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Cellulose and Callose Biosynthesis in Higher Plants[1]

I. Solubilization and Separation of (1→3)- and (1→4)-β-Glucan Synthase Activities from Mung Bean

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(1→3)- and (1→4)-β-glucan synthase activities from higher plants have been physically separated by gel electrophoresis in nondenaturing conditions. The two glucan synthases show different mobilities in native polyacrylamide gels. Further separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a different polypeptide composition in these synthases. Three polypeptides (64, 54, and 32 kD) seem to be common to both synthase activities, whereas two polypeptides (78 and 38 kD) are associated only with callose synthase activity. Twelve polypeptides (170, 136, 108, 96, 83, 72, 66, 60, 52, 48, 42, and 34 kD) appear to be specifically associated with cellulose synthase activity. The successful separation of (1→3)- and (1→4)-β-glucan synthase activities was based on the manipulation of digitonin concentrations used in the solubilization of membrane proteins. At low dipitomin concentrations (0.05 and 0.1%), the ratio of the cellulose to callose synthase activity was higher. At higher digitonin (0.5–1%) concentrations, the ratio of the callose to cellulose synthase activity was higher. Rosette-like particles with attached product were observed in samples taken from the top of the stacking gel, where only cellulose was synthesized. Smaller (nonrosette) particles were found in the running gel, where only callose was synthesized. These findings suggest that a higher level of subunit organization is required for in vitro cellulose synthesis in comparison with callose assembly.

Higher plants contain membrane-bound glucan synthases that are responsible for the synthesis of (1→4)-β-glucans (cellulose). Membrane-bound enzymes also synthesize (1→3)-β-glucans (callose) in response to wounding, physiological stress, or infection (Delmer, 1987). Callose is also a component of specialized walls or wall-associated structures at a particular stage of growth and differentiation (e.g. in cell plates, seeds, leaf and stem hairs, plasmodesmatal canals, sieve plates, transient walls of microsporogenic and megasporogenic tissues, and in pollen and pollen tubes; Fincher and Stone [1981]; Stone and Clarke [1992]). In members of the Poaceae a (1→3),(1→4)-β-glucan is a characteristic component of cell walls, especially those of the endosperm (Fincher and Stone, 1981).

The nature of the enzymes responsible for the synthesis of cellulose and callose, including the control of their activities during wall formation, is not well understood. Although particles attached to growing cellulose microfibrils, which were hypothesized to contain cellulose synthases, were detected in freeze-fracture preparations of plant membranes as early as 1976 (Brown and Montezinos, 1976), it has not been possible to isolate and characterize these particles (Delmer, 1987).

The earliest investigations of in vitro β-glucan synthesis were directed toward understanding the mechanism of cellulose synthesis; however, preparations from a number of different plants catalyzed the incorporation of labeled Glc from UDP-Glc into water-insoluble polymers identified as (1→3)-β-glucans (Feingold et al., 1958). Almost all attempts at in vitro synthesis of cellulose have resulted in the formation of only (1→3)-β-glucans (Carpita and Delmer, 1980; Delmer, 1983, 1987) or only a very limited synthesis of (1→4)-β-glucans; in the latter case, there was insufficient proof for its characteristic crystallinity (Franz and Blaschek, 1990). Therefore, a persistent problem with in vitro cellulose synthesis has been a simultaneous assembly of multiple products from the same membrane preparation (Mullins, 1990; Delmer, 1991) or (1→3),(1→4)-β-glucans from certain plants (Ordin and Hall, 1967; Peaud-Lenoel and Axelos, 1970; Smith and Stone, 1973; Henry and Stone, 1982; Gibeaut and Carpita, 1993; Becker et al., 1995).

Since either crude or only partially purified membrane proteins were used for assaying cellulose synthase activity, it was not clear whether (1→3)- and (1→4)-β-glucans were synthesized by the same protein(s) or by a separate protein(s) (Jacob and Northcote, 1985; Delmer, 1987). To resolve this question, it was necessary to isolate and separate the (1→3)- and the (1→4)-β-glucan synthases from the membrane. This has proven to be very difficult. Gradient centrifugation and product entrapment have provided the highest increase in specific activity (Eiberger and Wasserman, 1987; Bulone et al., 1990; Fink et al., 1990). However, conventional purification techniques, like most forms of chromatography, usually give very low activity, possibly

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Abbreviations: CBH I, cellobiohydrolase I; TC, terminal complex; TEM, transmission electron microscope/microscopy.

because of a depletion of essential boundary lipids, nonspecific aggregation of strongly hydrophobic polypeptides, or dissociation of the polypeptides required for enzymatic activity (Wasserman et al., 1992).

Because of their relatively high and stable activity, membrane-bound (1→3)-β-glucan synthases from a wide array of higher plants have received the most attention and their multicomponent nature has been revealed (Fink et al., 1990; Frost et al., 1990; Mason et al., 1990; Amor et al., 1991; Delmer et al., 1991a; Dhugga and Ray, 1991, 1992, 1994; Fredrikson et al., 1991; Meikle et al., 1991; Wu et al., 1991; Girard et al., 1992; Kamat et al., 1992; Slay et al., 1992; Wasserman et al., 1992; Bulone et al., 1995). The precise polypeptide composition of callose synthase and the degree to which the enzyme is conserved between species and tissue sources is not yet clear. Between six and nine prominent polypeptides ranging in size from 25 to 92 kD were found (Dhugga and Ray, 1994). Since similarly sized polypeptides occur in callose synthase preparations from a variety of species, it has been suggested that these polypeptides may be components of a multisubunit enzyme complex (Fredrikson et al., 1991; Wasserman et al., 1992).

The first major advance in the area of (1→4)-β-glucan synthesis came from a prokaryotic organism, *Acetobacter xylinum* (Ross et al., 1987). Subsequent detailed studies confirmed that cellulose was synthesized in vitro by *A. xylinum* (Lin and Brown, 1989; Lin et al., 1990). These investigations led to the identification and cloning of the genes for the catalytic subunit (Saxena et al., 1990, 1991; Wong et al., 1990). Recently, a rice cDNA and two cotton cDNA clones were characterized that are homologous to the bacterial *celA* genes that encode the catalytic subunit of cellulose synthase (Pear et al., 1996). Three regions in the deduced amino acid sequence of the plant *celA* gene products are conserved with respect to the proteins encoded by bacterial *celA* genes. Within these conserved regions, there are four highly conserved subdomains previously suggested to be critical for catalysis and/or binding of the substrate (Pear et al., 1996). Recently, we reported a similar clone from *Arabidopsis thaliana*, which appears to be part of a truncated cellulose synthase (Saxena and Brown, 1997).

During the past several years the partial purification of separate (1→3)- and (1→4)-β-glucan synthases has been achieved by product entrapment (Lin and Brown, 1989; Mayer et al., 1989; Frost et al., 1990, 1997; Bulone et al., 1995). Another approach for the purification of β-glucan synthase complexes has involved the use of polyclonal (Nodet et al., 1988; Fink et al., 1990; Dhugga and Ray, 1991) and monoclonal antibodies (Delmer et al., 1991a; Meikle et al., 1991). Affinity-labeling methods using substrate analogs to identify UDP-Glc-binding polypeptides in glucan synthases have been widely applied (Read and Delmer, 1987; Lawson et al., 1989; Frost et al., 1990; Lin et al., 1990; Mason et al., 1990; Delmer et al., 1991b; Meikle et al., 1991).

We achieved the in vitro synthesis of a small quantity of cellulose II allomorph by digitonin-extracted cotton fiber membranes (Okuda et al., 1993). Later, a two-step digitonin-solubilization procedure was used to increase the ratio of cellulose to callose to more than 30% of the total glucan product (Kudlicka et al., 1995). Significantly, the product crystallized as the cellulose I allomorph, whereas the other preparations synthesized cellulose II, suggesting that the revised isolation procedure was less damaging to the native macromolecular complex (Kudlicka et al., 1995).

We now report further modifications to the solubilization procedures, which have led to a higher in vitro activity of glucan synthases and differential solubilization of cellulose and callose synthase activities. In addition, we describe a nondenaturing gel electrophoresis method that separates callose- and cellulose-synthesizing activities from mung bean (*Vigna radiata*) membranes.

MATERIALS AND METHODS

Chemicals

UDP-[Glc-U-^{14}C] (250 mCi/mmol) and yeast lytic recombinant (1→3)-β-glucanase were purchased from ICN. Digitonin (special grade, water soluble) was purchased from Biosynth (Naperville, IL). Cellobiose, cyclic-3′:5′-GMP, protease inhibitors, protein A-10 nm gold, and other chemicals were obtained from Sigma. Tinopal LPW was a gift from Ciba-Geigy (Greensboro, NC), and aniline blue was from Polysciences (Warrington, PA). CBH I enzyme was a gift from Dr. Martin Schulein of Novo Industri (Copenhagen, Denmark). (1→3)-β-glucan monoclonal antibody was obtained from Biosupplies (Parkville Victoria, Australia). The molecular mass standards were purchased from Bio-Rad. [β-^{32}P]5′-N_3-UDP-Glc was provided by Dr. Richard Drake (Little Rock, AR). Celluclast was obtained from Novo Nordic Bioindustrial (Danbury, CT).

Plants

Mung bean (*Vigna radiata* var Berken) seeds from Calco (Dallas, TX) were supplied by Dr. Sathasivan Sata. The seedlings were grown in the dark at 28 to 30°C in water-saturated vermiculite and harvested after 3 to 5 d. The leaves and roots were excised, and only hypocotyls were used for the extraction of proteins.

Extraction of Proteins

Tissues of hypocotyls from etiolated seedlings (about 50 g) were homogenized in 50 mL of extraction buffer containing 50 mM Mops, pH 7.5, 5 mM EDTA, 0.25 M Suc, and a combination of protease inhibitors (0.5 mM PMSF, 10 μM leupeptin, 0.1 mM *N*-α-*p*-tosyl-L-Lys chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) using a mortar and pestle at 4°C. The extract was filtered through a 210-μm mesh screen (Spectra, Sussex, UK) to remove cell walls, and the filtrate was designated as the crude factor, which was then centrifuged at 100,000*g* for 1 h. The resulting supernatant was discarded, and the pellet was suspended in resuspension buffer (50 mM Mops, pH 7.5, with 0.25 M Suc) to obtain approximately 4.5 mg protein per milliliter; this was designated as the membrane fraction. Solubilized proteins were obtained from membrane fractions using a progressive five-step solubilization protocol in which digitonin

concentrations of 0.05, 0.1, 0.25, 0.5, and 1% were applied, and the fractions were centrifuged at 100,000*g*.

The solubilized proteins were concentrated using a 10-kD cutoff concentrator (Centriprep, Amicon, Beverly, MA). The proteins from all fractions were assayed using a modification of the Lowry procedure (Markwell et al., 1978), which included a separate blank for each digitonin concentration.

To determine the inhibitory effects of digitonin on glucan synthesis, 2% digitonin was added to fractions previously solubilized with 0.05, 0.1, 0.25, and 0.5% digitonin, and the final concentration was adjusted to 1%.

Enzyme Activity Assay

Reaction conditions found to favor cellulose synthesis in cotton fiber enzyme preparations (Li and Brown, 1993; Li et al., 1993; Okuda et al., 1993) were used to monitor the incorporation of Glc from UDP-Glc into a polymer product. The reaction mixture contained 0.5 mM UDP-[U-^{14}C[Glc (specific activity 12.5 mCi/mmol) with 10 mM Bis-Tris-propane-Hepes buffer, pH 7.6, 20 mM cellobiose, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM c-3′-5′-GMP, 0.05% digitonin, and 40 μg of proteins in enzyme fractions in a final volume of 100 μL (Kudlicka et al., 1995). The reaction was carried out for 30 min at 25°C and terminated by placing the reaction mixture in a boiling-water bath for 1 min. The radioactive products were collected by filtration on a glass microfiber filter (Whatman) and washed three times with distilled water and once with methanol. The radioactivity retained on the filters was determined with a scintillation counter (model LS 6800, Beckman).

Native Gel Electrophoresis and Detection of β-Glucan Synthase Activity

After electrophoretic separation of proteins in the solubilized fractions by nondenaturing PAGE, the products of β-glucan synthase activity can be observed as fluorescent bands under UV light after incubation of the gel with UDP-Glc and effectors, followed by treatment with Tinopal LPW, a fluorochrome (fluoroprobe) that binds both cellulose and callose (Thelen and Delmer, 1986; Dhugga and Ray, 1994).

Either an 8% running gel or a 6% running gel/3% stacking gel containing 62.5 mM Tris-HCl, pH 8.8, 0.05% ammonium persulfate, 0.025% *N,N,N′,N′*-tetramethylethylenedamine, and 0.02% digitonin was used to separate proteins from solubilized membrane fractions. The gels were precooled and run at 4°C at a constant current of 20 mA for about 3.5 h using a running buffer containing 25 mM Tris, pH 8.3, 192 mM Gly, and 0.02% digitonin. Bromphenol blue was added to solubilized fractions as a tracking dye and electrophoresis was continued until the dye migrated out of the gel. During this time the initial potential difference of 103 V increased to about 300 V.

It was important to continue the electrophoresis sufficiently to complete the separation of proteins. If the voltage is any lower than 300 V at the termination of the run, the separation will be incomplete, as evidenced by a uniform stain of Tinopal-positive material through the stacking gel.

For detection of enzyme activity, the gels were washed for 30 min at 4°C in 50 mM Mops buffer containing 0.25 M Suc and 0.02% digitonin. Gels were then incubated in an incubation mixture containing the same components as for activity assay. As a substrate 1 mM UDP-Glc (or 0.2 mM radioactive UDP-Glc, specific activity 0.625 mCi $mmol^{-1}$) was used for 30 min to 22 h at room temperature. The β-glucan products formed in the gels were stained in 0.01% Tinopal or 0.05% aniline blue in K_2PO_4 buffer, pH 8.2, for 10 min. The gels were then washed in water or 20% ethanol and stored in the same solution. The product bands were detected under UV light.

Gels incubated with radioactive substrate were washed several times in 20% ethanol until the radioactivity in the alcohol wash was reduced to the background. The radiolabeled product was detected by exposing the dried gel to Kodak X-Omat AR film and an intensifying screen (DuPont Cronex) for 10 to 36 h. Densitometric tracing of the exposed radiographic film was done using image-analysis software (IBAS, Kontron, Germany), and the areas under the curves were calculated.

Characterization of Proteins Separated by Native Gel Electrophoresis

For protein characterization the native gels were stained with 0.125% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 30 min and destained overnight. The protein bands from the stacking and running gel were excised. The gel slices were placed in electroelution tubes assembled in concentrators (Centricon, Amicon) and loaded into a microelectroelutor (Centrilutor, Amicon). Proteins were electroeluted for 2 h at 200 V and concentrated for 1 h at 6400 rpm. The concentrated proteins were run on an SDS-polyacrylamide gel.

SDS-PAGE

SDS-PAGE of proteins separated by the native gels and of nonseparated, solubilized fractions was used as described by Porzio and Pearson (1977). Acrylamide was used at 10% in the separating gel and at 4.5% in the stacking gel. The loading buffer contained 10% 2-mercaptoethanol. The gels were stained for 1 h with Coomassie blue.

Photoaffinity Labeling

The reaction mixture for the photolabeling reaction was the same as that used for the enzyme activity assay. The membrane fraction solubilized by 0.25% digitonin was assayed and [β-^{32}P]5′-N_3-UDP-Glc was used as the substrate. The reaction mixture was preincubated in an Eppendorf tube at room temperature for 1 min and then irradiated with a short UV wavelength (254 nm) using a hand-held UV lamp (UV Products, San Gabriel, CA) at a distance of 4 cm for 3 min on ice. The reaction was terminated by the addition of 0.4 mL of methanol, and protein pellets were

collected according to the methods of Wessel and Flugger (1984). Electrophoresis was performed as described by Porzio and Pearson (1977). Labeled polypeptides were detected by autoradiography at −80°C using Kodak X-Omat AR film and an intensifying screen (DuPont).

TEM of Proteins Separated by Native Gel Electrophoresis

The bands containing the products and protein complexes were excised from native gels and crushed in a small volume of distilled water using a Teflon pestle adjusted to an Eppendorf tube. The gel fragments were mounted on TEM grids, then labeled with CBH I-gold, negatively stained with 1% uranyl acetate, and examined with a TEM (model 420, Philips, Eindhoven, The Netherlands) operated at 100 kV.

Characterization of β-Glucan Products in the Native Gel

After incubating with substrate and effectors, the gel sections were placed in small Petri dishes containing enzymes for specific product analysis. For (1→3)-β-glucans, 25 units of recombinant yeast (1→3)-β-glucanase was placed in 3 mL of 50 mM K_2PO_4 buffer, pH 7.5, and 3 mM NaN_3. For (1→4)-β- glucans, 40 μg of CBH I was placed in 3 mL of 50 mM $NaC_2H_3O_2$ buffer, pH 4.8, and 3 mM NaN_3. As a nonspecific control, 3 mL of celluclast was diluted 1:1 with $NaC_2H_3O_2$ buffer, pH 4.8, containing 3 mM NaN_3.

In all of the procedures described above, the enzymes were changed twice during 72-h digestions at 40°C. After the gel sections were washed with water, they were stained with aniline blue or Tinopal and observed under UV light.

TEM of Products Synthesized in the Native Gel

The limited quantities of β-glucan product synthesized in native gels makes it difficult to achieve definitive chemical characterization. Since TEM does not require a large quantity of material, we examined the ultrastructure of the product synthesized in the native gels. In our earlier work we found that cellulose and callose have very characteristic, distinguishable morphologies. Cytochemical analysis with CBH I-gold complex labeling was very useful to differentiate these two products (Okuda et al., 1993; Kudlicka et al., 1995, 1996).

The bands containing the product and enzymes were excised from the native gels and placed in small glass bottles containing 1 mL of 30% H_2O_2. The mixtures were incubated at 60°C for about 5 h to remove the acrylamide. When gel slices were solubilized, the resulting liquid from each bottle was transferred into Eppendorf tubes and centrifuged for 10 min at 15,000*g*. The resulting supernatants were discarded, and the pellets were washed three times with distilled water and mounted on TEM grids. The products from separate bands were labeled with CBH I-gold or the (1→3)-β-glucan-specific monoclonal antibody (Meikle et al., 1991) and then with protein A-gold (10-nm particles; Voughn et al., 1996); the samples were negatively stained with uranyl acetate and then observed with a TEM.

RESULTS

Solubilization of Membrane Proteins

Membrane proteins solubilized by gradually increasing the concentration of digitonin show that solubilization of components incorporating Glc from UDP-Glc into an insoluble product is concentration dependent (Table I). Very low digitonin concentrations (0.05%) extract a large quantity of proteins from the membrane fractions. In this step it is probably not only solubilizing glucan synthase activities but also removing a large number of other proteins. The fraction solubilized by 0.1% digitonin shows an increase in specific activity. Digitonin at 0.25% seems to be optimal for glucan synthase activity. A further increase of the digitonin concentration to 0.5% caused a reduction of glucan synthase activity, and 1% digitonin caused an even greater reduction (Table I).

An additional increase of the digitonin concentration to 1% in the fractions solubilized originally by 0.05, 0.1, 0.25, and 0.5% digitonin supports the hypothesis of an inhibitory effect of high digitonin concentrations on glucan synthesis (Table II).

Table I. *Influence of digitonin concentrations on the solubilization of mung bean membrane proteins and β-glucan synthase specific activity*

Values are means ± SD of eight separate experiments.

Fraction	Total Protein	β-Glucan Synthase Specific Activity
	mg	*nmol min^{-1} mg^{-1}*
Crude factor	427.3 ± 7.3	16.4 ± 3.7
Supernatant fraction	292.7 ± 5.2	1.4 ± 0.2
Membrane fraction	228.6 ± 6.2	35.9 ± 4.6
First solubilization of membrane fraction (0.05)[a]	27.6 ± 3.3	4.8 ± 0.8
Second solubilization (0.1)	21.3 ± 3.1	16.9 ± 2.3
Third solubilization (0.25)	19.9 ± 2.0	86.2 ± 8.1
Fourth solubilization (0.5)	8.9 ± 1.7	50.2 ± 5.0
Fifth solubilization (1)	7.4 ± 1.8	19.9 ± 1.3
Pellet after solubilization	54.9 ± 7.2	15.3 ± 2.9

[a] Percentages in parentheses indicate concentration of digitonin used in solubilization.

Table II. *Influence of digitonin concentrations on the β-glucan synthase activity*

Fraction	β-Glucan Synthase Activity	β-Glucan Synthase Activity after Adding Digitonin to 1%
	nmol min^{-1} mg^{-1}	*nmol min^{-1} mg^{-1}*
First solubilization of membrane fraction (0.05)[a]	5.1	2.6
Second solubilization (0.1)	17.8	8.7
Third solubilization (0.25)	92.1	33.9
Fourth solubilization (0.5)	52.7	11.8

[a] Percentages in parentheses indicate concentration of digitonin used in solubilization.

The time course of the in vitro reaction suggests that the stability of individual enzyme fractions increases with higher digitonin concentrations (Fig. 1). The fractions solubilized by 0.05 and 0.1% digitonin incorporate the radioactive substrate only during the initial period of the reaction (30 min and up to 2 h, respectively), with the reaction rate leveling off after longer incubation times. The most active fractions, solubilized by 0.25 and 0.5% digitonin (Table I), show a rapid incorporation of radioactive substrate early in the reaction period and continued incorporation at a slower rate over the remaining period (22 h). The fraction solubilized by 1% digitonin showed a slower incorporation rate at the beginning of the reaction and continued to synthesize product at a reduced rate until the termination of the experiment.

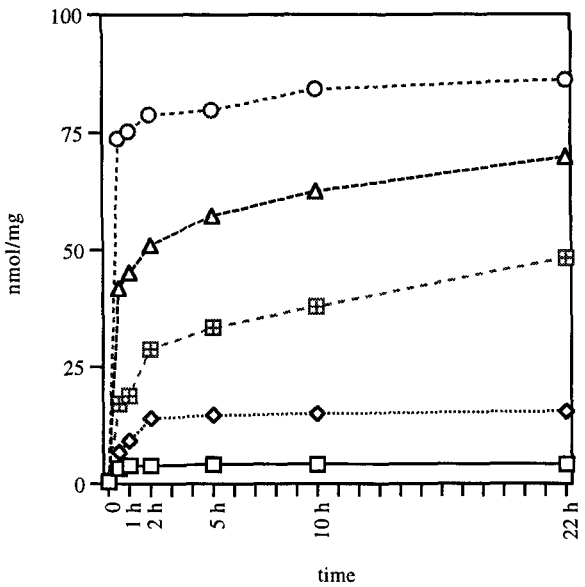

Figure 1. Radioactive substrate incorporation (nmol mg^{-1} protein) during the time course of the in vitro reaction of different enzyme fractions. Note that the enzyme fractions solubilized by 0.05% digitonin (□) show a low activity only during the initial 30 min of incubation. Fractions solubilized by 0.1% digitonin (◇) incorporate substrate during the first 2 h of the reaction, and the fractions solubilized by 0.25% digitonin (○) incorporate a high quantity of substrate at the beginning of the reaction and continue incorporation at a slower rate after 1 h. The enzyme fractions solubilized by 0.5% (△) and 1% digitonin (⊞) show a lower initial incorporation rate and continue incorporation during the entire reaction time (22 h).

Separation of Cellulose- and Callose-Synthesizing Activities by Native Gel Electrophoresis

The most active enzyme fraction of mung bean solubilized by 0.25% digitonin and run under nondenaturing conditions on the 8% running gel revealed a broad, intense protein band at the loading well (Fig. 2B). This band migrated very slowly into the gel compared with marker proteins of up to 205 kD, and it is predicted to be of a very high molecular mass. After incubation in a mixture of substrate and effectors and Tinopal staining, a single product band appeared (Fig. 2A) at the same location as the Coomassie blue-stained protein band (Fig. 2B). After the acrylamide was removed from the gel slices and the resulting products were analyzed with TEM, three different morphological structures, corresponding to callose, cellulose I, and cellulose II, were revealed (Fig. 2C; Okuda et al., 1993; Kudlicka et al., 1995, 1996).

When a 3% stacking/6% running gel was used for separation of proteins from the same membrane fraction (solubilized by 0.25% digitonin), two protein bands (Fig. 3B) and two corresponding product bands (Fig. 3A) appeared in different regions of the gel after incubation in the mixture of substrate and effectors and Tinopal staining. One band (S) was concentrated at the top of the loading well and the second band (R) barely entered the 6% separating gel.

Product Characterization

Reaction with Fluorochromes

Since Tinopal induces fluorescence with both cellulose and callose (Figs. 3A and 4A, bands S and R, respectively), we used aniline blue, a dye mixture containing fluorochrome specific for callose (Stone et al., 1984). This staining revealed a broad band at the top of the running gel (Fig. 4B, band R), indicating the presence of callose. After the gel section was incubated with (1→3)-β-glucanase and stained with Tinopal, band R disappeared (Fig. 4C). When the gel sections were incubated with CBH I and stained with aniline blue, band R was still present but band S was not visible (Fig. 4D). Celluclast digestion removed both bands from the gel; they were not visible after either aniline blue or Tinopal staining (data not shown).

TEM Analysis of Products Synthesized in the Native Gel

The material synthesized at the two different regions of the native gel showed very distinct morphologies (Figs. 3,

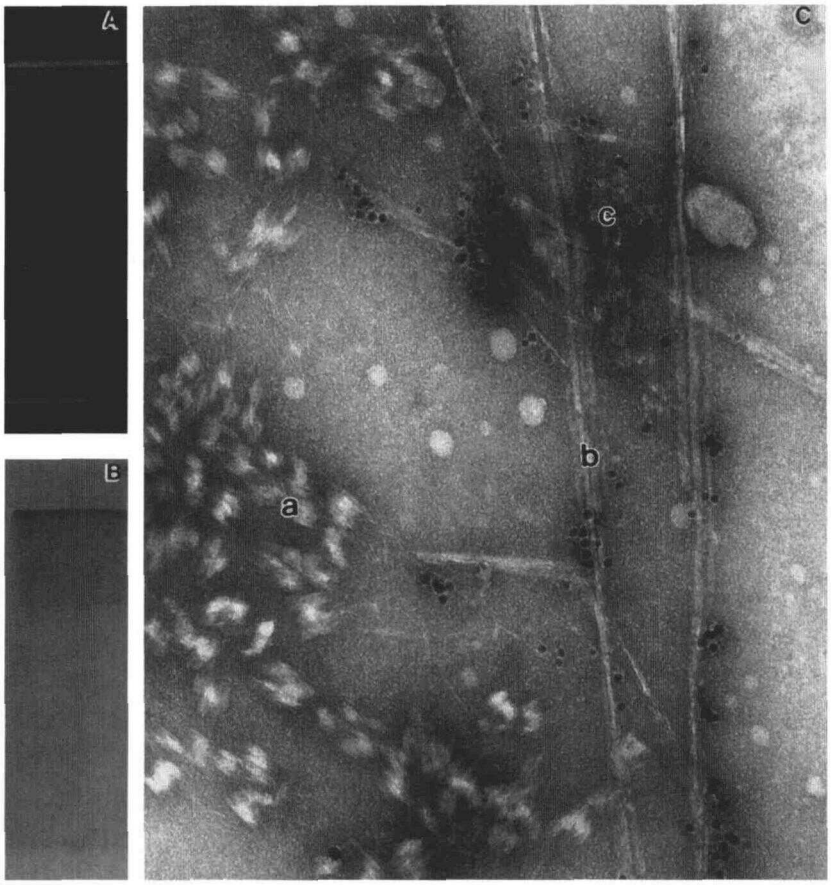

Figure 2. Native gel electrophoresis of mung bean enzyme fraction (solubilized by 0.25% digitonin) incubated with UDP-Glc for 20 h. A, The in vitro product at the boundary of the loading gel detected under UV light after staining with Tinopal. B, The same gel stained with Coomassie blue. C, Negatively stained and CBH I-gold-labeled product synthesized in the gel, after polyacrylamide was removed. a, Callose; b, cellulose I; c, cellulose II. Magnification = ×139,400.

D and E, and 4, E and F). The product extracted from the top of the stacking gel (band S) was fibrillar and was labeled with the CBH I-gold complex (Fig. 3D) but not with the (1→3)-β-glucan-specific antibody (Fig. 4E). This specific labeling demonstrates that only cellulose was synthesized by the enzyme complex, which remained in the loading well. The band extracted from the top of the running gel (band R) revealed a fibrillar material arranged into rodlets (Figs. 3E and 4F), a morphological feature typical of callose (Okuda et al., 1993), which was labeled with (1→3)-β-glucan-specific antibody (Fig. 4F).

Radiochemical Labeling of Product

The product band S (cellulose) was detected within 10 to 20 min of incubation with the substrate and reached a maximum intensity within 45 min to 1 h. After a prolonged incubation time (22 h) the fluorescent intensity did not change. The product band R (callose) was detected within 1 h of incubation with the substrate, and its intensity continued to increase until about 20 h.

The control reaction (without UDP-Glc) did not yield any product (cellulose or callose); however, a weak, Tinopal-positive band was present, suggesting that a glycoconjugate moiety of the electrophoresed proteins may have been stained (data not shown). To prove that the product bands were synthesized in situ and were not glycoproteins fluorescing with Tinopal, [^{14}C]UDP-Glc was used as a substrate for native gel incubation. After 20 h of radiographic exposure, the same two bands were clearly labeled on the gel (Fig. 3C).

The use of radioactive substrate in the incubation mixture for the native gel assay allowed quantitation of glucan synthase activities in the gel, giving a more sensitive assay than direct observation or the Tinopal-staining procedure. Figure 5 shows the separation of glucan synthase activities in five fractions solubilized by increasing concentrations of digitonin after incubation of the gel with radiolabeled substrate and exposure to radiographic film. It was surprising that the ratio of the product formed in bands S and R changed with increasing concentrations of digitonin (Fig. 5C). In the fraction solubilized by 0.05% digitonin (lane 1), band S was more intense than band R, suggesting that more cellulose is synthesized by this fraction. In the fraction solubilized by 0.1% digitonin (lane 2), band S was also more intense than band R. In the fraction solubilized by 0.25% digitonin (lane 3), both bands were equally intense as a result of the high activity of this fraction. In the fraction solubilized by 0.5% digitonin (lane 4), the intensity of band S decreased and that of band R increased. In the fraction solubilized by 1% digitonin (lane 5), band S was barely visible and band R was also characterized by a low intensity. These results show that increasing the concentration of digitonin reduces the cellulose synthase activity (band S) and increases callose synthase activity (band R; Figs. 5 and 6).

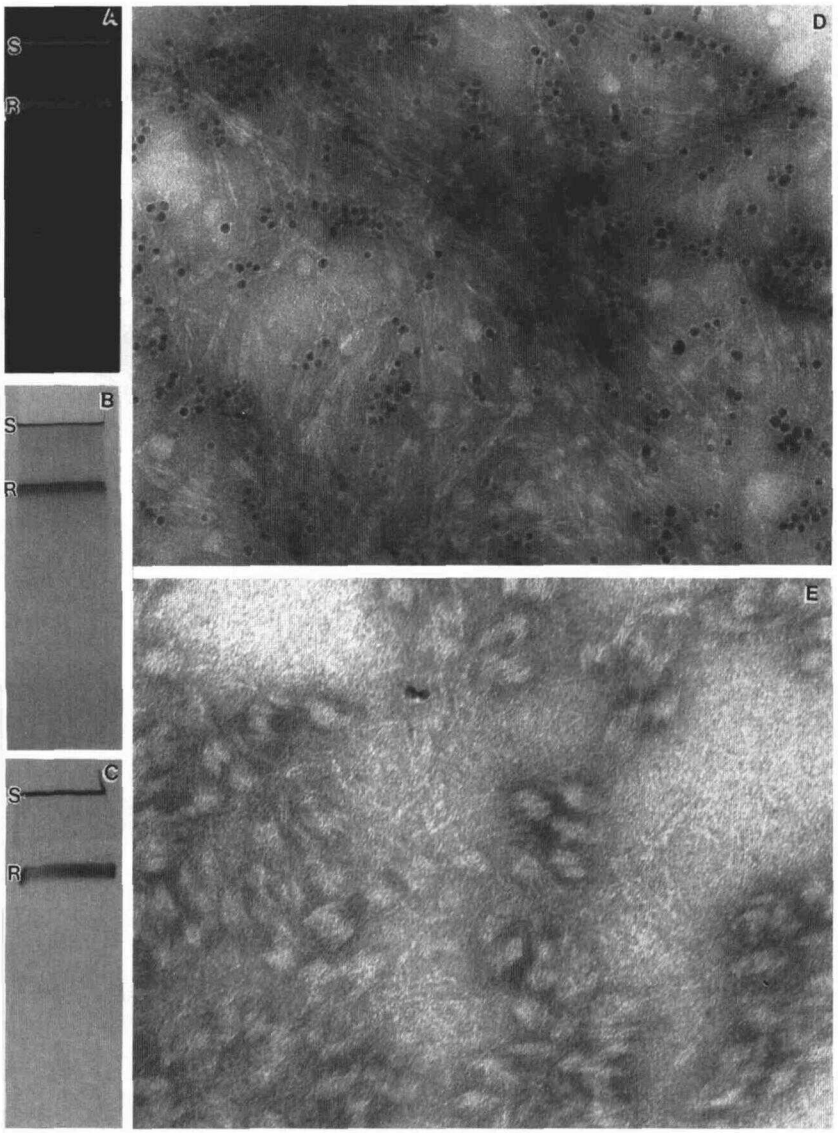

Figure 3. Native gel electrophoresis of mung bean enzyme fraction (solubilized by 0.25% digitonin) separated on two phases of stacking and running gel. A, Two product bands detected under UV light after staining with Tinopal. The band S in vitro product is in the stacking gel; the band R in vitro product is in the running gel. B, Two protein bands (S and R) in the same native gel detected after Coomassie blue staining. Note that these bands co-localize with the product bands. C, Two product bands (S and R) detected after exposure of the gel to radiographic film, indicating that these bands contained labeled Glc from [^{14}C]UDP-Glc. D, Electron micrograph of the product released from the top of stacking gel, negatively stained, and labeled with CBH I-gold. Note the characteristic CBH I-gold labeling indicating cellulose. Magnification = ×127,100. E, The product released from the running gel. Note the absence of CBH I-gold labeling and a rodlet appearance indicating callose. Magnification = ×209,250.

These results were confirmed by incubating separate pieces from the top of stacking and running gels in a liquid assay using activators and radioactive substrate (Table III). The fraction solubilized by 0.05% digitonin incorporated more radioactive substrate into the top part of the stacking gel (band S). On the contrary, the fraction solubilized by 1% digitonin incorporated more radioactive substrate into the top part of the running gel (band R). As in the gel assay, the fraction solubilized by 0.25% digitonin incorporated a similar quantity of substrate in the top parts of the stacking and running gels.

Polypeptide Composition of Protein Bands Separated by Native PAGE

SDS-PAGE of the protein bands separated by native gels showed the presence of 12 polypeptides associated with the cellulose synthase band (Fig. 7, lane S; Table IV); however, some of these polypeptides were found only in minor quantities. Two polypeptides appear to be present only in the callose synthase band (Fig. 7, lane R; Table IV), and three seem to be common to both activities (Table IV).

Photoaffinity labeling of the most active enzyme fraction (solubilized by 0.25% digitonin, Table I) using azido-UDP-Glc showed that the three polypeptides common to the cellulose and callose synthase bands (64, 54, and 32 kD) were labeled (Fig. 8B). The 78-kD polypeptide associated with the callose synthase band and the 136-kD polypeptide (one of many) found in the cellulose synthase band were also photolabeled (Fig. 8B), although they were poorly detected by Coomassie blue staining (Fig. 8A).

Structural Characterization of Cellulose/Callose Synthases

Most of the gel fragments were too thick for TEM observations; however, their marginal regions were sufficiently thin to reveal protein complexes and their products embedded in native gels. The top part of stacking gel (band S, the cellulose-synthesizing region) revealed numerous particles, among them a number of rosette-like structures

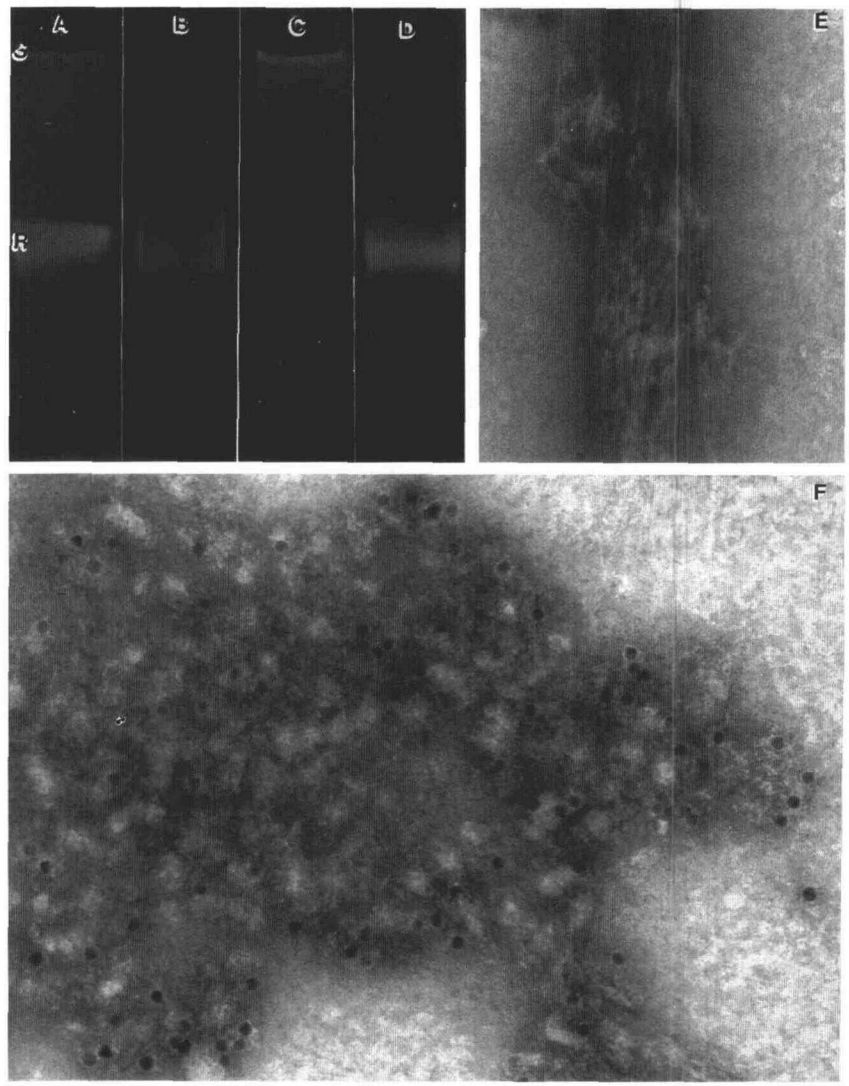

Figure 4. Product characterization in the native gel. A, Gel fragment stained with Tinopal. Note the presence of the two product bands (S and R). B, Gel fragment stained with aniline blue. Note the staining of only the broad band R. C, Gel fragment stained with Tinopal after digestion with β-1,3 glucanase. Note the absence of band R (which contains only callose) and the continuing presence of band S (which contains only cellulose). D, Gel fragment stained with aniline blue after CBH I digestion. S (cellulose) band is removed but band R (callose) remains. E, Electron micrograph of product released from band S (cellulose) and labeled with monoclonal antibody specific to callose. Note the absence of labeling, which verifies that callose is not present. The characteristic fibrils of cellulose I and aggregates of cellulose II are visible. Magnification = ×175,230. F, Electron micrograph of product released from band R (callose), labeled with a monoclonal antibody specific for callose, and detected by protein A gold complexes. Note the extensive labeling of this product throughout the mass. Magnification = ×222,750.

(63–82 nm in diameter; Fig. 9, A–D). These rosette complexes were composed of six subunits. Reinforcement analysis (not shown) indicated a 6-fold symmetry. The top part of the running gel (band R, the callose-synthesizing region) revealed abundant smaller protein complexes (29–38 nm), which never aggregated into rosette structures. In both samples, numerous examples of products associated with the particle complexes were found. Labeling of the products with CBH I-gold in the gel was sparse because of poor penetration.

DISCUSSION

Successful separation of β-glucan synthase activities depends on a number of factors such as efficient membrane solubilization and extraction of active proteins. In studies in which 1% digitonin was used to solubilize membrane-associated proteins, these fractions usually produced a large quantity of callose (Thelen and Delmer, 1986). Others have used 0.1% digitonin to purify solubilized enzyme fractions by removing peripheral proteins, but these fractions were usually discarded because of the small quantity of solubilized proteins (Dhugga and Ray, 1991, 1994).

An even lower concentration of digitonin (0.05%) in our earlier work gave, to our knowledge, the largest quantity of cellulose so far reported (Kudlicka et al., 1995). In the present report we have modified the solubilization protocol and obtained results clearly showing that the solubilization of active proteins incorporating Glc from UDP-Glc into a polymer product is dependent on the digitonin concentration. Digitonin at 0.25% appears to be optimal for total glucan synthase activity (Table I); however, 0.05 and 0.1% digitonin seem to be optimal for cellulose activity (Figs. 5 and 6). An understanding of the reduction in glucan synthase activity under higher detergent concentrations (Table II) remains to be elucidated, but the cause may be related to an initial disorganization of the enzyme complex structure during solubilization.

Solubilization of membrane proteins by digitonin coupled with in situ assays following electrophoresis in nondenaturing conditions have been applied previously to detect the activities of chitin synthase (Kang et al., 1984)

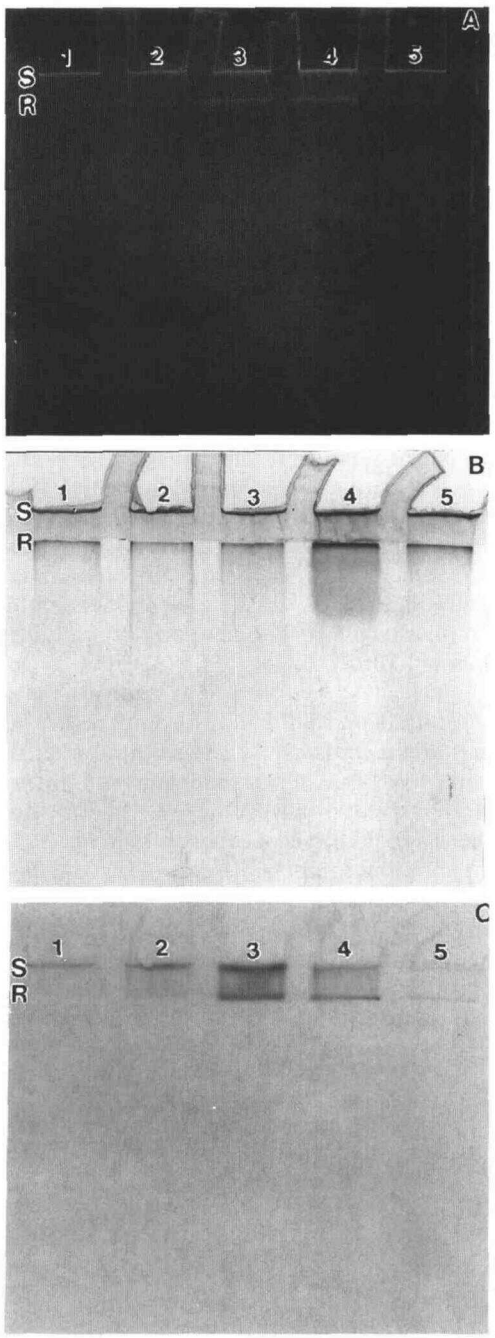

Figure 5. Native gel electrophoresis of five enzyme fractions solubilized by increasing concentrations of digitonin. Lanes 1, Solubilization by 0.05% digitonin; lanes 2, solubilization by 0.1% digitonin; lanes 3, solubilization by 0.25% digitonin; lanes 4, solubilization by 0.5% digitonin; and lanes 5, solubilization by 1% digitonin. The gel was incubated with radioactive substrate. A, The product bands visualized under UV light after the gel was stained with Tinopal. Band S is the product in the stacking gel; band R is the product in the running gel. B, Protein bands in the same gel visualized with Coomassie blue staining. C, Product bands in the same gel visualized after the gel was exposed to radiographic film. Note that the ratio of products in the stacking and running gels is different in various fractions (1–5).

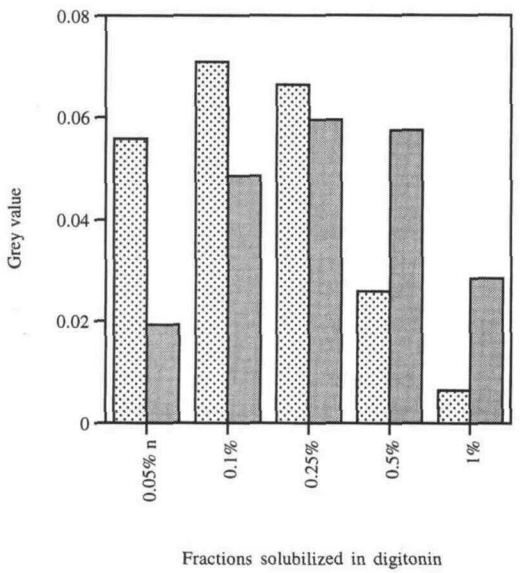

Figure 6. Relative quantity of radioactive substrate incorporation by glucan synthases separated by native gel electrophoresis. Stippled bars, Cellulose at the top of the loading well; gray bars, callose at the interface of the stacking and running gel. Gray values were created from Figure 5C.

and glucan synthase (Thelen and Delmer, 1986; Dhugga and Ray, 1994). In experiments measuring the (1→3)-β-glucan synthase activity of barley, soybean, mung bean, pea, and cotton, all proteins migrated as a broad band of very high molecular mass (Thelen and Delmer, 1986; Pederson et al., 1993; Dhugga and Ray, 1994).

Similar results were observed in our experiments when only one concentration (8%) of the running gel was used (Fig. 2, A and B). Application of a very low gel concentration (3% stacking/6% running) for the sequentially solubilized enzyme fractions run under nondenaturing conditions gave, for the first time to our knowledge, a complete separation of cellulose and callose synthase activities from a higher plant source (Figs. 3 and 5). Incubation of the gel with [^{14}C]UDP-Glc proved that radioactive Glc was incorporated into both product bands and confirmed separation of the two synthases (Fig. 3C). The techniques for product analysis used here have provided evidence that cellulose and callose have been synthesized as two separate product bands in the native gels (Figs. 3, D and E, and 4, B–F).

The major difference in our experiments appears to be the initial concentration of digitonin applied for membrane

Table III. *[^{14}C]UDP-Glc incorporation into the separated pieces from top of the stacking gel and from top of the running gel*

Fraction	Loading Well	Top of Running Gel
First solubilization (0.05)[a]	8,334	5,050
Second solubilization (0.1)	11,484	7,203
Third solubilization (0.25)	21,633	18,037
Fourth solubilization (0.5)	7,405	18,134
Fifth solubilization (1)	4,955	8,727

[a] Percentages in parentheses indicate concentration of digitonin used in solubilization.

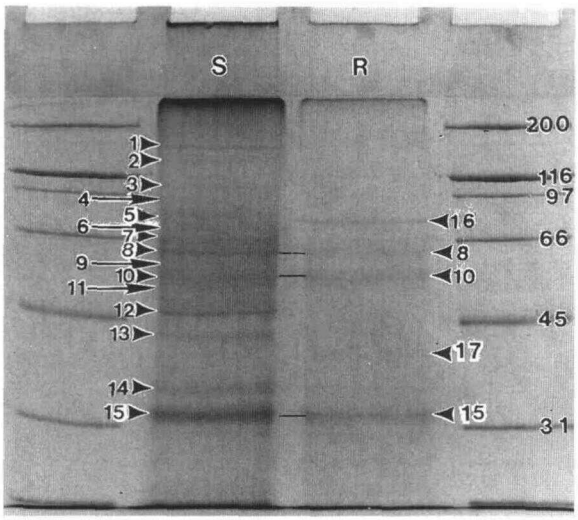

Figure 7. Protein profiles of separated polypeptides from native gel activities of cellulose (band S) and callose (band R) synthases. Note that polypeptides 1 (170 kD), 2 (136 kD), 3 (108 kD), 4 (96 kD), 5 (83 kD), 6 (72 kD), 7 (66 kD), 9 (60 kD), 11 (52 kD), 12 (48 kD), 13 (42 kD), and 14 (34 kD) are characteristic of the protein band eluted from the loading well, where only cellulose was synthesized. Polypeptides 16 (78 kD) and 17 (37 kD) are characteristic of the protein band eluted from the top of the running gel, where only callose was found. Polypeptides 8 (64 kD), 10 (54 kD), and 15 (32 kD) are common to both activities.

solubilization. Thelen and Delmer (1986) used only 1% digitonin to solubilize β-glucan synthase. The same 1% enzyme fractions in our experiments synthesized predominantly callose (band R) in the running gel (Fig. 5, lane 5), and in our earlier work only 4% cellulose was synthesized by such fractions from cotton fibers (Kudlicka et al., 1995) and only 2% from mung bean seedlings (Kudlicka et al., 1996). Data presented in this report clearly show that, together with the increasing concentrations of digitonin, the cellulose band intensity decreases, whereas the callose band intensity increases (Figs. 5C and 6).

Product band S focused at the loading well becomes detectable within 10 to 20 min of incubation with substrate and reaches maximum intensity within a short time (30 min–1 h). This could indicate a certain degree of instability of the proteins responsible for cellulose synthesis. On the other hand, the product band R located at the top of separating gels was detectable after a longer period (about 1 h) and its intensity continued to increase for about 20 h, reflecting a greater stability of callose synthase activity.

As noted in previous reports, 1% digitonin may disorganize the enzyme complexes during solubilization, causing separation of cellulose-binding components, whereas mild digitonin concentrations (0.05 and 0.1%) release the enzyme complexes relatively intact and similar to the native state, probably with more subunits associated. We believe that during separation using nondenaturing conditions, the components solubilized by the low digitonin concentrations may remain attached and highly integrated. As a result, the binding of these components could block callose-synthesizing sites, whereupon only cellulose could be synthesized (Brown et al., 1996). On the contrary, 1% digitonin may cause a severe separation of the enzyme complex subunits and the binding components. In such a conformation, the unblocked callose synthesis sites are hypothesized to be accessible to the substrate and compete more effectively than the cellulose synthesis sites. As a result, a massive synthesis of callose would occur. Subunits lacking the binding components are predicted to be smaller and could therefore migrate through the stacking gel.

It has been shown that glucan synthases are large protein complexes (>450 kD) and that several protein subunits ranging from 18 to 115 kD might be involved in glucan synthesis (Delmer, 1987; Eiberger and Wasserman, 1987;

Table IV. *Proteins fractionated by native PAGE and further resolved by SDS-PAGE*

Polypeptides Associated with Cellulose Synthase Activity	Polypeptides Associated with Callose Synthase Activity
170 kD-1	
136 kD-2 minor	
108 kD-3 minor	
96 kD-4 minor	
83 kD-5 minor	
	78 kD-16
72 kD-6 minor	
66 kD-7 minor	
64 kD-8 ⟷	64 kD-8
60 kD-9 minor	
54 kD-10 ⟷	54 kD-10
52 kD-11 minor	
48 kD-12	
42 kD-13	
	38 kD-17
34 kD-14	
32 kD-15 ⟷	32 kD-15

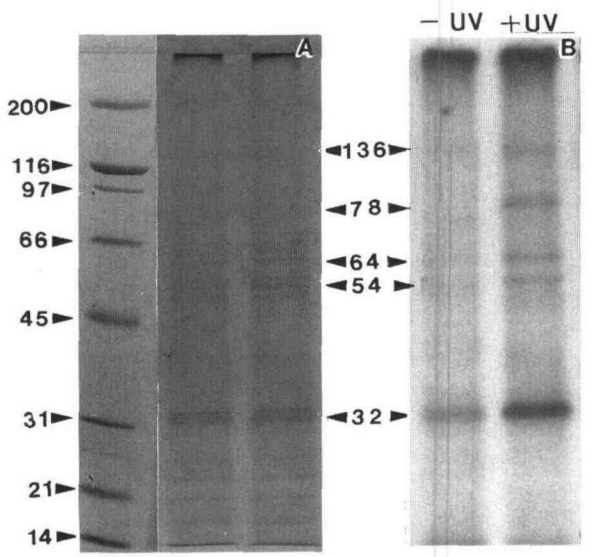

Figure 8. Photoaffinity labeling with [β-^{32}P]-5′-N_3-UDP-Glc of enzyme fraction solubilized by 0.25% digitonin. A, Protein profiles detected with Coomassie blue staining. B, Autoradiogram of gel A. Note that the 136-, 78-, 64-, 54-, and 32-kD polypeptides are specifically labeled with UV light.

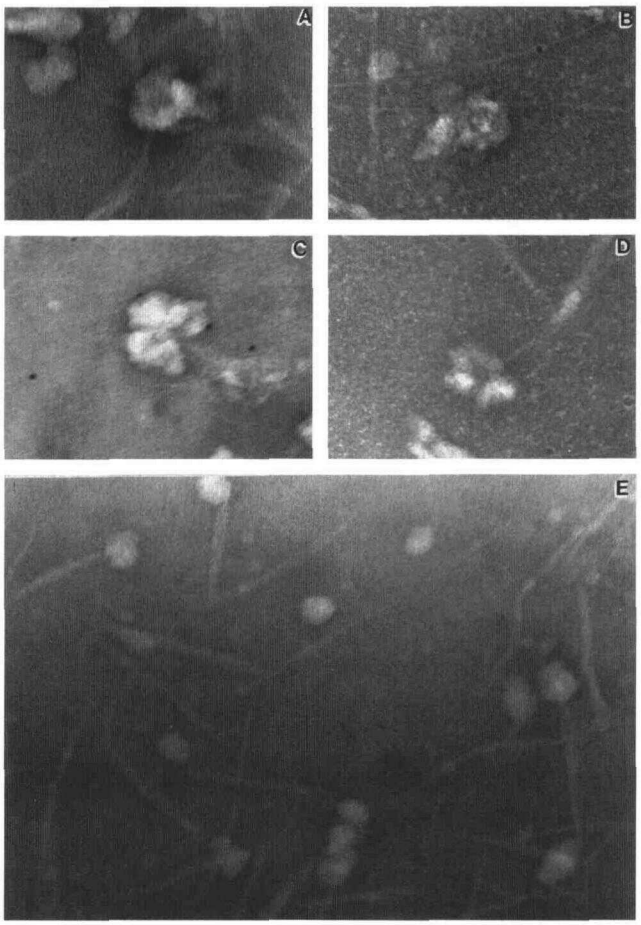

Figure 9. Electron micrographs of cellulose and callose synthase complexes (of fraction solubilized by 0.25% digitonin) and their products embedded in the native gel. The small gel fragments were negatively stained with 1% uranyl acetate. A to D, Rosette-like structures (63–84 nm) embedded in the top part of the stacking gel (band S), where only cellulose was synthesized. Note in particular that several rosette-like structures have products connected with them. The rosette structure in D is particularly well preserved and appears to have six subunits (about 24 nm in diameter). The dark particles are CBH I-gold complexes. Magnification = ×125,050. E, Particles (29–38 nm) associated with product synthesized in the top part of the running gel (band R), where only callose was synthesized. These structures are smaller than the rosettes found in A through D but are slightly larger than the individual subunits of the rosette-like particles. Magnification = ×125,050.

Read and Delmer, 1987; Bulone et al., 1990; Lin et al., 1990). Efforts have been directed at defining the relationship between the individual components in various synthase preparations and, in particular, at identifying which polypeptides might participate in (1→3)- and/or (1→4)-β-glucan synthesis. So far, only in the fungus *Saprolegnia monoica* has the separation of cellulose synthase and callose synthase been achieved (Bulone et al., 1990; Fevre et al., 1993; Bulone and Fevre, 1996). This (1→4)-β-glucan synthase was composed of 52-, 58-, and 60-kD polypeptides, whereas (1→3)-β-glucan synthase showed three different polypeptides (34, 48, and 50 kD; Bulone et al., 1990). These enzymes may have an oligomeric structure composed of different subunits.

Based on photoaffinity labeling, a number of polypeptides ranging from 31 to 83 kD have been suggested to be involved in cellulose and callose activity in different organisms (Read and Delmer, 1987; Mayer et al., 1989; Frost et al., 1990; Lin et al., 1990; Amor et al., 1991; Delmer, 1991; Delmer et al., 1991b; Meikle et al., 1991). Based on entrapment and immunoprecipitation reactions of callose synthase activity with antibodies, the 30- to 32-, 34-, and 55- to 58-kD polypeptides as well as a 60- and 62-kD doublet were identified as the most likely candidates for callose synthase polypeptides (Delmer, 1989; Fink et al., 1990; Dhugga and Ray, 1991; Meikle et al., 1991b; Bulone et al., 1995; Bulone and Fevre, 1996). If all of these identifications are valid, this would imply that the enzyme complex known to be of very high molecular mass could be composed of a number of nonidentical subunits.

Our results show that several polypeptides appear to be common to cellulose and callose synthase activities (64, 54, and 32 kD; Fig. 7; Table IV), and they are labeled with [β-^{32}P]5′-N_3-UDP-Glc (Fig. 8). This might suggest that the core region of glucan synthase may have an oligomeric structure composed of similar subunits, whereas the polypeptides specific for each separated fraction could play major, as yet unidentified, roles in the switching and regulation of β-glucan synthesis.

Numerous questions remain to be answered that are fundamental to a complete understanding of the structure and function of higher plant β-glucan synthases. For example, does the 78-kD polypeptide have a specific regulatory function for callose synthesis? Which polypeptides specifically regulate cellulose biosynthesis? Which are the UDP-Glc-binding proteins? Since the synthase bands lie at the interfaces of the gels, it is necessary to confirm that there are not trapped polypeptides unrelated to the synthase activities. Once the sequence data from all polypeptides are complete, we will have data available for comparison with sequences and functions that may be known.

It has been suggested that (1→4)-β-glucan synthase can be converted by conformational changes to (1→3)-β-glucan synthase and that the same enzyme might be able to catalyze the synthesis of both glucans (Delmer, 1987). Another hypothesis proposes that limited protease action might convert (1→4)-β-glucan synthase to (1→3)-β-glucan synthase (Girard and Maclachlan, 1987).

The first separation of in vitro activity for cellulose and callose synthesis, coupled with TEM of the particles involved, opens the way for understanding the nature of enzyme complexes responsible for cellulose and callose biosynthesis. We have found that particles with a rosette organization are exclusively associated with cellulose assembly in vitro (Fig. 9A), thus supporting the long-standing concept that rosette TCs are the sites for cellulose synthesis (Mueller and Brown, 1980). It is interesting that the rosette dimensions in the native gel are larger than those reported from the freeze-fracture data (Mueller and Brown, 1980; Herth, 1984). This could be understood based on the difference in preparation methods. During the freeze-fracture process membranes are split and the sample

is coated with Pt and C. The observed rosette TC subunits are found on the protoplasmic-fracture face. No views on the protoplasmic-surface have ever been noted; therefore, in the absence of this perspective, the in vitro whole-particle complex can now be much better appreciated.

The extracted complexes, when viewed by negative staining in the gel, have larger dimensions. The size difference could be due to the additional micelles associated during the solubilization phase with digitonin, or the size may represent the true, larger dimensions of the TC complex. For example, earlier sectioned views of linear TCs showed additional cytoplasmic components associated with the transmembrane complex (Kudlicka et al., 1987). Ordinarily, these would never have been observed in the freeze-fracture replicas. The isolated enzyme complexes require further extensive analysis. It is unknown how much the native protein dimensions may be modified by association with digitonin or endogenous lipids. However, the distinction between the particle complexes involved in cellulose and callose in in vitro synthesis is clear (Fig. 9, A–E).

Are rosette structures necessary for in vitro callose synthesis? According to our observations, the answer is no. Can cellulose be synthesized in vitro from single rosette TC subunits? Our observations indicate that the answer to this is yes. In spite of this advance, our observations still leave unanswered questions about the exact sites for cellulose and callose synthesis in situ; however, we can speculate that perhaps the TC rosette subunit contains the catalytic sites (hypothesized to be separate) for both (1→3)-β- and (1→4)-β-glucan synthesis. Since the SDS-separated proteins isolated from the cellulose- and callose-synthesizing bands have both common and specific peptide components, it is reasonable to hypothesize that the rosette particle complex is a multifunctional complex; however, sequencing analysis and identification of the genes for cellulose and callose synthase will be necessary to ultimately provide the answers to this vexing problem.

These studies have given some insight into the ways in which cellulose and callose may be switched on and off, developmentally, during wounding, or during extraction for in vitro studies. We have observed that enzymes synthesizing cellulose (at the top of stacking gel) are more labile, and the synthesis of cellulose band occurs at the early period of incubation with substrate. The enzyme particles frequently are associated with a multisubunit particle complex, often in the form of a rosette.

What prevents callose synthesis from overtaking cellulose assembly in vivo? It is possible that the catalytic site for callose is protected from access to the substrate when the rosette's conformation is tuned for cellulose synthesis. Wounding may cause rosette subunits to cleave into smaller particles, which then would have exposed callose catalytic sites (in addition to the native cellulose sites) (Brown et al., 1996).

Why would callose synthesis be so much more successful in competing for cellulose synthesis in vitro? A possible answer lies in the unique nature of the catalytic site predicted for cellulose synthesis (Saxena and Brown, 1997). If the multidomain hypothesis for dual simultaneous docking of two UDP-Glc for cellulose holds true, then the probability that both sites could receive the substrate at the same time is relatively low compared with a single site required for callose synthesis. Given an equal probability for exposure to UDP-Glc the callose site would have a much greater chance to capture the substrate and effectively compete with the cellulose site. Obviously, such conjecture will need to be supported by biochemical evidence; however, the suggestion of this type of unique regulatory mechanism for polysaccharide assembly does give some new insight into the years of mystery surrounding the synthesis of callose and cellulose.

Many questions remain concerning the regulatory mechanisms for cellulose and callose biosynthesis under natural conditions where callose is the major product, such as sieve plates and pollen tubes, versus the synthesis of callose under wounding conditions. A prediction can be made that the genes controlling the synthesis of callose in pollen tubes and sieve plates are different from the genes associated with cellulose/callose synthesis, such as the chitin synthases genes in fungi (Bulawa, 1993) and cellulose synthases genes in *A. xylinum* (Saxena and Brown, 1995).

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