

β -Glucan Synthesis in the Cotton Fiber¹

I. Identification of β -1,4- and β -1,3-Glucans Synthesized in Vitro

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In vitro β -glucan products were synthesized by digitonin-solubilized enzyme preparations from plasma membrane-enriched fractions of cotton (*Gossypium hirsutum*) fiber cells. The reaction mixture favoring β -1,4-glucan synthesis included the following effectors: Mg^{2+} , Ca^{2+} , cellobiose, cyclic-3':5'-GMP, and digitonin. The ethanol insoluble fraction from this reaction contained β -1,4-glucan and β -1,3-glucan in an approximate ratio of 25:69. Approximately 16% of the β -1,4-glucan was resistant to the acetic/nitric acid reagent. The x-ray diffraction pattern of the treated product favoring β -1,4-glucan synthesis strongly resembled that of cellulose II. On the basis of methylation analysis, the acetic/nitric acid reagent-insoluble glucan product was found to be exclusively β -1,4-linked. Enzymic hydrolysis confirmed that the product was hydrolyzed only by cellobiohydrolase I. Autoradiography proved that the product was synthesized in vitro. The degree of polymerization (DP) of the in vitro product was estimated by nitration and size exclusion chromatography; there were two average DPs of 59 (70%) and 396 (30%) for the β -1,3-glucanase-treated sample, and an average DP of 141 for the acetic/nitric acid reagent-insoluble product. On the basis of product analysis, the positive identification of in vitro-synthesized cellulose was established.

Cellulose is the most abundant macromolecule on earth (Brown, 1985). It serves a major structural role in the cell wall of plants, some algae, and certain fungi and is the primary component of economical products such as wood, cotton, and paper. Because of its tremendous abundance and its physiological and economical importance, many attempts at in vitro cellulose synthesis have been made with cell-free systems from various sources during the past three decades (Delmer, 1987; Brown, 1989b; Read and Delmer, 1991). The greatest progress has been made using *Acetobacter xylinum* as an experimental model (Ross et al., 1987; Brown, 1989a; Lin

and Brown, 1989; Lin et al., 1990; Saxena et al., 1990; Wong et al., 1990; Mayer et al., 1991; Saxena et al., 1991). In higher plants, however, numerous failures to detect activity for in vitro cellulose synthase have continued because it has remained impossible to make preparations from higher plant cells capable of synthesizing true microfibrillar cellulose or even appreciable quantities of β -1,4-glucan (Read and Delmer, 1991).

Several methods for the characterization of products synthesized in vitro, such as solubility properties, acid hydrolysis, acetolysis, specific enzymic digestion, and linkage analysis after periodate oxidation or methylation, have been used either alone or in various combinations (Fry, 1988). Insolubility in strong alkali (e.g. 24% KOH) has been used for the detection of β -1,4-glucan, but β -1,3-glucan synthesized in vitro may also be present in the alkali-insoluble fraction (Hayashi et al., 1987). The AN reagent (Updegraff, 1969) has been used for the selection of cellulose because only crystalline cellulose can remain after treatment with this reagent. The most reliable methods to prove the presence of crystalline cellulose are x-ray and electron diffraction analyses (Lin et al., 1985; Bureau and Brown, 1987). Highly purified cellulose conjugated to colloidal gold has been used as a probe to identify cellulose by TEM (Lin et al., 1985).

Cotton (*Gossypium hirsutum*) fiber cells have been used by many investigators for the study of cellulose biosynthesis (Delmer et al., 1984; Buchala and Meier, 1985). Experiments in which intact fibers from cotton fruit capsules (Pillonel and Meier, 1985), intact fibers from ovule cultures (Francey et al., 1989), and anucleate protoplasts isolated from fiber cells (Gould et al., 1986) were used have demonstrated cellulose synthesis. When fiber cells were detached from ovules in the presence of PEG, 50% of the capacity for cellulose synthesis was protected (Carpita and Delmer, 1980). In the absence of

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Abbreviations: AN reagent, acetic/nitric acid reagent; ANIP, AN reagent-insoluble product; C, experimentally determined correction factor; CBH I, cellobiohydrolase I; CB, cellobiose; DP, degree of polymerization; *DPctn*, the DP of cellulose trinitrate; EIP, ethanol-insoluble product; *F*_{1,3}, favoring the synthesis of β -1,3-glucan; *F*_{1,4}, favoring the synthesis of β -1,4-glucan; LB, laminaribiose; LT, laminaritriose; *Lctn*, extended chain length of cellulose trinitrate; *Lps*, extended chain length of polystyrene; MeOH, methanol; *MWctn*, mol wt of cellulose trinitrate; *MWps*, mol wt of polystyrene; *P/F*_{1,3}, product produced under conditions *F*_{1,3}; *P/F*_{1,4}, product produced under conditions *F*_{1,4}; PME, plasma membrane-enriched fraction; *Rf*_{GLC}, *Rf*_F factors relative to glucose; TEM, transmission electron microscopy.

PEG, only callose was synthesized (Heiniger and Delmer, 1977).

An early study in which membrane preparations from fiber cells were used in a completely cell-free system showed that glucan products synthesized in vitro were composed predominantly of callose, with less than 5% of 4-linked Glc identified by methylation analysis (Heiniger and Delmer, 1977). Later, it was found that certain ionophores, inducing a positive membrane potential inside the membrane vesicles, stimulated β -glucan synthesis in vitro, resulting in an increase of 4-linked Glc in ethanol-insoluble fractions to about 9% (Bacic and Delmer, 1981).

Delmer et al. (1984) improved the methods for isolation of plasma membranes from fiber cells and found that effectors such as CB, magnesium, and calcium ions may regulate glucan synthase activities and, consequently, may change the quantities and proportion of different glucans synthesized in vitro; however, there was no reasonably clear evidence for cellulose production.

In semi-intact cell systems (Pillonel and Meier, 1985; Gould et al., 1986; Francey et al., 1989) and in detached fibers with PEG (Carpita and Delmer, 1980), the AN reagent or hot DMSO (Pillonel et al., 1980) was used for cellulose isolation; however, less than 1% of glucan products synthesized in vitro were detected after treatment with the AN reagent (Bacic and Delmer, 1981).

In the series of studies of which this report is the first (Li and Brown, 1993; Li et al., 1993), we have demonstrated that (a) an ANIP is synthesized in vitro by cell-free membrane fractions from cotton fiber cells; (b) two different optimal reaction conditions, $F_{1,4}$ or $F_{1,3}$, were obtained by the combination of certain potent effectors, such as CB, cyclic nucleotides, magnesium, calcium, and digitonin; (c) among the polypeptides obtained by product entrapment, a 37-kD polypeptide is photolabeled only in the presence of Mg^{2+} , and it is primarily responsible for the synthesis of the ANIP, whereas the photolabeling of a 52-kD polypeptide is enhanced under the conditions with calcium but without magnesium. This polypeptide is postulated to be involved in the synthesis of β -1,3-glucan.

The goal of the present study was to characterize the products synthesized in vitro under conditions of $F_{1,3}$ and under conditions of $F_{1,4}$, using the following methods: solubility, total acid hydrolysis, enzymic digestion, methylation linkage analysis, x-ray diffraction, DP determination, and TEM coupled with autoradiography and CBH I-gold labeling.

MATERIALS AND METHODS

Plant Materials

The cotton line *Gossypium hirsutum* Texas marker 1 (TM-1) (Kohel et al., 1970) was used for the experiments. The seeds of this strain were provided by Barbara Triplett of Southern Regional Research Laboratory, U.S. Department of Agriculture, New Orleans, LA. TM-1 plants were grown in environmental chambers under incandescent and fluorescent lighting with a photoperiod and diurnal temperature variation of 14 h of daylight at 27°C and 10 h of darkness at 20°C. The plants were watered and fertilized, and on the

days of anthesis, flowers were tagged. Bolls were harvested 20 d after anthesis, and the locules were removed, quickly frozen in liquid nitrogen, and stored in a liquid nitrogen container until used for protein extraction.

Preparation of PME

The PME from cotton fibers was prepared by a modification of the procedure of Delmer et al. (1984). Cotton fibers were removed from seeds and ground to a fine powder in liquid nitrogen. This powder was then extracted with cold buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EGTA, 20% PEG (w/v), and a combination of protease inhibitors (0.5 mM PMSF, 10 μ M leupeptin, 0.1 mM *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone). The extract was filtered through a 210- μ m Spectra mesh screen (Spectrum Medical Industries, Inc., Los Angeles, CA) to remove cell walls, then carefully layered over a cushion of 60% Suc, and centrifuged at 5000g for 10 min. The membranes that concentrated at the buffer-Suc interface were carefully removed, resuspended in a 1:5 dilution of extraction buffer, and recentrifuged at 100,000g for 30 min. The membranes were resuspended in a small volume of 1:10 dilution of extraction buffer.

Solubilization of Membrane Proteins

The resulting membrane fraction was resuspended in a solubilization buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% digitonin, and 1.5 mg/mL of membrane preparation and sonicated on ice for 5 min. The solubilization mixture was layered over a 30% (w/v) glycerol cushion and centrifuged for 1 h at 100,000g at 4°C. The digitonin-solubilized enzymes in the buffer phase were carefully collected. The fractions of solubilized enzymes and residual membranes at the interface of the buffer and glycerol, the 30% (w/v) glycerol phase, and the bottom of the glycerol cushion were preserved and examined with TEM. The digitonin-solubilized enzymes were obtained free from contamination by native cotton fibers.

Preparation of the in Vitro Products

The reaction mixture $F_{1,4}$ contained 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μ M cyclic 3':5'-GMP, 0.05% digitonin, solubilized membrane proteins, 3 mM NaN_3 , and 1 mM UDP-Glc.

The reaction mixture $F_{1,3}$ contained 10 mM bis-trispropane-Hepes (pH 7.4), 20 mM CB, 2 mM $CaCl_2$, 0.05% digitonin, solubilized membrane proteins, 3 mM NaN_3 , and 1 mM UDP-Glc.

The reactions synthesizing in vitro glucan products were performed by incubation at 25°C for 4 h or overnight. The reaction mixtures were centrifuged at 7000g for 40 min. The pellets were washed with deionized water. Specific treatments of the in vitro products will be described in conjunction with particular experiments. For the synthesis of radioactive in vitro products, 0.5 mM UDP-[U- ^{14}C]Glc (specific activity: 12,500 cpm/nmol), diluted from the original UDP-[U- ^{14}C]Glc (200 mCi/mmol; ICN, Costa Mesa, CA) was used as the

substrate. Protein assay was performed using a modification of the Lowry procedure (Markwell et al., 1978).

Product Solubility

Each radioactive in vitro product was subjected to one of the following manipulations: (a) 30-min soaking in distilled water at room temperature, filtering through a GC/F glass filter, followed by rinsing three times with deionized water and once with MeOH; (b) 30-min treatment with 66% (v/v) ethanol at -20°C , filtering through a GC/F glass filter, followed by rinsing three times with 66% (v/v) ethanol and once with MeOH and chloroform (1:1, v/v); (c) 20-min treatment with water in a boiling water bath, filtering through a GC/F glass filter, followed by rinsing three times with deionized water and once with MeOH; (d) 20-min treatment with 24% (w/v) KOH and 0.5 M NaBH₄ in a boiling water bath, filtering through a GC/F glass filter, followed by neutralizing with HCl (0.5 N), then rinsing three times with deionized water and then once with MeOH; or (e) 30-min treatment in AN reagent in a boiling water bath, filtering through a GC/F glass filter, followed by neutralizing with 0.5 N NaOH, then rinsing three times with deionized water and once with MeOH. The radioactivity retained on the filter was dissolved in Ready Organic cocktail (Beckman Instruments, Inc., Fullerton, CA) and was counted with an LS 6800 Liquid Scintillation System (Beckman Instruments, Inc., Irvine, CA).

Total Acid Hydrolysis

Total acid hydrolysis was performed according to the Seaman procedure (Fry, 1988). The radioactive in vitro EIP or ANIP was treated with 58% (v/v) H₂SO₄ for 1 h at room temperature with shaking. It was then diluted with distilled water to give a final concentration of 1 M H₂SO₄ and heated at 120°C (autoclaved) for 1 h. Sulfate ions were removed by titration to neutrality with 0.2 M Ba(OH)₂ with bromophenol blue as the indicator. The resulting BaSO₄ was removed by centrifugation. After the mixture was concentrated by freeze drying, the acid hydrolysates were chromatographed on a Whatman silica gel 60A TLC plate (Whatman International Ltd., Maidstone, England), developed with a solvent containing isopropanol:acetone:0.1 M lactic acid (4:4:2), and visualized by spraying with 4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, 30 mL of 80% H₃PO₄ and heating for 30 min in an oven at 105°C as described by Hansen (1975). To detect the radioactive products, the TLC plate was sprayed with EN3HANCE spray (NEN Research Products, Boston, MA) and then exposed to Kodak X-Omat AR x-ray film (Eastman Kodak Co., Rochester, NY) for 3 d at -80°C .

Enzymic Digestion

Radioactive in vitro products were incubated for 72 h at 40°C with either buffer (buffer I: 25 mM acetate, pH 5.0, with 3 mM NaN₃; buffer II: 50 mM maleate, pH 6.5, with 3 mM NaN₃) or 2 units of *Trichoderma reesei* CBH I (Novo Industri A/S, Copenhagen, Denmark) in acetate buffer or with 2 units of *Rhizopus arrhizus* β -1,3-endoglucanase (a gift

from Dr. E.T. Reese of the U.S. Army Laboratories, Natick, MA) in acetate buffer (Reese, 1977) or with 2 units of *Bacillus subtilis* (1-3)/(1-4)- β -glucan hydrolase (Biosupplies Australia, Parkville, Victoria, Australia) in 50 mM maleate buffer (pH 6.5) (Anderson and Stone, 1975). Enzyme solutions were renewed every 24 h. Released oligosaccharides were extracted with 66% ethanol, and the supernatant was collected by centrifugation. Under the above conditions, almost no radioactive product was released during the third 24-h incubation. The percentage of the product digested by each enzyme was calculated by counting radioactivity in the aliquot of each hydrolysate. The remainder of the hydrolysates was dried in vacuo at 40°C and then resuspended in a small amount of distilled water. Chromatograms of the enzymic hydrolysates were run on Whatman silica gel 60A TLC plates and visualized as described above. Nonradioactive in vitro products also were prepared for HPLC analysis. Positive control experiments were performed under the same conditions with known substrates: Sigmacell, type 100 (Sigma Chemical Co., St. Louis, MO), pachyman (from *Poria cocos*; Calbiochem, La Jolla, CA), barley glucan (Biosupplies Australia), and lichenin (from *Cetraria islandica*; Sigma). Glc and known oligosaccharides CB, LB, LT, G4G3GR, and G4G4G3GR were used as controls for hydrolysates (LT, G4G3GR, and G4G4G3GR were supplied by Dr. B. Stone, La Trobe University, Melbourne, Australia). A carbohydrate HPX-42A column (300 \times 7.8 mm; Bio-Rad, Richmond, CA) was used for HPLC.

Methylation Analysis

The in vitro products were treated with methanol:chloroform (1:1, v/v) for 30 min at room temperature to remove lipids and then treated with 10 mg/mL of protease, type 14 (Sigma), in 50 mM bis-trispropane-Hepes (pH 7.6) containing 3 mM NaN₃ overnight at 40°C to remove proteins, and the products were divided into aliquots that were then treated with either 66% ethanol or AN reagent. The treated in vitro products were thoroughly washed with distilled water and then freeze dried.

Methylation analysis was performed using Hakomori's methylation procedure as described by York et al. (1985), except that butyllithium was used to prepare the lithium DMSO anion rather than potassium hydride or sodium hydride. The freeze-dried samples were suspended in DMSO and then butyllithium was added, and the samples were stirred in the lithium-DMSO anion for several hours. An excess of methyl iodide was added and the samples were allowed to stir overnight. The permethylated samples were then purified using a C₁₈ Sep Pak cartridge. The methylation procedure was repeated with the same samples. The resulting purified permethylated samples were then hydrolyzed using 1 N TFA, reduced with sodium borodeuteride, and acetylated using acidic anhydride-pyridine. The permethylated alditol acetates were analyzed by GC-MS using a Hewlett-Packard benchtop GC-MS system. A 30-m capillary SP2330 column from Supelco was used for this analysis. This methylation analysis was performed at the Complex Carbohydrate Research Center (Athens, GA).

X-Ray Diffraction

The in vitro product was synthesized under conditions favoring β -1,4-glucan synthesis and then treated with methanol-chloroform and protease as described above. Next, the product was treated either with AN reagent or with 24% KOH-0.5 M NaBH₄, followed by AN reagent, or with *R. arrhizus* β -1,3-glucanase, yielding three classes of in vitro products. These products were washed three times with water, once with methanol, freeze-dried, and packed into a 0.3-mm glass capillary. The x-ray diffraction patterns were recorded with Ni-filtered Cu K α radiation (35 kV, 25 mA, 45-min exposure time) using a Philips PW 1729 x-ray generator and a Debye Scherrer camera (PW 1024/30; Philips Electronics Instruments, Eindhoven, Holland).

EM

A colloidal gold suspension was used to prepare a complex with CBH I (AuroBeads G5, average particle size 5 nm; Amersham, Arlington Heights, IL). The CBH I-gold complex was prepared as described by Chanzy et al. (1984). Gold labeling of the in vitro products for TEM was performed by floating grids with the product on a drop of a CBH I-gold complex solution on ice for 3 min and washing with water; they were then negatively stained (1.5% uranyl acetate) and examined with a Philips EM 420 transmission electron microscope.

For the autoradiographic studies, the reaction mixture was essentially the same as described above, except that 0.5 mM UDP-[1-³H]-Glc (20 mCi/mmol) (NEN Research Products, Boston, MA) was used as the substrate. Reactions were performed for 90 min at 25°C. Three control experiments were conducted: (a) zero time incubation, (b) without substrate, and (c) with unlabeled UDP-Glc as the substrate. After incubation, the in vitro products were collected and treated as described above. The water suspensions of these products were mounted on Formvar-coated grids, allowed to dry, treated with CBH I-gold complex solution, and then coated with Ilford L-4 photographic emulsion as described by Kopriva (1973). Coated samples were exposed for 10 d, developed with Kodak D-19 (diluted 1:10) for 1 min, and then negatively stained by 1.5% uranyl acetate.

DP Determination

The in vitro products treated with β -1,3-glucanase or AN reagent were dried for 30 min at 80°C in vacuo and converted to cellulose trinitrate by treating with an ice-cooled mixture of nitric acid and phosphorus pentoxide (Alexander and Mitchell, 1949). The nitrated product was collected on a fritted glass filter and washed thoroughly with distilled water. The samples were dried, dissolved in tetrahydrofuran, and analyzed by size exclusion chromatography columns (TSK-GEL GMHXL 300 mm and G2500 H8 300 mm; Supelco, Inc., Bellefonte, PA). The column set was calibrated by a series of monodisperse polystyrenes (Tosoh, Inc., Tokyo, Japan; Meyerhoff and Jovanovic, 1967; Timpa, 1991). A cellulose sample of an average DP of 200 (Filter Pulp; Advantec, Inc., Tokyo, Japan) was also used as the standard.

The *DPctn* was estimated as follows: the *MWps* with the same retention time as the cellulose nitrate sample in question

was obtained by using the calibration shown in Figure 8. The *Lps* is given by

$$Lps = MWps/(104/2.54) = MWps/40.94$$

where 104 and 2.54 are the mol wt and length of the repeating unit of polystyrene, respectively. The *Lctn* with the same retention time is given by

$$Lctn = Lps/C$$

where *C* = 2.5 (at low mol wt range). *C* is the experimentally determined correction factor accounting for the difference in the relation between the hydrodynamic volume and the *Lps* and *Lctn* (Meyerhoff and Jovanovic, 1967). The value of *C* was determined from graphic analysis of figure 1 of Meyerhoff and Jovanovic (1967). Combining these equations, we obtain

$$DPctn = Lctn/5.15 = MWps/527 \quad (1)$$

where 5.15 is the length per anhydroglucose unit of cellulose. This relationship holds regardless of the type of column or other conditions of size exclusion chromatography. In fact, our result for the standard cellulose we have used (*DP* = 247 from Eq. 1) agreed well with the value based on viscometry by cupraethylenedamine solvent for the same material (*DP* = 200).

RESULTS

Total Acid Hydrolysis

Complete acid hydrolysis of the radioactive in vitro product followed by TLC gave Glc and trace amounts of Man or possibly Ara in the ethanol-insoluble products (EIPs) and only Glc in the acetic-nitric acid reagent-insoluble product (ANIP), suggesting that the EIPs are composed essentially of glucans, and the ANIPs are exclusively glucans (Fig. 1).

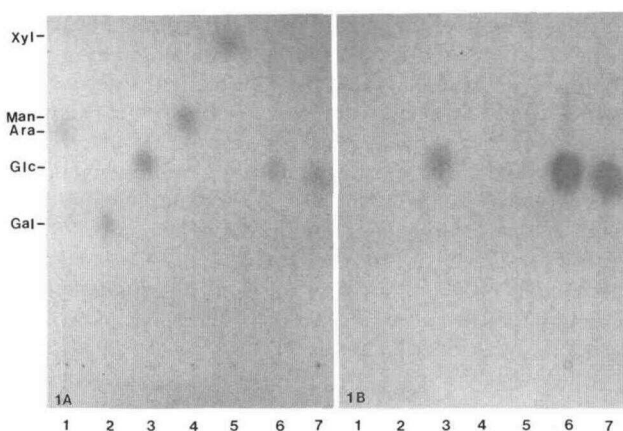


Figure 1. Total acid hydrolysis (TLC). Lanes 1, Ara; lanes 2, Gal; lanes 3, unlabeled Glc and [¹⁴C]Glc; lanes 4, Man; lanes 5, Xyl; lanes 6, hydrolysate of radioactive in vitro product insoluble in 66% ethanol; lanes 7, hydrolyses from the ANIP. A, Visualized by the spraying reagent (1 mL of aniline, 1 g of diphenylamine, 50 mL of acetone, 7.5 mL of 80% H₃PO₄). B, Visualized by autoradiography.

Table I. Solubility properties of P/F_{1,4} and P/F_{1,3} synthesized in vitro by cotton solubilized enzymes

Treatment	P/F _{1,4}	P/F _{1,3}
	% of retained radioactivity	
66% ethanol, -20°C, 30 min	100.0	100.0
H ₂ O, room temperature, 30 min	61.9 ± 1.0	91.5 ± 0.9
H ₂ O, 100°C, 20 min	34.0 ± 0.1	29.5 ± 0.9
24% KOH-0.5 M NaBH ₄ , 100°C, 20 min	22.9 ± 0.4	5.9 ± 0.8
AN reagent, 100°C, 30 min	4.0 ± 0.7	Trace

Product Solubility

The solubility of either P/F_{1,4} or P/F_{1,3} in various extractions is summarized in Table I. It should be noted that compared with P/F_{1,4}, P/F_{1,3} had a higher solubility in hot water and alkali solution and was almost completely dissolved in the AN reagent. In contrast to P/F_{1,3}, the P/F_{1,4} fraction contained about 4% insoluble material in the AN reagent.

Enzymic Digestion

Representative chromatograms of sugar standards are shown in Figure 2A. R_fGlc of sugar standards obtained with this solvent system are CB, 0.84; LB, 1.25; LT, 1.32; G4G3GR, 0.96; and G4G4G3GR, 0.76. The identification of Glc and G4G3GR was further confirmed by HPLC because R_fGlc of G4G3GR was close to that of Glc. The results of positive control experiments with the known substrates are shown in Figure 2, B and C: Sigmacell (type 100), pachyman, barley glucan, lichenin, and three enzymes, *T. reesei* CBH I, *R.*

arrhizus β -1,3-glucanase, and *B. subtilis* (1-3)/(1-4)- β -glucan hydrolase. These results are summarized in Table II. *T. reesei* CBH I hydrolyzed both β -1,4-glucans and mixed linked glucans, *R. arrhizus* β -1,3-glucanase hydrolyzed both β -1,3-glucans and mixed linked glucans, and *B. subtilis* (1-3)/(1-4)- β -glucan hydrolase hydrolyzed only mixed linked glucans (Table II) (for properties of these enzymes, also consult Anderson and Stone, 1975; Reese, 1977; Henrissat et al., 1985).

Three types of in vitro products were analyzed by enzymic digestions (Table III): (a) EIP/F_{1,3}, (b) EIP/F_{1,4}, and (c) ANIP/F_{1,4}. The percentage of β -1,4-glucan was obtained from the difference between the radioactivity released by *T. reesei* CBH I and that by *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase, and the percentage of β -1,3-glucan was obtained from the difference between the radioactivity released by *R. arrhizus* β -1,3-glucanase and that by *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase. Therefore, the composition of EIP/F_{1,4} was shown to be about 25% β -1,4-glucans, 69% β -1,3-glucans, and 6% unidentified glucans. EIP/F_{1,3} is essentially composed of β -1,3-glucan (*T. reesei* CBH I released only a trace amount of radioactivity from this product). ANIP/F_{1,4} contained exclusively β -1,4-glucans, which were degraded only by CBH I.

Enzymic hydrolysates of EIP/F_{1,4} and ANIP/F_{1,4} were characterized by TLC (Fig. 2D). Glc, CB, and LB were released by *T. reesei* CBH I from EIP/F_{1,4} (lane 2); Glc, LB, LT (trace), and probably laminarotetraose and laminaropentaose were released by *R. arrhizus* β -1,3-glucanase (lane 3); LB, LT, Glc (trace), and CB (trace) were released by *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase (lane 4). CB (majority) and Glc were released from ANIP/F_{1,4} by *T. reesei* CBH I (lane 5). Nothing was released by the other two enzymes indicated in lanes 6 and 7.

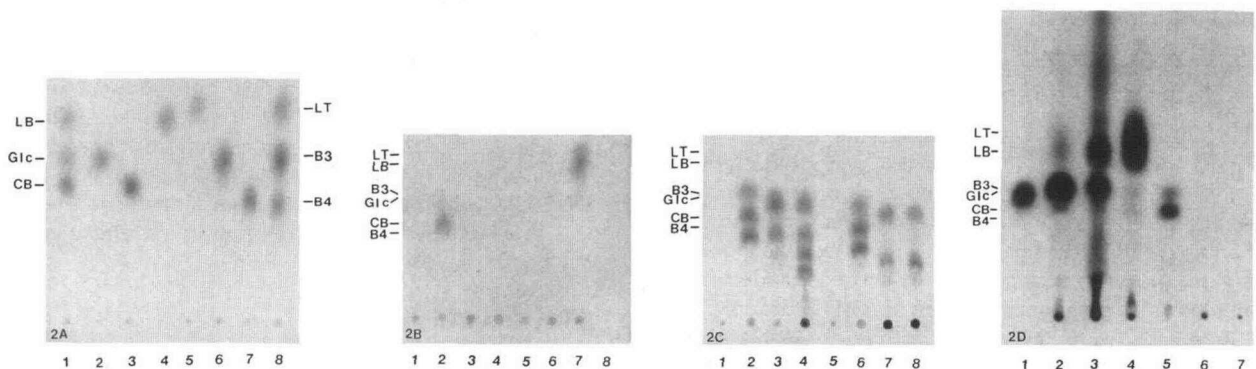


Figure 2. TLC of sugar standards and enzymic hydrolysis of known glucans, EIP/F_{1,4}, and ANIP/F_{1,4}. A, TLC of sugar standards: lane 1, Glc, CB, and LB; lane 2, Glc; lane 3, CB; lane 4, LB; lane 5, LT; lane 6, G4G3GR; lane 7, G4G4G3GR; lane 8, LT, G4G3GR, G4G4G3GR. B, Hydrolysis of Sigmacell and pachyman: lane 1, Sigmacell plus buffer; lane 2, Sigmacell plus *T. reesei* CBH I; lane 3, Sigmacell plus *R. arrhizus* β -1,3-glucanase; lane 4, Sigmacell plus *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase; lane 5, pachyman plus buffer; lane 6, pachyman plus *T. reesei* CBH I; lane 7, pachyman plus *R. arrhizus* β -1,3-glucanase; lane 8, pachyman plus *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase. C, Hydrolysis of barley glucan and lichenin: lane 1, barley glucan plus buffer; lane 2, barley glucan plus *T. reesei* CBH I; lane 3, barley glucan plus *R. arrhizus* β -1,3-glucanase; lane 4, barley glucan plus *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase; lane 5, lichenin plus buffer; lane 6, lichenin plus *T. reesei* CBH I; lane 7, lichenin plus *R. arrhizus* β -1,3-glucanase; lane 8, lichenin plus *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase. D, Enzymic digestion of EIP/F_{1,4} and ANIP/F_{1,4}: lane 1, [¹⁴C]Glc; lane 2, EIP/F_{1,4} hydrolyzed by *T. reesei* CBH I; lane 3, EIP/F_{1,4} hydrolyzed by *R. arrhizus* β -1,3-glucanase; lane 4, EIP/F_{1,4} hydrolyzed by *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase; lane 5, ANIP/F_{1,4} hydrolyzed by *T. reesei* CBH I; lane 6, ANIP/F_{1,4} hydrolyzed by *R. arrhizus* β -1,3-glucanase; lane 7, ANIP/F_{1,4} hydrolyzed by *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase.

Table II. Enzymic hydrolysates of known β -glucans released by *T. reesei* CBH I, *R. arrhizus* β -1,3-glucanase, and *B. subtilis* (1,3)/(1,4)- β -glucan hydrolase in positive control experiments

B3, G4G4GR; B4, G4G4G3GR; B5, G4G4G4G3GR; B6, G4G4G4G4G3GR; B7, G4G4G4G4G4G3GR, +, small amount; ++, large amount; +++, extremely large amount; \pm , trace; n.d., not detected; ?, because no standards for B5, B6, and B7 were available, the oligosaccharides denoted in front of the question mark are inferred.

Substrates	Enzymes		
	<i>T. reesei</i> CBH I	<i>R. arrhizus</i> β -1,3-Glucanase	<i>B. subtilis</i> (1,3)/(1,4)- β -Glucan Hydrolase
Sigmacell (type 100)	CB+++; Glc+	n.d.	n.d.
Pachyman	n.d.	LB+++; Glc+	n.d.
Barley glucan	B4+++; CB+++; Glc+++; B3 \pm	B3+++; B4+++; LB+; B5? \pm	B3+++; B4+++; B5?+; B6?+++; B7?+++
Lichenin	Glc+++; CB+++; B4+++; B3 \pm	B3+++; B5?++++; B4 \pm	B3+++; B5?++++; B4+

Methylation Analysis

The results of linkage determination by methylation analysis of the in vitro products are shown in Table IV. EIP/F_{1,3} consisted of 3-linked and 2,3-linked Glc; 4-linked Glc was not detected in EIP/F_{1,3}. Both 3-linked Glc and 4-linked Glc were found in EIP/F_{1,4} in the ratio of 61:26. The ANIP/F_{1,4} consisted of only 4-linked Glc, which supports the results of enzymic digestion.

EM

The in vitro EIP fraction was so strongly aggregated with proteins that it was difficult to distinguish any product labeled by gold particles (not shown). After protease treatment, two different structures could be identified (Fig. 3): (a) extended fibrils that maintain a constant dimension not larger than 1.3 to 1.6 nm (Fig. 3, B and E), this probably being due to the helical arrangement of the β -1,3-glucan chains; (b) extensive subfibrils of 1.3 nm aggregated or fasciated with other subfibrils to form dimensions of 3 to 5 nm (Fig. 3, C and D). These aggregates were strongly labeled by CBH I-gold particles, indicating that they are composed of β -1,4-glucan chains. The β -1,3-glucan in vitro product was observed more often than the β -1,4-glucan in vitro product. Furthermore, CBH I-gold particles were never attached to the β -1,3-glucan in vitro product (Fig. 3E). The extended fibrils observed in (a) were similar to those of β -1,3-glucan synthesized in vitro by mung beans (Hayashi et al., 1987), *Neurospora* (Jabri et al., 1989), and *Saprolegnia* (Fevre and Rougier,

1981). These data suggest that the product in (a) is composed of β -1,3-glucans, that is, callose.

In the fraction treated with the AN reagent, spine-like fibrils consisting of overlapping rodlets (40–100 nm long, 5–15 nm wide) (Fig. 4B) were observed together with elongated fibrils (20–200 nm long and 5–10 nm wide) (Fig. 4A). The rodlets (Fig. 4B) consisted of very fine filaments (approximately 1 nm in diameter) that are associated with each other by lateral fasciation. The CBH I-gold probe was bound to these rodlets. These observations are in accord with the results from the enzymic digestions and the methylation analysis and support the conclusion that only β -1,4-glucan remained after treatment with the AN reagent.

The incorporation of [³H]Glc into the in vitro product, followed by AN reagent treatment, CBH I-gold labeling, autoradiography, and negative staining, shows that colloidal gold and silver grains both coincide with the needle-like fibrils of the in-vitro product (Fig. 5). The radioactive labeling provides evidence that the product is a glucan derived from the supplied UDP-Glc, and it does not represent any preexisting or contaminating fibrils in the preparation. CBH I-gold particles labeled the same material, indicating that it is β -1,4-glucan. The silver grains were not observed in controls, when UDP-Glc was omitted or when it was added and the reaction was immediately terminated by adding AN reagent (0-min incubation time).

X-Ray Diffraction

The x-ray diffraction patterns of cotton in vitro products are shown in Figure 6, A to C. The in vitro product treated

Table III. Enzymic digestion of the in vitro products synthesized by cotton-solubilized enzymes

Fractions	Percent of Released Radioactivity by		
	<i>T. reesei</i> CBH I	<i>R. arrhizus</i> β -1,3-Glucanase	<i>B. subtilis</i> (1,3)(1,4)- β -Glucan Hydrolase
EIP/F _{1,3}	Trace	100.0	0
EIP/F _{1,4}	31.0 \pm 0.6	74.9 \pm 1.0	5.8 \pm 0.5
ANIP/F _{1,4}	100.0	0	0

Table IV. Methylation analysis of three different in vitro products from the digitonin-solubilized enzymes of cotton fiber cells

Glycosyl Residue	EIP/F _{1,3}	EIP/F _{1,4}	ANIP/F _{1,4}
relative area % of GC peaks			
3-Linked Glc	65	61	0
4-Linked Glc	0	26	80
2,3-Linked Glc	16	0	0
4,6-Linked Glc	0	0	Trace
Terminal Glc	19	13	20

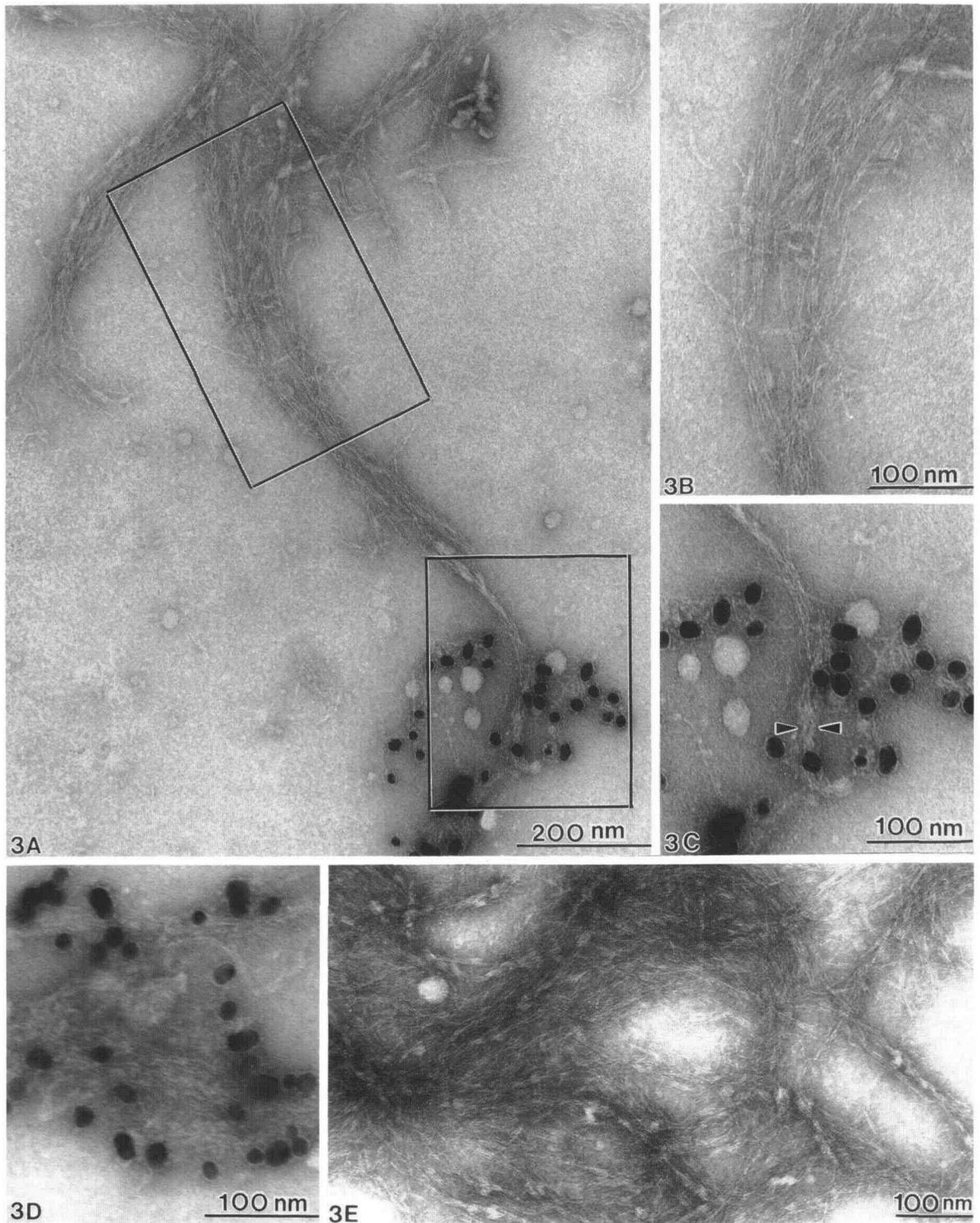


Figure 3. A to D, Negatively stained and CBH I-gold-labeled *in vitro* EIP synthesized under conditions of $F_{1.4}$. A (upper), Long, extended fibrils arranged linearly or overlapping to form a fibrous mass not labeled with CBH I-gold particles, indicating β -1,3-glucan chains. A (lower), Extensively laterally fasciated fibrils labeled with CBH I-gold particles, indicating β -1,4 glucan chains. B, Higher magnification view of upper box of A. Note the distinct separated elongated fibrils of the β -1,3-glucan. C, Higher magnification view of lower box of A. Note extensive lateral fasciation that produced a 3- to 5-nm aggregate (two or more shown between arrowheads). These dimensions are sufficient to generate a crystalline product. D, Another region showing a mass of β -1,4-glucan that is highly fasciated and tightly associated with the CBH I-gold label. E, Negatively stained and CBH I-gold labeled *in vitro* EIP synthesized under conditions of $F_{1.3}$. No single CBH I-gold particle attached to the product is visible. This product is similar in its structure to the product shown in B.

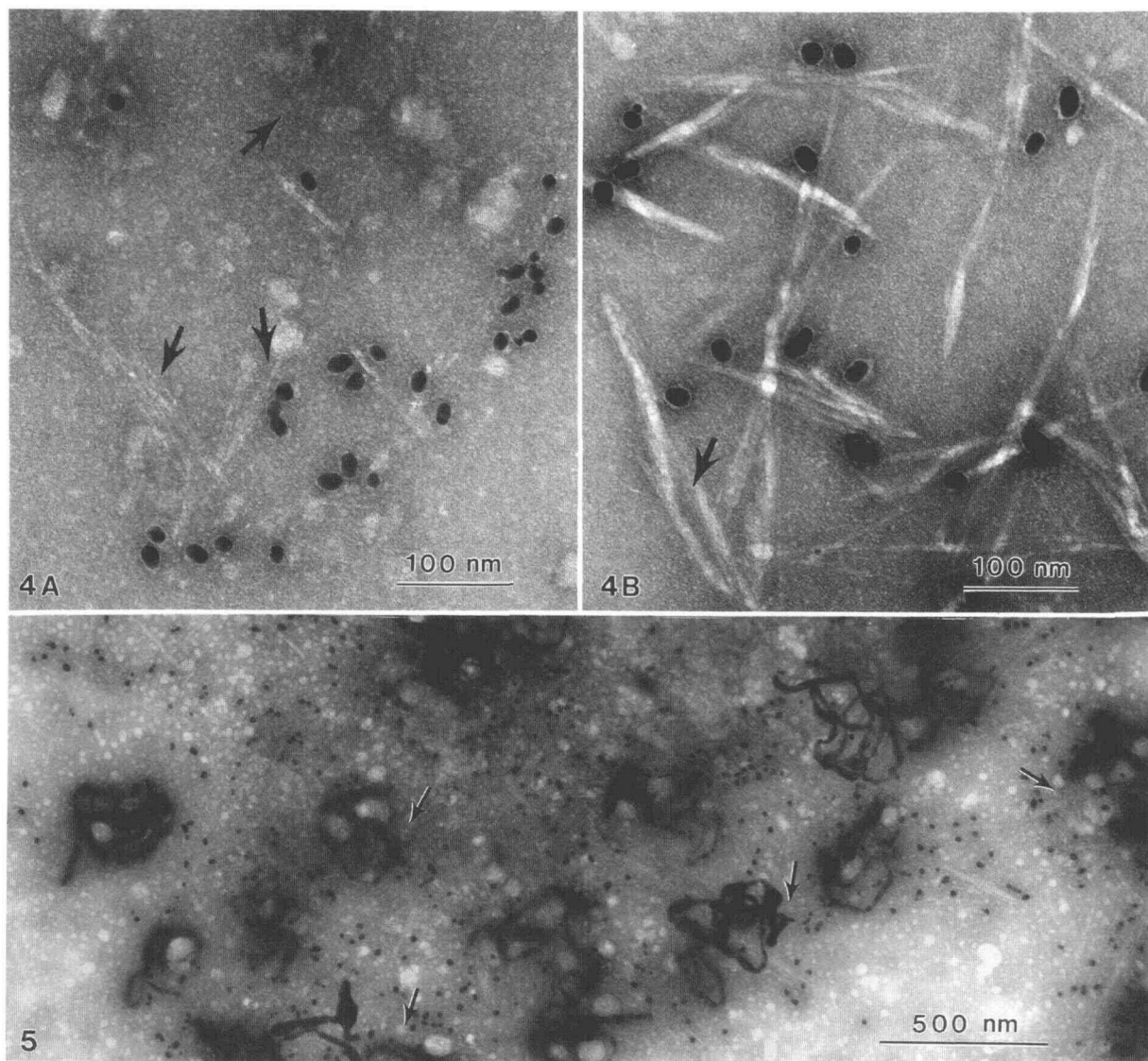


Figure 4. Electron micrographs of in vitro ANIPs. Note elongated fibrils (A) and spine-like fibrils (B) labeled by CBH I-gold complexes. Arrows indicate fine filaments.

Figure 5. Autoradiography of in vitro ANIP. Note silver grains that look like fibrous electron-dense masses and CBH I-gold particles associated with fibrils (arrows).

with *R. arrhizus* β -1,3-glucanase is shown in Figure 6A. The in vitro product treated directly with the AN reagent is shown in Figure 6B. The sample shown in Figure 6C was treated first with 24% KOH, followed by AN reagent. A native cotton fiber fraction treated with methanol-chloroform, followed by AN reagent, is shown in Figure 6D. This is a control showing a typical diffraction pattern for cellulose I synthesized in vivo by cotton. The x-ray diffraction pattern of a native cotton sample treated with KOH, followed by AN reagent, is shown in Figure 6E. This pattern is similar to that of rayon or cellulose II. The diffraction pattern of an authentic rayon sample (i.e. cellulose II) is shown in Figure 6F. The diffraction patterns also are presented for pachyman, a noncrystalline

β -1,3-glucan (Fig. 6G), and paramylon, a highly crystalline β -1,3-glucan (Fig. 6H).

A comparison was made of the patterns produced by the in vitro-synthesized cotton samples (Fig. 6, A–C) with that in Figure 6D, which is from native cellulose I, and Figure 6E, which is from mercerized cotton cellulose. The sample treated with *R. arrhizus* β -1,3-glucanase (Fig. 6A) shows three reflections characteristic of cellulose II, namely the 7.60 Å (=a), 4.36 Å (=b), and 3.90 Å (=c) reflections. The AN reagent-treated sample (Fig. 6B) shares two reflections characteristic of cellulose II, namely the 4.22 Å (b) and 3.90 Å (c) reflections. The sample treated with KOH followed by the AN reagent (Fig. 6C) shows a weak reflection of 7.44 Å (a) and stronger

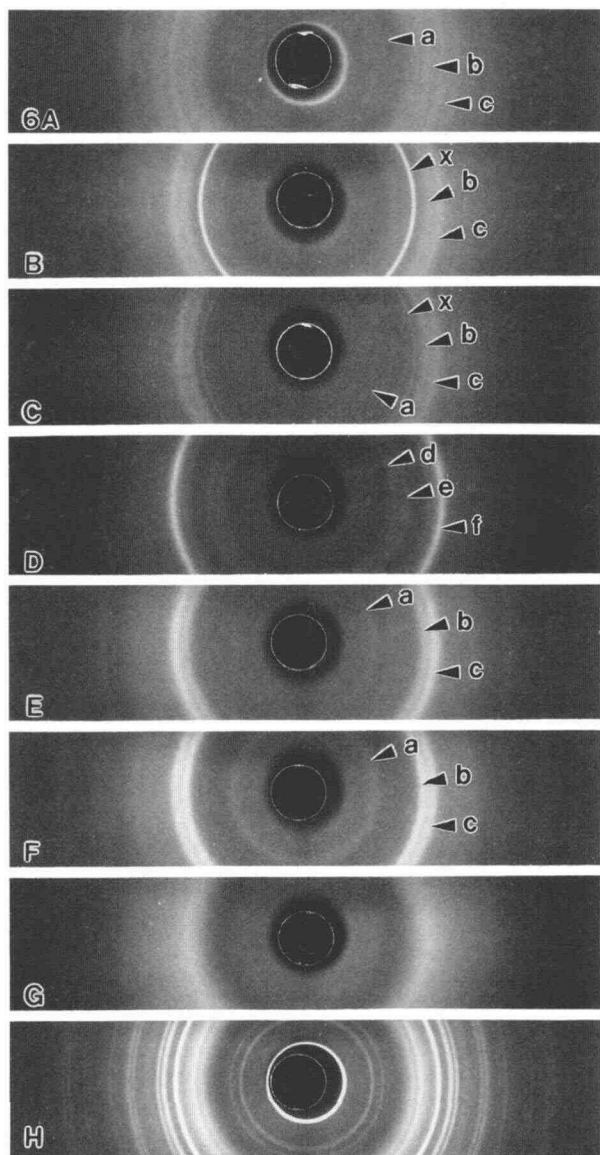


Figure 6. X-ray diffraction patterns of cotton in vitro products and appropriate controls. A, Cotton in vitro product treated with *R. arrhizus* β -1,3 glucanase; B, cotton in vitro ANIP; C, cotton in vitro product treated with 24% KOH, followed by AN reagent; D, native cotton fiber treated with AN reagent (as the cellulose I control); E, mercerized cotton fiber treated with KOH, followed by AN reagent (as the cellulose II control); F, bona fide sample of rayon treated with KOH, followed by AN reagent (as the cellulose II control); G pachyman, a noncrystalline sample of β -1,3-glucan; H, paramylon, a highly crystalline sample of β -1,3-glucan. Reflections for samples: A, a = 7.60 Å, b = 4.36 Å, c = 3.90 Å; B, x = 4.86 Å, b = 4.22 Å, c = 3.90 Å; C, a = 7.44 Å, b = 4.36 Å, c = 4.06 Å, x = 4.86 Å; D, d = 5.95 Å, e = 5.30 Å, f = 3.88 Å; E, a = 7.44 Å, b = 4.34 Å, c = 4.01 Å; F, a = 7.34 Å, b = 4.38 Å, c = 4.05 Å.

reflections at 4.86 Å (x), 4.36 Å (b), and 4.06 Å (c). Note that the 4.86-Å reflection is unique in both B and C of Figure 6.

DP of in Vitro Products

Size exclusion chromatography of the β -1,3-glucanase-treated in vitro product showed a peak (about 70% of the total area) with a retention time of 27.7 min and a shoulder fraction (about 30% of total area) with a retention time of 25.2 min (Fig. 7D). The *MW*ps corresponding to the peak

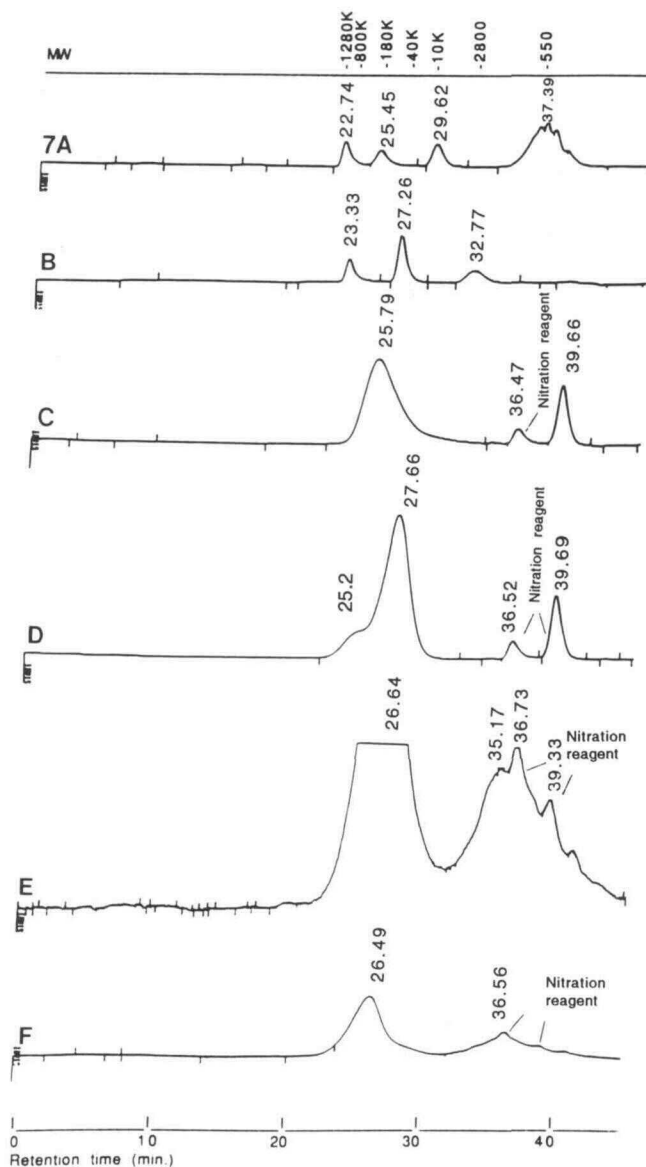


Figure 7. A to E, Size exclusion chromatogram. A, Polystyrene mixture I (mol wt: 1,280,000; 180,000; 10,000; 550). B, Polystyrene mixture II (mol wt: 800,000; 40,000; 2,800). C, Filter pulp, a cellulose sample with average DP of 200. D, The in vitro product treated with *R. arrhizus* β -1,3-glucanase. E and F, The in vitro product treated with AN reagent. E, Overloaded sample; F, sample with adjusted volume.

position was 30,900, conforming to a DP of 59 for cellulose trinitrate, whereas that corresponding to the shoulder fraction was 208,900, or a DP_{ctn} of 396 (Fig. 8). The in vitro product was severely degraded by treatment with the AN reagent, and most of the material was lost during this treatment. Furthermore, the mol wt of the remaining product was reduced, and size exclusion chromatography showed a major peak (about 88% of the total area) with a retention time of 26.6 min and a small peak (about 12% of the total area) with a retention time of 35.2 min (Fig. 7E). This small peak was fused with a peak having a retention time of 36.7 min, which came from the nitration reagent when the volume of the injected sample was adjusted (Fig. 7F). The $MWps$ corresponding to the major peak was 74,100, conforming to a DP of 141 (Fig. 8).

DISCUSSION

From the results of enzymic digestion, about 25% of EIP/ $F_{1,4}$ was β -1,4-glucan. About 4% of the total glucan in EIP/ $F_{1,4}$ was resistant to AN reagent, which was confirmed to be exclusively β -1,4-glucan by methylation analysis and enzymic digestion. We noted that only 16% of β -1,4-glucan in the EIP/ $F_{1,4}$ fraction remained after treatment with the AN reagent. This means that the β -1,4-glucans produced in vitro have different degrees of crystallinity, and most of them are of low crystallinity. Methylation analysis and enzymic degradation indicated that the ANIP had no 3-linked Glc, indicating that no other product except for the 4-linked glucan synthesized in vitro remained after treatment with the AN reagent. P/ $F_{1,3}$ products were more soluble in water and alkali and were completely soluble in the AN reagent.

Recently, Meikle et al. (1991) reported that an immunopre-

cipitated β -glucan synthase complex from *Lolium multiflorum* was able to synthesize about the same amount of β -1,3- and β -1,4-glucans based on enzymic degradation. The ratio of β -1,4-glucan in their experiment was higher than that reported here; however, we noted that Meikle et al. terminated their reactions by heating samples at 100°C for 5 min. Under these conditions, the hot water could remove large amounts of β -1,3-glucan according to our solubility tests (Table II). Therefore, a simple comparison of the percentage of β -1,4-glucan in the two in vitro systems may not be useful. A better procedure to determine the ratio of β -1,4-glucan in the total glucan fraction would be to use EIPs or the cold water-insoluble product as a starting point for the analysis.

According to our positive control experiments for enzymic analysis (Table II) and previous work by Anderson and Stone (1975) the major hydrolysis products by (1,3)/(1,4)- β -glucan hydrolase from *B. subtilis* are G4G3GR and G4G4G3GR. In our case, however, oligosaccharides with mobilities consistent with LB and LT rather than G4G3GR and G4G4G3GR were detected by TLC (Fig. 2D) and HPLC (data not shown). On this basis, we question whether EIP/ $F_{1,4}$ actually contains any mixed linked glucans. Therefore, we denote that the 6% glucans constitute an unidentified glucan fraction.

The in vitro product treated with protease after ethanol extraction showed laterally fasciated β -1,4-glucan fibrils labeled by CBH I-gold complexes. They were distinguishable from the other materials that were elongated fibrils and were regarded as β -1,3-glucans, suggesting that these two different glucans have been synthesized separately and not intermingled with each other.

For both samples treated directly with AN reagent and treated sequentially with KOH followed by AN reagent, the x-ray diffraction patterns showed a unique reflection of 4.86 Å. The origin of this reflection is unknown.

The DP of the in vitro product treated with β -1,3-glucanase was found to be within two mol wt ranges: 70% of the product had an average DP of 59 and 30% had an average DP of 396. These mol wt determinations indicate that at least some of the β -glucans produced by cotton solubilized enzymes had a reasonably high mol wt. After treatment with AN reagent, however, most of the in vitro product was lost and the remaining product also suffered from degradation. In addition to a major peak with an average DP of 141 (Fig. 7, E and F), some fragments corresponding to a DP 7 to 19 were observed in some experiments (data not shown). This indicates that extensive acid hydrolysis takes place during the treatment with AN reagent. We have also found that severe degradation of in vitro cellulose produced from *A. xylinum* membrane fractions is caused by the AN reagent (data not shown).

In this study we have made considerable progress over earlier research concerning in vitro cellulose synthesis. In our methylation analysis, 26% of EIP/ $F_{1,4}$ is 4-linked Glc, whereas in the methylation analysis reported by Bacic and Delmer (1981), 4-linked Glc was 9.4% of the in vitro EIP that was synthesized by isolated cotton fiber membranes. Furthermore, the in vitro ANIPs were identified exclusively as β -1,4-glucan by enzymic digestion, methylation, CBH I-gold labeling, and x-ray diffraction.

Can our in vitro product be defined as cellulose? According

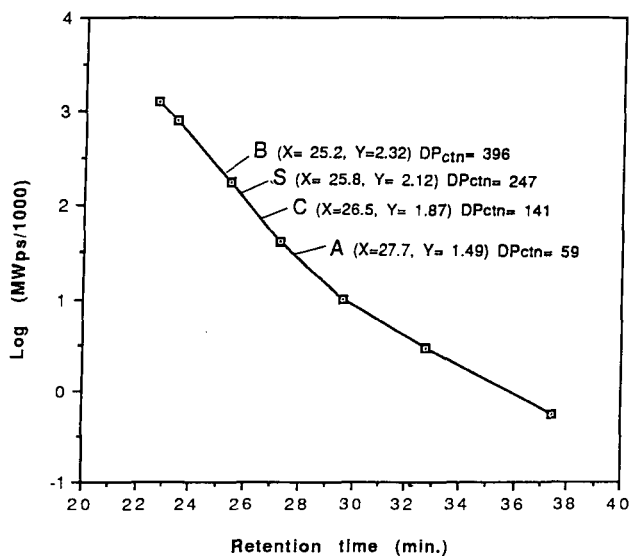


Figure 8. Semilogarithmic plot of the relationship between retention time and mol wt. A, The major peak in β -1,3-glucanase-treated in vitro product. B, The shoulder fraction in β -1,3-glucanase-treated in vitro product. C, The major peak in AN reagent treated in vitro product. S, The DP 200 cellulose standard.

to Blaschek et al. (1983), the cell-free synthesis of cellulose should meet three major criteria: (a) exclusive β -1,4-linkages, (b) a DP of several hundred, and (c) a high crystallinity. The ANIP described here has been demonstrated to be exclusively β -1,4-linked glucan. At least one fraction of the in vitro product has an average DP of 396 before treatment with the AN reagent. This product yields an x-ray diffraction pattern characteristic for cellulose II. Thus, according to the guidelines for the definition of cellulose of Blaschek et al., we have met all of these criteria. Furthermore, we can rule out contamination and thus verify that we have made cellulose in vitro because: (a) radioactive CB was produced from ANIP/ $F_{1,4}$ by CBH I; (b) autoradiography and gold-labeled CBH I both specifically label the cellulose products; and (c) the β -1,3-glucanase-treated sample and the AN reagent-treated sample yield cellulose with a typical x-ray diffraction pattern for cellulose II. This rules out possible contamination from the preexisting native cellulose, which would be only cellulose I.

We offer four suggestions regarding how to achieve in vitro synthesis of cellulose:

(a) Cellulose synthase is very labile. We have found that long-term storage at -80°C gradually results in the loss of activity. Therefore, we have changed storage conditions to liquid nitrogen.

(b) Bolls are first removed from the cotton plant and immediately placed into water. Within minutes after the bolls are harvested, the locules are excised and placed into liquid nitrogen. We believe that this strict time frame is important for maintaining enzyme activity.

(c) From the moment the enzymes are extracted they are subjected to proteolysis. We have used a greater diversity of protease inhibitors that may have prevented enzymic proteolysis of the labile cellulose synthase.

(d) Although not yet approaching cellulose synthesis rates in vivo, we have clearly detected a cellulose product synthesized in vitro. We have found an optimal combination of effectors that has produced a clear distinction between β -1,3-glucan synthesis and β -1,4-glucan synthesis.

In the other two reports in this series, we have identified, described, and characterized the polypeptides responsible for β -glucan synthesis in vitro, and we have elaborated the first kinetic parameters. Taken together, these investigations will now pave the way to the eventual purification of the proteins involved in cellulose synthesis in the cotton fiber and the cloning of the genes for the process.

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