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Assembly of synthetic cellulose I

(enzymatic polymerization/cellulase/*Trichoderma viride*/β-cellobiosyl fluoride)

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ABSTRACT **Cellulose microfibrils with an electron diffraction pattern characteristic of crystalline native cellulose I have been assembled abiotically by means of a cellulase-catalyzed polymerization of β-cellobiosyl fluoride substrate monomer in acetonitrile/acetate buffer. Substantial purification of the *Trichoderma viride* cellulase enzyme was found to be essential for the formation of the synthetic cellulose I allomorph. Assembly of synthetic cellulose I appears to be a result of a micellar aggregation of the partially purified enzyme and the substrate in an organic/aqueous solvent system favoring the alignment of glucan chains with the same polarity and extended chain conformation, resulting in crystallization to form the metastable cellulose I allomorph.**

Cellulose is a major component of plant cell walls and is the most abundant macromolecule on earth. The two major allomorphs of this biopolymer are cellulose I and cellulose II. Cellulose I, the dominant form in nature (1), consists of a microfibrillar crystalline array of linear β-1,4-glucan chains, all of which are oriented parallel to one another with the same polarity (2). The extended chain conformation of cellulose I allows the formation of microfibrils having extraordinary mechanical strength. Normally, cellulose II is formed from cellulose I through chemical treatments that alter the crystal structure (e.g., mercerization) (3, 4). The cellulose II allomorph also is produced by a few organisms in nature (3, 5, 6). A special instance is the anomalous microbial cellulose from which strong evidence has been obtained to indicate an antiparallel structure for cellulose II (7). Previous studies have led to the conclusion that cellulose II is the more thermodynamically stable allomorph (3). Until now, no *in vitro* or abiogenic process has been reported that produces cellulose I, either by recrystallization or by polymerization. Cellulose synthesis *in vitro* from uridine diphosphate glucose always results in the formation of cellulose II (8–10). Therefore it has been argued that living organisms which synthesize cellulose I must control glucan chain crystallization in a manner not duplicated under acellular conditions (3, 4, 11, 12).

Recently the abiogenic synthesis of cellulose II has been achieved through an enzymatic polymerization utilizing β-cellobiosyl fluoride (β-CBF) substrate monomer, a crude cellulase mixture (Onozuka R-10) as catalyst, and an organic/aqueous solvent system (13–17) (Fig. 1). The cellulose produced in this manner has been called "synthetic cellulose" to distinguish it from cellulose produced *in vivo* or *in vitro* by enzymes typical of the natural biogenic pathway (13). Formation of the stereoregular β-1,4 linkage is explained in terms of the formation of a cellobiosyl-enzyme intermediate or a cellobiosyl oxocarbenium ion at an active site of cellulase. This reactive intermediate is then attacked by a 4′-hydroxyl group of another monomer or propagating polymer which locates in a subsite of the enzyme leading to the stereoselective formation of the β-1,4 linkage (16). An examination of synthetic cellulose produced this way showed that the product consisted of irregular rodlets characteristic of the cellulose II allomorph (17).

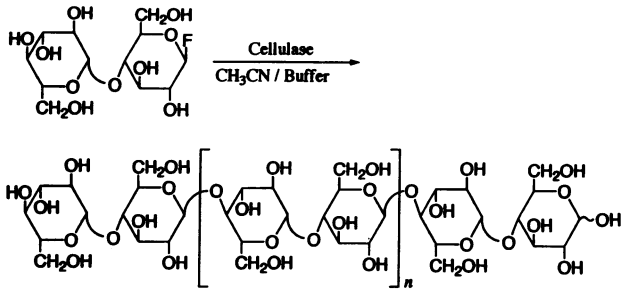

FIG. 1. Synthesis of cellulose by a condensation polymerization catalyzed by cellulase with use of β-CBF as a substrate monomer.

In this study, the assembly of synthetic cellulose I has been accomplished by means of a partially purified cellulase-catalyzed polymerization of β-CBF in an optimized acetonitrile/acetate buffer system. This cellulose has been characterized by transmission electron microscopy (TEM), electron diffraction (ED), enzymatic hydrolysis followed by thin-layer chromatography (TLC), and cellobiohydrolase I–colloidal gold (CBH I-Au) binding.

MATERIALS AND METHODS

Purification Methods. *The P-100 active fraction.* Two grams of commercially available crude cellulase Onozuka R-10 (Kinki Yakult, Osaka) was added to 15 ml of 0.01 M sodium acetate buffer (pH 5.0), and the insoluble material was removed by centrifugation at 50,000 × *g* for 30 min. The supernatant fraction was loaded onto a Bio-Gel P-10 Fine column (30 × 500 mm; Bio-Rad) with flow rate of 25 ml/hr at 4°C, and the void volume fractions were pooled and concentrated by using a stirred ultrafiltration cell with a PM-30 membrane (Amicon). Then 2.6 ml of Bio-Lyte Ampholyte pH 3–10 (40% wt/vol; Bio-Rad) and deionized water were added to make a final volume of 55 ml. This solution was loaded into a Rotofor preparative isoelectric focusing cell (Bio-Rad) operating at 12 W for 4 hr at 1°C. Twenty fractions of about 2.5 ml each were collected and the pH and A_{280} were measured. Fractions with the highest cellulose-producing activity were pooled and concentrated by using a Centricon-30 (Amicon). This concentrated material was applied to a Bio-Gel P-100 column (20 × 900 mm) with a flow rate of 7.5

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Abbreviations: β-CBF, β-cellobiosyl fluoride; TEM, transmission electron microscopy; ED, electron diffraction; CBH I-Au, cellobiohydrolase I–colloidal gold; TC, terminal complex.

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ml/hr at 4°C. Eluted fractions from the P-100 were analyzed, and the active fraction was determined. This active fraction is called the P-100 fraction.

The P-60 active fraction. Two grams of the crude cellulase was added to 40 ml of 0.01 M sodium acetate buffer, pH 5.0, and the insoluble material was removed by centrifugation at 50,000 × *g* for 30 min. Ammonium sulfate fractionation led to a precipitate (between 37% and 45% saturation) which was pelleted by centrifugation at 50,000 × *g*. The pellet was dissolved in 10 ml of 0.01 M sodium acetate buffer, pH 5.0, and loaded into a Bio-Gel P-10 desalting column (30 × 500 mm) with a flow rate of 25 ml/hr at 4°C. The void volume fractions were pooled and concentrated. One milliliter of a Bio-Lyte Ampholyte pH 3–10 (40% wt/vol), 1 ml of Bio-Lyte Ampholyte pH 3–5 (20% wt/vol), and deionized water were added to make a final volume of 55 ml. This solution was loaded into a Rotofor preparative isoelectric focusing cell. Fractions showing the greatest cellulose productivity were pooled and concentrated by using a Centricon-30. This concentrated material was applied to a Bio-Gel P-60 column (20 × 900 mm) with a flow rate of 7.5 ml/hr at 4°C. Fractions eluted from the P-60 column were analyzed, and the most active fraction was taken as the P-60 fraction.

Enzyme Activity Assay. For the activity assay, enzymatic polymerization conditions were essentially similar to those used by Kobayashi *et al.* (13) (Fig. 1), although the reaction temperature (25°C), time (1 hr), and scale (5 mg of monomer in 0.584 ml or 0.288 ml), as well as the catalyst condition (20 μg of purified enzyme), were changed. The 5:1 methanol/water-insoluble product (cf. Table 1) was pelleted and washed with 1 ml of 5:1 methanol/water and collected by centrifugation. For the hot-SDS-insoluble product, the pellet was boiled for 10 min with 10% SDS to deactivate the enzyme. The product was centrifuged and vigorously washed three times with deionized water.

For yield measurements, the phenol/sulfuric acid method (18) and the anthrone reagent method (19) were utilized with glucose as the standard. The micro Lowry method (20) was employed for all protein concentration assays, except that for the Rotofor fractions A_{280} was used to measure the protein concentration, with bovine serum albumin as the standard.

SDS/PAGE. Slab-gel electrophoresis was performed as described by Porzio and Pearson (21). Samples were boiled for 3 min with sample buffer. Gels were stained for protein with Coomassie brilliant blue. Low molecular mass standards were used (Bio-Rad).

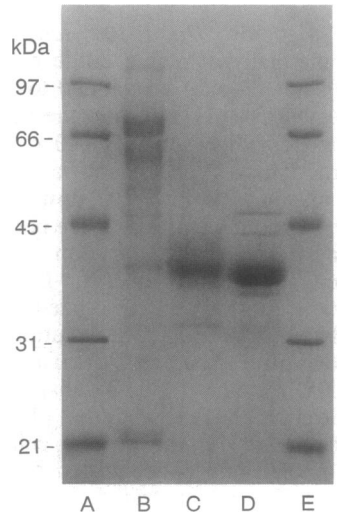

FIG. 2. Coomassie blue stain of the protein bands separated by SDS/10% PAGE. Lanes: A and E, molecular mass markers; B, 40 μg of the crude enzyme; C, 20 μg of the P-100; D, 20 μg of the P-60. The proteins in lane D had the highest synthetic activity among the three enzyme preparations.

Table 1. Polymerization of β-CBF catalyzed by enzyme at various stages of purification

Enzyme fraction	Yield, %*	
	5:1 methanol/water-insoluble precipitate	Hot 10% SDS-treated and water-insoluble precipitate
Crude	0.07	0.04
P-100	1.12 (16-fold)	0.60 (15-fold)
P-60	32.37 (462-fold)	9.46 (237-fold)

Polymerization was at 25°C for 1 hr; β-CBF was 25 mM and cellulase was 0.34 wt %, in 5:1 (vol/vol) acetonitrile/50 mM sodium acetate buffer, pH 5.

*In parentheses are the fold increases in the yield compared with crude enzyme reaction yield.

Product Analysis. The sample obtained from the activity assay also was collected for product characterizations. For this, we used TEM, ED, CBH I-Au binding, and time-course observations. The reaction products were subjected to 30-sec ultrasonication before analysis.

For TEM observations and ED analysis, samples were mounted on copper grids coated with thin carbon films or Formvar films. For morphological analysis, these grids were negatively stained with 2% uranyl acetate containing 0.01% bacitracin and examined with a Philips 420 TEM. For ED analysis, grids were not stained and a camera length of 107 mm was employed, using an accelerating voltage of 120 kV, and the diffraction pattern was recorded on Kodak Tri-X film by using a 35 mm camera. The intermediate aperture delineated the irradiated area of the specimen from which the electron diffraction pattern was recorded. A minimal beam dosage technique was employed, using a Philips low-dose unit. Rayon and native cellulose I were used as control standards for comparison.

Enzymatic hydrolyses of the synthetic products were accomplished by using a complete cellulase mixture, Celluclast (Novo Industries, Bagsvaerd, Denmark), for 48 hr at 37°C at a concentration of 0.25 μg/ml (pH 5.0, 0.01 M acetate buffer). After centrifugation to remove the water-insoluble components, the soluble sugars were chromatographed on a Whatman silica gel 60A TLC plate, as described by Hansen (22).

A colloidal gold suspension (AuroBeads G5, average particle size, 5 nm; Amersham) was used to prepare a CBH I-Au complex as described by Chanzy *et al.* (23). CBH I-Au labeling of the synthetic products for TEM was performed by

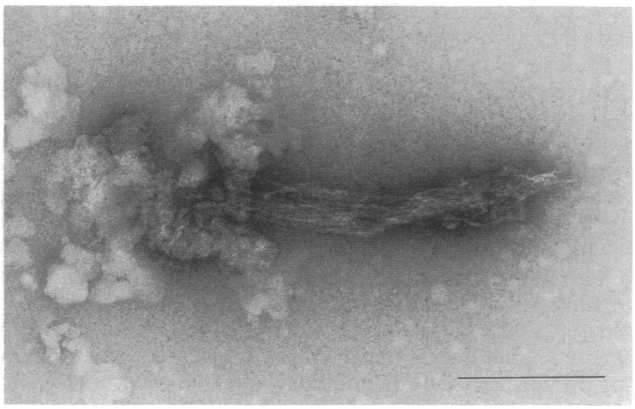

FIG. 3. The P-100 fraction product in 2:1 acetonitrile/buffer incubated for 3 min. Note the prominent unidirectionally elongated bundle of extended microfibrils which appear to originate from a depression or channel within the protein aggregate. (Scale bar, 250 nm.)

floating grids with the product on a drop of a CBH I-Au complex solution on ice for 3 min and washing with water, then negatively staining with 2% uranyl acetate.

The 2:1 acetonitrile/buffer reaction mixture was prepared by dissolving 10–50 μg of protein from the P-100 fraction or the P-60 fraction in 0.05 M acetate buffer (pH 5.0) to give a total volume of 0.032 ml; 5.0 mg of β-CBF was dissolved in 0.064 ml of same buffer and 0.192 ml of acetonitrile. Reactions were conducted at room temperature for 20 min.

RESULTS

Partial Purification of the Synthetic Cellulose I Catalyst. In attempts to isolate from the crude enzyme mixture the catalyst(s) which was responsible for the polymerization of β-CBF, two different purification methods resulted in two distinct partially purified enzyme preparations, namely the P-100 (Fig. 2, lane C) and P-60 fractions (Fig. 2, lane D).

During the purification, some minor synthetic activity appeared in several different Rotofor fractions in both P-100 and P-60 purification steps; however, the most active fractions were nos. 1 and 2 (pI 3.0–3.5) from P-100 and nos. 5 and 6 (pI 4.2–4.6) from P-60. When these fractions were loaded onto their respective gel chromatographic columns, only one peak with activity appeared. These fractions were used for product synthesis. The progressive purification through the P-100 and P-60 protocols from the crude enzyme resulted in higher yields for both 5:1 methanol/water-insoluble and hot 10% SDS/water-insoluble products (Table 1).

Product Characterization. *Enzymatic hydrolysis.* Enzymatic hydrolysis of the synthetic products with Celluclast yielded glucose and cellobiose with a trace of cellotriose on the TLC plate (not shown).

TEM: The P-100 fraction. The product produced with the P-100 fraction in the 5:1 acetonitrile/buffer yielded a small quantity of elongated microfibrils (cellulose I) dispersed

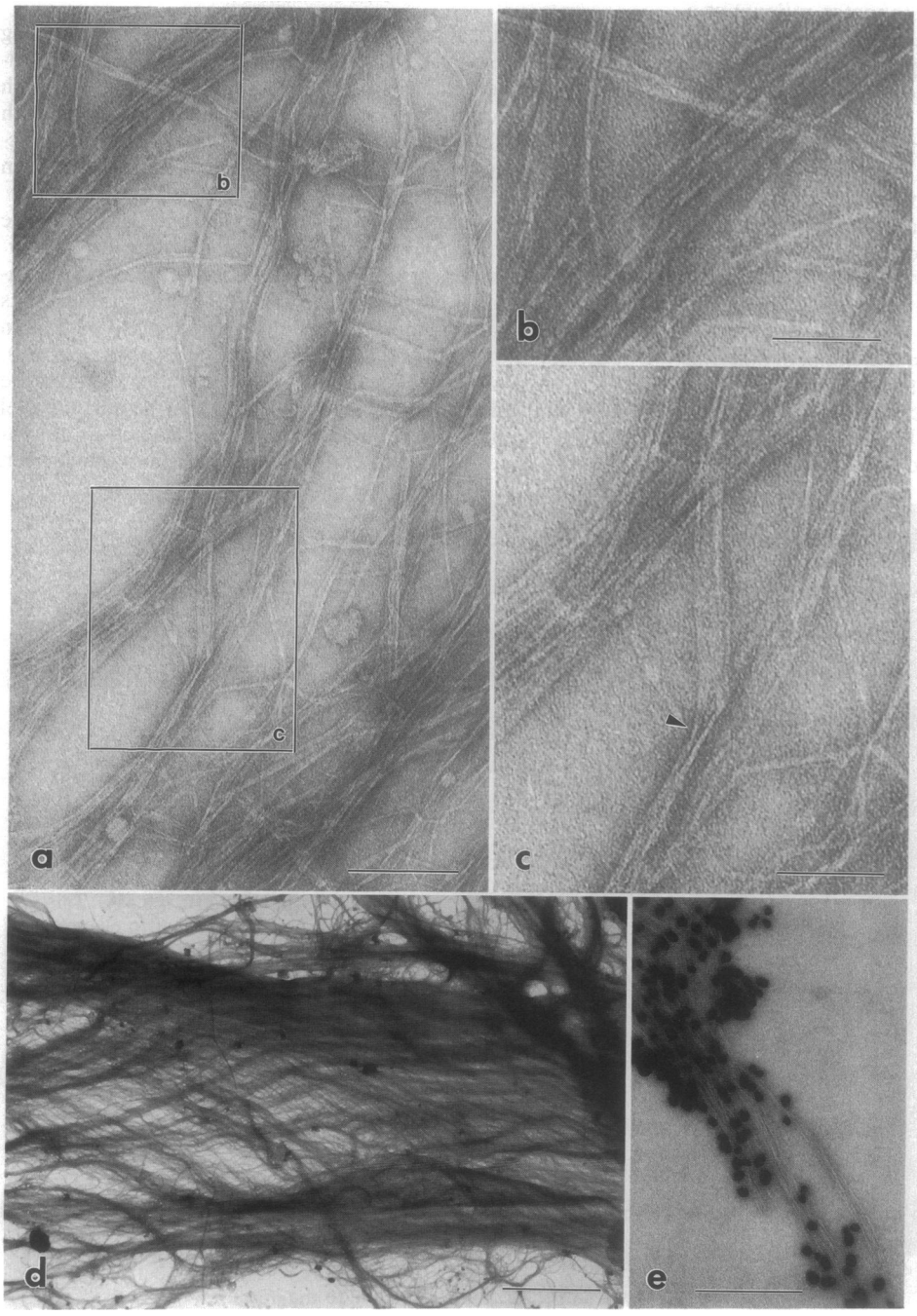

FIG. 4. Transmission electron micrographs of cellulose synthesized from β-CBF by the P-60 fraction with an acetonitrile/buffer ratio of 2:1. The production of microfibrillar material is significantly enhanced in comparison with the P-100 reaction product in the same solvent. (*a*) High-magnification electron micrograph of the product. Note highly oriented extended microfibrils. (Scale bar, 100 nm.) (*b*) Higher-magnification view of box *b* of *a*. Note the microfibril width is presented in the narrow axis (11–13 Å) when the microfibrils cluster. (Scale bar, 50 nm.) (*c*) Higher-magnification view of box *c* of *a*. Note the wide axis of individual unclustered microfibrils (up to 130 Å) on the grid. Most of these ribbonlike microfibrils in negatively stained specimens have distinctive pleats or longitudinal foldings (indicated with an arrow). (Scale bar, 50 nm.) (*d*) Low-magnification electron micrograph of the same product as in *a*. Note highly oriented extended microfibrils (cellulose I) aggregating into a large bundle. The reaction product is similar to the P-100 reaction product in 2:1 acetonitrile/buffer, but the ratio between cellulose I and cellulose II seems to have increased. (Scale bar, 2.5 μm.) (*e*) CBH I-Au binding to the product made under the same reaction conditions as for *a–d*. (Scale bar, 200 nm.)

among shorter irregular rodlets (cellulose II) (not shown). An enhanced yield of the microfibrillar product was attained by decreasing the ratio of acetonitrile to buffer (2:1 from 5:1) (not shown).

When the time-course study of the reaction using the P-100 fraction was conducted, the progress of microfibril elongation could be observed with TEM. One control was the P-100 fraction alone dissolved in acetate buffer. This control revealed particles about 4 nm in diameter with some larger aggregates (not shown). A second control (the P-100 fraction added to the 2:1 acetonitrile/buffer system minus substrate) showed distinct micellar aggregates of proteins but no microfibrils or rodlets (not shown). Three minutes after the addition of β-CBF to this mixture, a cluster of extended microfibrils elongated and became more organized (Fig. 3).

TEM: The P-60 fraction. When the P-60 fraction was used for synthesis (2:1 acetonitrile/buffer), the microfibril production increased. Microfibrils aggregated to form large bundles on the grid (Fig. 4*d*). In morphology, the microfibrils were similar to native cellulose I from *Acetobacter xylinum* at a low magnification; however, at a high magnification the dimensions were different from any reported native cellulose I (Fig. 4*a*). The microfibrillar dimensions varied, depending upon the orientation of the structures. Individual microfibrils were ribbonlike, with the wide axis ranging from 12 to 130 Å. Most of these ribbonlike microfibrils in negatively stained specimens had pleats or longitudinal foldings (Fig. 4*c*; a pleat is indicated with an arrow). These microfibrils were labeled with CBH I-Au (Fig. 4*e*). When the microfibrils were stacked vertically upon clustering, the narrow axis dimension could be determined. This appeared to be uniform and narrow (11–13 Å) (Fig. 4*b*).

ED. ED studies provided additional evidence that the product was cellulose I. ED reflections from the crude enzyme reaction product gave only cellulose II reflections (not shown) (17). ED analysis of the P-100 product synthesized in 2:1 acetonitrile/buffer showed both abundant cellulose II and infrequent cellulose I reflections. ED analysis of the P-100 product produced in 5:1 acetonitrile/buffer showed only cellulose II reflections, as in the case of the crude enzyme product (not shown).

ED studies of the P-60 fraction product synthesized in 2:1 acetonitrile/buffer showed both cellulose II and cellulose I, but here, highly oriented cellulose I reflections were observed without cellulose II reflections appearing in the same area (Fig. 5*c*). Most frequently, however, the cellulose I ED pattern lacked a 6.0-Å reflection (as was also the case with the P-100 above), but occasionally the 6.0-Å reflection for cellulose I appeared (Fig. 5*c*). When the stage was tilted 30°, the 6.0-Å reflections became more apparent (not shown). Reflections of the synthetic product were similar to those of the *Acetobacter* control cellulose (Fig. 5*a*), although the latter often seemed somewhat more resistant to electron beam irradiation.

DISCUSSION

In the identification of the synthetic product, the following criteria were considered: (*i*) enzymatic hydrolysis with Celluclast yielded glucose, cellobiose, and cellotriose; (*ii*) both extended microfibrils and irregular rodlets were labeled with CBH I-Au; and (*iii*) ED analysis showed reflections characteristic for cellulose I and II. Additional criteria were given in refs. 13–17.

Synthetic cellulose assembly should be viewed in the context of cellulose I biosynthesis *in vivo*. Since the discovery of organized presumptive cellulose-synthesizing complexes (terminal complexes or TCs) (24), the literature has recorded many examples of cellulose synthesized by TCs in bacteria (25), algae (24, 26–28), and vascular plants (29, 30). Thus, the synthesis of cellulose I in nature appears to require an organized array of catalytic subunits to assemble parallel glucan chains unidirectionally (29). Our production of synthetic cellulose I indicates that conditions may have been

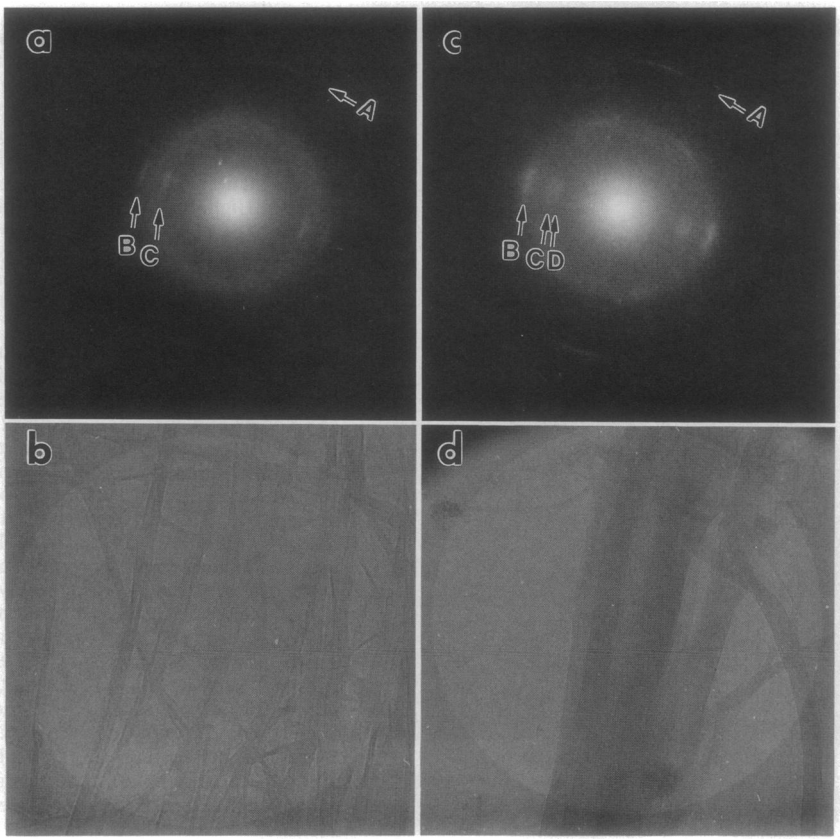

FIG. 5. EDs and corresponding areas of the cellulose I control from *Acetobacter* and the product synthesized in 2:1 acetonitrile/buffer with the P-60 fraction. (*a*) ED of the cellulose I control from *Acetobacter*. Note highly oriented cellulose I ED reflections (meridional-A = 2.6 Å, equatorial-B = 4.0 Å; C = 5.4 Å). This cellulose was more resistant to electron beam damage in comparison with the synthetic product. (*b*) Defocus contrast image of corresponding area of *a*. The diameter of the area irradiated for diffraction (bright circle in *b*) is 0.8 μm. The pattern is properly aligned relative to the image. (*c*) ED of the P-60 product. Note highly oriented cellulose I ED reflections (meridional-A = 2.6 Å, equatorial-B = 4.0 Å; C = 5.4 Å; D = 6.0 Å) appearing without cellulose II reflections. (*d*) Defocus contrast image of corresponding area of *c*. The diameter of the area irradiated for diffraction (bright circle in *d*) is 0.8 μm. The pattern is properly aligned relative to the image.

fortuitously found in which this allomorph is assembled. We hypothesize that a microscopic phase separation in this system with sufficient purification of the enzyme may have simulated in some way the organized structure of the natural cellulose I-synthesizing TC.

Because the substrate has a hydrophobic group (fluoride) attached to a hydrophilic region (the cellobiose moiety), the complex interactions of the substrate in the acetonitrile/aqueous buffer are thought to lead to an accumulation of an oriented substrate at the micelle surface interface. Such conditions of oriented substrates interacting with concentrated catalysts may favor the reaction to form extended β-1,4 glucan chains, particularly if the micelles are conducive in bringing the substrate efficiently in contact with the catalytic sites. With an acetonitrile/buffer ratio of 2:1, the entire solution became turbid upon the addition of substrate. This indicates that microscopic micelles may have formed. Under these conditions, the P-100 fraction produced an enhanced yield of cellulose I, while the crude enzyme produced only cellulose II under both 5:1 and 2:1 conditions. It should be noted that with an acetonitrile/buffer ratio of 5:1, the reaction mixture immediately underwent a macroscopic phase separation resulting in the formation of separate and distinct layers in the reaction tube. Such conditions do not favor synthetic cellulose I formation even if the partially purified P-100 or P-60 fractions are used. Clearly, more work on phase behavior and its effects on cellulose allomorph assembly is required.

These observations lead us to conclude that a crystalline synthetic cellulose I allomorph has been produced due to: (*i*) partial purification and enrichment of the enzyme(s) responsible for the polymerization; and (*ii*) micelle formation through optimization of the organic solvent/aqueous buffer ratio. The microscopic micellar aggregates appear to be functioning as organized catalytic subunits to assemble synthetic cellulose I although they are not as perfectly organized as native TCs. This is evidenced by the morphologies of the product, occasional lack of the 6.0-Å ED reflection, and greater susceptibility of the product to electron beam damage. The very thin, 11- to 13-Å, dimensions of the microfibril indicate that it may be only 1 or 2 glucan chains thick. Pleats or longitudinal foldings of the product (Fig. 4*c*) also indicate that the microfibrils are thin, resulting in flexibility of the microfibrils parallel to the longitudinal axis. One possible explanation for the occasional lack of the 6.0-Å ED reflection may be that microfibrils are thin and that there is a preferential orientation of such microfibrils on the grid, as known from the microfibrils of *Vaucheria* (28). Of obvious interest is the question of cellulose Iα/Iβ content of synthetic cellulose. The ED pattern for synthetic cellulose (Fig. 5*c*) includes a second-layer meridional spot characteristic of the monoclinic crystal form (Iβ) (31).

Further studies are necessary to understand exactly how crystallization can occur in the abiogenic production of synthetic cellulose I. The present findings may provide clues for further understanding of *in vivo* biosynthetic mechanisms leading to native cellulose I formation. In the future, it might be possible to engineer, on an industrial scale, novel synthetic cellulose materials with controlled crystal structure, molecular weight, and morphology.

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