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Factors Influencing β-Glucan Synthesis by Particulate Enzymes from Suspension-Cultured *Lolium multiflorum* Endosperm Cells[1]

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ABSTRACT

Particulate enzymes from suspension-cultured ryegrass (*Lolium multiflorum* Lam.) endosperm cells incorporated glucosyl residues from UDP-glucose and GDP-glucose into β-glucans. Three types of β-glucans were produced from UDP-glucose: 1,3-β-glucan; 1,4-β-glucan; and mixed-linkage 1,3;1,4-β-glucan. As in other systems, relatively more 1,4-β-glucan was produced from a low (10 micromolar) UDP-glucose concentration, and relatively more 1,3-β-glucan was produced from a high (1 millimolar) UDP-glucose concentration. However, in ryegrass, 1,3;1,4-β-glucan represented a major proportion of the products at both low and high UDP-glucose concentrations. The arrangement of linkages in the 1,3;1,4-β-glucan was different at the two concentrations; at the low UDP-glucose concentration, more sequences of three consecutive 1,4-linkages were produced.

The effects of pH, temperature, and metal ion concentrations on incorporation were dependent on the UDP-glucose concentration. At the low UDP-glucose concentration, incorporation into all three types of β-glucan increased with increasing pH. At the high UDP-glucose concentration, 1,3-β-glucan was the major product at pH 7 and below; 1,4-β-glucan synthesis was optimal at pH 8; and synthesis of 1,3;1,4-β-glucan was greatest above pH 8.

With 10 micromolar GDP-glucose as substrate, 1,4-β-glucan, but no 1,3;1,4-β-glucan, was produced. Incorporation from either UDP-glucose or GDP-glucose was not influenced by the presence of the other.

Cell-free preparations from higher plant sources incorporate glucosyl residues from nucleoside diphosphate glucose into β-glucans containing 1,3- and 1,4-linkages. Some preparations produce both 1,3-β-glucan and 1,4-β-glucan, while other also produce mixed-linkage 1,3;1,4-β-glucan (23). Evidence for the synthesis of these mixed-linkage β-glucans has come from identification of 1,3;1,4-β-oligoglucosides in enzymic hydrolysates of the products formed by oat coleoptile (*Avena sativa*) (17) and mung bean hypocotyl (*Phaseolus aureus*) (15) preparations, from Smith degradation studies of the products from wheat root preparations (18), and from the susceptibility of β-glucans produced by preparations from ryegrass (*Lolium multiflorum*) endosperm (7, 23) to hydrolysis by the 1,3;1,4-β-glucan hydrolase from *Bacillus subtilis* (1).

β-Glucans produced *in vitro* from μM concentrations of UDP-glucose are rich in 1,4-β-glucan linkages, but those formed from mM concentrations of UDP-glucose, by the same preparations, contain mostly 1,3-β-linkages (22, 29). In systems that produce 1,3;1,4-β-glucan in addition to 1,4- and 1,3-β-glucan, the proportion of 1,4- and 1,3-β-glucosidic linkages is similarly influenced by the UDP-glucose concentration. Thus, at 4 μM UDP-glucose and pH 8, the products of the ryegrass β-glucan synthetases were shown by specific enzymic hydrolysis to be composed of equal proportions of 1,4-β-glucan and 1,3;1,4-β-glucan, whereas, at 1 mM UDP-glucose, the products were 70% 1,3;1,4-β-glucan, 24% 1,3-β-glucan, and 6% 1,4-β-glucan (7).

This paper reports the effect of temperature, pH, metal ions, and several inhibitors and activators on total β-glucan synthesis and the influence of pH on the relative amount of each of the three types of β-glucans produced at low and high UDP-glucose concentrations. The size of the water-soluble products has been investigated, and the β-glucans formed from GDP-glucose have been characterized.

MATERIALS AND METHODS

Chemicals. Ammonium salts of UDP-[^{14}C]glucose (8.88 Giga bq/mmol) and GDP-[^{14}C]glucose (9.95 Giga bq/mmol) (Amersham Australia Ltd.) were diluted to the appropriate concentration with unlabeled sodium salts (Sigma Chemical Co.). The purity of both ^{14}C- and unlabeled nucleoside disphosphate sugars was verified by TLC on poly-(ethyleneimine)-cellulose (20) using 0.5 M LiCl in 0.1 M sodium acetate buffer (pH 4.6) as solvent. Bacitracin and coumarin were from Sigma Chemical Co.

Plant Material. Liquid suspension cultures of ryegrass (*Lolium multiflorum* Lam.) endosperm cells were grown, at 27°C in the dark on a modified White's medium with sucrose as carbon source (24), and subcultured every 2 weeks. Particulate enzymes were prepared from mid-log phase (7-d) cultures.

Preparation of Particulate Enzymes. Up to 300 g wet weight of cells were collected by filtration and washed with 1 L of cold (4°C) distilled H_2O. All subsequent steps were performed at 4°C. The washed cells were resuspended in an equal volume of 100 mM Mops[2] (pH 7.5) containing 4 mM EDTA and 1 mM DTT and disrupted in a French pressure cell (Aminco Bowman Corp., Silver Spring, MD) at 60 MPa. The disrupted cells were centrifuged at 1,000*g* for 6 min, and the supernatant was filtered through Miracloth and centrifuged at 100,000*g* for 1 h. The 1,000- to 100,000*g* pellet was resuspended in 20 ml of 10 mM Mops (pH 7.5) containing 2 mM DTT, using a Dounce homogenizer, and used as the particulate enzyme source in assays for β-glucan synthesis.

Assay Procedure. Final concentrations in the standard assay were 50 mM Mops (pH 7.5), 20 mM $MgCl_2$, 0.5 mM DTT, and 10 μM (low concentration) or 1 mM (high concentration) UDP-glucose in 0.4 ml total volume. Each assay contained approximately 3 kBq (1.8×10^5 dpm) of radioactive substrate. The specific radioactivity of UDP-glucose used in 10 μM and 1 mM incubations was 4.5×10^4 dpm/pmol and 4.5×10^2 dpm/pmol, respectively. The reaction

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[2] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; DP, degree of polymerization.

was started by addition of 0.1 ml of particulate enzyme to 0.3 ml of buffered substrate. The assay mixture was incubated at 25°C for 15 min with gentle shaking, and the reaction was stopped by heating at 100°C for 10 min.

Incorporation of radioactivity into products insoluble in 66% (v/v) ethanol was measured by drying 0.1 ml of the reaction mixture on a 2.1-cm diameter glass fiber disc (Whatman GF/A). Unreacted substrate was removed by washing each disc successively with 10 ml cold (4°C) 66% ethanol containing 0.85 mM EDTA, 66% ethanol, 70% ethanol, absolute ethanol, and, finally, acetone (23). This procedure also removes chloroform-methanol-soluble products (23).

In control assays, the particulate enzyme was heated for 10 min at 100°C before incubation with substrate. Radioactivity in products insoluble in 66% ethanol was determined for duplicate aliquots from each of two or three replicate tubes.

Assay Conditions. As shown in a previous study (23), the incorporation of glucose from UDP-glucose into 66% ethanol-insoluble products by the mixed-membrane preparation at 25°C was not linear with time during the 15-min incubation. However, there was a linear increase in incorporation from 1 mM UDP-glucose up to at least 12 mg protein/ml in the assay mixture and with 10 μM UDP-glucose up to 9 mg protein/ml. All experiments were performed at a protein concentration of less than 9 mg/ml.

The particulate preparation could be stored at 10°C for 24 h without appreciable loss of activity, but the most effective long-term storage was at −70°C. Under these conditions, approximately 80% of activity was retained after 28 d. This was marginally improved by the addition of 40 mg/ml BSA.

Characterization of Products. To characterize the products, the total reaction mixture was freeze-dried and washed twice with 10 ml of chloroform:methanol:water (10:10:3), and then it was successively washed with the five solvents used in the standard assay. The washed products were hydrolyzed by incubating three replicates separately for 24 h with the 1,3;1,4-β-glucan endohydrolase (EC 3.2.1.73) from *B. subtilis* (1), the 1,3-β-glucan exo-hydrolase (EC 3.2.7.58) from *Euglena gracilis* (4), or the 1,4-β-glucan endohydrolase (EC 3.2.1.4) from *Steptomyces sp.* (7), which had been freed of 1,3-β-glucan hydrolase contaminant by DEAE-Sepharose chromatography (F. M. Grave and B. A. Stone, unpublished).

When no further radioactivity became soluble in 66% ethanol, the extent of hydrolysis was determined by drying the hydrolysates on glass fiber discs, washing, and counting, as described for the standard assay procedure. The amount of 1,3- and 1,3;1,4-β-glucan was given directly by the decrease in radioactivity of the 66% ethanol-insoluble fraction following treatment with *Euglena* and *Bacillus* enzymes, respectively. Since the *Streptomyces* enzyme hydrolyzes both 1,4- and 1,3;1,4-β-glucan, and the *Bacillus* enzyme hydrolyzes only 1,3;1,4-β-glucan, the content of 1,4-β-glucan was calculated by difference. Oligosaccharides released by the hydrolases either from radioactive β-glucan products or from endogenous β-glucans were separated by gel filtration on Bio-Gel P-2 (1). Radioactive oligosaccharides in the fractions were measured by scintillation counting, and total carbohydrate was estimated using a Multiref 902 differential refractometer (Optilab, Vallingby, Sweden).

RESULTS

Characterization of Products.

Products from UDP-Glucose. Radioactive glucosyl residues from UDP-glucose were transferred to two main fractions, one soluble in chloroform-methanol and the other insoluble in 66% ethanol. Previous studies have shown that steryl glucosides are the major radioactive components of the chloroform-methanol-soluble fraction (23). At 10 μM UDP-glucose, almost 3 times as much radioactivity appears in the chloroform-methanol fraction, as in the ethanol-insoluble fraction. In contrast, at 1 mM UDP-glucose, almost 4 times as much radioactivity is transferred to products insoluble in 66% ethanol, as compared with chloroform-methanol fractions.

To test for the synthesis of low mol wt oligosaccharides from 1 mM UDP-glucose, the water-extractable components of the fraction solubilized by chloroform:methanol:water (10:10:3) (2) were chromotographed on Bio-Gel P-2. No radioactive, low mol wt oligosaccharides were found, but a component corresponding to monosaccharide and representing 1.5% of the radioactivity in the reaction mixture was observed. Porcine pancreatic α-amylase treatment of the 66% ethanol-insoluble products formed from 1 mM UDP-glucose released 3.2% of the radioactivity in this fraction as oligosaccharides separable on Bio-Gel P-2, indicating the formation of a small amount of α-glucan (7, 23).

β-Glucan Products. The proportion of the three β-glucans in the 66% ethanol-insoluble products formed at pH 7.5 was determined by hydrolysis with specific β-glucan hydrolases. With 10 μM UDP-glucose, the products were 6% 1,3-β-glucan, 56% 1,4-β-glucan, and 38% 1,3;1,4-β-glucan; with 1 mM UDP-glucose, the products were 50% 1,3-β-glucan, 18% 1,4-β-glucan, and 32% 1,3;1,4-β-glucan.

The patterns of oligosaccharides released by the individual β-

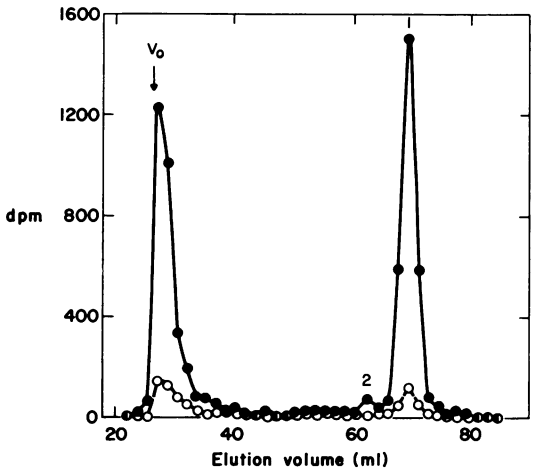

FIG. 1. Gel filtration of 1,3-β-glucan exo-hydrolase (*E. gracilis*) hydrolysate on Bio-Gel P-2. β-Glucans produced from 10 μM UDP-glucose, O- - -O; from 1 mM UDP-glucose, ●——●. The numbers on the profile refer to the DP of the oligosaccharides released by enzymic hydrolysis.

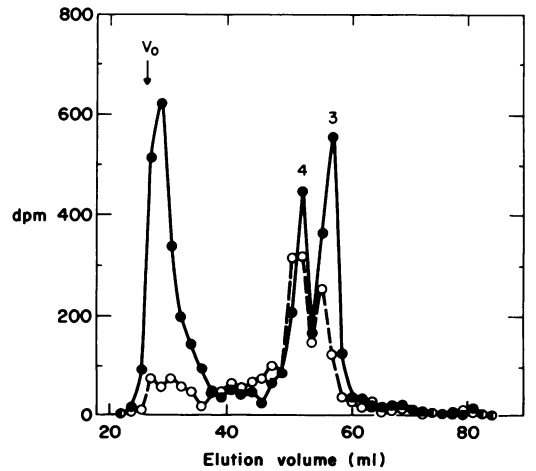

FIG. 2. Gel filtration of 1,3;1,4-β-glucan endo-hydrolase (*B. subtilis*) hydrolysate on Bio-Gel P-2. β-Glucans produced from 10 μM UDP-glucose, O- - -O; from 1 mM UDP-glucose, ●——●. The numbers on the profile refer to the DP of the oligosaccharides released by enzymic hydrolysis.

glucan hydrolases shown in Figures 1, 2 and 3 were consistent with the known specificity of the hydrolases. Thus, the products of the *Euglena* 1,3-β-glucan exo-hydrolase (Fig. 1) were glucose and a trace of disaccharide. The *Bacillus* 1,3;1,4-β-glucan endo-hydrolase (Fig. 2) released tri- and tetrasaccharides from products formed at both high and low UDP-glucose concentrations. The ratio of tri- to tetrasaccharides was different at the two substrate concentrations. With 1 mM UDP-glucose, 54% of the oligosaccharide released was trisaccharide, while, with 10 μM UDP-glucose, only 43% was trisaccharide. The trisaccharide originates from regions of the polymer containing two consecutive 1,4-linkages and the tetrasaccharides from regions containing three consecutive 1,4-linkages. Thus, in the mixed-linkage β-glucan produced from 10 μM UDP-glucose, three consecutive 1,4-linkages are more common than two consecutive 1,4-linkages, whereas in the products from 1 mM UDP-glucose, the reverse is true.

The *Streptomyces* endo-hydrolase (Fig. 3) cleaves both 1,4-β-glucans and 1,3;1,4-β-glucans. This enzyme released, in addition to tri- and tetrasaccharides, significant amounts of mono- and disaccharides. The higher proportion of mono- and disaccharides released from the products at low UDP-glucose concentration indicates the formation of more 1,4-β-glucan under these conditions.

Endogenous β-Glucans. Endogenous β-glucans and other polysaccharides (T. Mascara and G. B. Fincher, personal communication) are present in the enzyme preparation. The β-glucans were detected by separately digesting the enzyme preparation with each of the three β-glucan hydrolases, separating the oligosaccharides released on Bio-Gel P-2, and quantifying them by refractive index monitoring. The presence of this endogenous glucan is reflected in the low specific activities of products from 10 μM UDP-glucose (0.006 to 0.03% of the specific activity of the UDP-glucose substrate). The specific activities at 1 mM UDP-glucose were somewhat higher (1.2–8.3% of the UDP-glucose substrate).

Effect of pH. Incorporation was greatly influenced by the choice of buffer at both 10 μM and 1 mM UDP-glucose. At pH 7.0 and 1 mM UDP-glucose, incorporation was 52% higher in cacodylate than it was in Mops, whereas, in the widely used Tris and phosphate buffers, incorporation was about 30% lower than it was in Mops. At pH 8.0, greatest incorporation was observed with carbonate buffers. All amine-type buffers tested resulted in lower incorporation than did cacodylate or carbonate. Since both ionic strength and buffer ions influenced incorporation, pH dependence was studied in Mops buffer adjusted to constant ionic strength.

The proportion of the three β-glucan products varied with the pH of the incubation mixture. At low UDP-glucose concentrations (Fig. 4), increasing pH resulted in enhanced total β-glucan synthesis. At high UDP-glucose concentrations (Fig. 5), a more complex pH dependence was observed. The incorporation into 1,3-β-glucan was maximal at pH 7.0; at pH 5.5 and 6, almost all the product was 1,3-β-glucan. 1,3;1,4-β-Glucan synthesis increased as the pH increased and was maximal at the highest pH tested. 1,4-β-Glucan was produced maximally at pH 8.0. The influence of pH on the type of β-glucan formed has not been previously studied, but the optimum pH reported for β-glucan synthesis by preparations from other sources ranges from 6.5 (15) to 8.0 (11, 13). The results with the ryegrass system indicate that, at high UDP-glucose concentrations, 1,3-β-glucan synthesis is optimal at the lower end of this pH range and that 1,4-β-glucan synthesis is optimal at the higher pH values.

Effect of Temperature. The influence of reaction temperature on total incorporation was also quite different at the two substrate concentrations. With 1 mM UDP-glucose, maximum incorporation was observed at 20 to 25°C, while, at 30°C, activity was only 50% of the maximum and was only slightly greater than the activity at 6°C. With 10 μM UDP-glucose, the incorporation was much less sensitive to temperature, and the activity at 40°C was 81% of the maximum incorporation found between 20 and 30°C.

Effect of Metal Ions. Incorporation from 10 μM UDP-glucose was stimulated by Ca^{2+}, Mn^{2+}, and Zn^{2+} but was independent of

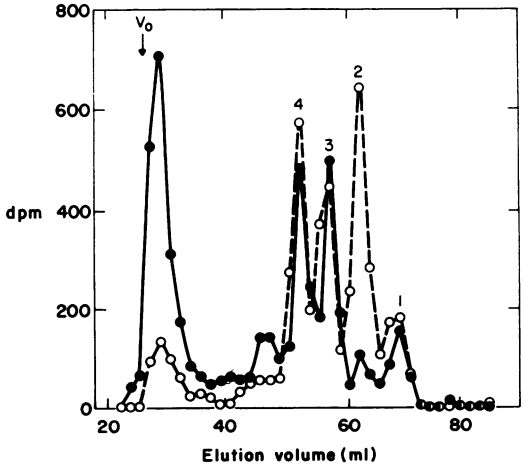

FIG. 3. Gel filtration of 1,4-β-glucan endo-hydrolase (*Streptomyces sp.*) hydrolysate on Bio-Gel P-2. β-Glucans produced from 10 μM UDP-glucose, O– – –O; from 1 mM UDP-glucose, ●——●. The numbers on the profile refer to the DP of the oligosaccharides released by enzymic hydrolysis.

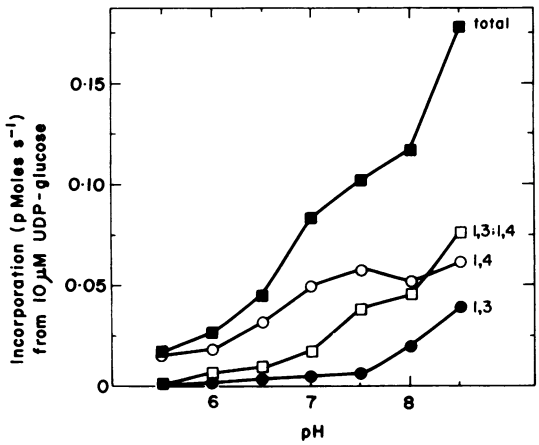

FIG. 4. pH Dependence of β-glucan synthesis from 10 μM UDP-glucose: 1,4-β-glucan, O; 1,3-β-glucan, ●; 1,3;1,4-β-glucan, □; total β-glucan, ■.

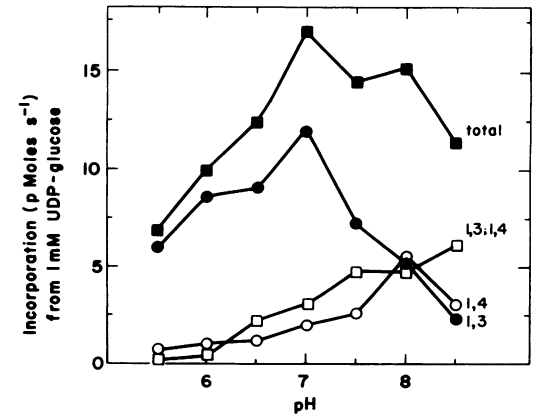

FIG. 5. pH Dependence of β-glucan synthesis from 1 mM UDP-glucose: 1,4-β-glucan, O; 1,3-β-glucan, ●; 1,3;1,4-β-glucan, □; total β-glucan, ■.

Table I. *Influence of Carbohydrates on the Formation of Products Insoluble in 66% Ethanol*

Addition[a]	Glucose Incorporated into Products Insoluble in 66% Ethanol	
	UDP-Glucose, 10 μM	UDP-Glucose, 1 mM
	pmol/s	
None	0.269 (0.046)[b]	25.6 (3.74)[b]
Glycerol	0.340 (0.080)	38.2 (3.92)
Glucose	0.368 (0.047)	98.2 (5.50)
Galactose	0.290 (0.084)	25.3 (4.58)
Fructose	0.157 (0.032)	37.8 (8.26)
Cellobiose	0.739 (0.041)	158.7 (8.78)
Maltose	0.439 (0.084)	67.5 (1.98)
Sucrose	0.354 (0.098)	46.3 (3.02)

[a] All carbohydrates were present at a final concentration of 20 mM in the assay mixture, protein 5.6 mg/ml.

[b] Numbers in parentheses, SD of four replicates.

Mg^{2+} concentration. At 1 mM UDP-glucose incorporation was stimulated by Mg^{2+} in addition to Ca^{2+}, Mn^{2+}, and Zn^{2+}. Synthesis was maximal with 10 μM Mg^{2+}. β-Glucan synthesis in other systems is also stimulated by Mn^{2+} (3, 15, 25, 27) and Ca^{2+} (3, 8, 27), and a requirement for Mg^{2+} has been reported for 1,4-β-glucan synthesis but not for 1,3-β-glucan synthesis (10, 13, 25, 28, 29). On the basis of these observations, Mg^{2+} often has been included in assays at low, but not at high, UDP-glucose concentrations (21). The stimulation of the ryegrass enzymes by Mg^{2+} at 1 mM UDP-glucose shows that it is necessary to determine the effect of Mg^{2+} for each system independently. The specific influence of Mg^{2+} on 1,3;1,4-β-glucan synthesis has not been investigated; however, since total β-glucan synthesis from 1 mM UDP-glucose in ryegrass is increased and 1,3-β-glucan synthesis in other systems is not enhanced, Mg^{2+} may increase incorporation into 1,3;1,4-β-glucan.

Activators and Inhibitors. Table I shows the effect of the presence of different polyols, mono-, and disaccharides on β-glucan synthesis. These carbohydrates showed some specificity; thus, glucose was an activator, but galactose had no effect. Cellobiose (20 mM) stimulated almost 3-fold at 10 μM and 6-fold at 1 mM UDP-glucose. Cellobiose stimulated incorporation from both low and high UDP-glucose concentrations, and enzymic analysis of β-glucans formed indicated that the synthesis of all three types of β-glucans was increased. Stimulation of incorporation by cellobiose and other sugars has been reported for β-glucan synthetases from many sources (5, 6, 10, 13, 14, 25, 27, 28), but the mechanism is unknown.

The possibility that cellobiose exerts its effect through a role as an acceptor or primer was investigated using [^{14}C]cellobiose, but no incorporation could be detected. Similarly, there was no incorporation of glucose (10), laminaribiose (14), or cellobiose (27) in other systems. However, interpretation of these experiments is complicated by lack of evidence concerning the DP of the reaction products. If their DP is very high and relatively few chains are formed, incorporation of acceptor would be difficult to detect.

Bacitracin, an inhibitor of dolichol phosphate sugar formation in plant systems (9), is reported to block cellulose synthesis in cotton fiber preparations at high concentration (16). In the ryegrass system, 10 mM or 30 mM bacitracin did not inhibit β-glucan synthesis from low or high UDP-glucose concentrations. The failure of bacitracin to inhibit the formation of β-glucan suggests that dolichol phosphate intermediates are not involved in the pathway of β-glucan synthesis in ryegrass. A similar result has been reported for 1,3-β-glucan synthesis by mung bean preparations (9). Coumarin, an inhibitor of *in vivo* cellulose synthesis (16), was ineffective at 0.1 mM and 1 mM concentrations.

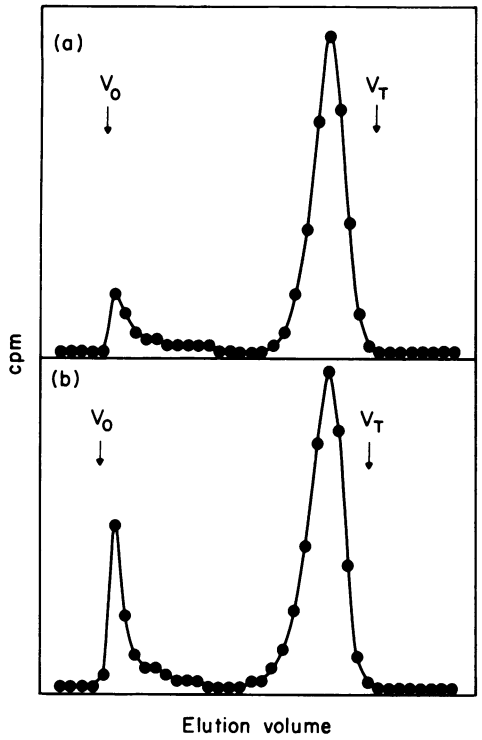

FIG. 6. Gel filtration on Bio-Gel P-200 of water-soluble products from 10 μM UDP-glucose (a) and from 1 mM UDP-glucose (b).

The reaction product, UDP, inhibited incorporation by 51% when both UDP and the substrate, UDP-glucose, were at 1 mM concentrations. Dialdehyde-UDP, which has been shown to react with and inhibit UDP-galactose-dependent galactosyl transferases from bovine colostrum (19), was not an inhibitor.

Size of Water-Soluble Products. The size of the radioactive, water-soluble products was determined by gel filtration on Sepharose 4B, Bio-Gel P-200 (Fig. 6), P-30, and P-10.

The low mol wt products formed at both UDP-glucose concentrations (Fig. 6) contained components that were not excluded by Bio-Gel P-10. The products from 10 μM UDP-glucose had sizes at least up to the exclusion limit of Bio-Gel P-200 (mol wt 0.2×10^6 for globular proteins) and, from 1 mM UDP-glucose, up to the exclusion limit of Sepharose 4B (mol wt 20×10^6 for globular proteins; 5×10^6 for dextran).

Substrate Specificity. Incorporation from 10 μM GDP-glucose under standard assay conditions was 42% of that from 10 μM UDP-glucose. The incorporation of [^{14}C]glucose from 10 μM GDP-[^{14}C]glucose was not decreased by the presence of the 1 mM unlabeled UDP-glucose. Similarly, incorporation from 10 μM UDP-[^{14}C]glucose was not altered by the presence of 1 mM unlabeled GDP-glucose. These experiments indicate that independent enzymes transfer glucosyl residues from GDP-glucose and UDP-glucose.

Substrate Concentrations. When the GDP-glucose concentration was increased 100-fold from 10 μM to 1 mM under standard reaction conditions, there was a 63-fold increase in incorporation. By contrast, when the concentration of UDP-glucose was increased 100-fold, with the same reaction conditions, there was a 110-fold increase in incorporation. At low pH, where 1,3-β-glucan synthesis is favored, the increase in incorporation was up to 200-fold. A similar 'substrate activation' effect has been reported for 1,3-β-glucan synthesis in other systems (22, 25, 29).

Products from GDP-Glucose. With 10 μM GDP-glucose as substrate under the standard assay conditions, 87% of the β-glucan produced was 1,4-linked. The remainder was 1,3-β-glucan, and

there was no detectable 1,3;1,4-β-glucan product. This contrasts with the formation of 38% 1,3;1,4-β-glucan from 10 μM UDP-glucose under identical conditions.

DISCUSSION

The finding that [^{14}C]glucose is incorporated into high mol wt glucan products may be taken as evidence for the *de novo* synthesis of β-glucan. Alternatively, the radioactivity associated with high mol wt water-soluble polymers could simply be due to transfer of a limited number of terminal glucosyl residues to preexisting molecules, such as the endogenous β-glucan present in the enzyme preparation. However, the size of the newly formed 1,3-β-glucan molecules can be determined directly from the ratio of [^{14}C]-glucose (from 1,3-β-glucosyl chain residues) to [^{14}C]laminaribiose (from the reducing ends of 1,3-β-glucan chains) released by the *Euglena* 1,3-β-glucan exo-hydrolase (Fig. 1). The DP calculated from these data was 37 and is somewhat higher than that calculated from earlier experiments (7). This result indicates that some complete 1,3-β-glucan chains are synthesized *de novo*. The production of characteristic ^{14}C-labeled tri- and tetrasaccharides by the endo-hydrolase from *B. subtilis* in proportions similar to that found in high mol wt 1,3;1,4-β-glucan is also consistent with the synthesis of a high mol wt β-glucan by the ryegrass preparations.

The number of enzymes involved in the synthesis of the three types of β-glucans by ryegrass endosperm membranes is not known, but the effect of reaction conditions on the identity of the products indicates the presence of several specific synthetases. It seem likely, from the different requirements for substrate concentration and pH, that separate synthetases for 1,3- and 1,4-β-glucans are present, but direct evidence awaits their dissociation from membranes and their separation.

Two 1,4-β-glucan synthetases are present in the ryegrass preparations, one specific for UDP-glucose and the other for GDP-glucose. In the chlorophycean alga, *Prototheca zopfii*, a pathway for cellulose synthesis requiring both UDP-glucose and GDP-glucose has been described (12). If such a pathway operates in ryegrass, UDP-glucose might be expected to stimulate incorporation from GDP-glucose, but this was not found. Also, the failure of bacitracin to inhibit β-glucan synthesis suggests that dolichol phosphate glucose, which is apparently an intermediate in the algal pathway, is probably not an intermediate in 1,4-β-glucan synthesis in ryegrass.

The synthetase which produces 1,4-β-glucan from 10 μM GDP-glucose is not involved in the synthesis of 1,3;1,4-β-glucan, but the role, if any, of the UDP-glucose-dependent synthetases for 1,3- and 1,4-β-glucan in mixed-linkage β-glucan formation is unclear. The preferential synthesis of 1,3;1,4-β-glucan at high pH suggests that 1,3;1,4-β-glucan may be formed by a system separate from those responsible for the synthesis of 1,3- or 1,4-β-glucan.

The mechanism of formation of β-glucans containing different linkages in the same polymer is not known, but enzymes synthesizing 1,3;1,4-β-glucans do not produce the same arrangement of linkages at different UDP-glucose concentrations. The 1,3;1,4-β-glucan products contain cellotriosyl and cellotetraosyl sequences joined by single 1,3-β-linkages at both UDP-glucose concentrations, but relatively more cellotetraosyl sequences were formed at the low UDP-glucose concentration. The ratio of these two sequences in the 1,3;1,4-β-glucan produced at high UDP-glucose concentrations is more characteristic of mixed-linkages β-glucans from grasses (26).

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LITERATURE CITED

1. ANDERSON MA, BA STONE 1975 A new substrate for investigating the specificity of β-glucan hydrolases. FEBS Lett 52: 202–207
2. BAILEY DS, M DURR, J BURKE, G MACLACHLAN 1979 The assembly of lipid-linked oligosaccharides in plant and animal membranes. J Supramol Struct 11: 123–138
3. BARBER GA, AD ELBEIN, WZ HASSID 1964 The synthesis of cellulose by enzyme systems from higher plants. J Biol Chem 239: 4056–4061
4. BARRAS DR, BA STONE 1969 β-1,3-Glucan hydrolases from *Euglena gracilis*. II. Purification and properties of the β-1,3-glucan exo-hydrolase. Biochim Biophys Acta 191:342–353
5. BELTRÁN JP, J CARBONELL 1978 1,3-β-Glucan synthase from citrus phloem. Phytochemistry 17: 1531–1532
6. BRUMMOND DO, AP GIBBONS 1965 Enzymatic cellulose synthesis from UDP-(^{14}C)-glucose by *Lupinus albus*. Biochem Z 342: 308–318
7. COOK JA, GB FINCHER, F KELLER, BA STONE 1980 The use of specific β-glucan hydrolases in the characterization of β-glucan synthetase products. *In* JJ Marshall, ed, Mechanisms of Saccharide Polymerization and Depolymerization. Academic Press, New York, pp 301–315
8. DELMER DP, U HEINIGER, C KULOW 1977 UDP-Glucose:glucan synthetase in developing cotton fibres. Kinetic and physiological properties. Plant Physiol 59: 713–718
9. ERICSON MC, J GAFFORD, AD ELBEIN 1978 Bacitracin inhibits the synthesis of lipid-linked saccharides and glycoproteins in plants. Plant Physiol 62: 373–376
10. FEINGOLD DS, EF NEUFELD, WZ HASSID 1958 Synthesis of a β-1,3-linked glucan by extracts of *Phaseolus aureus* seedlngs. J Biol Chem 233: 783–788
11. HELSPER UP, JH VEERKAMP, MM SASSEN 1977 β-Glucan synthetase activity in Golgi vesicles of *Petunia hybrida*. Planta 133: 303–308
12. HOPP HE, PA ROMERO, GR DALEA, R PONT LEZICA 1978 Synthesis of cellulose precursors: the involvement of lipid-linked sugars. Eur J Biochem 84: 561–571
13. LARSEN GL, DO BRUMMOND 1974 β-(1→4)-D-Glucan synthesis from UDP-[^{14}C]-D-glucose by a solubilized enzyme from *Lupinus albus*. Phytochemistry 13: 361–365
14. MARÉCHAL LR, SH GOLDEMBERG 1964 Uridine disphosphate glucose-β-1,3-glucan β-3-glucosyltransferase from *Euglena gracilis*. J Biol Chem 239: 3163–3167
15. MIYAMOTO C, K TAMARI 1973 Synthesis of β-1,3-glucan and β-1,3 and β-1,4-mixed glucan from UDP-α-D-glucose. Agric Biol Chem 37: 1253–1260
16. MONTEZINOS D, DP DELMER 1980 Characterization of inhibitors of cellulose synthesis in cotton fibres. Planta 148: 305–311
17. ORDIN L, MA HALL 1968 Cellulose synthesis in higher plants from UDP-glucose. Plant Physiol 43: 473–476
18. PÉAUD-LENOËL C, M AXELOS 1970 Structural features of the β-glucans enzymatically synthesized from uridine diphosphate glucose by wheat seedlings. FEBS Lett 8: 224–228
19. POWELL JT, K BREW 1976 Affinity labelling of bovine colostrum galactosyltransferase with a uridine 5′-diphosphate derivative. Biochemistry 15: 3499–3504
20. RANDERATH K, E RANDERATH 1964 Ion-exchange chromatography of nucleotides on poly(ethyleneimine)-cellulose thin layers. J Chromatog 16: 111–125
21. RAY PM 1979 Maize coleoptile cellular membranes bearing different types of glucan synthetase activity. *In* E Reid, ed, Plant Organelles. Ellis Horwood, Chichester, England, pp 135–146
22. RAYMOND Y, GB FINCHER, GA MACLACHLAN 1978 Tissue slice and particulate β-glucan synthetase activities from *Pisum* epicotyls. Plant Physiol 61: 938–942
23. SMITH MM, BA STONE 1973 β-Glucan synthesis by cell-free extracts from *Lolium multiflorum* endosperm. Biochim Biophys Acta 313: 72–94
24. SMITH MM, BA STONE 1973 Studies on *Lolium multiflorum* endosperm in tissue culture. Aust J Biol Sci 26: 123–133
25. SOUTHWORTH D, DB DICKINSON 1975 β-1,3-Glucan synthase from *Lilium longiflorum* pollen. Plant Physiol 56: 83–87
26. STINARD PS, DJ NEVINS 1980 Distribution of noncellulosic β-glucans in grasses and other monocots. Phytochemistry 19: 1467–1468
27. TSAI C 1975 Isolation of UDP-glucose β-(1→4) glucan glucosyltransferase from oat and its properties. J Carbohydr Nucleosides Nucleotides 2: 419–431
28. TSAI CM, WZ HASSID 1971 Solubilization and separation of uridine diphospho-D-glucose: β-(1→4) glucan and uridine diphospho-D-glucose: β-(1→3) glucan glucosyltransferases from coleoptiles of *Avena sativa*. Plant Physiol 47: 740–744
29. TSAI CM, WZ HASSID 1973 Substrate activation of β-(1→3) glucan synthetase and its effect on the structure of β-glucan obtained from UDP-glucose and particulate enzyme of oat coleoptiles. Plant Physiol 51: 998–1001