CELLULOSE BIOSYNTHESIS IN HIGHER PLANTS

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ABSTRACT

Knowledge of the control and regulation of cellulose synthesis is fundamental to an understanding of plant development since cellulose is the primary structural component of plant cell walls. *In vivo*, the polymerization step requires a coordinated transport of substrates across membranes and relies on delicate orientations of the membrane-associated synthase complexes. Little is known about the properties of the enzyme complexes, and many questions about the biosynthesis of cell wall components at the cell surface still remain unanswered. Attempts to purify cellulose synthase from higher plants have not been successful because of the liability of enzymes upon isolation and lack of reliable *in vitro* assays. Membrane preparations from higher plant cells incorporate UDP-glucose into a glucan polymer, but this invariably turns out to be predominantly a β-1,3-linked rather than a β-1,4-linked glucans. Various hypotheses have been advanced to explain this phenomenon. One idea is that callose and cellulose-synthase systems are the same, but cell disruption activates callose synthesis preferentially. A second concept suggests that a regulatory protein as a part of the cellulose-synthase complex is rapidly degraded upon cell disruption. With new methods of enzyme isolation and analysis of the *in vitro* product, recent advances have been made in the isolation of an active synthase from the plasma membrane whereby cellulose synthase was separated from callose synthase.

KEY WORDS: glucan chains, microfibrils, β-1,4-glucan (cellulose), β-1,3-glucan (callose), cellulose synthase, callose synthase, enzyme complex.

CELLULOSE STRUCTURE

Cellulose is assembled by representatives of most living organisms, including bacteria and animals. It is the major component of plant cell walls. In general, primary cell walls contain 10-20% cellulose, secondary cell walls up to 50% and only specialized walls such as cotton fibers contain up to 98% cellulose. Besides providing the mechanical strength of the plant cell, cellulose is a protective component against external attack by mechanical forces or microorganisms. The occurrence of cellulose is remarkably broad in terrestrial plants, always displaying the same structure. No other cell-wall polysaccharide is known to be so widespread.

It is generally accepted that cellulose is an unbranched polymer of β-1,4-linked glucose residues. This creates a linear, extended glucan chain where every other glucose residue is rotated approximately 180°. This means that cellobiose, and not glucose, is the basic repeating unit of the cellulose molecule. This is in contrast with other glucan polymers such as starch (a-1,4-glucan) or callose (β-1,3-glucan), where the disaccharide is not the repeating unit and the chains are not perfectly extended but are less-ordered, usually with helical configurations.

The extended cellulose molecule forms a flat ribbon which is further stiffened by intra- and intermolecular hydrogen-bonds of the neighboring units. These bonds produce a regular crystalline arrangement of the glucan molecules in a very precise manner to form a rigid structure. In nature, cellulose never occurs as a single chain, but exists from the time of synthesis as a composite of many parallel oriented chains called microfibrils, which are the fundamental structural units of native cellulose (=cellulose I). Microfibril size can vary among organisms, ranging from the so-called elementary fibrils of about 36 chains, to the very large microfibrils of the cellulosic algae which can contain more than 1200 chains and are so highly organized that they can diffract as a pure single crystal (Sugiyama and Okano, 1990; Kuga and Brown, 1991).

The chain length (degree of polymerization) varies among different organisms, from a low of about 2000 to more than 25000 glucose residues, and nothing is known about how molecular weight is determined. The most important form of cellulose is the native cellulose I allomorph with parallel chains and strong intramolecular hydrogen bonds. Cellulose I is abundant in nature (bacteria, algae, fungi, plants, and some animals such as tunicates). The giant marine alga *Valonia*, synthesizes cellulose I which is accepted as the native cellulose standard, having the highest crystallinity and perfection. In nature, cellulose I exists as two different suballomorphs, called I α and I β with two different crystalline forms. Pure cellulose I β has been isolated from an animal (tunicates), and, to date, no pure α has been found (Atalla and Vanderhart, 1984). Additionally, cellulose can exist in various allomorphs such as cellulose II, III and IV, which exhibit different arrangements of the glucan chains and thereby produce

different X-ray or electron diffraction patterns (Sarko, 1978; Sugiyama and Okano, 1990).

CELLULOSE AND CALLOSE BIOSYNTHESIS

The description of cellulose seems to be relatively simple, as it is composed of just one type of sugar residue joined in one kind of repeating linkage. Yet open questions still exist concerning its biosynthesis. For a long period it has been a matter of discussion as to how the aggregation of glucan chains assembles into the microfibrils and ultimately how the degree of polymerization is controlled. There is probably no other biochemical process in plants that is so important, yet so poorly understood.

The process of cellulose biosynthesis involves not only chain polymerization but also crystallization, the mechanism that determines how and to what extend the chains associate to form microfibrils. In higher plants, complex mechanisms are also required to regulate the orientation of cellulose deposition.

There is a general agreement that in most algae and in higher plants, cellulose synthesis originates at the plasma membrane by membrane-integrated or associated enzyme complexes which are responsible for the production of 1,4-glucan (cellulose). However, membrane bound β-glucan synthases also synthesize β-1,3-glucan (callose) that is deposited at the plasma membrane surface in response to wounding, physical stress, or infection. Callose also occurs as a component of specialized walls or wall-associated structures at particular stages of growth and differentiation, e.g. in cell plates, on sieve plates, or in the micro- and megasporocyte wall. Also, a mixture of β-1,3 and β-1,4-glucans is found in grasses, as well as major components of endosperm walls of barley, rye, rice and wheat (Stone and Clarke, 1992; Schlupmann et al., 1993).

The nature of the enzymes involved in the synthesis of these two important polysaccharides and the control of their activities during wall formation are not well understood. Although particles attached to growing cellulose microfibrils, believed to be cellulose synthases, were detected in freeze-fracture preparations of plant plasma membranes as early as 1976 (Brown and Montezinos, 1976), these assemblies have proven to be very difficult to isolate and characterize because of their hydrophobic nature and instability. The demonstration of cellulose synthesis in vitro by membrane preparations from higher plants has been particularly difficult. Any preparation protocols in which membranes are perturbed results in a significant loss of 1,4-glucan synthesis. In contrast to the low activity of β-1,4-glucan synthase observed in microsomal preparations of higher plants, the normal latent, plasma membrane bound 1,3-glucan synthases is activated to produce high levels of β-1,3-glucan (Kauss, 1991: Trewavas and Gilroy, 1991).

Very high rates of *in vitro* synthesis of callose by enzymes localized on the same membrane system and using the identical substrate as cellulose synthase led to speculation that callose synthase may, in fact, also be the cellulose synthase, whereupon unknown factors change its specificity during cell damage (Delmer, 1987). Cellulose synthase is active in intact cells, and callose synthase is generally latent in intact cells and becomes rapidly activated in response to variety of perturbations. It is not known how these two glucan synthase activities are controlled. Perhaps there exists only one glucosyl transferase which can be switched, e.g. by conformational changes between the formation of both linkage types, depending on the requirements of the cell.

MODEL SYSTEMS FOR *IN VITRO* CELLULOSE BIOSYNTHESIS STUDIES

No single system has emerged as ideal for the study of cellulose biosynthesis, but some have proven particularly useful. Among a few bacteria that synthesize relatively large amounts of cellulose, *Acetobacter xylinum* has been used as the model system. This bacterium synthesizes cellulose from a row of synthetic sites along the longitudinal axis of the cell. The microfibrils from each synthetic site merge to form a large ribbon of cellulose in the growth medium. These ribbons and associated cells tangle and form a floating pellicle which allows the non-mobile, strictly aerobic bacterium to grow in the higher oxygen tension at the surface. The great advantage of *Acetobacter* cellulose is its high purity (Brown, 1886; Brown et al., 1976).

Agrobacterium tumefaciens synthesized cellulose from all regions of the cell surface during the contact with host plant, a process that aids in cell attachment and promotes virulence (Matthysse et al. 1995a, 1995b). Both *Acetobacter* and *Agrobacterium* can be grown in a large quantities and can be transformed. Mutants of both bacteria that are impaired in cellulose biosynthesis have been selected, and genes and enzymes involved in this process have been identified in both bacteria (Saxena et al., 1990; Wong et al., 1990; Matthysse et al., 1995 a, 1995 b).

The cellulosic algae have been quite useful for freeze-fracture studies to visualize terminal complexes (Brown and Montezinos, 1976; Itoh and Brown, 1984; Itoh et al., 1984), but they have proven difficult to study at biochemical and molecular levels. Cellulose synthase activity has been demonstrated in cellular slime mold *Dictyostelium discoideum* (Blanton and Northcote, 1990), and many genetic approaches are available that make it attractive for studies of cellulose biosynthesis.

Cotton, the most important commercial source of pure cellulose, has been intensively investigated (Basra and Malik, 1984; Ryser, 1985). Cotton fibers are single cells which elongate from the epidermis of the ovule, and their secondary walls have been considered as a source of pure cellulose. In contrast with other plant cells, cotton fiber cellulose constitutes more than 98% of the dry weight of the mature cell. In addition many investigators have used mung bean seedlings to study glucan synthesis (Feingold et al., 1958; Read et al., 1986; Thelen and Delmer, 1986; Hayashi et al., 1987; Read and Delmer, 1987; Callagan et al., 1988a, b).

Recently, scientists have been turning their attention to *Arabidopsis*. Indications are that some mutants are impaired in cellulose synthesis at high temperature (Betzner, et al., 1995). These mutants which map to distinct loci, are most likely impaired in primary wall synthesis. The potential to clone the genes exists, and further characterization of this mutant should provide valuable new insights into the process of cellulose and callose synthesis.

STRUCTURE OF THE CELLULOSE SYNTHASE COMPLEX

In bacteria, the complexes are stationery, and in algae and plants, they move in the fluid mosaic membrane. The organized direction of movement determines the pattern of cellulose deposition. This process is somehow guided by microtubules that are either adjacent to, or directly connected with the synthase complex. Among the diversity of plant forms, only two

basic cellulose synthesizing complexes exist: (a) the complex of subunits that form a linear pattern which is found among certain algae that synthesize highly crystalline ordered microfibrils (*Valonia* and *Boergesenia*); and (b) another type of TC where subunits are arranged in a hexagonal arrangement to form rosette. Rosette TCs are the evolutionary culmination leading to cellulose assembly in vascular plants. So far there have been no exceptions to this arrangement.

Purification of the enzymes responsible for cellulose synthesis was first accomplished using entrapment technique for digitonin-solubilized cytoplasmic membrane proteins from *Acetobacter* (Lin and Brown, 1989). Photoaffinity studies led to labeling of an 83 kD polypeptide, indicating that this polypeptide was the catalytic subunit for UDP-glucose binding (Lin et al, 1990). N-terminal sequencing of this polypeptide allowed the gene to be cloned (Saxena et al. 1990). It has been found that the first gene in the operon codes for the β-glucan synthase catalytic subunit that binds UDP-glucose (the 83 kD polypeptide), and the second gene codes for a polypeptide (93 kD) that binds an activator for cellulose synthase, c-di-GMP. These two polypeptides are very tightly associated as they have always been observed from entrapment-purified cellulose synthase. The amino acid sequence of the polypeptide coded by the third gene of the operon shows the presence of a signal sequence characteristic of bacterial lipoproteins (Saxena et al., 1994). This suggests that the polypeptide may be localized in the cell envelope and probably functions in the organization of the nascent glucan chains. Only lately the fourth gene in the operon has been found, which codes for a polypeptide of 17 kD and may function in the determination of the crystallization phase of cellulose biosynthesis (Saxena et al., 1994).

In the absence of any purified cellulose synthase from higher plants, it is difficult to provide any biochemical evidence to model the synthase complex. Amor et al., (1991) identified two polypeptides in membrane of cotton fibers that bind c-di-GMP with high specificity, but these have been difficult to purify and characterize. Several reports indicate that polypeptides in the 30 kD range co-purify with callose synthase activity, and these have been suggested as possible channels for export of glucan chains (Qi et al., 1995). Additional evidence suggests that purified cotton fiber annexin binds to, and influences the activity of callose synthase (Andravis et al., 1993). It is possible that plant annexin may turn out to serve as a pore for callose secretion or, more likely, for allowing entry of Ca^{++}, which would then cause localized activation of the callose synthase complex.

It has been hypothesized that a single β-glucan synthase complex may be capable of synthesizing both β-1,3- and 1,4-glucans (Delmer, 1987). To resolve the question of multiple activities residing in a single enzyme complex, the polypeptides involved in both 1,3- and 1,4-glucan synthesis need to be identified and purified.

IN VITRO CELLULOSE SYNTHESIS

In order to understand the mechanisms of cellulose biosynthesis, many investigations have been performed with cell-free systems from various organisms. UDP-glucose is considered to be the glucosyl donor for cellulose synthesis. Membrane preparations usually are incubated in the presence of various concentrations of UDP-glucose in buffers optionally supplemented with divalent cations such as Mg^{++} or Ca^{++}, β-linked disaccharides, and detergents. A major complication in all searches for *in vitro* cellulose synthase activity in plasma membranes delivered from higher plants arises from the fact that such membranes have very high levels of callose synthase activity. Because callose often has been confused with cellulose in non-rigorous determination of glucan structure, there have been a number of erroneous claims of cellulose synthesis that were subsequently disproved (Callaghan and Benziman, 1984, 1985).

In studies with membranes derived from developing cotton fibers, it was demonstrated for the first time that reproducible but low rates of β-1,4-glucans are synthesized simultaneous with callose (Li and Brown, 1993, Okuda et al., 1993). At that time only 4% of the total glucans synthesized was cellulose which was characterized as cellulose II. Recently, the conditions to change the ratio of cellulose to callose were found, and the quantity of cellulose was increased to more than 30% of the total glucans synthesized *in vitro* (Kudlicka et al., 1995), (Fig. 1). For the first time, extended microfibrils characterized as cellulose I, very strongly labeled with CBH-I-gold complex (the probe is very specific for cellulose) were synthesized from a solubilized enzyme fraction (Figs 2 and 3). It was interesting to discover that only the fractions solubilized in very low concentrations of digitonin (0.05%) synthesized cellulose I. This is probably due to the gentle solubilization process during which the enzyme complex may have remained intact whereby sufficient packing and organization of the catalytic subunits could ensure parallel extended glucan chain formation. This close association of catalytic subunits may be a possible explanation for a similar arrangement of these components *in vivo* into an organized terminal complex where the synthesis of parallel β-1,4-glucan chains leads to the crystallization of cellulose I. The fraction solubilized in higher concentrations of digitonin (1%) produced cellulose II (Fig. 4). This indicates that the enzymes may have been sufficiently disorganized, such that extended parallel chain aggregation would have been prevented during the polymerization process. In this case chain folding probably may have occurred, leading to the antiparallel arrangement, which is more thermodynamically favorable (Rånby, 1952).

It is interesting to note that the cellulose I allomorph recently has been assembled in an abiotic reaction mixture containing cellobiosyl fluoride as the substrate and a highly purified endoglucanase as the catalyst in a non aqueous environment which favors synthesis rather than hydrolysis of the polymer (Lee et al. 1994). The details of these new findings are truly novel.

In spite of the progress we have made toward *in vitro* cellulose synthesis from higher plants, it seems that we still are lacking an understanding of the critical factors necessary for obtaining substantial synthesis *in vitro*. With the recent discovery of a membrane-bound form of sucrose synthase, the question has been addressed of whether *in vitro* synthesis by disrupted and detergent-permeabilized cotton fibers might occur more readily if the substrate supplied were sucrose instead of UDP-glucose (Amor et al. 1995; Delmer and Amor 1995).

IDENTIFICATION OF CELLULOSE SYNTHASE

The only catalytic subunit of cellulose synthase that has been clearly identified is that of *A. xylinum*. There are still no firm indications of the nature of the catalytic subunits of plant cellulose synthase. Li et al., (1993) using photoaffinity labeling with azido-UDP-glucose, identified a polypeptide of 37 kD which was suggested to be a likely candidate for the cata-

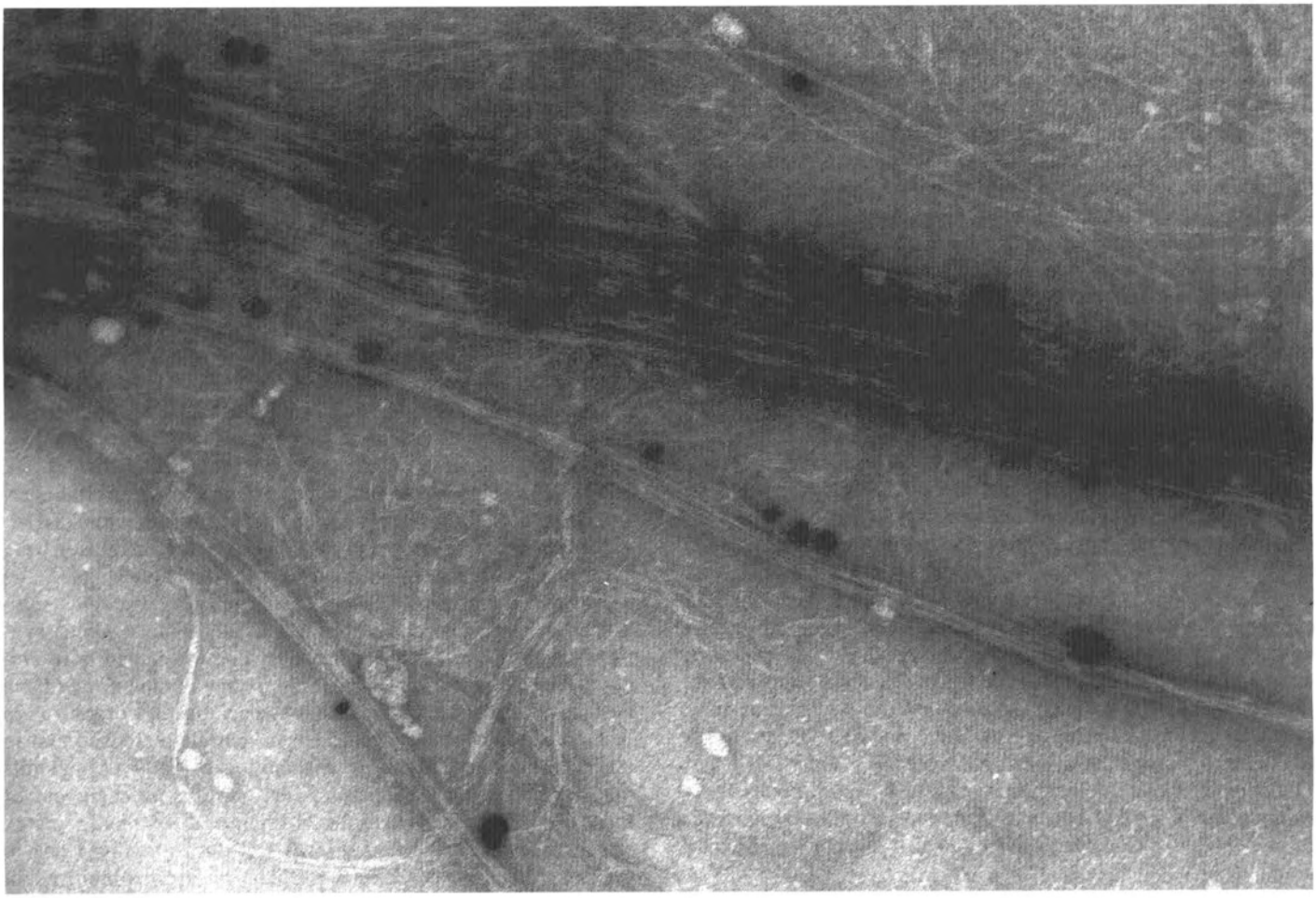

Fig. 1. Negatively stained and CBH I-gold labeled *in vitro* products synthesized by a cotton enzyme fraction solubilized in a low concentration of digitonin (0.05%). The β-1,4 glucans (labeled with CBH I-gold) and β-1,3-glucans (not labeled) are clearly distinguishable. β-1,4-glucan in the form of long, extended fibrils is characteristic of cellulose I. x 122,500.

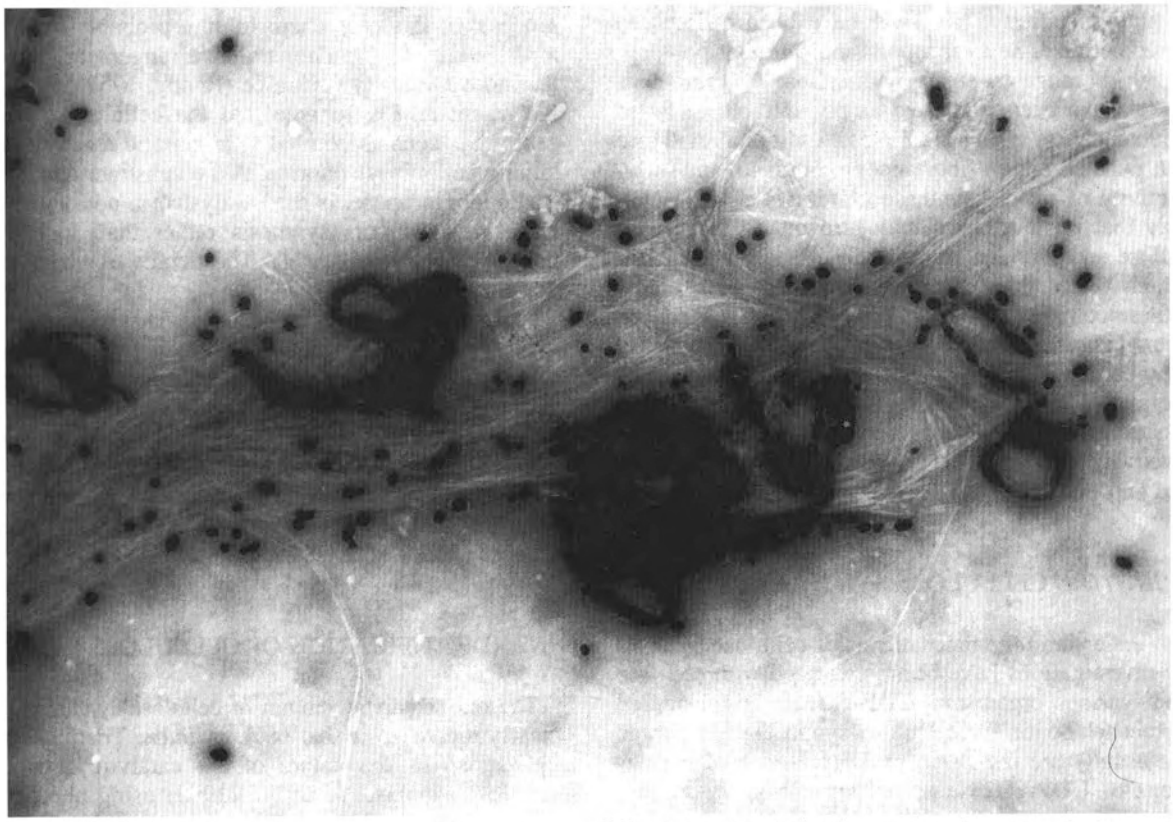

Fig. 2. Autoradiography of *in vitro* cellulose synthesized by enzyme solubilized in a low digitonin concentration, treated with acetic/nitric acid reagent to remove β-1,3-glucans. Silver grains and CBH I-gold particles specifically label the fibrillar product. x 122,500.

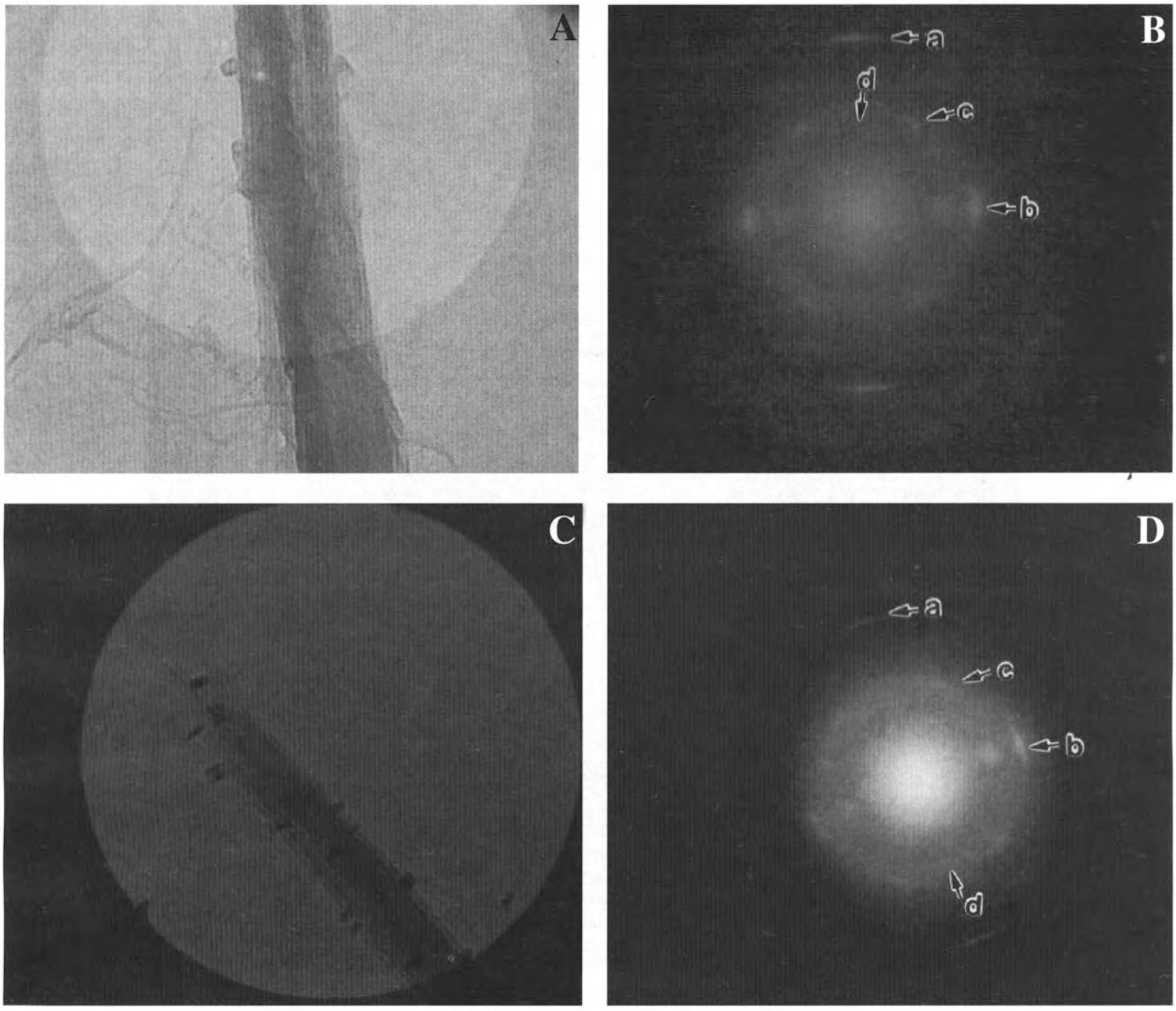

Fig. 3. Electron diffraction patterns and the irradiated fields of the exposed area selected for ED. **A.** Defocused image of native cotton. **B.** ED pattern of native cotton with sharp 004 meridional (arrowhead a) and three equatorial reflections (arrowhead b = 200, 110 and 110) indicating a high orientation and high crystallinity. The week 002 reflection (arrowhead d) is characteristic for the cellulose I β allomorph. **C.** Defocused image of *in vitro* product synthesized by cotton fraction solubilized in low digitonin concentration, after acetic/nitric acid reagent treatment and labeled with CBH I-gold complex. **D.** ED pattern of the same product as in C. Note the similarity of these reflections to those of the native cellulose I pattern in Fig. B.

lytic subunit. However, these results still remain correlative, because they were not done with purified cellulose synthase. In a similar correlative study with crude or partially purified cellulose synthase, polypeptides in the range of 52 to 57 kD have been suggested as likely candidates for the catalytic subunit of this enzyme (Frost et al., 1990; Delmer et al., 1991; Fredrikson et al., 1991; Dhugga and Ray, 1994).

Recently, a comparative study of cellulose synthesized *in vitro* from cotton and mung bean has indicated two morphological forms of cellulose I synthesized by two different organisms (Kudlicka et al., 1996, in press). Perhaps mung bean cellulose synthases may be specific for a given developmental stage within the plant, or different among various plant species (e.g. mung bean vs. cotton or primary vs. secondary wall stages of development). The greater resistance to acetic/nitric acid reagent (which solubilized all other glucan polymers except cellulose) treatment of the *in vitro* cellulose synthesized by cotton suggests the possibility of a higher molecular weight product. If so, this would parallel the distribution of *in vivo* synthesis of cellulose in cotton secondary walls. This raises the interesting question that one or more different cellulose synthases may be operative at various developmental stages of life cycle in given organisms.

Very recently, we found also that native gel electrophoresis of solubilized fractions of mung bean followed by incubation with UDPG, produced a single band of polymeric product at the top of gel. The single band of glucan activity in the native gel corresponds to a single Coomassie blue stained protein band. After removing the acrylamide from the band, and observing the product in the electron microscopy, it was

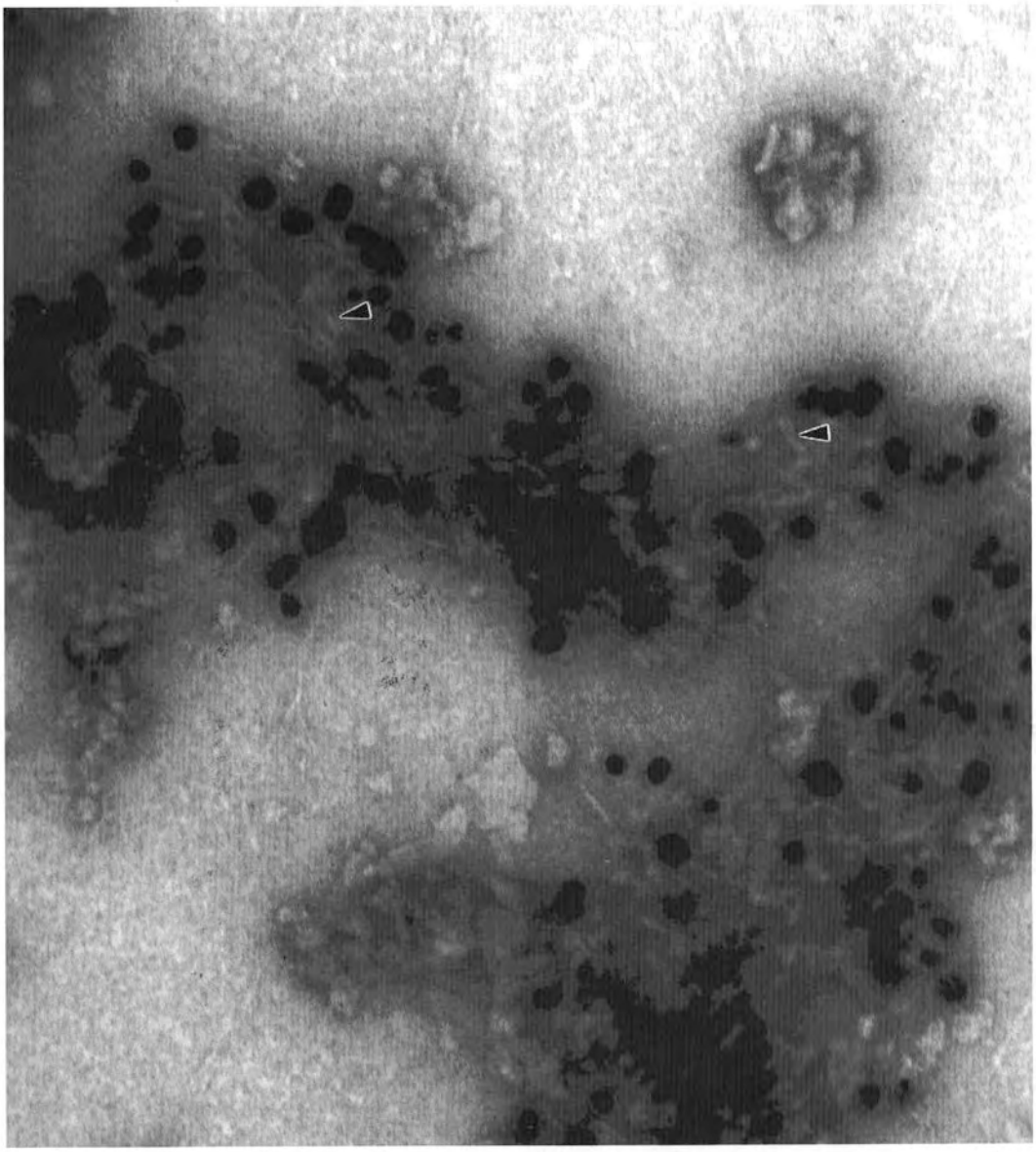

Fig. 4. Negatively stained and CBH I-gold labeled *in vitro* product synthesized by cotton enzyme fraction solubilized in 1% digitonin. The product was subjected to acetic/nitric acid reagent treatment. Rod-like structures (arrows) indicate morphological characteristic of cellulose II. x 205,000.

evident that it is comprised of three different structures corresponding to callose, cellulose I and cellulose II (data not published).

When a separate 3% stacking/6% running gel was used in mung bean for separation of the most active fraction under nondenaturating conditions, two bands appeared after incubation with UDPG: (1) a sharp band at the top of the stacking gel; and, (2) a broad band at the top of running gel. The product from the stacking gel was exclusively fibrillar material strongly labeled with CBH-I-gold complex indicating only cellulose. Interestingly, the band from the running gel revealed only callose. This appears to be the first result of an exclusive separation of β–1,4- and β-1,3-glucan synthesis activity *in vitro*. It will be interesting to compare the proteins from these two bands. This work is in progress (data not published).

FUTURE DIRECTIONS

There are some interesting areas in which the future studies should be directed. First, the overall regulation of cellulose and callose synthesis as it relates to cell perturbation deserves further study. Identification of polypeptides for *in vitro* assembly of cellulose and callose by separating them in nondenaturating conditions followed by their sequencing, is a major goal. Rapid progress is being made in signal transduction in plants, and some of the knowledge gained from these advances should be applied to the regulation of cellulose and callose synthesis. The evidence for a coupled reaction between sucrose synthase and glucan synthesis offers a new approach for characterization of cellulose synthase activity. Finally, the power of genetics is beginning to show possibilities for dissecting the process of cellulose synthesis by selection of mutants impaired in cellulose deposition. This mutant approach is beginning to bear fruit in studies of β-glucan synthesis in fungi (Diaz, 1993; Douglas et al., 1994). The key to success in plants undoubtedly lies in the use of proper selection and screening techniques. Looking to the long term, once critical genes are identified, the possibility exists for genetic modification of plants in ways that could alter the structure or crystallinity of cellulose produced, leading to improved cellulose-bearing crops.

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BIOSYNTEZA CELULOZY U ROŚLIN WYŻSZYCH

STRESZCZENIE

Poznanie procesu kontroli i regulacji syntezy celulozy jest podstawą zrozumienia rozwoju roślin, gdyż celuloza jest podstawowym składnikiem ścian komórek roślinnych. Pierwszym etapem syntezy celulozy jest proces polimeryzacji cząsteczek glukozy do łańcuchów glukanowych. *In vivo* proces ten wymaga skoordynowanego transportu substratu przez błonę komórkową i odpowiedniego ułożenia kompleksów enzymatycznych w błonie. W następnym etapie krystalizacji, łańcuchy glukanowe łaczą się i tworzą mikrofibrylle. U roślin wyższych złożony mechanizm kieruje odpowiednim ułożeniem mikrofibrylli w ścianie komórkowej. Jak dotąd wiedza dotycząca właściwości kompleksów enzymatycznych jest bardzo uboga i ciągle wiele pytań dotyczących syntezy celulozy na powierzchni komórki pozostaje bez odpowiedzi. Próby oczyszczenia enzymów odpowiedzialnych za syntezę celulozy u roślin wyższych nie powiodły się, ponieważ enzymy te są bardzo mało stabilne po wyizolowaniu z błony komórkowej. W reakcji *in vitro* wyizolowane enzymy pobierają substrat (UDP-glukoza) i tworzą łańcuchy glukanowe, jednak głównym produktem jest kaloza, a nie celuloza. Różne hipotezy były wysuwane w celu wytłumaczenia tego zjawiska. Jedna z nich tłumaczy, że ten sam enzym jest odpowiedzialny za syntezę obu polisacharydów, a uszkodzenie komórki uaktywnia priorytetowo syntezę kalozy. Inna hipoteza sugeruje, że białka regulujące proces syntezy celulozy, będące częścią kompleksu enzymatycznego, są gwałtownie niszczone w czasie izolacji. Zastosowanie łagodnej metody ekstrakcji enzymów oraz odpowiednia analiza produktu, pozwoliły ostatnio na osiągnięcie syntezy celulozy *in vitro*. Warunki syntezy nie są jeszcze optymalne, gdyż ilość syntetyzowanej celulozy stanowi tyko 30% całkowitego produktu. Najnowsze wyniki uzyskane z rozdziału białek w warunkach niedenaturujących są bardzo zachęcające. Metodą tą prawdopodobnie uda się rozdzielić enzymy odpowiedzialne za syntezę celulozy i kalozy.

SŁOWA KLUCZOWE: łańcuchy glukanowe, mikrofibryle, β-1,4-łańcuchy glucanowe (celuloza), β-1,3-łańcuchy glukanowe (kaloza), kompleksy terminalne, kompleksy enzymatyczne.