substrate glucan synthesis is about 0.82 mM UDP-glucose (Fig. 5). On the other hand, we would never able to obtain the Km for UDP -glucose under low substrate assay condition because the LGS was largely masked by HGS (data not shown).

The effects of divalent cation, $Mg^{2+}$, were also examined for HGS and LGS. Both enzyme activities were strongly activated by $Mg^{2+}$ with a Km of 0.52 mM (Fig. 6A) and 0.62 mM (Fig. 6B) respectively. Cellobiose strongly activated both the glucan synthase activity under the high substrate condition (Fig. 7A) and the low substrate condition (Fig. 7B). Km for HGS and LGS is 2.1 mM and 3.1 mM respectively.

*Reaction Product Analysis*

Periodate oxidation followed by borohydrate reduction, complete acid hydrolysis of the glucan product yielded primarily glucose, either under high substrate assay condition (Fig. 8, lane 2) or low substrate condition (Fig. 8, lane 4). In no case could a significant peak of erythritol (indicative of 1-4 linkages) be detected, however erythritol was largely produced from cellulose (Fig. 8, lane 3). A duplicate of the paper chromatogram was cut into 0.5 cm strip and was counted for the radioactivity, the major peak was found to be coincided well with the position of glucose (Fig. 9). This is because 1-3 linked glucans which are resistant to per-

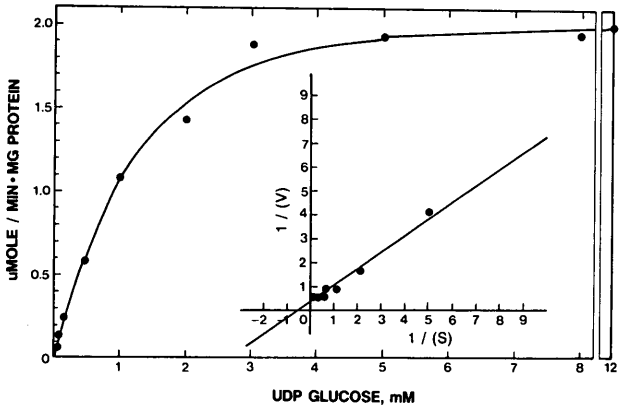


Fig. 5. Lineweaver-Burk plot, showing the relationship between the reciprocals of the initial substrate concentration (UDP-glucose) and initial rate of reaction for the glucan synthase under high substrate assay condition (see "Materials and Methods").

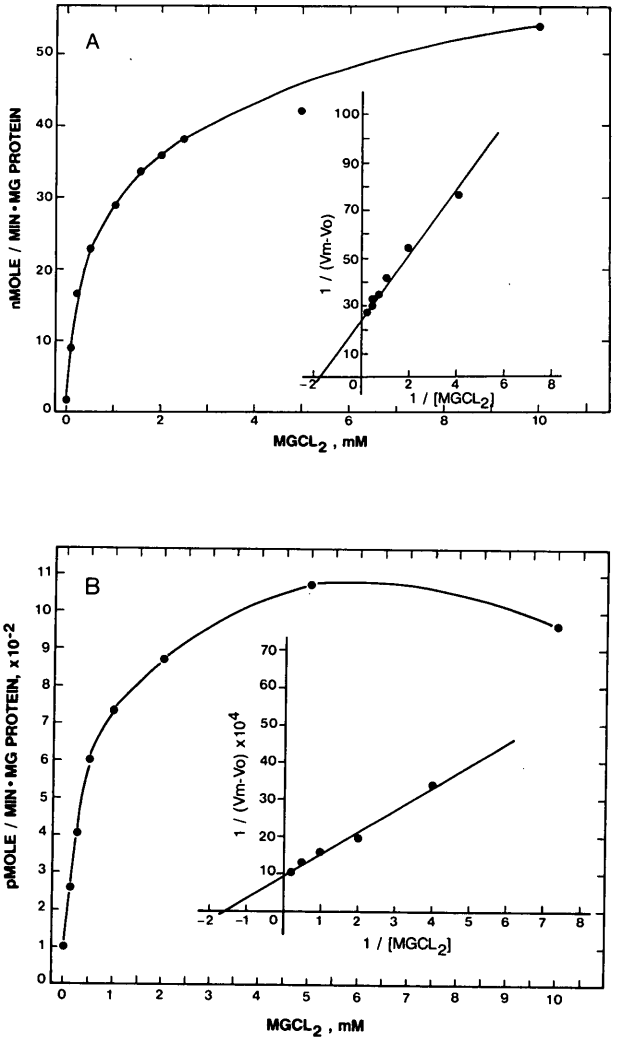


Fig. 6. The activation of glucan synthase by $MgCl_2$ under high (A) and low (B) substrate condition. The synthesis reaction mixture is described under "Materials and Methods" except that the concentration of $MgCl_2$ was varied as indicated.

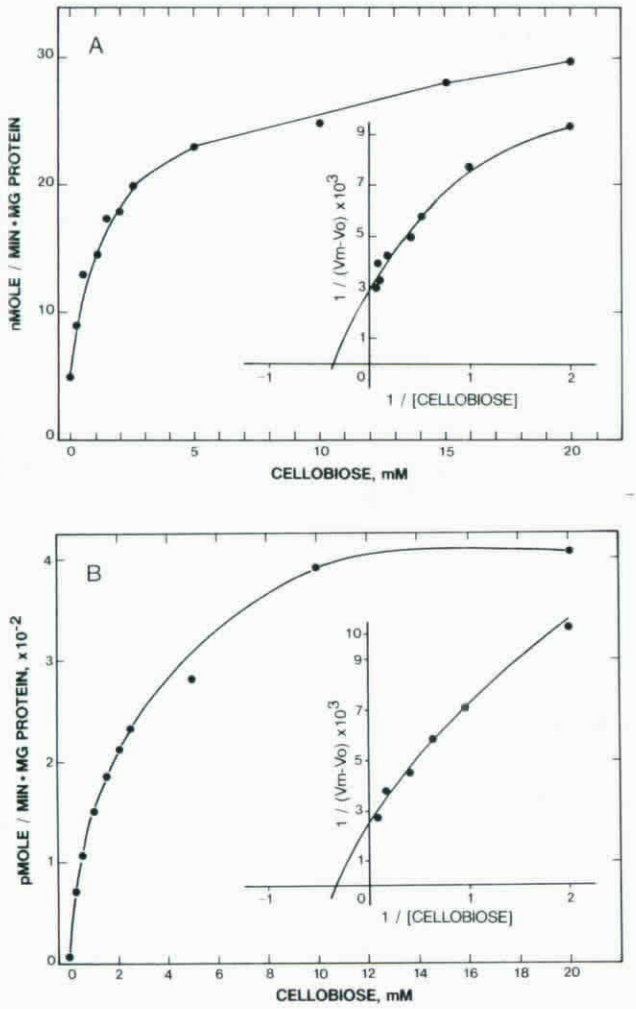


Fig. 7. Effects of cellobiose on stimulation of glucan synthase activity under high (A) and low (B) substrate assay condition. The reaction mixture is described under "Materials and Methods" except the concentration of cellobiose was varied as indicated.

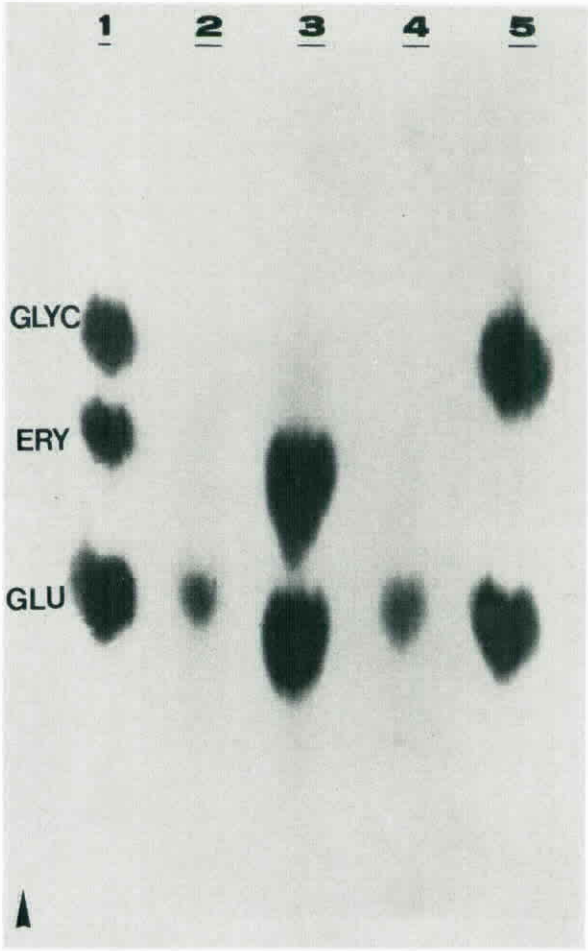


Fig. 8. Chromatogram showing the products formed resulting from periodate oxidation, reduction and hydrolysis of glucan formed during the action of high (lane 2) and low (lane 4) substrate condition. Lane 3, cellulose. Lane 1 and lane 5, monosaccharide standards. Glu, glucose; Ery, erythritol; Glyc, glycerol.

iodate oxidation so that glucose can be released after complete acid hydrolysis. A trace of glycerol also can be detected. It is assumed that glycerol arises from the terminal of glucan chain. A similar result was also obtained for the LGS (data not shown) indicating (1—3) glycosidic linkage of the glucan product.

The linkage of glucan product was also identified by methylation analysis. The glucan product was methylated, hydrolysed, and acetylated, and the resulting alditol acetate derivatives were separated by gas chromatography. A single peak was detected in the derivatized reaction product of the high substrate assay condition (Fig. 10 middle panel). This peak corresponded with the peak for 1,3,5,-tri-O-acetyl-2,4,6-tri-O-methyl glucitol, indicating a (1—3) glycosidic linkage. No peak was detected for the low substrate assay glucan product even a 40 times scale-up assay was done (data not shown). The synthesized product from LGS was very low in quantity (<0.1 nmole).

*Glucan Synthase Distribution in Membrane System*

To reveal the association of glucan synthase with membrane system, bamboo shoot extracts were subjected to isopycnic centrifugation and membranes were fractionated on a linear sucrose gradient. Markers for plasmalemma, UDP-glucose sterol glucosyltransferase, and for Golgi apparatus, latent IDPase, were shown in Figure 11. The peak for LGS coincided with the plasmalemma marker, UDP-glucose sterol glucosyltransferase, and HGS showing several peaks has the major activity roughly co-equilibrated with plasmalemma fractions too. This results strongly suggest that two glucan synthases are not associated with Golgi membrane vesicles.

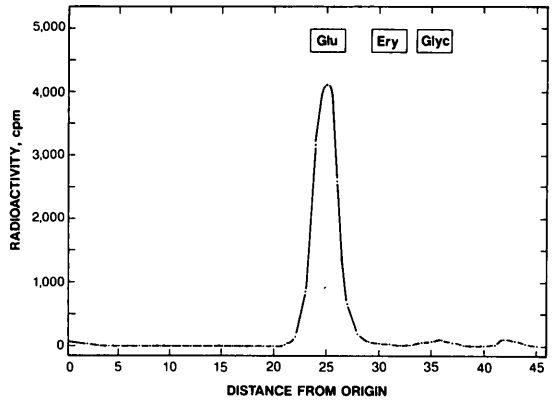


Fig. 9. Separation of high substrate glucan products resulting from periodate oxidation. Glucan products were separated by descending paper chromatography for 17 hours on Whatman No. 1 paper using n-butanol-propanol-water (6:4:3) as solvent. The experiments were repeated at least twice with the same results. Glu, glucose; Ery, erythritol; Glyc, glycerol.

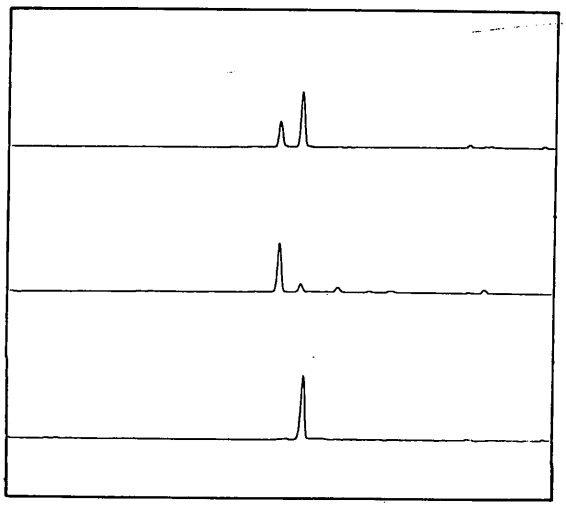

Fig. 10. Gas chromatographic separation of the alditol acetate derivatives of methylated lichenen (upper panel); cellulose (lower panel) and the radioactive product of high substrate glucan synthase (middle panel). Two peaks can be traced for the derivatized standards, the left peak, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol and right peak, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol.

Two glucan synthases, one with low Km for UDP-glucose that synthesizes $\beta$-1, 4-linkages, and the other with high Km that forms $\beta$-1, 3-linkages, have been failed to be separated physically, despite many attempts (Maclachlan, 1983). There was evidence from sucrose gradient analyses of pea membranes that glucan synthetase activities were separatable into Golgi and plasma membrane-enriched fraction (Ray *et al.*, 1969). It is clear that two glucan synthases were also detectable in the shoot of *Dendrocalamus latiflorus*. They can be separated at the same preparation basing upon the observation of the differences of pH optimum (Fig. 1), reaction temperature (Fig. 2), solubility in detergents (Fig. 3 and 4), etc. Therefore these two enzymes can be separated functionally instead of physically. It is still not clear are they the same enzyme molecule? Association of LGS with the plasma membrane was also reported in other monocots, i.e., in onion (Van der Woude *et al.*, 1974) and in sugar cane (Robinson and Glas, 1983) and physically separatable from the HGS.

The physiological significance of such a highly active $\beta$-(1-3)-glucan synthetase in bamboo shoot is possible that the activity is normally latent and is observed as a wound response to damage just like the case in celery petioles (Jacob and Northcote, 1985). The 1,3-linkage predominates at high substrate levels has been found in all preparation that have been tested (see refs in Raymond *et al.*, 1978). There is speculation, but no existing proof, that *in vivo* this system many be capable of synthesizing cellulose (Sloan *et al.*, 1987). Putative cellulose-synthetase complexes have been observed by freeze-etch techniques on the plasma membrane of higher plants (Mueller and Brown, 1980). Lability of such a complex such as has been observed in cotton fiber (Bacic and Delmer, 1981) indicates that the cell may need to be intact for such a complex to be functional.

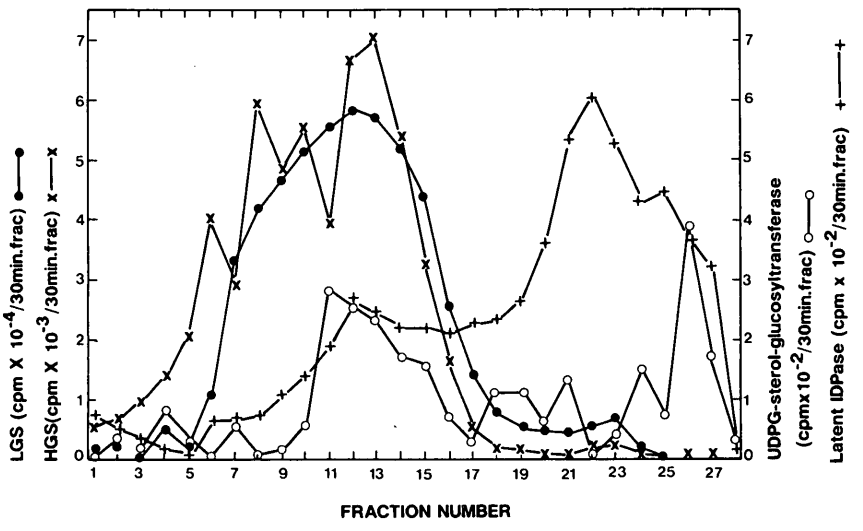


Fig. 11. Linear 20-55% (W/W) sucrose gradient of the homogenate of shoot of *Dendrocalamus latiflorus* centrifuged for 3 h at 130,000 g. (○), UDP-glucose sterol glucosyltransferase; (+), latent IDPase; (×), HGS; (●), LGS.

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# 麻竹竹筍二種聚葡萄醣合成酶之特性研究

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經由二相配位法做麻竹竹筍之細胞膜之部份純化獲致二種聚葡萄醣合成酶並研究其特性。其中之一可在高受質濃度(1 mM UDP-glucose)下行反應，其 pH 最適值爲 7.2，最適反應溫度 30-32°C。另一種酶可在低受質濃度(3 μM UDP-glucose)下行反應，其 pH 最適值爲 6.5，最適反應溫度爲 15-20°C。Cellobiose 與氯化鎂爲酵素反應所必需，有促進活性之效。氯化鈣不能取代氯化鎂的功能。在高受質聚葡萄醣合成條件下，受質 UDP-glucose, $Mg^{2+}$和 Cellobiose 之動力學常數分別爲 0.82 mM，0.53 mM 與 2.1 mM。在低受質聚葡萄醣合成條件下，$Mg^{2+}$與 Cellobise 之動力學常數分別爲 0.62 mM 與 3.1 mM。二種聚葡萄醣合成酶之產物在 24% KOH 中之溶解度並不相同；高受質條件下之產物可溶而低受質條件下之產物則不可溶。Digitonin 與 CHAPS 二種介面活性劑在某一特定濃度時對聚葡萄醣合成酶具有促進活性之作用。過碘酸氧化作用與甲基化分析顯示二種聚葡萄醣合成酶均具有 1～3 醣苷鍵聯結。蔗醣梯度離心則顯示二種聚葡萄醣合成酶均落在細胞膜之位置上，雖然高受質聚葡萄醣合成酶出現了一個以上的高峰。由以上結果可看出此二種聚葡萄醣合成酶在功能上是完全不同的酶。