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UDP-glucose: Glucan Synthetase in Developing Cotton Fibers

II. STRUCTURE OF THE REACTION PRODUCT[1]

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ABSTRACT

The solubility properties, composition, and structure of the radioactive product synthesized from UDP-[^{14}C]glucose by a highly active cotton fiber glucan synthetase have been determined. Product obtained under the following three different conditions was analyzed: at high and low substrate concentrations by detached fibers, and at high substrate concentrations with an isolated particulate preparation. The results of acetic and nitric acid digestion, enzyme digestion, total acid hydrolyses, periodate oxidation, partial acid hydrolyses, and methylation analyses all support the conclusion that the product of the glucan synthetase produced under all three assay conditions is a linear β-(1→3)-glucan.

In the preceding article (4), we have shown that detached cotton fibers as well as a particulate fraction from such fibers incorporate glucose from UDP-[^{14}C]glucose into hot water-insoluble, chloroform-methanol-insoluble product(s) via a highly active glucan synthetase. In this paper, we report on the linkage properties of the product formed by detached fibers at both high and low substrate concentrations, as well as those of the product formed by the isolated particulate fraction. The results show that the products obtained under these different conditions are indistinguishable from one another, and are not cellulose but rather a glucan with predominately β-(1→3) linkages.

MATERIALS AND METHODS

General. The growth conditions of the plants, and the preparation and assay of the enzymes were as described previously (4). Except where indicated, all products[4] of the detached fiber assay were prepared from incubation of fibers harvested between 17 and 19 days postanthesis. Product of the particulate fraction was produced from enzyme isolated from fibers harvested 16 or 17 days postanthesis. Standard reactions were scaled up appropriately to produce sufficient product for analysis. General techniques for radioactive counting were as described previously (4). *Streptomyces* cellulase (S199g) and *Rhizopus* endo-β-(→3)-glucanase QM 1032 were the gifts of E. T. Reese, U.S. Army Laboratories, Natick, Mass. 4-O-β-Laminaribiosyl glucose and 3-O-β-cellobiosyl glucose were the gifts of A. Kivilaan, Dept. of Botany and Plant Pathology, Michigan State University. Acetic-nitric reagent was prepared according to the method of Updegraff (18).

α-Amylase and Pronase Digestion. Lyophilized samples of water-insoluble product were incubated at 37 C with 1 ml 0.1 M K-phosphate buffer (pH 7) containing 400 μg *Bacillus subtilis* α-amylase (type II-A, Sigma Chemical Co.) at 25 C, or with 0.05 M Na-phosphate buffer (pH 7.4) containing 1 mg pronase CB (Calbiochem) at 37 C. To prevent bacterial contamination, 3 drops of toluene were added to each sample. After 24 hr, another ml of buffer with α-amylase or pronase, respectively, was added. The reaction was terminated after 72 hr by placing the test tubes in a boiling water bath for 5 min. The samples were then filtered on glass filters, thoroughly washed with water, and the retained radioactivity was counted. Control samples were incubated in buffer lacking enzymes.

Partial Acid Hydrolysis. Partial acid hydrolysis of lyophilized samples was carried out by a modification of the procedure of Miller (9). The samples were hydrolyzed for 3 hr at 22 C in 1 ml of concentrated HCl to which 0.1 ml of fuming HCl at 0 C was added. Following repeated evaporation, the samples were then resuspended in water and subjected to descending paper chromatography for 20 hr on Whatman No. 4 paper using 1-propanol-ethyl acetate-H_2O (7:1:2, v/v) as solvent.

Periodate Oxidation. Periodate oxidation was performed by a modification of the method of Hay *et al.* (6). The lyophilized samples were incubated with 2.5 ml 0.05 M sodium metaperiodate in the dark for 4 days at 4 C. Sodium metaperiodate (2.5 ml) was then added again and the incubation continued for 5 days. Ethylene glycol (100 μl) was then added. After 30 min, the samples were filtered, the filters were washed, and the product was removed and resuspended in 5 ml H_2O. One hundred mg $NaBH_4$ was added and the incubation continued for 9 hr at 22 C. The $NaBH_4$ was destroyed by addition of glacial acetic acid, and the treated products of isolated fibers were filtered and washed again whereas the treated product of the particulate fraction was dialyzed. Total hydrolysis was performed in 2 N trifluoracetic acid at 121 C for 1 hr. The solution was concentrated, spotted on Whatman No. 1 paper, and chromatographed for 21 hr in 1-propanol-ethyl acetate-H_2O (7:1:2).

Enzymic Digestion of Radioactive Products. High U and Low U products were first treated with three successive incubations in 1 N NaOH at 100 C for 10 min. (One experiment done with products not treated with alkali gave similar results.) The resulting hot alkali-insoluble products were washed to neutrality with water and then incubated at 50 C in 2 ml 0.05 M sodium acetate (pH 5) containing 3 mM NaN_3 and 80 μg *Rhizopus* β-(1→3)-glucanase. After 2 hr, no further digestion was observed, and addition of further enzyme did not result in further solubilization of radioactivity. The percentage of product solubilized was mon-

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[4] Abbreviations: High U: radioactive product produced by detached fibers incubated in presence of 1 mM UDP-glucose and 10 mM cellobiose; Low U: radioactive product produced by detached fibers incubated in presence of 1.2 μM UDP-glucose, 10 mM cellobiose, and 0.6 mM UTP; High U-(P): radioactive product produced by isolated membrane preparations incubated in presence of 1 mM UDP-glucose and 10 mM cellobiose. $MgCl_2$ was present at 5 mM in all cases.

itored by determining the radioactivity in an aliquot of the supernatant surrounding the fiber product from the reaction at different time intervals. Whenever a portion of the supernatant was removed, an equal volume of enzyme plus buffer was added back to the reaction so that the amount of enzyme present was constant. After no further digestion with β-(1→3)-glucanase occurred, the product of isolated fibers was filtered, washed with acetate-azide buffer, and resuspended in 2 ml acetate-azide buffer containing 1 mg/ml *Streptomyces* cellulase preparation. Sampling for digestion was performed as for the β-(1→3)-glucanase digest. When the product of the particulate fraction was digested, it was incubated as described above, but in this case an aliquot was removed at different time intervals, filtered onto Whatman GF/A filters, washed with H_2O, dried, and counted. Loss of radioactivity which could be retained on the filters was used to measure solubilization by the enzyme preparation. When the products of the enzyme hydrolysis were to be analyzed by chromatography, the digestion solutions were filtered, the filtrate was placed in a boiling H_2O bath for 5 min, centrifuged, and the supernatant was lyophilized, resuspended in a small amount of H_2O, and chromatographed as described in the text.

Analysis of ^{14}C-Trisaccharides Released during Enzyme Digestion. Products of the detached fiber assay were subjected to enzyme digestion with β-(1→3)-glucanase for 5 hr at 50 C and then with cellulase for 5 hr at 50 C as described above. Following each digestion, the reactions were terminated by heating at 100 C, filtered, and the filtrate was applied to a Bio-Gel P-2 column (200–400 mesh, 1.5 × 105 cm) equilibrated in H_2O. The trisaccharide region, which eluted as a distinct peak, was pooled, lyophilized, and resuspended in a small volume of water, and analyzed by paper electrophoresis. Electrophoresis was performed by spotting samples on Whatman 3MM paper preequilibrated in 0.2 M sodium borate buffer (pH 10), and running them for 10 hr at 20 v/cm and 5 C using the same buffer. Standard reference compounds were detected with alkaline silver nitrate-pentaerythritol reagent (19).

Methylation Analyses. All products were extracted with water and chloroform-methanol (2:1, v/v) and lyophilized to complete dryness before use. Because of their insolubility in the first step of the Hakamori methylation procedure, the products were first methylated with dimethyl sulfate in NaOH by the Hayworth method (7). These partially methylated products were then methylated by the Hakamori method (5) as described by Sandford and Conrad (13). Following methylation, 3 ml chloroform-methanol (1:1) was added to the methylated products and the solution dialyzed extensively against water for 2 days. The products were then reextracted into chloroform-methanol, reevaporated under N_2, and resuspended in chloroform. At this stage in most of the experiments, about 90% of the original radioactivity was soluble in chloroform. The solutions were then evaporated under N_2, and hydrolyzed for 90 min in an autoclave in 1 ml 2 N trifluoroacetic acid containing internal inositol standard, and the resulting partially methylated derivatives were reduced and acetylated by the method of Albersheim *et al.* (1). Before separation on GLC, the final solutions were concentrated 5- to 10-fold by blowing a stream of N_2 over them at room temperature. Control experiments showed that no loss of a terminal glucose standard (2,3,4,6.-0-tetra-methyl glucose) resulted from this procedure. Separation of the derivatized sugars was performed on a Varian 2100 gas chromatograph. Glass columns (180 × 0.2 cm i.d.) were packed with a mixture of 0.2% poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate), and 0.4% silicone XE-1150 on Gas-chrom Q (80–100 mesh). The column to be injected was equipped with a stream splitter which apportioned 1 part of the effluent to the detector and 10 parts to a port where the effluent was collected and condensed into chilled Pasteur pipettes. The Pasteur pipettes were then rinsed with 5 ml toluene scintillation fluid (containing 2.2 g PPO and 176 mg POPOP/l toluene) into scintillation vials and the solubilized derivatives were counted.

Chromatography was performed with temperature programming at 1 C/min from 110 to 180 C. The peaks of methylated derivatives obtained with detached fibers corresponded well to those previously identified by GLC-MS from cell wall preparations of fibers harvested 20 days postanthesis (8), and thus served as internal standards for various methylated derivatives. Permethylated laminaribiose and cellobiose served as additional markers for 3-linked and 4-linked glucose, respectively. Standards for terminal glucose, 2,3-linked glucose, and 3,6-linked glucose were purchased from Supelco. Control experiments using alditol acetate preparations from ^{14}C-labeled fiber cell walls indicated that radioactivity eluted from the column essentially coincident with its respective peak on the recorder. Approximately 5 sec was required to change Pasteur pipettes during peak collections.

RESULTS

In this report, we analyze the structure of the product of cotton fiber UDP-glucose:glucan synthetase obtained under three different conditions: (*a*) at low substrate concentrations (1.2 μM UDP-glucose) in the presence of 10 mM cellobiose and 0.6 mM UTP using detached fibers as enzyme source; (*b*) at high substrate concentrations (1 mM UDP-glucose) with 10 mM cellobiose only present as activator, also using detached fibers; and (*c*) at high substrate concentration (1 mM UDP-glucose) plus 10 mM cellobiose using an isolated particulate fraction from detached fibers as enzyme source. The three different products are referred to hereafter, respectively, as the Low U, High U, and High U-(P) products. It should be noted that if our previous explanation of the requirement for UTP at low substrate concentrations is correct (4), the low U product is, in reality, a product produced at concentrations of UDP-glucose which we can estimate to be in the range of 10 to 30 μM.

Solubility Properties of the Products. Following extraction with water and chloroform-methanol (1:1, 1:2, or 2:1), the products were subjected to hot alkali or hot acetic acid-nitric acid as described by Updegraff (18). For all three products, at least 90% of the product was solubilized by the acetic acid-nitric acid reagent under conditions where α-cellulose is not solubilized (100 C, 30 min). Over 70% of the products from the detached fiber assay were insoluble in hot alkali (1 N NaOH, 100 C, 10 min; three successive treatments), but the High U-(P) product was much more susceptible to solubilization by this treatment (only 26% of this product remained insoluble).

Evidence for β-linked Glucans. Digestion of all three products with pronase or α-amylase released less than 5% of the incorporated radioactivity whereas all products were up to 95% digested by a mixture of β-(1→3)-glucanase and cellulase. This indicates that the products are β-linked polysaccharides and not α-linked polysaccharides or glycoproteins. The occurrence of glycolipids was ruled out by previous extraction with chloroform-methanol. About 95% of the total radioactivity of all three products are solubilized by hydrolysis in 2 N trifluoroacetic acid (121 C, 1-2 hr), and all radioactivity released was present exclusively as [^{14}C]glucose.

Partial Acid Hydrolysis of the Products. Partial acid hydrolysis of the Low U, High U, and High U-(P) products resulted in solubilization of 80, 95, and 50%, respectively, of the original radioactivity. The distribution of this solubilized radioactivity on paper chromatograms of a partial acid hydrolysate from High U product is shown in Figure 1A. Although data are not shown, the results for all three products were quite similar. Besides glucose, a disaccharide can be detected the R_F of which corresponds to that of laminaribiose. No cellobiose, which would be indicative of β-(1→4) linkages, could be detected even though

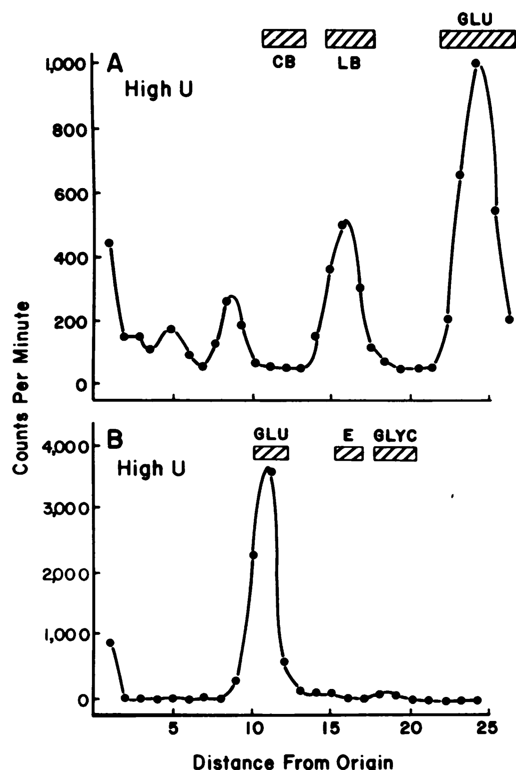
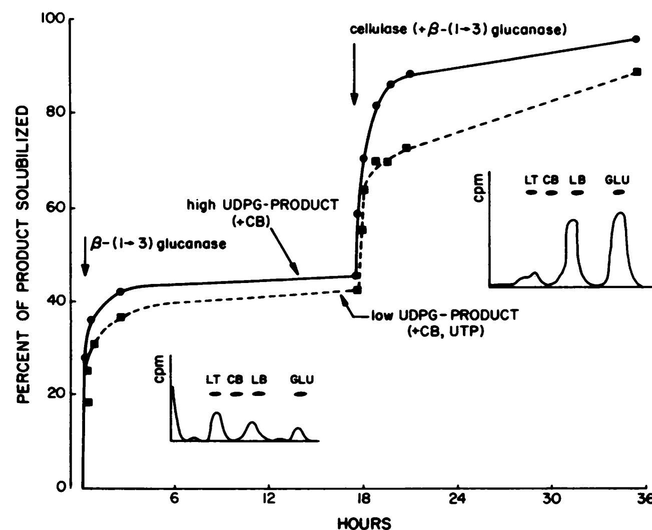


FIG. 1. A: Partial acid hydrolysis of high U product. Product was subjected to partial acid hydrolysis as described under "Materials and Methods" and the resulting hydrolysis products were separated by descending chromatography on Whatman No. 4 paper for 20 hr in 1-propanol-ethyl acetate-H_2O (7:1:2). Standards were co-chromatographed with samples. CB: cellobiose; LB: laminaribiose; GLU: glucose. B: Separation of products resulting from periodate oxidation, reduction, and complete hydrolysis of high U product. Products were separated by descending chromatography for 21 hr on Whatman No. 1 paper in 1-propanol-ethyl acetate-H_2O (7:1:2). Standards were co-chromatographed with samples. GLU: glucose; E: erythritol; GLYC: glycerol. The experiments were repeated at least twice with similar results.

controls indicated that cellobiose could be produced from α-cellulose under the conditions used. Radioactivity in the trisaccharide region co-chromatographed with laminaritriose and, upon prolonged separation in a separate analysis, was clearly separated from cellotriose, 3-O-β-cellobiosyl glucose, and 4-O-β-laminaribiosyl glucose.

Periodate Oxidation. Periodate oxidation followed by borohydride reduction and complete acid hydrolysis of the three products yielded primarily glucose, a trace of glycerol, and some material which remained at the origin (Fig. 1B; data shown only for the High U product). In no case could a significant peak of erythritol (indicative of 1-4 linkages) be detected, even though erythritol was produced from cellulose under our experimental conditions. This result is in agreement with the results of partial acid hydrolysis, and is consistent with the three products being predominately 1→3-linked glucans which are resistant to periodate oxidation. A small peak of glycerol was detected which amounted to a low percentage (1-3%) of the radioactivity of the major glucose peaks. Assuming that this glycerol arises from terminal glucose residues, this indicates that the radioactive products are not highly branched, and that the glucan synthetase(s) seems to be producing long chains and not to be a chain-terminating enzyme, adding just single glucose residues to preexisting polysacchardies.

Enzyme Digestion of Radioactive Products. When the High U or Low U products were incubated with *Rhizopus* β-(1→3)-glucanase, between 30 and 50% (the absolute value varied somewhat from experiment to experiment) of the radioactivity could be rapidly solubilized (Fig. 2, upper). If further glucanase was added after the plateau, no additional radioactivity could be released. If, at this point, the remaining insoluble product is incubated with a *Streptomyces* cellulase preparation (which is known to be contaminated with β-[1→3] and β-[1→6]-endoglucanase activity [E. T. Reese, personal communication]), the remainder of the product is rapidly solubilized. A similar digestion pattern is obtained for the High U-(P) product (Fig. 2, lower). Such results may indicate that a portion of the product is β-(1→3)-linked while another fraction may be β-(1→4)-linked glucan. When the products of either the β-(1→3)-glucanase *or* the subsequent cellulase digestion are analyzed by paper chromatography, we find that the predominant products in either case are glucose, laminaribiose, and a radioactive trisaccharide



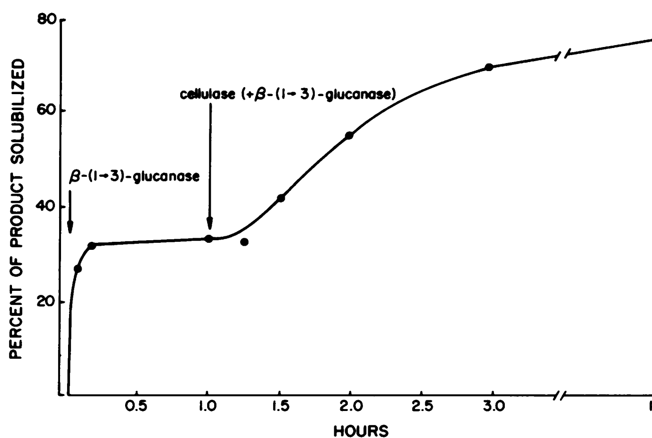


FIG. 2. Time course of enzyme digestion of radioactive glucan products. Upper graph shows the results for products of detached fiber assays; the lower graph, for the high U-(P) product. Insets show radioactive profiles of the digestion products separated by descending chromatography on Whatman No. 1 paper using 1-propanol-ethyl acetate-H_2O (7:1:2) as solvent. Lower inset was obtained from chromatography of an 18-hr β-(1→3)-glucanase digestion. Upper inset corresponds to chromatography of the subsequent cellulase digestion shown on the graph at 35 hr. The experiments were repeated at least three times with similar results. LT: laminaritriose; LB: laminaribiose; CB: cellobiose; GLU: glucose.

which migrates in the region of laminaritriose. When [14C]trisaccharide from either enzyme digestion was isolated on a Bio-Gel P-2 column and separated by borate electrophoresis, the radioactivity was clearly separated from 3-O-β-cellobiosyl glucose and 4-O-β-laminaribiosyl glucose and in all cases migrated with laminaritriose. In no case did we detect any radioactivity released that co-chromatographed with cellobiose.

The enzyme digestion data seem at least partly inconsistent with the previous results which indicated that the radioactive products were primarily linear β-(1→3)-glucans. If so, one would expect that the products would be more completely digested with β-(1→3)-glucanase, and no cellulase would be needed to complete the digestion. Nevertheless, the products of the cellulase digestion were only 3-linked oligosaccharides. One possible explanation is that a portion of the product is inaccessible to β-(1→3)-glucanase digestion unless endogenous cellulose is first digested. This possibility could be put forth for the detached fiber products, but seems less likely for the high U-(P) product. Another possibility is that the product is partially branched, but that the branched residues are synthesized from some endogenous unlabeled substrate. Some enzyme in the cellulase preparation could be cleaving the branched residues, thus making the remainder of radioactive β-(1→3)-glucan backbone accessible to digestion. An indication of such a structure could be obtained by a methylation analysis in which one should detect radioactive 3-linked glucose, and a certain fraction of radioactive doubly linked glucose without the corresponding amount of radioactive terminal glucose residues.

Methylation Analysis of the Products. Figure 3 shows the radioactive elution profile from GLC separations of the permethylated sugars resulting from permethylation, hydrolysis, reduction, and acetylation of the three U products. The recorder tracing shown is that obtained for the High U product and the peaks obtained correlate well with those obtained for cell walls of cotton fibers of this age for which we have assigned structural identifications by the use of combined GLC/MS techniques (8). The recorder tracing is identical for the methylated Low U preparations. For the High U-(P) preparation, only very small peaks of methylated sugars are detectable on the recorder. The elution profiles of radioactivity for product prepared under three different conditions were quite similar. The major fraction of the radioactivity elutes coincident with 3-linked glucose. Very little radioactive terminal glucose is obtained and no significant radioactivity is found in the region corresponding to branched hexoses. Thus, the possibility of branched residues on the glucan seems unlikely. Less than 5% of the total radioactivity is found under the region of 4-linked glucose, providing final confirmation of the fact that more than 95% of the product is not cellulose. The small peak of radioactivity eluting in the region of 4-linked xylose is unexplained. The size of this peak is variable (8–17% of total radioactivity) from preparation to preparation. Since total acid hydrolysis of the nonmethylated products releases only [14C]glucose, this peak most probably represents some rearranged or degraded product from the glucan.

Characteristics of Reaction Products Produced from Fibers of Various Ages. The products analyzed in the previous sections were formed by fibers harvested at the time of the rapid onset of secondary well cellulose deposition, a time which should be favorable for cellulose production. However, it seemed important to check whether the product formed at other ages might contain cellulose and/or β-(1→3)-glucan. High U product produced *in vitro* by fibers varying in age from 8 to 32 days postanthesis is susceptible to similar solubilization by acetic acid-nitric acid treatment, suggesting that little cellulose is produced in this assay at any fiber age. Because *in vivo* cellulose synthesis is maximal at 27 days postanthesis (8), we felt that it was important to confirm that the product formed at this age was not cellulose. Results of methylation analysis of either High U or

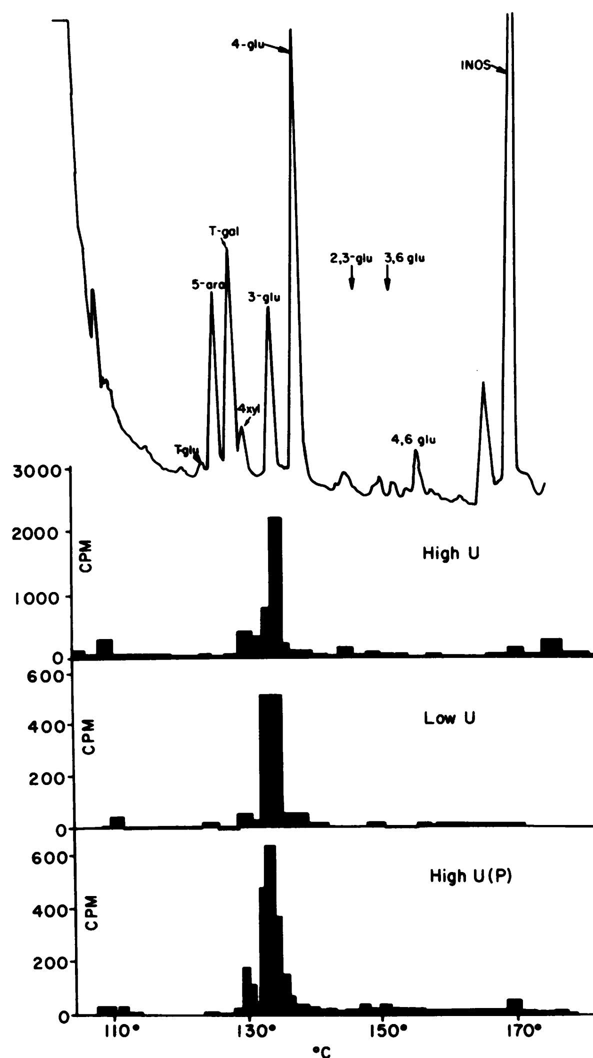


FIG. 3. GLC separation of the permethylated derivatives of the radioactive glucan products. Details of derivitization and separations are given under "Materials and Methods." Upper curve shows the recorder tracing of the elution profile of the permethylated derivatives obtained from the High U preparation. Peaks obtained correspond to derivatives of the neutral sugar residues present in the cell walls of the fibers. Identifications listed were determined both from retention times of standard derivatives and fragmentation patterns observed with combined GLC-MS (8). Lower bar diagram represents the elution profile of radioactivity from the chromatograph, for the three different products. Chromatography was performed with temperature programming at 1 C/min. The experiments were repeated at least three times with similar results. INOS: internal inositol standard.

Low U product formed by 27-day-old fibers show that the product is not cellulose, but also a predominately 3-linked glucan.

Characteristics of the Reaction Product of Pea Stem Cell Surface Glucan Synthetase. The fact that the characteristics of the cotton fiber glucan synthetase closely resembled those of a recently reported "cell surface cellulose synthetase" in pea stem sections (14, 15) prompted us to investigate the linkage of this product in more detail than that reported previously. Using product synthesized under conditions as close as possible to those described in the literature (14, 15), we find that the majority of this product does not appear to be cellulose but rather a β-(1→3)-glucan. This conclusion is based upon our finding that the product is >95% susceptible to solubilization by acetic acid-nitric acid treatment and that upon digestion with

Streptomyces cellulase or upon partial acid hydrolysis, >80% of the radioactivity is released as glucose and laminaribiose. Less than 8% of the radioactivity is found in cellobiose.

DISCUSSION

All of the data presented are consistent with the product synthesized by the cotton fiber glucan synthetase under all three conditions being a linear β-(1→3)-glucan. In contrast to reports in other plant systems (10, 16, 17), the concentration of UDP-glucose used in the assay has no effect on the type of product synthesized. These results also reemphasize the need to abandon alkali insolubility as a criterion for cellulose and against β-(1→3)-glucan. These data also indicate that differences in hot alkali solubility of certain polysaccharides are not necessarily indicative of basic structural differences but could reflect differences in degree of polymerization of the polymers or differences in localization and association with other polymers. One other notable example of this is the observation that a β-(1→3)-glucan from yeast cell walls is only soluble in hot alkali after other wall polymers are removed (2).

What is the physiological significance of such a highly active β-(1→3)-glucan synthetase in the developing cotton fiber? It is possible that the activity is normally latent and is observed as a "wound response" to fiber damage. However, recent results in our laboratory on the structure of the cotton fiber cell wall (8), indicate another possible role of this enzyme. In these studies, we have observed a large rise in the content of noncellulosic glucose in cell wall preparations from cotton fibers which occurs between 12 and 16 days postanthesis and coincides with the earliest rise in secondary well cellulose deposition. Methylation analyses of these cell wall preparations show a corresponding rise in 3-linked glucose in the wall fractions indicating that this noncellulosic glucan is a 3-linked polymer. As further support for this, the data of Figure 3 show a significant peak of 3-linked glucose in the methylated detached fibers (harvested 19 days postanthesis) used in these analyses. The amount observed in these analyses is far more than could be accounted for by the *in vitro* synthesis from UDP-[^{14}C]glucose, indicating its presence prior to assay. Thus, the glucan synthetase studied here is very probably responsible for catalyzing the synthesis *in vivo* of this naturally occurring glucan.

For reasons outlined previously (3), we feel that UDP-glucose is the most logical candidate to serve as precursor to secondary wall cellulose in the cotton fiber. Using this substrate under a variety of assay conditions with the cotton fiber system, more than 95% of the product formed is not cellulose. Furthermore, results of acetic-nitric treatment of methylation analyses indicate that the product is 3-linked regardless of fiber age. There is some reason to question whether *in vitro* synthesis of true microfibrillar cellulose has ever been achieved with any higher plant enzyme preparation, as it has recently been achieved without question for chitin in fungal preparations (11, 12). This situation would seem to indicate that conventional enzymological approaches to the problem of cellulose synthesis in higher plants are inadequate, and that the understanding of some fundamental and perhaps unique property of the enzyme for cellulose synthesis is still lacking.

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