



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12P 19/18, 19/04, C12N 9/10, 11/12, 11/06	A2	(11) International Publication Number: WO 95/04155 (43) International Publication Date: 9 February 1995 (09.02.95)
(21) International Application Number: PCT/US94/08731 (22) International Filing Date: 2 August 1994 (02.08.94) (30) Priority Data: 08/100,868 2 August 1993 (02.08.93) US (71)(72) Applicants and Inventors: BROWN, R., Malcolm, Jr. [US/US]; 305 Skyline Drive, Austin, TX 78746 (US). KOBAYASHI, Shiro [JP/JP]; 1-8-21, Yagiyama, Minami, Taihaku-ku, Sendai, Miyagi (JP). KUDLICKA, Krystyna [PL/US]; 1401 St. Edwards Drive #149, Austin, TX 78704 (US). KUGA, Shigenori [JP/JP]; 3-4-7, Mizukino, Moriya-Machi, Ibaraki-Ken 302-01 (JP). LEE, Jong [US/US]; 3455 Lake Austin Boulevard #D, Austin, TX 78703 (US). LI, Likun [-/US]; Apartment #1, 1908 Carroll Street, Houston, TX 77030 (US). OKUDA, Kazuo [JP/JP]; 10-41, Ozu-Cho #424, Kochi 780 (JP). SHODA, Shin-Ichiro [JP/JP]; 4-7-25-601, Chuo, Aoba-Ku, Sendai, Miyagi (JP). (74) Agent: HODGINS, Daniel, S.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: A METHOD FOR SYNTHESIS OF POLYSACCHARIDES (57) Abstract Methods of synthesizing cellulose I <i>in vitro</i> are disclosed. One method comprises contacting an activated saccharide substrate with an endoglucanase in an appropriate organic solvent/buffer ratio. The invention also encompasses a purified endoglucanase. A second method comprises contacting a nucleotide sugar with a purified glycosyl transferase in an appropriate buffer medium to insure polymerization and crystallization of parallel chains from the enzyme/micelle complex to form cellulose I.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

DESCRIPTION

A METHOD FOR SYNTHESIS OF POLYSACCHARIDES

5

BACKGROUND OF THE INVENTION

Field of the Invention

10

The present invention relates generally to the *in vitro* production of polysaccharides, and more particularly to the synthetic production of cellulose I.

15

Description of the Related Art

Cellulose is the most abundant macromolecule on earth (Brown, 1985). It serves a major structural role in the cell wall of plants, some algae, and certain fungi and is the primary component of economically important products such as wood, cotton, and paper. Because of its tremendous abundance and its physiological and economic importance, many attempts at *in vitro* cellulose synthesis have been made with cell-free systems from various sources during the past three decades (Delmer, 1987; Brown, 1989b; Read and Delmer, 1991). The greatest progress has been made using *Acetobacter xylinum* as an experimental model (Ross et al., 1987; Brown, 1989a; Lin and Brown, 1989; Lin et al., 1990; Saxena et al., 1990; Wong et al., 1990, Lin Mayer et al., 1991; Saxena et al., 1991). However, until very recently (Okuda et al., 1993) no preparation from higher plant cells has ever been shown to be capable of synthesizing true microfibrillar cellulose or even appreciable quantities of β -1,4-glucan (Read and Delmer, 1991).

35

- 2 -

Native cellulose predominately occurs as a fibrillar crystalline allomorph designated cellulose I. Cellulose I consists of a crystalline array of glucan chains, all of which are oriented parallel to one another (Preston, 1974). Cellulose I can be transformed to the more stable allomorph, cellulose II, via chemical treatments that alter the crystal structure (Rånby, 1952; Sarko, 1976). This change is strictly irreversible, and no process has been known that gives cellulose I either by recrystallization or by polymerization *in vitro*. Cellulose II recently has been found to have a folded chain, antiparallel conformation (Kuga, et al., 1993). It has been argued that cellulose I is thermodynamically metastable and, therefore, that living organisms control crystallization in a manner not duplicated under acellular conditions (Rånby, 1952; Sarko, 1976; Blackwell, 1982; Sawyer and George, 1982).

The cellulose I polymorph is characteristic of almost all native celluloses synthesized by living systems to date. Cellulose II is only rarely synthesized under natural conditions. *Halicystis* (an alga) (Rånby, 1952; Sisson, 1938; Roberts, 1991), *Sarcina* (a bacterium) (Roberts, 1991; Roelfsen, 1959) and a mutant strain of *Acetobacter xylinum* (a bacterium) that produces folded-chain cellulose II (Kuga et al., 1993) are the few known organisms producing native cellulose II. Cellulose I is a metastable polymorph; it exists in a more thermodynamically unstable form than that of the cellulose II polymorph. In this form, the glucan chains are antiparallel to one another, and there is one additional hydrogen bond linking each glucose residue. Cellulose II is formed when cellulose I is dissolved and reprecipitated.

35

Electron diffraction analysis has shown that native celluloses are synthesized in a crystalline form. The

- 3 -

electron diffraction patterns are characteristic for each type of cellulose polymorph or allomorph (both words are used interchangeably in this disclosure). That is, cellulose I is a polymorph that has characteristic
5 electron diffraction patterns different from those of cellulose II. At the ultrastructural level, the crystalline form of cellulose can be visualized by a variety of electron microscope techniques, among them negative staining. This technique is useful in that it
10 can identify small aggregates of glucan chains down to a mean diameter of approximately 12-15 Å.

In vitro synthesis of cellulose has been one of the most difficult, yet important and challenging topics of
15 the early stages of macromolecular science. Much effort has been devoted to regio- and stereoselective preparations of cellulose, i.e., construction of stereoregular polysaccharides having β -1,4 glycosidic linkages. The chemical approaches so far attempted,
20 however, have failed to solve the problem in spite of the remarkable development of modern synthetic methods (Klar, 1963; Husemann, et al., 1966; Hirano, 1973; Schuerch, 1972; Uryu, et al., 1985).

25 In one attempt, the condensation of 2,3,6-glucose tricarbanilate with phosphorus pentoxide in a mixture of chloroform/dimethyl sulfoxide gave branched products, however, the molecular weight of the resulting polysaccharide after removing the protecting group was
30 low (Husemann, 1966). In a Lewis acid catalyzed reaction, 1,4-Anhydro-2,3,6-tri-O-benzyl- α -D-glucose has been polymerized giving rise to polymers having mixed structures of β -1,4 (cellulose-type) and α -1,4 (amylose-type) linkages. Uryu and coworkers investigated the
35 possibility of synthesizing polysaccharides having β -1,4 linkages (cellulose) by the cationic ring-opening polymerization of 1,4-anhydroglucose derivatives.

- 4 -

However, stereoregular polysaccharides having the desired structure were not obtained due to the lack of regioselective ring opening (Uryu, et al., 1985).

5 The *in vitro* synthesis of cellulose utilizing biosynthetic pathways has been reported exploiting *Acetobacter xylinum* (Colvin, 1959) or *Phaseolus aureus* extracts (Elbein, 1964) with a nucleoside diphosphate sugar (ADP, CDP, or GDP-glucose) as the substrate. An
10 alternate method for synthesizing cellulose *in vitro* utilizes cotton fibers. The plasma membrane from cotton fibers is extracted with 50mM Tris-HCl buffer (pH 7.5), containing 5mM EGTA, 20% PEG and combination of protease inhibitors. The resulting plasma membrane fraction is
15 solubilized in 10mM Tris-HCl (pH 7.4), containing 1% digitonin, 1mM EDTA, 1mM EGTA 10% glycerol. The *in vitro* product is synthesized under conditions favoring β -1,4-glucan synthesis. The resulting *in vitro* product is characterized by solubility, enzymatic digestion, degree
20 of polymerization (DP) determination, methylation linkage analysis, x-ray diffraction and transmission electron microscopy (TEM) coupled with autoradiography and CBHI-gold labeling. Although the product synthesized by this method is cellulose, it is cellulose II. Thus, until the
25 present invention described herein, there has been no method known to synthesize the cellulose I polymorph *in vitro* from higher plant extracts.

Two of the present inventors have reported a
30 different approach for the synthesis of cellulose, i.e., by a transglycosylation reaction (condensation polymerization) catalyzed by cellulase, an extracellular cellulose hydrolytic enzyme, with β -D-cellobiosyl fluoride as a glycosyl donor (Kobayashi, et al., 1991).
35 β -D-cellobiosyl fluoride was chosen as the activated glycosyl donor because a disaccharide is the smallest molecule recognized by the enzyme. The configuration of

- 5 -

the C1 fluorine atom of the starting material was designed to form a reactive intermediate leading to a β -1,4 product (cellulose) via a "double displacement mechanism" (Lai, 1974) at the active site of the enzyme.

5 The major advantage of this approach was that it did not involve protection and deprotection of the hydroxyl groups. Several organic solvents/buffers were investigated with an acetonitrile/acetate buffer being the most preferred, and a 5:1 ratio was found to be the
10 best ratio for the production of cellulose. Although cellulose was produced by this method, the product was cellulose II. So, unfortunately, there was still no *in vitro* method to synthesize the more useful cellulose I polymorph. In European Patent Specification application
15 No. 87307891.9 (Brown et al., 1987) *in vitro* cellulose synthesis was reported. However such cellulose was not synthesized but created only after washing a synthesized product with strong base or other solubilizing agents to remove contaminants.

20

The present invention overcomes these and other drawbacks inherent in the prior art by providing a method of producing cellulose I either synthetically by a non-biological reaction, or by using a biological catalyst *in*
25 *vitro*.

Abbreviations:

AN reagent: acetic/nitric acid reagent
ANIP: acetic/nitric acid reagent insoluble product
30 CB: cellobiose
CBH I: cellobiohydrolase I
c-3':5'-GMP: cyclic 3':5'-GMP
ED: electron diffraction
EIP: ethanol insoluble product
35 ES1: protein fraction released into supernatant
after entrapment
F1,4: condition favoring synthesis of β -1,4-glucan

- 6 -

Mops: 3-[N-morpholino]-propanesulfonic acid

PM: plasma membrane

SE1: enzyme solubilized in the first solubilization
step by using 0.05% digitonin

5 SE2: enzyme solubilized in the second solubilization
step by using 1% digitonin

TEM: transmission electron microscopy

10 SUMMARY OF THE INVENTION

One method of the present invention is a method for
producing cellulose I, the method comprising contacting a
cellobiose bearing a displaceable substituent at the C1
15 position with a partially purified cellulolytic enzyme
contained in micelles. Cellobiose is a glucose
disaccharide in which the two glucose molecules are bound
in a β -1,4 configuration. The displaceable substituent
is an active group or a leaving group in a double
20 displacement reaction in which the activated glucan dimer
is added to the growing cellulose polymer. The
displaceable substituent or active group may be a halogen
atom or a methyl, allyl, trifluoroethyl or acetyl group,
or any other group that is displaceable in the double
25 displacement reaction. The most preferred group is a
halogen and particularly preferred is fluorine. The most
preferred substrate for the synthesis reaction is β -D-
cellobiosyl fluoride.

30 Other activated saccharide moieties may be used as
substrates in the present invention. Such substrates
include, but are not limited to a monosaccharide,
disaccharide, oligosaccharide, polysaccharide or
combinations thereof. For example, a preferred substrate
35 is a disaccharide. However, a monosaccharide or a
monosaccharide joined by a glycosidic bond to another
saccharide moiety having substrate activity is usable.

- 7 -

Other possible substrates include glycoproteins, glycolipids and oligosaccharides. The particularly preferred activating group on the saccharide substrate is an agent, such as a fluoride atom chemically bonded to the reducing end of the saccharide (Brauns, 1929). Other preferred activating groups are generally halogens, halogen derivatives such as iodide, methyl, allyl, trifluoroethyl and acetyl. The activating group is also in the desired anomeric configuration to yield the desired anomeric glycosidic bond.

The enzyme to be used in the method of the present invention may be glycosyltransferase, an endoglucanase, a hydrolase or a cellulolytic enzyme and the hydrolase may be used in combination with one or more glycosyltransferases. For synthetic cellulose I assembly, preferably, a cellulolytic enzyme is allowed to react with an activated cellobiosyl substrate under conditions favoring the reverse reaction, resulting in the formation of new glycosidic bonds rather than their hydrolysis.

A preferred enzyme is an endoglucanase with transglycosylation activity of the retaining type. A retaining type enzyme catalyzes glycoside hydrolysis with retention of the stereoisomeric configuration about the anomeric carbon. For example, an active group in the β position would be bonded in a β linkage in the reaction. A preferred enzyme may be a retaining type endoglucanase selected from the group consisting of a 38 kD endoglucanase isolated from *Trichoderma viride*, Endoglucanase I from *Trichoderma reesei*, Endoglucanase II from *Trichoderma reesei* and Endoglucanase from *Chrysosporium lignorum*, however it is understood that other endoglucanases with the necessary activity would also be effective in the practice of the invention and would be encompassed within the spirit and scope of the

- 8 -

claimed invention. A more preferred enzyme is a partially purified endoglucanase from Onazuka R-10 Cellulase. Cellulase Onazuka R-10 (Kinki Yakult, Osaka, Japan), "Onazuka Cellulase" is a commercial preparation
5 of crude cellulases from *Trichoderma viride* containing several dozen polypeptides.

The preferred enzyme can then be defined as a cellulolytic enzyme that is a fraction of *Trichoderma*
10 *viride* cellulase prepared by a procedure comprising any of the steps of salting out, gel permeation chromatography, preparative isoelectric focusing and ion exchange chromatography. The enzyme may also be defined as exhibiting a specific activity of cellulose synthesis
15 of from 0.1491 to 3.8814 Units/mg in a 5:1 ratio of acetonitrile to sodium acetate buffer at room temperature. A unit is defined as μM glu/min/mg protein. It is important to note that under these conditions the cellulose synthesis product may include the cellulose II
20 allomorph, but the activity is total cellulose synthesis. The most preferred enzyme may also be defined as a single band of 38 kD when analyzed on an SDS polyacrylamide gel.

The preferred micelles in this method comprise
25 substrate dissolved in an aqueous buffer and an organic solvent. The organic solvent may be acetonitrile, 1,4-dioxane, nitromethane, or isooctane, with the most preferred organic solvent being acetonitrile. It is also understood that other organic solvents that are capable
30 of forming micelles in an aqueous buffer to contain the enzymes and substrate would also be useful in the practice of the present invention. The micelles are preferably formed in an organic solvent/aqueous buffer with a ratio between 5:1 and 2:1. Most preferably the
35 micelles are formed at a 5:1 acetonitrile:aqueous buffer ratio and even more preferably in a 2:1 acetonitrile:aqueous buffer ratio.

- 9 -

The method of cellulose synthesis using endoglucanase and cellobiosyl substrate may also include the further step of terminating the polymerization reaction with the addition of cellobiose. The

5 unsubstituted disaccharide, cellobiose will cause the polymerization to stop as there will be no activating group, or displaceable substituent available to react with the substrate. Therefore, the length of the polymers produced by the method disclosed herein may be

10 controlled by the addition of certain proportions of cellobiose in the substrate mixture.

An important aspect of the present invention is a purified cellulolytic enzyme preparation capable of

15 synthesizing cellulose I from an activated disaccharide substrate. This is preferably a purified fraction of cellulase from *Trichoderma viride*, purified by the steps of ammonium sulfate precipitation at 35%-45%, centrifugation at 50,000 x g, desalting over a Bio-Gel P-

20 10 column, 2 preparative isoelectric focusing fractionations, Bio-Gel P-60 chromatography, DEAE-650M chromatography and chromatography over SEPHADEX G-50.

Another embodiment of the present invention is a

25 method of cellulose I synthesis comprising contacting an isolated terminal complex with UDP-glucose under conditions effective to produce cellulose I, wherein the terminal complex comprises cellulose synthase and retaining its cellulose I synthesis activity. Terminal

30 complexes or TCs have been discovered in freeze fractured membranes and comprise organized cellulose synthesizing complexes. Terminal complexes are known to occur in bacteria, algae and vascular plants. The most preferred terminal complex is isolated from a cotton plant cell.

35

The present invention is also a method of synthesizing cellulose I, the method comprising the steps

- 10 -

of obtaining a plasma membrane enriched fraction from cotton fibers, mixing the plasma membrane enriched fraction with an equal volume of solubilization buffer comprising 0.1% digitonin to obtain a mixture, layering
5 the mixture on a 30% (w/v) glycerol cushion, centrifuging and collecting the supernatant to form a solubilized enzyme fraction. The enzyme fraction is then contacted with UDP-glucose in a reaction mixture.

10 In a preferred embodiment, the plasma membrane enriched fraction may be obtained by grinding cotton fibers to a fine powder in liquid nitrogen and extracting said powder with cold extraction buffer comprising 50 mM
15 3-[N-morpholino]-propanesulfonic acid (Mops), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a combination of protease inhibitors (0.5 mM PMSF, 10 μ M leupeptin, 0.1 mM
N-a-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) to
20 obtain membranes, and resuspending the membranes in resuspension buffer comprising 50 mM Mops, pH 7.5 and 0.25 M sucrose. In this embodiment, the solubilization buffer may comprise 50 mM Mops, pH 7.5, and 0.25 M
sucrose, centrifugation may be at 100,000g for 1 hour at 4°C, and the reaction mix may comprise 10 mM
25 bis-trispropane-Hepes buffer (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 1mM $CaCl_2$, 100 μ M cyclic 3':5'-GMP), 0.05% digitonin, 3 mM NaN_3 , and 1 mM UDP-Glc.

Solubilizing the membranes as described is thought
30 to keep the structure of the terminal complexes intact so that cellulose I synthesis activity is retained. Therefore, the present invention is also a method of solubilizing a terminal complex so that the terminal complex retains its cellulose I synthesis activity. This
35 method comprises the steps of grinding cotton fibers to a fine powder in liquid nitrogen and extracting said powder with cold extraction buffer comprising 50 mM

- 11 -

3-[N-morpholino]-propanesulfonic acid (Mops), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a combination of protease inhibitors (0.5 mM PMSF, 10 μ M leupeptin, 0.1 mM N-a-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) to obtain membranes; and resuspending said membranes in resuspension buffer comprising 50 mM Mops, pH 7.5 and 0.25 M sucrose.

Another aspect of the invention is a composition comprising cellulose I obtained by the methods disclosed herein. In particular, a form of cellulose I consisting essentially of β -glucan sheets comprising no more than two layers of glucan chains in thickness is an embodiment of the present invention. The crystalline cellulose I allomorph consists of two glucan chain sheets in thickness but consists of 5 or more glucan chains within said glucan chain sheets. The novel, thin form of cellulose I is thought to be due to the organization of the enzymes in the micelle complex, in that the organization controls glucan sheet stacking to form the crystalline allomorph.

A further aspect of the invention is a micelle comprising acetonitrile, aqueous buffer, cellobiose having a displaceable substituent and an endoglucanase enzyme. The preferred aqueous buffer is sodium acetate buffer. The micelle is preferably obtainable by agitating a mixture of acetonitrile and sodium acetate buffer at a ratio of 5:1, for example by vortexing for 1-3 seconds, and is more preferably obtainable by agitating a mixture of acetonitrile and sodium acetate buffer at a ratio of 2:1.

The P-100 and P-60 enzyme fractions described herein are partially purified preparations from the crude Onazuka R-10 Cellulase. Partial purification of the

- 12 -

cellulase has been achieved through a combination of gel permeation chromatography, preparative isoelectric focusing, salting out, and preparative electrophoresis. These steps have led to the partial purification and
5 identification of a single 38 kDa polypeptide which appears to be responsible for synthetic cellulose production.

The "P-100" Fraction

10

An initial attempt to identify and isolate the factor/s from the *Trichoderma viride* cellulase system that are responsible for synthesizing cellulose resulted in a partially purified preparation denoted as "P-100"
15 based on the use of a P-100 gel filtration column as the ultimate step in the purification scheme. The P-100 fraction was found to contain about 8 polypeptide bands representing major polypeptides, and minor polypeptides appearing as smears in the region between about 66 kDa
20 and 35 kDa on the sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Cellulase activity was determined by quantifying water-insoluble synthetic cellulose yields of each collected fraction throughout the purification procedure. The P-100 fraction exhibited a 10 fold
25 increase in synthetic activity over the crude enzyme. Protein concentrations were determined using a modification of the Lowry procedure (Markwell, 1978).

The "P-60" Fraction

30

It was not possible to identify which of the 8 or more proteins in the P-100 fraction was responsible for synthesizing cellulose; therefore, an ammonium sulfate fractionation was added to the purification regime.
35 Furthermore, the P-100 column was changed to a P-60 column in order to achieve better separation. The most active fraction collected from the P-60 gel purification

- 13 -

also appeared to contain about 8 polypeptides when analyzed on an SDS-PAGE gel, however, there was one major polypeptide band at 38 kDa. This 38 kDa polypeptide comprised about 80% of the total protein of the most active fraction, while in comparison, the P-100 fraction did not contain any single polypeptide band that was more concentrated than the others. There was a 31-fold increase in synthetic activity from the crude enzyme to the most active P-60 fraction. A cellulytic activity assay (Wood, 1988) using carboxymethylcellulose and Avicel as substrates indicated that the P-60 fraction contained endoglucanase activity, but cellobiohydrolase activity was lacking.

There has also been a further purification of the P-60 enzyme fraction achieved by preparative electrophoresis. More than 90% of this newly purified fraction from the Prep Cell-preparative electrophoresis was the 38 kDa polypeptide and there were 2 or 3 very minor bands. This fraction also produced synthetic cellulose I. This is further evidence that the 38 kDa protein is the enzyme responsible for synthetic cellulose. This band is related to other endoglucanases by the criteria used in the literature to define endoglucanases. The criteria are based on the ability of the hydrolase to completely degrade carboxymethylcellulose, but with a very low activity toward crystalline cellulose. The endoglucanases have a random cleavage pattern, in contrast to the exoglucanases (e.g. cellobiohydrolase I) which systematically attacks from the non-reducing end to cleave out cellobiose.

Synthetic cellulose was also produced with cellulase preparations from other sources, *A. niger*, *P. tulipiterae*, and *P. chrysaspi*, for example. Preliminary results have also been obtained indicating that all retaining type endoglucanases are active in synthesizing

- 14 -

cellulose. The P-60 fraction that contains the 38 kDa polypeptide has the highest endoglucanase activity and also has the highest cellulose synthesis activity. But so far, endoglucanase I and II from *T. viride* and
5 endoglucanase from *P. chrysaspi* also have good synthetic activity (cellulose I).

It is an important aspect of the purification, that the exoglucanase activity be enzyme fraction and higher
10 specific activity is obtained when the inversion type of endoglucanase activity is removed. Table I demonstrates the various activities of the enzyme fractions produced in the purification of the most preferred 38 kD endoglucanase disclosed herein. Other endoglucanases
15 that are shown to be active in synthesizing cellulose by the inventors include e.g. endoglucanase I and endoglucanase II, both from *T. reesei*, and an endoglucanase from *P. chrysaspi*. Therefore, other endoglucanases do synthesize cellulose under the
20 conditions described herein, however none tested by the inventors exhibits activity as high as the 38 kDa polypeptide disclosed herein.

The concentration of the enzyme is also an important
25 aspect in the method of the present invention, as the packing, or orientation of the enzymes in the micelles appears to depend on the concentration of enzyme. The proper packing or orientation of the catalytic sites of the enzymes, then directs the proper crystallization of
30 cellulose I. Of course, it is understood that different solvent/buffer combinations or substrate concentration may form different sized micelles and that the important embodiment of the present invention is the formation of correctly oriented enzymes in a micelle for example, and
35 that any variation in enzyme concentration that is able to synthesize cellulose I is encompassed within the scope of the present invention.

- 15 -

It is recognized that each organic solvent/buffer combination will require a different ratio in order to achieve maximum efficiency of the reaction. These ratios may also be adjusted in order to achieve the desired products, for example different polymer lengths and different crystallization patterns may be achieved by adjusting the solvent/buffer ratios. A preferred solvent for the production of cellulose I is acetonitrile in a solvent/buffer ratio of from about 2:1 to about 5:1 and most preferably about 2:1. With only a reasonable amount of experimentation, for example with different solvents and/or different solvent ratios, one skilled in the art could follow cellulose I production and arrive at optimal synthetic conditions.

15

A certain embodiment of the present invention is that the highly polar substrate, cellobiosylfluoride can have a marked effect on the nature of micelle formation. It is understood that each different ratio will require different concentration of cellobiosylfluoride. For example, even under a 5:1 solvent ratio condition, by adjusting the concentration of substrate, macro-biphasic separation can be prevented.

25

Although preferred for use in certain embodiments, there is no general requirement that the cellulolytic enzyme always be provided in its most purified state. Indeed, it is contemplated that less substantially purified enzymes, which are nonetheless enriched in certain cellulolytic activity relative to the natural state, will have utility in certain embodiments. These include, for example, the production of cellooligosaccharides and alternate crystallization states of cellulose and other glucan polymers. Partially purified cellulase fractions for use in such embodiments may be obtained by subjecting a crude extract to one or a combination of the steps described above.

30
35

- 16 -

It will be appreciated that once the cellulolytic enzyme is purified, it can be sequenced and/or replicated by techniques well-known to those skilled in the art. For example, replication might be accomplished by

5 recombinant techniques involving the isolation of genetic material coding for the cellulolytic enzyme and the preparation of an immortal cell line capable of producing the cellulolytic enzyme. For example, the inventors have digested the 38 kD protein with V8 protease (EC 3.4.21.9)

10 from *Staphylococcus aureus* (Sigma), resulting in five polypeptides. These SDS-PAGE bands were transferred to ProBlott® and cut out for N-terminal amino acid sequencing. Two sequences were obtained, TTNNIDGAFAP, SEQ ID NO:1 and IGVQXXIQD, SEQ ID NO:2. These sequences

15 can be utilized to design oligonucleotide primers by methods well known in the art, including the production of degenerate primers to probe a cDNA or genetic library produced from *Trichoderma*. Through standard hybridization techniques, the gene encoding this protein

20 can be isolated without undue experimentation. The cellulolytic enzyme, once the gene is cloned, can be overexpressed in for example an *E. coli* strain and can be modified, for example by site directed mutagenesis. It is understood that the use of such modified enzymes is

25 within the scope of the present invention.

The molecular weight of the polymers synthesized by the method of the present invention might also be controlled by mechanical agitation of an immobilized

30 catalytic complex or by the addition of cellobiose. After determination of the rate of synthesis in each *in vitro* system, the mechanical agitation or periodic ultrasonic bursts could be programmed to reach the desired molecular weight. Alternatively, after routine

35 optimization, proportions of cellobiose to activated cellobiose can be adjusted to achieve polymers of certain preferred lengths.

- 17 -

It is also understood that by the use of alternate activated substrates, specific polysaccharides could be synthesized. Other saccharide substrates that could be used include, but are not limited to glucose, galactose, arabinose, xylose, rhamnose, etc. or oligosaccharides thereof as analogs of the preferred cellobiosyl fluoride. This method would include the use of other hydrolases, endoglucanases as well as glycosyltransferases such as xylosyltransferase, e.g., that are specific for the desired glycosidic bonds and could include one or more endoglucanases and/or one or more glycosyltransferases. The substrates mentioned above may also be used in an activated form and as such would be included within the scope of the present invention. A specific hydrolase may be used for a specific substrate, xylanase for xylan, mannosidase for mannans, galactosidase for galactose, etc.

A certain embodiment of the present invention is a new and novel composition of synthetic cellulose I which is obtainable by the method described in the present disclosure. This composition can be further defined as a crystalline isomorph of cellulose I wherein said cellulose I is comprised of β -glucan sheets, said β -glucan sheets being no more than two layers of glucan chains in thickness. All known naturally occurring cellulose I crystals are at least 3 chains thick resulting in electron diffraction patterns that are distinguishable from the data obtained with the cellulose I synthesized with the method of the present invention. Morphological studies of the synthetic cellulose indicate crystal dimensions of $12\text{\AA} \times 25\text{\AA}$, a dimension never found in nature. It is also contemplated that cellulose I fibrils with a larger degree of polymerization than found in nature are possible in light of the present disclosure. DP. of 23,000 may be the upper limit from native cellulose I (Kuga et al., 1989). It is understood

- 18 -

that these long polymers would also be encompassed by the present invention.

Cellulose, the main component of wood, paper,
5 cotton, rayon and other yarns is one of the world's most important polymers. The ability to synthesize synthetic cellulose is thus an extremely important invention which has been sought by researches over many years. The ability to synthesize new forms of cellulose also creates
10 the possibility of the creation of stronger, denser, or even lighter fibers than those that occur in nature. Therefore, this invention answers a long felt and important need in many fields of materials research and utility.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts cellulose synthesized by the
20 cellulase P-100 fraction, with cellobiosyl fluoride as substrate, in 5:1 acetonitrile/acetate buffer, showing mostly non-fibrillar cellulose II, with occasional bundles of long fibrils. Both forms are strongly labeled with colloidal gold-cellobiohydrolase I, a specific
25 marker of cellulose.

Figure 1B shows cellulose produced as in Figure 1A, in an acetonitrile/buffer ratio of 2:1. Under these conditions, the production of fibrillar material was
30 significantly enhanced.

Figure 1C depicts cellulose II and cellulose I synthesized as in Figure 1A in a 5:1 organic solvent/buffer ratio. (mag=122,00X).

35

- 19 -

Figure 1D shows cellulose I at high magnification (=92,000X) which was produced as in Figure 1A in a 2:1 organic solvent/buffer ratio.

5 Figure 2A is a more highly magnified (mag=76,000X) electron micrograph of the fibrillar cellulose produced in a 2:1 acetonitrile/acetate buffer ratio with the P-100 enzyme fraction.

10 Figure 2B is the electron diffraction pattern of the product described in Figure 2A.

15 Figure 2C is the electron micrograph of the cellulose I product as in Figure 2A, produced with the P-60 enzyme fraction under 2:1 acetonitrile/aqueous buffer ratio.

20 Fig 2D is the electron diffraction pattern of cellulose I produced with the P-60 enzyme fraction in a 2:1 acetonitrile/aqueous buffer ratio. The 6.0 Å reflection is absent from this diffraction pattern, which is indicative of a two glucan chain sheet layer.

25 Figure 3A is a schematic diagram of conditions favoring cellulose II synthesis (with the 5:1 acetonitrile/aqueous buffer ratio). Note that with this solvent/buffer ratio and 5 mg of substrate, the reaction mixture undergoes a macroscopic phase separation.

30 Figure 3B is a schematic diagram of conditions favoring cellulose I (with the 2:1 acetonitrile/aqueous buffer ratio). Note that with this ratio and 5 mg of substrate, the reaction mixture does not undergo a macroscopic phase separation, but rather forms inverted
35 micelles.

- 20 -

Figure 4 is a flow chart for producing the homogenous 38 kDa enzyme fraction.

Figure 5 is an SDS-PAGE of the purified 38 kD protein (Lane 2). Lanes 1 and 3 are size standards.

Figure 6 is a graph of the protein concentration and activity profile of the product of the second isoelectric focusing after fractionation over a P-60 column.

10

Figure 7A is an SDS-PAGE showing the protein profiles of different fractions stained with Coomassie Blue. Lane 1 - molecular weight standards, lane 2 - plasma membranes, lane 3 - SE1, lane 4 - SE2, (lanes 2 - 4 - 20 μ g).

15

Figure 7B is a gel as in Figure 7A stained with silver. Lane 1 - molecular weight standards, lane 2 - 1ES1=protein released from SE1 fraction after entrapment and containing a prominent 37 kD band; lane 3 - 2ES1=protein released after entrapment from SE2 fraction. The 37 kD band is attenuated. In both lanes 2 and 3, the 52 kD polypeptide is weakly stained. This may be due to the callose synthase remaining entrapped in the pellet fraction of the entrapped protein/oligosaccharide complex.

20

25

Figure 8A is the negatively stained *in vitro* product synthesized by the SE1 enzyme fraction, treated with protease, and labeled with CBHI-gold particles. This view is a low magnification view showing long fibrils in the form of linear aggregates, labeled with CBH I-gold particles embedded in β -1,3-glucan (dark mass of material), which is not labeled with CBH I-gold complex.

30

35

- 21 -

Figure 8B is a higher magnification view of Figure 8A. The structures of the β -1,3-glucans and β -1,4-glucans are clearly distinguishable. x72,000, (bar 0.2 μ).

5

Figure 8C is a high magnification view of the fibrils arranged into a large bundle, spanning the background of β -1,3-glucan. x164,000, (bar 0.1 μ).

10

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

An important embodiment of the present invention is the *in vitro* synthesis of cellulose I by several different methods and using various concentrations of different catalysts and different substrates. This list does not limit the scope of the invention, nor does it imply that these are the only substrates, catalysts or physical methods possible to assemble cellulose I. It is important however, to select an appropriate catalyst, suitably purified, and an appropriate substrate. Then it is important to make certain that the glucan chains are polymerized in close proximity to one another. Under these conditions, the glucan chains will spontaneously and irreversibly form parallel glucan chain aggregates which can further associate with other glucan chain aggregates to form larger parallel glucan chain clusters having the crystallinity typical of the cellulose I allomorph. The parallel chain orientation insures higher molecular weight polymers and concomitantly longer fibrils.

An embodiment of the present invention is the synthesis of cellulose I via an enzymatic polymerization of cellobiosyl fluoride, catalyzed by an at least partially purified cellulase preparation in an optimized organic solvent/aqueous buffer system and concentration

35

- 22 -

of substrate. This production of microfibrillar cellulose I in this system appears to be the result of a micellar aggregation of the synthesis-active components, a condition that promotes fasciation of the glucan chains with the same polarities and extended chain conformations.

In vitro cellulose synthesis by cellulose synthetase from the natural monomer, uridine diphosphate glucose, always has resulted in the formation of cellulose II (Lin et al., 1985; Bureau and Brown, 1984; Okuda et al., 1993). Until now, the synthesis of cellulose by the reaction involving a cellobiosyl fluoride substrate, crude cellulase, and an organic solvent system also have led to the formation only of cellulose II in the form of irregular rodlets (Kobayashi et al., 1991). Cellulose II is nonfibrillar, while all known forms of cellulose I have a fibrillar morphology.

The unique characteristics of cellulose I and its predominance in nature relate to the fact that an extended chain conformation confers mechanical strength as well as flexibility through fibrillar forms. With this arrangement, cellulose molecules can aggregate and crystallize into submicroscopic reinforcing rods known as microfibrils. The arrangement and orientation of cellulose microfibrils within the cell wall control the growth expansion axis and give the plant tissue the strength necessary for survival in turbulent environments.

Cellulose I is one of nature's most orderly biopolymers. Thus, it is of great interest to duplicate this perfection in non-living systems. In the method of chemical synthesis cited in Kobayashi et al. 1991, cellobiosyl fluoride is polymerized through catalysis by Onazuka R-10 Cellulase in an acetonitrile/buffer ratio of

- 23 -

5:1. The reverse of the normal hydrolysis reaction is attained by the combination of an environment of reduced water activity and the fluorinated monomer which has a strong inclination to polymerize. The Onazuka Cellulase is a commercial preparation of crude cellulases from *Trichoderma viride* containing at least several dozen polypeptides. In an attempt to isolate the enzyme/s responsible for the reaction, a partially purified preparation (denoted here as the P-100 fraction) was obtained. This partially purified fraction contains 8 major polypeptide bands by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In a further purification, a fraction denoted as P-60 was obtained which contains a major 39kDa protein with several minor proteins when visualized on SDS-PAGE.

When testing the partially purified P-100 fraction for cellulose synthesizing activity, it was found that the product contained fibrillar materials dispersed randomly among rodlets. These fibrils were similar to those of cellulose I formed *in vivo*, and could be labeled with a specific cellulose probe, colloidal gold-cellobiohydrolase (Fig. 1A).

To enhance the production of the fibrillar material, various conditions were studied, especially the acetonitrile/buffer ratio. An enhanced cellulose synthesis was attained by an optimal ratio of 2:1 acetonitrile/buffer. Under these conditions, large aggregates of fibrillar material were synthesized (Figure 1B). This permitted an electron diffraction analysis of the fibrils, which clearly indicated a cellulose I pattern combined with a cellulose II pattern (Fig. 2A) from the rodlets in the surrounding area (Fig. 2B).

To eliminate the possibility that the observed fibrils might be contaminants, various controls were

- 24 -

performed, including one control which lacked substrate. Fibrils or rodlets were never found during the incubations without substrate, indicating that the observed fibrils are the enzymatic reaction products.

5

A time-course of the reaction was visualized with electron microscopy. The P-100 enzyme fraction, when dissolved in acetate buffer, yielded particles of about 4 nm in diameter, which are believed to be individual enzyme molecules with some larger aggregates of enzyme molecules. When the P-100 fraction was introduced into the acetonitrile/buffer (2:1), distinct micellar aggregates of proteins were observed. Thirty seconds after addition of cellobiosyl fluoride to this mixture, short disorganized fibrils were seen emanating from a depression within an aggregated complex. At 3 minutes incubation, the extended fibrils became elongated and more organized. Continued incubation up to 20 minutes resulted in larger and longer fibrillar aggregates. Because of excessive aggregation, the product needed intense ultrasonic treatment to produce fibrils thin enough for electron microscopic observations (Fig. 1B).

During the past 16 years since the discovery in freeze fractured membranes of presumptive organized cellulose synthesizing complexes known as terminal complexes or TCs (Brown & Montezinos, 1976) the literature is replete with examples of organized TCs from bacteria (Brown & Montezinos, 1976) algae (Brown & Montezinos, 1976), (Itoh & Brown, 1984), (Brown, 1985) and vascular plants (Mueller & Brown, 1980). Thus, the synthesis of cellulose I in nature appears to require an organized array of catalytic subunits in order to assemble parallel glucan chains unidirectionally. As a result of cellulose synthesis, these TCs move in the plane of the fluid membrane (in the case of plant cells), or push forward the cellulose microfibrils into the

- 25 -

environment (in the case of extracellular secretion by bacteria).

Synthesis of cellulose I in the present system
5 indicates that the conditions of the present invention
have reproduced some of the natural conditions under
which this allomorph is produced. The microscopic phase
separation in this system, i.e., the micellar aggregation
of enzyme molecules appears to have mimicked the
10 organized structure of the natural cellulose I
synthesizing system.

Because of the poor solubility of the enzyme and the
substrate in acetonitrile/buffer, the reaction mixture
15 undergoes a complex phase separation at the initial
stage. With an acetonitrile/buffer ratio of 5:1 and 5 mg
of substrate, the reaction mixture immediately undergoes
a macroscopic phase separation resulting in the formation
of a single, separated layer (Fig 3A), with most of the
20 reaction product formed at the interface between the
aqueous and hydrophobic phases. The product under these
conditions is mostly cellulose II rodlets.

However, with an acetonitrile/buffer ratio of 2:1
25 and 5 mg of substrate, no distinct phase boundary is
formed; instead, the entire solution becomes turbid upon
the addition of substrate (Fig 3B). The resulting
microscopic micelles which formed were visualized by
electron microscopy. Under these conditions the P-100
30 enzyme fraction produced an enhanced amount of cellulose
I, while the crude enzyme produced cellulose II only.

A surprising discovery of the present invention is
that the combination of the partially purified enzyme
35 preparation and the lower acetonitrile to buffer ratio
allows the glucan chains to grow simultaneously and
unidirectionally into an extended chain conformation,

- 26 -

achieving an artificial terminal complex. Another important discovery lies in the purification of the 38 kD protein from *Trichoderma viride*. As the exonuclease activity is removed during the purification steps, the cellulose I synthesis increases. Another discovery is that the retaining type endoglucanases are responsible for the cellulose I synthesis and the removal of inverting type increases the specific activity.

Another important aspect of the present invention is the discovery of the conditions essential for *in vitro* cellulose I assembly using the solubilized terminal complexes from cotton plants. Use of Mops buffer with sucrose and two digitonin extraction protocols led to an effective extraction of the 37 kD polypeptide, concomitantly increasing the quantity of β -1,4-glucan produced *in vitro*. Although a low concentration of digitonin (0.05%) in the first solubilization protocol released a small quantity of proteins, this SE1 fraction synthesized a larger quantity of ANIP (31.77%), than previously reported by the inventors (4% by SE fraction) (Okuda et al, 1993). Entrapment purification of the SE1 fraction led to substantial purification of the 37 kD polypeptide. This fraction also is a prerequisite for the synthesis of cellulose I. Higher concentrations of digitonin (1%) solubilized more membrane proteins, but this (SE2) fraction produced much less cellulose (8.4%), which is mostly cellulose II.

The present disclosure demonstrates that by a modification of the extraction and solubilization procedures, cellulose I can be synthesized *in vitro* from cotton membrane extracts. Evidence that the fibrillar material synthesized *in vitro* from the SE1 fraction is cellulose I comes from morphological studies of the ANIP and from ED analysis. The morphology of the product synthesized by this fraction is completely different from

- 27 -

the product synthesized by the SE2 fraction. Cellulose produced by the SE1 fraction is in the form of extended fibrils, which are labeled with CBH I-gold. In contrast, the cellulose produced from the SE2 fraction is similar to cellulose II reported earlier (Okuda et al., 1993), and it is also similar to the folded glucan chains from an *Acetobacter xylinum* mutant identified recently as cellulose II (Kuga et al., 1993). Conclusive evidence for the identity of cellulose from the *in vitro* product comes from the ED studies. A diffraction pattern of native cotton fibers revealed the following diagnostic reflections of cellulose I, according to the indexing convention of Woodcock and Sarko (1980): the 004 meridional reflection (labeled "a"); three equatorial reflections, 200, 110 and 110 (labeled "b"), the 012 reflections (labeled "c") and the 002 reflection (labeled "d").

The diffraction pattern of the *in vitro* product synthesized by the SE1 fraction is virtually identical to that of native cotton. Furthermore, a reflection characteristic for cellulose I β is present which suggests that the *in vitro* cellulose probably contains a fraction of this sub-allomorph (Sugiyama et al., 1991). Whether or not cellulose I β is enriched over that found in native cellulose is unknown at present; however, these results are not unlike those obtained from synthetic cellulose I recently produced in the laboratory of several of the inventors.

30

It is interesting that only the SE1 fraction produces cellulose I while the SE2 produces cellulose II. This is probably due to the gentle solubilization process in the first step using a low concentration of digitonin. In this case, the enzyme complex may remain intact, with its subunits organized in such a manner that sufficient packing and organization of the catalytic subunits could

35

- 28 -

insure 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 or TC where the synthesis of parallel β -1,4-glucan chains leads to the crystallization of cellulose I (Kuga et al., 1993; Okuda et al., 1994; Mizuta and Brown, 1992). The final concentration of digitonin to solubilize SE1 fraction was only 0.05%, which is very low, and seems to suggest that the proteins released in this conditions are only peripherally associated with membrane.

Cellulose synthases extracted by 1% digitonin (the SE2 fraction) may be sufficiently disorganized to prevent extended parallel chain aggregation during the polymerization process. In this case, chain folding may occur, leading to the antiparallel arrangement which is more thermodynamically favorable (Rånby, 1952) and is indicative of cellulose II.

20

The percentage of cellulose produced by the SE1 fraction (31.77%) is greater than that produced by the SE2 fraction (8.4%) (Table 2). This latter percentage is greater than that reported previously (4%) (Okuda et al., 1993). These results are in accordance with the protein profiles in each fraction (Fig. 7A). In the SE1 fraction, the 37 kD polypeptide is more prevalent than in the SE2 fraction. In the latter fraction, the 52 kD polypeptide is much more abundant, while in the SE1 fraction, the 52 kD polypeptide is weakly stained. This may be one of the reasons why the SE2 fraction produces more abundant β -1,3-glucan. Since the solubilized fractions (SE1 & SE2) are somewhat crude, it can be only assumed at this point that the 37 kD protein band is the polypeptide implicated as cellulose synthase; however, this evidence, combined with that from the earlier work (Okuda et al, 1993, Li and Brown, 1993, Li et al, 1993),

- 29 -

strengthens the argument that the 37 kd polypeptide plays a key role in cellulose synthesis in cotton.

That the cellulose produced *in vitro* was not a
5 contaminant has been assured by: (a) three different
controls; (b) additional centrifugation of all the
components used in the *in vitro* reaction mixture; and,
(c) additional centrifugation of enzyme fractions before
addition to other components. No fibrillar structures
10 were observed when UDP-Glc was omitted from the reaction
mixture, or when enzyme was not added to the other
components of the reaction mixture. In the zero-time
control, single CBH I-gold particles and single silver
grains were visible, indicating that the reaction mixture
15 placed for 1 minute in the boiling water bath did not
completely block the enzyme activity immediately, but it
was sufficient to prevent continued synthesis of product.
Contamination was ruled out by observing the solubilized
enzyme fractions with EM, in which case, no fibrillar
20 material was ever detected.

The addition of tritium labeled UDP-Glc to the
reaction mixture, followed by CBH I-gold labeling,
autoradiography, and negative staining, shows that the
25 CBH I-gold and silver grains both coincide with the
product. The dual labeling provides reasonable evidence
that the product is a glucan derived from the supplied
UDP-Glc, and that this product does not represent any
pre-existing fibrils in the preparation. Taken together,
30 these observations confirm that cellulose I has been
synthesized *in vitro* using new extraction and
solubilization protocols.

The present disclosure further confirms that
35 cellulose is synthesized *in vitro* using improved
solubilization protocols. The percentage of cellulose
synthesized *in vitro* has been increased from 4% to at

- 30 -

least 31.77% of the total *in vitro* product. Even this latter figure is conservative since only the ANIP was measured, which does not retain low molecular weight or poorly crystallized β -1,4-glucans.

5

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow
10 represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many
15 changes can be made in the specific embodiments which are disclosed and still obtain a like or similar results without departing from the spirit and scope of the invention.

20

EXAMPLE I

Synthetic Cellulose I Assembly

The 5:1 acetonitrile/acetate buffer reaction mixture was prepared as described elsewhere (Kobayashi, 1991).
25 The 2:1 acetonitrile/buffer reaction mixture was prepared by dissolving 50 mg of the P-100 fraction (See Example II, *infra*) in 0.05 M sodium acetate buffer (pH 5) to give a total volume of 0.032 ml. Five milligrams of substrate was dissolved in 0.064 ml of the same buffer and 0.192 ml
30 of acetonitrile. Reactions were run at room temperature (25°C) for 20 minutes. The reaction product was repeatedly washed with water at 4°C, and subjected to 30 seconds of ultrasonication with a Fisher Dismembrator-Model 300.

35

For electron microscopy, the samples were mounted on copper grids coated with thin carbon films, negatively

- 31 -

stained with a 2% uranyl acetate containing 0.01%
bacitracin (except for the electron diffraction
specimens), and examined with a Philips 420 TEM operating
at 100 KV or 120 KV (Kobayashi et al., 1993). For the
5 time-course study, a drop of the control solution or the
reaction mixture was directly placed on the grid, excess
solution was removed and the preparation was dried.

When the acetonitrile/buffer ratio was adjusted to
10 2:1 and the concentration of the P-100 enzyme fraction
was 50 micrograms, keeping the substrate, cellobiosyl
fluoride at 5 milligrams, huge fibers of cellulose I were
produced. The fibers were abundant in the entire EM
grid. The bundle was up to 1mm, and the individual
15 microfibrils within the bundle were in very close
alignment with each other. Individual microfibrils were
approximately 12 Å x 25 Å. CBH I-Gold sparingly labeled
these microfibrils, indicating that there were relatively
few non reducing ends for labeling.

20

As seen in Fig 2B, the cellulose I pattern is
intermixed with the cellulose II pattern. However, when
the enzyme fraction is changed from the P-100 to the P-60
fraction, surprisingly, the cellulose II is almost non-
25 existent (Fig. 2D). The cellulose I pattern appears
alone with some of the cellulose II pattern appearing at
other areas of the grid. This indicates that by
increasing the purity of the enzyme and by preventing
phase separation, the micelles are further saturated,
30 thus achieving a denser multiple array of catalytic sites
for parallel extended glucan chain formation.

EXAMPLE II

Purification of Cellulolytic Activity

35

It is a surprising embodiment of the present
invention that with a partially purified cellulase,

- 32 -

cellulose I is synthesized under conditions that only produce cellulose II when crude cellulase preparations are used. The inventors' studies, based on numerous trials, indicate that a substantial degree of catalyst purification is required before cellulose I can be synthesized *in vitro* using cellulase as the catalyst. It is believed that the purification must be such as to remove the enzyme activity which hydrolyses the cellulose I (exoglucanase) in competition with the desired reverse cellulose synthesis reaction. It is also contemplated that the purer enzyme preparation may allow more orderly packing of the enzymes in the micelles, thus producing the more ordered cellulose I product.

15 Purification Method

Cellulase Onazuka R-10 (Kinki Yakult, Osaka, Japan) was dissolved in 0.01 M sodium acetate buffer (pH 5), and insoluble material was removed by centrifugation at 50,000 x g. Fifteen milliliters of a 13.3% solution of this preparation was loaded into a Bio-Gel P-10 column (30 x 500 mm) and the void fraction was collected. Using an Amicon ultracel- with a PM 30 membrane, the fraction was concentrated into 10 ml. To 42.4 ml of deionized water and 10 ml of concentrated enzyme solution were added 2.6 ml of a 40% w/v Bio-Lite ampholyte blend (pH 3-10). This mixture was loaded into a Rotoform™ Preparative Isoelectric Focusing Cell (Bio-Rad, California) operating at 12 W constant power for 4 h at 1°C. Twenty fractions of about 2.5 ml each were collected and assayed for cellulose synthesizing activity by measuring the water insoluble product using the anthrone reagent (Fry, 1988). The fraction (pH 3.6) with the highest activity was collected and further purified over a Bio-Gel P-100 column (20 x 900 mm). The majority of the activity was associated with the shoulder fraction of only one peak. This fraction was used in this study and described as the

- 33 -

P-100 fraction. An SDS-PAGE analysis of the P-100 fraction revealed eight broad bands with some evidence of minor bands.

5 In a further purification, an ammonium sulfate fractionation step was added and the P-100 column was replaced by a P-60 column. The SDS-PAGE of the product of this purification step revealed a major band at 38 kDa comprising about 80% of the total protein. When a Prep
10 Cell™ (Bio-Rad, California) was employed the 38 kDa protein was >90% of the total protein and there were only 2 or 3 minor bands. This fraction exhibited a 31-fold higher specific activity than the crude extract and lacked any cellobiohydrolase activity (Wood et al.,
15 1988).

EXAMPLE III

Enzymes Other Than Cellulase

20 Enzymes other than cellulase are encompassed by the present invention. Preliminary studies have been done with different endoglucanases, cellobiohydrolase I, β -glucosidase and particularly cellobiohydrolase II. Others which may be used in the present invention
25 include, but are not limited to hydrolases such as chitinase, β -1,3 glucanase, β -1,2 glucanase, or xylanase to synthesize, mannosidase to synthesize mannans, or other appropriate candidates. This invention would also include the use of any of the glycosyl transferases
30 already discovered to function in cellulose assembly in *Acetobacter*, *Dictyostelium*, *Saprolegnia*, or cotton, for example.

- 34 -

EXAMPLE IV

Other Substrates for Cellulolytic Enzymes

In certain embodiments of the present invention,
5 other oligosaccharides such as cellotetrasyflouride,
cellopentasyflouride, etc. could serve as substrates for
the cellulase enzyme. The invention would also encompass
substrates with active groups other than fluorine,
including, but not limited to iodide, methyl, allyl,
10 trifluoroethyl and acetyl groups. In general, the
organic solvent would be water miscible up to a certain
point, but should not be too highly dipolar aprotic which
will cause inactivation of the enzyme. Since it is
envisioned that cellulose synthases may be used in the
15 method of the present invention, cellobiose or possible
even glucose could also be used as substrates.

The effect of the monomer structure on the
polymerization was investigated using various cellobiose
20 derivatives. α -D-Cellobiosyl fluoride, whose fluorine
atom at the anomeric position has the opposite (α)
configuration did not polymerize. This result shows that
the anomeric stereochemistry plays an important role in
this enzymatic polymerization. Other cellobiose
25 derivatives, alkyl cellobioside and 1-O-acylclobiose
derivatives were found to be recognized by cellulose
catalyst. However, these cellobiose derivatives showed
poor polymerizability in comparison with that of β -D-
cellobiosyl fluoride under the conditions tested. It is
30 understood that specific conditions may be necessary for
each particular substrate/enzyme combination and that
these conditions can be determined without undue
experimentation in light of the present disclosure. For
xylan and mannan synthesis, it is expected that activated
35 monomer derivatives such as xylobiosylfluoride or
mannobiosylfluoride can be used.

- 35 -

EXAMPLE V**The Use of Other Solvents**

Other possible solvents may include, but are not
5 limited to 1,4-dioxane, nitromethane, dimethyl sulfoxide
(DMSO), isooctane, and propionitrile. In general, the
organic solvent should be water miscible up to a certain
point, but should not be too highly dipolar aprotic which
may cause inactivation of the enzyme.

10

Since it has been demonstrated by the inventors that
the crystalline structure of the product as well as the
degree of polymerization are dependent on the packing of
the enzyme complex, it is contemplated that different
15 structural arrangements of the enzymes in the catalytic
complexes will result in different products. Therefore,
in light of the present disclosure, the organic
solvent/buffer combinations can be varied to produce
unique crystallization patterns and polymer lengths.

20

EXAMPLE VI**Immobilization of the Enzymes**

In order for a cellulose I allomorph to be assembled
25 *in vitro*, the cellulose polymerization catalytic sites
from several enzymes must be brought into close
proximity. One way this might be accomplished would be
by careful extraction of native cellulose synthase
complexes so that the multimeric organization of the
30 catalytic sites remains relatively undisturbed.

Alternative, either the enzyme complex must be
immobilized, or it must be free and unrestricted so as to
be able to move away from its product and have access to
35 incoming substrate. This is exemplified in natural
systems by the enzyme complexes, known as terminal
complexes or TCs which have been visualized in cell

- 36 -

membranes using the freeze fracture technique. In this natural system, the enzyme complex is always associated with the terminus of the cellulose microfibril it is assembling. The TC is free to move in the plane of the fluid membrane, thus continually moving away from its product.

In past attempts at *in vitro* cellulose synthesis, the enzyme complex, if intact, has been disrupted and separated from the membrane. As the catalysis proceeds, the enzyme complex becomes entrapped within its product, and eventually the reaction slows or stops altogether. This is because the product cannot be moved away from the catalytic site resulting in product inhibition or possibly conditions favorable to the reverse reaction through mass action.

Thus, immobilization of the enzyme complex in such a way that the product could be extruded away from the catalytic complex would permit, on an industrial scale, the continuous generation of polymers. The interface formation and/or micellar formation of the present invention is favorable for the *in vitro* assembly of cellulose since it is an intrinsically dependable method for separation of the substrate/catalyst/product during the reaction. For example, the enzyme complex is held in the micelle and the product is extruded into the aqueous medium.

The immobilization of the many catalysts on or within a micelle promotes the crystallization of many polymer chains. The degree of order of the enzymes determines the ultimate chain packing, either parallel or antiparallel. During micelle formation, it is important that enzyme or catalyst immobilization takes place for the directed synthesis of glucan chains to form cellulose I. If the isolated catalytic subunits were merely

- 37 -

solubilized in suspension, they would created free β -1,4-glucan chains which would then collapse into the more thermodynamically stable folded chain cellulose II crystalline structure.

5

Fibers as an immobilization substrates

Immobilization of purified cellulolytic enzymes allows the nascent cellulose product to move away from
10 the enzyme while the reaction proceeds in a hydrophilic environment. For example, since proteins bind to nitrocellulose, a nitrocellulose fiber could be used to immobilize the endoglucanase. Also, any fiber, either
15 natural or synthetic which has a high affinity for binding proteins or which can be modified to chemically bind the catalyst without loss of activity, is a good candidate for the immobilization.

A specific cross linking agent could be
20 glutaraldehyde or another bifunctional agent which would not damage the catalytic site. A protein fiber (hair, silk, collagen, etc.) would be a preferred choice as an immobilization substrate because the efficiency of cellulose production could be monitored by using a dye
25 such a Tinopal which binds to the cellulose product and which can be monitored through its fluorescence.

Optimal reaction conditions for the immobilized enzyme reaction would be established empirically without
30 undue experimentation. Variables would include various combination of acetonitrile/buffer ratios and temperatures as well as substrate concentration, etc. Other variables include fiber orientation to optimize substrate flow and accessibility to the catalytic sites
35 as well as the product removal downstream. One important consequence of the immobilized enzyme for cellulose synthesis is that the hydrolysis can be minimized or

- 38 -

prevented as long as the product continually is removed from the catalytic sites.

Immobilization in a cylindrical chamber

5

It is envisioned that cellulose can be assembled continuously or intermittently using a confined cylindrical structure within which is placed a membrane which immobilizes the catalyst/s and one which also is permeable to the substrate. As the fluid carrying the substrates plus activators passes through the membrane, these components come in association with the catalyst whereupon the substrates are converted into the growing polymer. The polymer is directed away from the catalyst by the fluid flow. The polymer chains being directed away also are in parallel association with each other so that when they crystallize, the cellulose I allomorph is made. The idea is unique in that to develop such a functional system will require a precise method for bringing the catalytic enzymes, cellulose synthases or cellulases or others into close proximity so that the glucan chains do not get entangled and thus entrap the catalyst. If this happens, the reaction will auto-terminate. As long as the substrate is provided in excess and as long as the product is continuously removed from the catalyst, the reactions will proceed smoothly.

10
15
20
25

It is important to note that the enzymes must be positioned on the membrane in such a way that they are organized on an approximately spherical surface (= micelle-like component). This can be accomplished possibly by using an organic solvent with the water solvent, adjusting the ratio of solvents or varying the concentration of substrate so that a very fine spherical droplet suspension with the enzymes on the surface is produced. The hydrophobic and hydrophilic groups on the enzyme will determine the nature and degree of

30
35

- 39 -

positioning of enzymes on the droplet surface at the interface of the micelle.

The glucan polymer chains initially grow in an
5 unrestricted manner in all directions. As the growing
chains come into contact with their nearest neighbors,
this contact establishes the directionality of collapse,
thus leading all the other glucan chains growing from the
surface to adhere and bond with the elongating
10 crystalline microfibril or sub-microfibril or glucan
chain aggregate. Implicit in this invention is the idea
that the size of the spherical surface area can
contribute specifically to the structural characteristics
of the cellulose I allomorph produced. For example, a
15 large microfibril might be produced if many different
spherical droplets with their enzymes were sufficiently
closely packed, hexagonally for example, so that the
glucan chain aggregates themselves would unite and bond
and crystallize with their nearest neighbors. The result
20 of this hierarchical assembly is that the crystalline
structure would literally "grow" thus possibly achieving
the largest crystals of cellulose ever made.
Theoretically, the highly crystalline structure could be
as large as several microns or even approach the
25 dimensions of individual yarns such as cotton fibers, or
polyester fibers.

Colloidal Gold

30 It is also contemplated that the endoglucanase or
catalyst may exhibit bifunctionality. For example, if
the catalyst is a glycoprotein, the carbohydrate moiety
may serve to hold the product in the organic solvent
micelle phase during the synthesis of cellulose I. This
35 may serve to orient the glucan chains in positions
favorable for the metastable crystallization to form
cellulose I.

- 40 -

Extending this bifunctionality, the carbohydrate groups on the enzyme may effect the observed high frequency of clumping of enzymes upon activation in the aqueous phase. Adding colloidal gold and
5 electrostatically binding the gold to the enzyme favors clumping. This should initiate an excellent immobilization platform for the enzyme complex to generate cellulose I. The gold would be in the center of the complex binding the endoglucanases by their
10 carbohydrate trails and the polypeptide catalytic heads would be positioned to the surface. This would make a TC for generating parallel glucan chains. Since the gold is dense, the gold enzyme complex could be loaded in an organic phase and by gravity, would descend through the
15 fluid during synthesis, thus producing oriented cellulose. The evidence that this might work is based on data that CBH-gold complexes still retain their cellulose binding activity as well as their catalytic activity (Chanzy et al., 1984).

20

This concept is not limited to simple physical clumping of the colloidal gold. The colloidal gold could be specifically deposited into many known geometrical arrangements (rows, arcs, cones, triangles, etc.) to
25 provide specific geometric sites for subsequent binding with endoglucanase or the catalytic component. Thus synthetic TCs could be made to organize glucan chains to assemble cellulose microfibrils with specific shapes, sizes, and crystalline perfection.

30

EXAMPLE VIII

New Forms of Cellulose

One isomorph of the cellulose I synthesized by the
35 method of the present invention appears to be a new form not seen in nature. The synthetic cellulose fibril appears to be composed of only two glucan chain sheets

- 41 -

and the closest natural cellulose from *Vaucheria* (Mizuta, et al., 1989) and *Erythrocladia* (Okuda, et al., personal communication) has at least 3 layers per sheet.

5 Many types of new synthetic celluloses may be made by this invention. Cellulose with extremely high molecular weight could possibly be produced with a molecular weight of 10 million or more. This has never been achieved by natural living systems or by *in vitro*
10 systems. The inventors' anticipate many different crystalline microfibril shapes to be made by this invention. Square or rectangular cross sections, or even almost membrane-like sheets of cellulose I could be made. The synthesis of these sheets could be controlled by
15 varying the ratio of organic solvent to buffer since it is known that in a 5:1 acetonitrile/aqueous buffer, mostly cellulose II is produced, while in a 2:1 acetonitrile/aqueous buffer ratio, cellulose I is much more abundant. By varying the organic solvent and buffer
20 along with temperature or concentration of substrate, it could be possible to form truly unique thin films with novel glucan chain orientations. Composites with other polymers and the novel cellulose described in this invention are also possible. Some examples of other
25 polymers are xylan, mannan, and chitin. Cellulose also could be substituted with various side chains, leading to novel polymer compounds such as cellulose acetate, cellulose butyrate, and even methylcellulose. Detergent-like celluloses, soluble in both organic and hydrophilic
30 environments might be possible.

EXAMPLE IX

Characterization of Products

35 Several methods for the characterization of products synthesized *in vitro*, such as solubility properties, acid hydrolysis, acetolysis, specific enzymatic digestion, and

- 42 -

linkage analysis after periodate oxidation or methylation, may be used either alone or in various combinations (Okuda et al., 1993).

5 Insolubility in strong alkali (e.g., 24% KOH) may be used for the detection of β -1,4-glucan, but β -1,3-glucan synthesized *in vitro* may also be present in the alkali-insoluble fraction (Hayashi et al., 1987). The acetic/nitric acid reagent (Updegraff, 1969) may be used
10 for the selection of cellulose because only crystalline cellulose can remain after treatment with this reagent. The most reliable methods to prove the presence of crystalline cellulose are x-ray and electron diffraction analyses (Sarko, 1976). Highly purified cellulase
15 conjugated to colloidal gold has also been used as a probe to identify cellulose by TEM (Lin et al., 1985).

EXAMPLE X

Combinations of Glycosyl Transferases

20

Cellulase or any polysaccharide hydrolase may be used in combination with different glycosyl transferases in the aqueous mode to make heteropolymers with novel properties. For example, a heteropolymer could be
25 synthesized by a pulsed synthesis of the β -1,4 glucan chain backbone in an organic solvent, followed by a pulsed hydrolysis or addition reaction to add a branch polysaccharide chain, then possibly back to a pulsed synthesis in organic solvent using a different enzyme to
30 produce a linear polymer on the side chain. The combination of this type of procedures would produce a novel polysaccharide. This is similar to the method of Roth (US Patent 5,180,674, incorporated herein by reference) except that by combining organic and aqueous
35 phases, this method can be extended to other polysaccharides such as mannans, xylans, callose, starch, etc.

- 43 -

Another unique property of the present invention is the use of the activated monomer in the cellulose synthesis. For example, a glucosyltransferase may be used to attach the activated monomer to the

5 oligosaccharide. This activated terminus could then serve as a hydrolase site for further reactions. Active functional groups could include fluorine or other halogens, or methyl, allyl, trifluoroethyl and acetyl groups.

10

A limitation of using hydrolases exclusively is that the active site for branch formation cannot be controlled. But, having the specific glycosyltransferase available, the active side chain can be added and then a

15 hydrolase can be used to continue the homopolymeric growth of the branch. Cyclodextrins could then be added to the ends of the branches or perhaps even proteoglycans or glycoproteins could be added to the dendritic structure.

20

EXAMPLE XI

Purification of the Endoglucanase to Homogeneity

25

As an important embodiment of the present invention, the endoglucanase from *Trichoderma viride* has been purified to homogeneity.

30

Ten grams of crude enzyme was dissolved in 200 ml of 0.01 M, pH 5.0 sodium acetate buffer. Ammonium sulfate was added to 35% to 45% and the pellet was precipitated by centrifugation. The resulting pellet was dissolved in 20 ml of buffer solution and then loaded onto a P-10

35 column.

- 44 -

The void volume from the P-10 column was concentrated with an Ultracell concentrator using a PM-10 membrane and then loaded to the Rotofor. For preparative isoelectric focusing, a pH 3-10 electrolyte was used.

- 5 Fractions 4-10 were selected based on their synthetic activity and loaded to the Rotofor for refractionation.

- 10 Fractions 4-10 from the first Rotorfor fractionation were diluted with deionized water to make 55 ml volume and loaded onto the Rotofor. Fractions 5-9 were selected based on their synthetic activity and loaded onto the P-60 column after being concentrated using a Centricone (P-10) apparatus.

- 15 Concentrated fractions 5-9 from the second Rotorfor fractionation were loaded onto a P-60 column (Figure 6). Fractions from 59 to 65 were selected based on their synthetic cellulose activity and loaded onto a DEAE-650M column and eluted with 0.01M sodium acetate buffer, pH
20 5.0. A linear concentration gradient of NaCl was used for further elution. Synthetic cellulose activity was only present in the first peak, which contained almost no BMC-cellulase activity. The first peak was concentrated and loaded onto a Sephadex G-50 column, from which only
25 one peak was obtained. SDS-PAGE analysis also revealed a single protein band at 38 kDa (Figure 5).

Table 1 Purification of Endoglucanase, 38 kD Polypeptide.

Fractions	Volume (ml)	Total protein (mg)	CM-cellulase activity		
			Total activity (Units)	Specific activity (Units/mg)	Activity yield (% of total)
Crude enzyme	200	9560	1068.12	0.112	100.00
(NH ₄) ₂ SO ₄ precipitate	20	3016	2702.46	0.896	253.00
Bio-gel P-10	15	2124	2011.40	0.947	188.31
Rotofor #1	35	495.91	975.44	1.985	91.32
Rotofor #2	12.5	104.13	344.07	3.413	32.21
Bio-Gel P-60	7.0	13.37	82.73	6.257	7.75
DEAE-650M	5.8	8.27	74.969	8.450	7.02
Sephadex G-50	6.0	3.68	47.727	12.823	4.47

-46-

Table 1 (Continued)

Fractions	Volume (ml)	Total protein (mg)	BMC-cellulase activity		
			Total activity (Units)	Specific activity (Units/mg)	Activity yield (%) of total)
Crude enzyme	200	9560	159.84	0.0167	100.00
(NH ₄) ₂ SO ₄ precipitate	20	3016	60.42	0.0200	37.54
Bio-gel P-10	15	2124	41.07	0.0193	25.69
Rotofor #1	35	495.91	23.564	0.0480	14.74
Rotofor #2	12.5	104.13	2.190	0.0208	1.37
Bio-Gel P-60	7.0	13.37	0.02018	0.001603	0.0126
DEAE-650M	5.8	8.27	0.00384	0.000552	0.0024
Sephadex G-50	6.0	3.68	0.00216	0.000581	0.0014

-47-

Table 1 (Continued)

Fractions	Volume (ml)	Total protein (mg)	Syn. cellulose activity		
			Total activity (Units)	Specific activity (Units/mg)	Activity yield (%) of total)
Crude enzyme	200	9560	110.46	0.0116	100.00
(NH ₄) ₂ SO ₄ precipitate	20	3016	198.38	0.0658	179.59
Bio-gel P-10	15	2124	148.43	0.0699	134.37
Rotofor #1	35	495.91	72.65	0.1491	65.77
Rotofor #2	12.5	104.13	38.98	0.4020	35.29
Bio-Gel P-60	7.0	13.37	37.45	2.7173	33.90
DEAE-650M	5.8	8.27	27.70	3.3811	25.08
Sephadex G-50	6.0	3.68	14.45	3.8814	13.08

- 48 -

CM-cellulase (Carboxymethyl) activity was measured in a mixture containing 0.4 ml of a 0.5% CM-cellulose solution in 0.01 M sodium acetate, pH 5.3 and 0.1 ml of enzyme solution. After incubation at 48°C for 1 h, the reaction was terminated. The reducing sugars were measured by the Somogyi-Nelson method at $\lambda 650$. BMC-cellulase (Bacterial microcrystalline) activity was measured in a reaction mixture consisting of 0.5 ml of a 1% suspension of finely granulated BMC-cellulase in 0.01 M sodium acetate buffer, pH 5.0 and 0.5 ml of enzyme solution. Incubation took place in a shaking incubator at 37°C for 20 h. After centrifugation, 0.5 ml of the supernatant was analyzed for soluble reducing sugars by the Somogyi-Nelson method at $\lambda 650$. For synthetic cellulose producing activity assay, we measured the 5:1 MeOH/water insoluble product from the 5:1 acetonitrile/buffer reaction at room temperature for 1h. The 5:1 MeOH/water insoluble product was centrifuged and dried. The dried pellet was dissolved in concentrated H_2SO_4 and then the anthrone reagent method was utilized with glucose as the standard at $\lambda 620$.

EXAMPLE XII

In Vitro Synthesis of Cellulose I

MATERIALS AND METHODS

Chemicals

UDP-[Glucose-U- ^{14}C] (318 mCi/mmol) was purchased from ICN Biochemical Inc. (Costa Mesa, CA). UDP-[Glucose-1- ^3H] (15.3 Ci/mmol) was purchased from NEN Research Products, (Dupont Company, Doraville, GA). Digitonin was purchased from Serva (Heidelberg, Germany). Cellobiose, cyclic-3':5'-GMP, protease inhibitors and other chemicals were obtained from Sigma (St. Louis, MO).

- 49 -

Gold suspension (Auro Beads G5) average particle size 5 nm was purchased from Amersham, (Arlington Heights, IL). CBH I enzyme was a gift from Dr. Martin Schulein of Novo Industri. The molecular mass standards, which contain
5 phosphorylase b (97 kD), BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21 kD), and hen egg white lysozyme (14 kD) were purchased from Bio-Rad (Richmond, CA).

10 Plant Materials

The cotton strain, *Gossypium hirsutum* Texas marker 1 (TM-1) (Kohel, 1970) was used for the present examples. Bolls were harvested, and the locules were removed and
15 stored in liquid nitrogen as described previously (Okuda et al., 1993).

Preparation of the Plasma Membrane Enriched Fractions

20 The plasma membrane enriched fraction from cotton fibers was prepared as described previously (Okuda et al., 1993), except for use of the extraction buffer comprising 50 mM 3-[N-morpholino]-propanesulfonic acid (Mops), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a
25 combination of protease inhibitors (0.5 mM PMSF, 10 μ M leupeptin, 0.1 mM N-a-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone). The membranes were resuspended in a small volume of resuspension buffer comprising 50 mM
30 Mops, pH 7.5 and 0.25 M sucrose.

Solubilization of membrane proteins

Solubilized enzyme fractions were obtained
35 separately through a two-step digitonin solubilization. In the first step, the plasma membrane enriched fraction

- 50 -

was mixed with an equal volume of the first solubilization buffer comprising 50 mM Mops, pH 7.5, 0.25 M sucrose, and 0.1% digitonin. The mixture was carefully layered on a 30% (w/v) glycerol cushion and centrifuged at 100,000g for 1 hour at 4°C. The supernatant was collected and denoted as the first solubilized enzyme fraction (SE1). In the second solubilization step, the pellet below the glycerol cushion was resuspended in the second solubilization buffer containing 50 mM Mops, pH 7.5, 0.25 M sucrose, and 1% digitonin, and the mixture was sonicated on the surface of ice for 5 minutes, then incubated on ice (total solubilization time was 1 hour). After solubilization, the mixture was layered again on a glycerol cushion and centrifuged under the same conditions as in the first solubilization step. The supernatant was collected and was denoted as the second solubilized enzyme fraction (SE2). SE1 and SE2 were concentrated with a Centriprep-10 Concentrator (Amicon Division of W. R. Grace & Co., Beverly Massachusetts). The proteins in each fraction were assayed using a modification of the Lowry procedure (Markwell et al., 1978). Electrophoresis was performed as described by Porzio and Pearson (1977).

25 Preparation of the *in vitro* product for EM

The F1,4 reaction mixture contained 10 mM bis-trispropane-Hepes buffer (pH 7.6), 20 mM CB, 8 mM MgCl₂, 1mM CaCl₂, 100 μM cyclic 3':5'-GMP, 0.05% digitonin, the solubilized enzyme fractions, 3 mM NaN₃, and 1 mM UDP-Glc (in 1 ml volume), and was incubated at 25°C for about 15 hours (overnight). The synthesized product was centrifuged at 15,000g for 10 minutes. For the total product analysis, the pellet was washed with deionized water, treated with protease for 30 minutes at room temperature, washed 3 times in water, resuspended in

- 51 -

a small volume of water, sonicated for 30 seconds (Fisher Sonic Dismembrator Model 300) and for EM studies. For ANIP, the pellet was resuspended in AN reagent, placed for 30 minutes in a boiling water bath (Updegraff, 1969),
5 then washed 3 times with deionized water, resuspended in a small volume of water, sonicated for 30 seconds. Both product suspensions were placed on EM grids which were shielded between two Petri dishes (to avoid any possible lint from the air) and allowed to dry. In addition, the
10 room air was filtered with 0.2 μ m HEPA filters. Gold labeling of the *in vitro* products and the negative staining were performed as described by Okuda et al., (1993).

15 For autoradiographic studies, the reaction mixture was essentially the same as described above, except that 0.5 mM UDP-(1-³H)-Glc (10 mCi/mmol) was used as the substrate. Reactions were performed overnight at 25°C. Three control studies were conducted: (a) zero time
20 incubation; (b) incubation without substrate; and, (c) incubation with all components but without enzyme. After incubation, the *in vitro* product was collected by centrifugation for 10 minutes at 15,000g. For total product analysis, the pellet was washed 3 times with
25 water. For ANIP, the pellet was resuspended in a small volume of AN reagent, placed for 30 minutes in a boiling water bath, then washed 3 times with water. The water suspensions of both radioactive products (total and ANIP) were mounted on Formvar-coated grids, allowed to dry,
30 treated with CBH I-gold complex solution, and then coated with Ilford L-4 photographic emulsion as described by Kopriwa (1973). Coated samples were exposed for 10 days, developed with Kodak D-19 developer (diluted 1:10) for 1 minute, and then negatively stained by 1.5% uranyl
35 acetate.

- 52 -

Product entrapment

The reaction mixture used for entrapment was the same as described above, but it was incubated at 25°C for 2 hours. The reaction was terminated by placing the reaction mixture on ice for 30 minutes, followed by centrifugation at 15,000g for 10 minutes at 4°C. The pellet was resuspended in 10 mM bis-trispropane-Hepes buffer, (pH 7.6) to 1/10 of the original reaction volume, and the suspension was recentrifuged at 15,000g for 10 minutes at 4°C. The resulting supernatant was designated as ES1 fraction and was used for electrophoresis. The pellet was resuspended in a small volume of deionized water and used for EM observations.

Enzyme activity assay

For the UDPG binding capacity of SE1 and SE2, 0.5 mM UDP-[U-¹⁴C]-Glc (specific activity 6.25mCi/mmol), diluted from the original UDP-[U-¹⁴C]-Glu (200 mCi/mmol) was used as a substrate in a final volume of 100 μ l. The concentration of the other components in the reaction mixture was the same as described above, and the reaction was incubated at 25°C for 30 - 60 minutes. For UDPG incorporation into the total product, the reaction was terminated by placing the reaction mixture for one minute in a boiling water bath. For ANIP, the reaction was terminated by addition of 1 ml of AN reagent and incubated in a boiling water bath for 30 minutes. Both radioactive products were collected by filtration on a GF/C glass filter, washed three times with distilled water (ANIP was washed first once with 0.5 N NaOH), and once with methanol. The radioactivity retained on the filters was dissolved in a Ready Organic Cocktail (Beckman Instruments, Inc., Fullerton, CA) and counted

- 53 -

with an LS 6800 Liquid Scintillation System (Beckman Instruments, Inc., Irvine, CA).

RESULTS

5

A comparison of protein profiles and β -1,4-glucan synthase activity was made for the plasma membrane, solubilized enzyme fractions, and the enzymes released after the entrapment. Figure 7A shows a prominent 52 kD polypeptide in the plasma membrane fraction (lane 2,); as well as in the second solubilized enzyme fraction (SE2) (lane 4,). In these two fractions, a 37 kD band is weakly stained. By contrast, in the first solubilized enzyme fraction (SE1, lane 3), a prominent 37 kD band is present, while the 52 kD band is diminished. After entrapment purification from the SE1 fraction, a prominent 37 kD polypeptide band is revealed (Fig 7B, lane 2), while the 37 kD polypeptide is much weaker from the product entrapped by the SE2 fraction (lane 3). These results are in accordance with the UDP-[U-¹⁴C]-Glc incorporation data (Table 3). The plasma membrane and SE2 enzyme fractions had higher specific activity (Table 2), and each incorporated more radioactive UDP-Glc into the total product than did the SE1 fraction; however, when the incorporation into ANIP is compared with the total product produced by each fraction (Table 3), it is evident that the SE1 fraction produced the greatest amount of cellulose. Table II shows that about 31.77% of the total product produced by the SE1 fraction consists of cellulose, whereas with the SE2 enzyme fraction, only about 8.4% of the total product is cellulose, compared to 4.8% for cellulose in the PM.

The SE1 and SE2 fractions were mixed together for the *in vitro* product synthesis. When a drop of this reaction product mixture was placed on an EM grid,

- 54 -

reacted with CBH I-gold, and negatively stained, it was difficult to distinguish any differences among the products. The product was very disperse and relatively homogeneous, and CBH I-gold complexes also were found
5 dispersed in this material. When the same product was centrifuged and resuspended in water, it became so strongly aggregated with proteins that it was difficult to distinguish in the TEM any product labeled with gold particles. After treating this reaction product with
10 protease, three different structures could be identified: i) fibrillar material aggregating into long rodlets, not labeled with CBH I-gold; ii) long fibrils; and, iii) disorganized aggregates. The long fibrils and disorganized aggregates were both very strongly labeled
15 with CBH I-gold, indicating that there were two different structural forms of cellulose synthesized.

The incorporation of UDP-[³H]-Glc into the *in vitro* product synthesized from mixed SE1 and SE2 fractions,
20 followed by ethanol treatment, CBH I-gold labeling, autoradiography, and negative staining, indicates that the CBH I-gold particles and silver grains both coincide with the EIP products whereas the background is free of labeling. Similar results were obtained when the
25 radioactive products were treated with the AN reagent, followed by autoradiography, CBH I-gold labeling, and negative staining. In this case, there were two different types of aggregates: a) long fibrils labeled with CBH I-gold particles and silver grains, and b)
30 disorganized aggregates also strongly labeled with CBH I-gold and silver.

To verify that the cellulose in the reaction product was not due to contamination, the mixed enzyme fractions
35 were examined with TEM. No fibrillar material was observed. Additional evidence that the fibrillar product

- 55 -

was synthesized *in vitro* came from autoradiographic studies of three controls: a) all components mixed together without any of the enzyme fraction added; b) all components mixed together, but without substrate; and c) zero-time reaction conditions.

In the control without enzyme, silver grains were dispersed on the grids, indicating a typical background of applied radioactive material, but no aggregation of silver grains was observed. In the second control in which the substrate was omitted from the reaction mixture, it was difficult to find any silver grains at all. In the case of the zero-time control, single gold particles from CBH I-gold labeling and single silver grains were occasionally visible, indicating that the reaction mixture placed for 1 minute in the boiling water bath did not immediately completely block the enzyme activity, but this treatment was sufficient to inhibit the continued synthesis of the product.

Electron microscopy of the product synthesized *in vitro* by digitonin-solubilized mixed fractions (SE1 + SE2) clearly indicated that there were three different kinds of morphologies indicative of β -1,3-glucans as well as β -1,4-glucans corresponding to cellulose I and cellulose II. The relative amounts of these allomorphs was difficult to assess. Only the ratio between β -1,3- and β -1,4-glucans could be estimated by UDP- ^{14}C -Glc incorporation into the total product and into ANIP (Table 2 and 3).

In order to more accurately pinpoint which extraction protocol was active in cellulose I assembly, the two solubilized fractions were used independently. Surprisingly, the SE1 fraction produced not only a relatively small quantity of β -1,3-glucan (Table 2 and

- 56 -

3), but it also made a considerable quantity of the cellulose I allomorph. The SE2 fraction produced more β -1,3-glucan and 8.4% of the total product of this fraction was cellulose II.

5

The morphology of products synthesized by the separate SE1 and SE2 fractions was analyzed. The amount of β -1,3-glucan synthesized by the SE2 enzyme fraction (material not labeled with CBH I-gold) is much greater
10 than the quantity of cellulose. Higher magnification views of the cellulose produced by the SE2 fraction shows that this product is very similar to the cellulose II reported earlier (Okuda et al., 1993).

15 Autoradiography of the product produced by the SE2 fraction shows that EIP is very specifically associated with silver grains, and CBH I-gold particles are relatively highly dispersed in between grains. ANIP
20 produced by the SE2 fractions shows a small quantity of disorganized aggregates abundantly labeled by CBH I-gold and silver grains. It was very difficult to localize any product on the grids for ED studies after a typical 30 minute boiling with AN reagent, therefore the treatment time was reduced to 5 minutes only. After a 5 minute
25 treatment with AN, the recorded ED pattern shows a similarity to the pattern of cellulose II, but it is not very clear. The region from which the ED pattern was recorded shows that there is still an abundant quantity of other material present, which probably is β -1,3-glucan
30 not completely removed after the short treatment with AN. This material obscures the pattern of cellulose II.

In contrast, the product synthesized by the SE1 enzyme has a different morphology (Figs. 8A - 8C). Low
35 magnification views (Fig. 8A) show that the ratio of β -1,3-/ β -1,4-glucan is not as high as that produced by

- 57 -

the SE2 fraction, and that the material associated with CBH I-gold particles has a distinctive fibrillar structure and appears dispersed among β -1,3-glucans. Higher magnification views of the product show that the fibrils are very long. Their dimensions are 1.2 - 1.8 nm (Figs. 8B and 8C) and are slightly larger than the size of native cotton fibrils.

Autoradiography of the product synthesized by the SE1 fraction after ethanol and AN reagent treatments shows that in both cases the product is very specifically labeled with silver grains and CBH I-gold particles. High magnification of the EIP reveals that in addition to long fibrils with attached CBH I-gold particles, there is also a product not labeled with CBH I-gold indicating β -1,3-glucan. After AN reagent treatment, only the elongated fibrils remain, all labeled with CBH I-gold and silver. The ED pattern of the same ANIP shows a pattern typical of cellulose I comparing favorably with the ED pattern of native cotton cellulose.

Table 2. Glucan synthase activity in different enzyme fractions.

Fractions	Total Product*	ANIP
PM	22.65 $\pm$ 1.30	0.71 $\pm$ 0.05
SE1	4.06 $\pm$ 0.99	1.22 $\pm$ 0.37
SE2	15.95 $\pm$ 2.68	0.85 $\pm$ 0.09

* nmol of Glu incorporated/min/mg protein

Table 3. Percentage of retained radioactivity for different fractions after treatment with AN reagent.

- 58 -

Fraction	Total Product	ANIP
PM	100	4.8±1.08
SE1	100	31.77±1.77
SE2	100	8.4±0.93

5

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that
10 variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both
15 chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of
20 the invention as defined by the appended claims.

The following citations are incorporated in pertinent part by reference herein for the reasons cited in the above text.

- 59 -

REFERENCES

- Aloni Y, Cohen R, Benziman M, Delmer DP (1983)
Solubilization of the UDP-glucose:
- 5 1,4- β -D-glucosyltransferase (cellulose synthase) from
Acetobacter xylinum. J Biol Chem 258: 4419-4423
- Blackwell (1982) The macromolecular organization of
cellulose and chitin. In RM Brown Jr, ed, Cellulose and
10 Other Natural Polymer Systems. Biogenesis, structure, and
degradation, Plenum Press, New York, pp 403-427
- Blaschek W, Haass D, Koehler H, Semler U, Franz G (1983)
Demonstration of a β -1,4-primer glucan in cellulose-like
15 glucan synthesized in vitro. Z. Pflanzenphysiol 111:
357-364
- Brauns, D. H., J. Am. Chem. Soc. 1, 1820, (1929).
- 20 Brown, R. M. Jr., J. Cell Sci. Suppl. 2, 13-32, (1985).
- Brown, R. M. Jr., & Montezinos, D., Proc. Natl. Acad.
Sci. U.S.A. 73, 143-147, (1976).
- 25 Brown, R. M. Jr., in *Cellulose and Wood-Chemistry and
Technology*, (ed. Schuerch, C.) 473-492, John Wiley and
Sons, New York, (1989a).
- Brown, et al., 1987, E.P. application No. 87307891.9.
- 30 Brown, R. M. Jr., in *Cellulose: Structural and Functional
Aspects*, (ed. Kennedy, J.F.), 145-151, Ellis Horwood Ltd,
London, UK, (1989b).

- 60 -

- Bureau TE, Brown RMJr (1987) In vitro synthesis of cellulose II from a cytoplasmic membrane fraction of *Acetobacter xylinum*. Proc Natl Acad Sci USA 84: 6985-6989
- 5 Carpita NC (1982) Cellulose synthsis in detached cotton fibers. In RM Brown Jr , ed, Cellulose and other natural polymer systems. Biogenesis, structure and degradation. Plenum Press, New York, pp 225-242
- 10 Chanzy H, B Henrissat, R Vuong (1984) Colloidal gold labelling of 1,4- β -D-glucan cellobiohydrolase adsorbed on cellulose substrates. FEBS 172: 193-197
- Colvin, J. R., Nature, 183, 1135, (1985).
- 15 Delmer DP (1991) The biochemistry of cellulose synthsis. In CW Lloyd ed, The cytoskeletal basis of plant growth and form. Academic Press, Limited, London, pp 101-107
- 20 Delmer DP, Ohana P, Gonen L, Benziman M (1993) In vitro synthesis of cellulose in plants: Still a long way to go! Plant Physiol 103: 307-308
- Delmer DP (1987) Cellulose biosynthesis. Ann Rev Plant
- 25 Physiol 38: 259-290
- Dhugga KS, Ray PM (1991) A 55 kDa plasma membrane-associated polypeptide is involved in β -1,3-glucan synthase activity in pea tissue. Plant.
- 30 Physiol 278: 283-286
- Elbein, A. D., Barber, G. A., & Hassid, W. Z. J. Am. Chem. Soc. 86, 309, (1964).

- 61 -

- Franz G, Heininger U (1981) Biosynthesis and metabolism: Cellulose and non-cellulosic cell wall glucans. In FA Loewus, W Tanner, eds, Encyclopedia Plant Physiol 13, Springer Verlag, Berlin, Heidelberg, New York, pp 47-67
- 5 Frey-Wyssling A (1976) The Plant Cell Wall, GrebrYder BorntrSger, Stuttgart, pp 1-294
- 10 Fry, S. C., The Growing Plant Cell Wall: Chem. & Metabolic Analysis, Longman Scientific and Technical, Harlo, Essex., 102-187 (1988).
- 15 Hayashi, T., Read, S. M., Drake, R. R., Haley, B. E. & Wasserman, B. P., Plant Physiol. 83, 1054-1062, (1987).
- Hirano, S., Agric. Biol. Chem. 37, 187, (1973).
- Husemann, E., & Muller, G., J. M. Makromol. Chem. 91, 212, (1966).
- 20 Itoh, T., & Brown, R. M. Jr., Planta 160, 372, (1984).
- Klar, J., Chem.-Ztg. 87, 731, (1963).
- 25 Kobayashi, S., Shoda, S., Lee, J. H., Okuda, K., Brown, R. M., Jr., & Kuga, S., Die Makromol. Chemie, manuscript submitted, (1993).
- Kobayashi, S., Kashiwa, K., Kawasaki, T., & Shoda, S., J. 30 Am. Chem. Soc. 113, 3079-3084, (1991).
- Kohel RJ (1970) Texas Marker-1. Description of a genetic standard for Gossypium hirsutum L. Crop Sci 10: 670-671

- 62 -

Kopriwa BM (1973) A reliable, standardized method for ultrastructural electron microscopic radioautography. *Histochemie* 37: 1-17

- 5 Kuga S, Takagi S, Brown RMJr (1993) Native folded-chain cellulose II. *Polymer* 34: 3293-3297

Kuga, S. and R. M. Brown in *Cellulose and Wood-Chemistry and Technology*, (ed. Schuerch, C.), 677-688, John Wiley
10 and Sons, New York, (1989).

Lai, H.-L., Butler, L. G., & Axelrod, B., *Biochem. Biophys. Res. Commun.* 60, 635, (1974).

- 15 Li L, Drake RJr, Clement S, Brown RMJr (1993) β -glucan synthesis in the cotton fiber. III. Identification of UDP-glucose-binding subunits of β -glucan synthases by photoaffinity labeling with [β - ^{32} P]5'-N3-UDP-glucose. *Plant Physiol* 101: 1149-1156

20

Li L, Brown RMJr (1993) β -glucan synthesis in the cotton fiber. II. Regulation and kinetic properties of β -glucan synthases. *Plant Physiol* 101: 1143-1148

- 25 Lin, F. C., Brown, R. M. Jr., Drake, R. R., Jr. & Haley, B. E., *J. Biol. Chem.* 265, 4782-4784, (1990).

Lin FC, Brown RMJr, Cooper JB, Delmer D (1985) Synthesis of fibrils in vitro by a solubilized cellulose synthase
30 from *Acetobacter xylinum*. *Science* 230: 822-825

- Lin FC, Brown RMJr, Drake RRJr, Haley BE (1990) Identification of the uridine 5'-diphosphoglucose (UDP-Glc) binding subunit of cellulose synthase in
35 *Acetobacter xylinum* using the photoaffinity probe 5-azido-UDP-Glc. *J Biol Chem* 265: 4782-4784

- 63 -

- Lin FC, Brown RMJr, (1989) Purification of cellulose synthase from *Acetobacter xylinum*. In C Schuerch, ed, Cellulose and Wood - Chemistry and Technology. John Wiley and Sons, New York, pp 473-492
- 5
- MacLachlan G (1982) Does β -glucan synthesis need a primer? In RM Brown Jr, ed, Cellulose and other natural polymer systems, New York: Plenum Press, pp 327-339
- 10 Markwell MAK, Hass SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87: 206-210
- 15 Mayer R, Ross P, Weinhouse H, Amikam D, Volman G, Ohana P, Calhoon RD, Wong HC, Emerick W, Benziman M (1991) Polypeptide composition of bacterial cyclic diguanylic acid-dependent cellulose synthase and the occurrence of immunologically cross-reacting proteins in higher plants.
- 20 Proc Natl Acad Sci USA 88: 5472-5476
- Micheel, F. Brodde, O.-E. & Reinkin, K., *Leibigs Ann. Chem.* 124, (1974).
- 25 Mizuta S, Brown RMJr (1992) High resolution analysis of the formation of cellulose synthesizing complexes in *Vaucheria hamata*. Protoplasma 166: 187-199
- Muller, S. C. & Brown, R. M. Jr., *J. Cell Biol.* 84, 315, 30 (1980).
- Nakatsubo, F., Takano, T., Kawada, T., & Murakami, K. in *Cellulose, Structural and Functional Aspects*, (eds. Kennedy, J. F., Philips, G. O. Williams P. A.), Ellis 35 Horwood, Sussex, 201-206. (1989).

- 64 -

- Okuda K, Li L, Kudlicka K, Kuga S, Brown RMJr (1993)
 β -glucan synthesis in the cotton fiber. I. Identification
of β -1,4- and β -1,3-glucans synthesized in vitro. Plant
Physiol 101: 1131-1142
- 5 Porzio MA, Pearson AM (1977) Improved resolution of
myofibrillar proteins with sodium dodecyl
sulfate-polyacrylamide gel electrophoresis. Biochim
Biophys Acta 490: 27-34
- 10 Preston, R.D., The Physical Biology of Plant Cell Walls,
Chapman & Hall, London, (1974).
- Rånby BG (1952) The mercerisation of cellulose: III. A
15 phase transition study using electron diffraction. Acta
Chemica Scandinavica 6: 128-133
- Rånby, B., *Acta Chemica Scandinavica*, 6, 101, (1952).
- 20 Read SM, Delmer DP (1991) Biochemistry and regulation of
cellulose synthesis in higher plants. In CH Haigler, P
Weimer, eds, Biosynthesis and biodegradation of
cellulose. Marcel Dekker, New York, pp 177-200
- 25 Roberts, E. M. Biosynthesis of Cellulose II and Related
Carbohydrates, The Univ. of Texas at Austin, U.S.A.
(1991).
- 30 Roberts EM, Saxena IM, Brown RMJr (1989). Biosynthesis of
cellulose II in *Acetobacter xylinum* In C Schuerch, ed,
Cellulose and Wood - Chemistry and Technology. John Wiley
and Sons, New York, pp 689-704
- 35 Robinson DG, Quader H (1981) Structure, synthesis, and
orientation of microfibrils. IX. A freeze-fracture

- 65 -

- investigation of the Oocystis plasma membrane after inhibitor treatment. J. Cell Biol. 25: 278-288
- Roelofson, P.A., The Plant Cell, Gebruder Bornstraeger, Berlin-Nikolassee, (1959).
- Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M (1987) Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. Nature 325: 279-281
- Roth, U.S. Patent 5, 180, 674.
- Sarko, A., Appl. Polym, Symp. 28 (2), 729, (1976).
- Sarko A (1978) What is the crystalline structure of cellulose? TAPPI 61: 59-61
- Sawyer LH, George W (1982) Comparison between synthetic and natural microfiber systems. In RM Brown Jr, ed, Cellulose and Other Natural Polymer Systems, Plenum Press, New York, pp 429-453
- Sawyer, L. H. & George, W., in ref. (6), 429-455, (1982).
- Saxena IM, Lin FC, Brown RMJr (1990) Cloning and sequencing of the cellulose synthase catalytic subunit gene of Acetobacter xylinum. Plant Mol Biol 15: 673-683
- Saxena IM, Lin FC, Brown RMJr (1991) Identification of a new gene in an operon for cellulose biosynthesis in Acetobacter xylinum. Plant Mol Biol 16: 947-954
- Schuerch, C., Adv. Polym. Sci. 10, 173, (1972).

- 66 -

Sisson, W., Science 87, 350, (1938).

Stipanovic AJ, Sarko A (1976) Packing analysis of
carbohydrates and polysaccharides. VI. Molecular and
5 crystal structure of regenerated cellulose II.
Macromolecules 9: 851-857.

Sugiyama J, Person J, Chanzy H (1991) Combined infrared
and electron diffraction study of the polymorphism of
10 native celluloses. Macromolecules 24: 2461-2466

Updegraff DM (1969) Semimicro determination of cellulose
in biological materials. Anal Biochem 32: 420-424

15 Uryu, T., Yamaguchi, C., Morikawa, K., Terui, K., Kanai,
T., & Matsuzaki, K., *Makromolecules* 18, 599, (1985).

Wong HC, Fear AL, Calhoon RD, Eichinger GH, Mayer R,
Amikam D, Benziman M, Gelfand DH, Meade JH, Emerick AW,
20 Bruner R, Ben-Bassat A, Tal R (1990). Genetic
organization of the cellulose synthase operon in
Acetobacter xylinum. Proc Natl Acad Sci USA 87:
8130-8134.

25 Wood, T. M., & Bhat, K.M., in *Methods for Measuring
Cellulase Activities* (eds. Wood, W.A. and Kellogg, S.
T.), Academic Press, Inc. San Diego, 87-112, (1988).

Woodcock C, Sarko A, (1980) Packing analysis of
30 carbohydrates and polysaccharides. 11. Molecular and
crystal structure of native ramie cellulose,
Macromolecules 9: 1183-1187

- 67 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5
- (i) APPLICANT: Brown, R. M.
Kobayashi, Shiro
Kudlicka, Krystyna
Kuga, Shigenori
10 Lee, Jong
Li, Likun
Okuda, Kazuo
Shoda, Shin-Ichiro
- 15 (ii) TITLE OF INVENTION: A Method for Synthesis of
Polysaccharides
- (iii) NUMBER OF SEQUENCES: 2
- 20 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Arnold, White & Durkee
(B) STREET: P. O. Box 4433
(C) CITY: Houston
(D) STATE: Texas
25 (E) COUNTRY: USA
(F) ZIP: 77210-4433
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
30 (B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.25
- 35 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/170,868

- 68 -

(B) FILING DATE: 02-AUG-1993

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- 5 (A) NAME: Hodgins, Daniel S.
(B) REGISTRATION NUMBER: 31,026
(C) REFERENCE/DOCKET NUMBER: BRWZ:002\HOD

(ix) TELECOMMUNICATION INFORMATION:

- 10 (A) TELEPHONE: (713) 787-1400
(B) TELEFAX: (713) 789,2679
(C) TELEX: 79-0924

15 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
(B) TYPE: amino acid
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

25

Thr Thr Asn Asn Ile Asp Gly Ala Phe Ala Pro
1 5 10

30 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
35 (D) TOPOLOGY: linear

- 69 -

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

5      Ile Gly Asn Val Gln Xaa Xaa Ile Gln Asp
      1              5              10

```

10 It is understood that those skilled in the art may practice the following claimed invention by substitution of various equivalents and that such substitutions are within the spirit and scope of these claims.

- 70 -

CLAIMS:

1. A method of producing cellulose I, the method comprising contacting a cellobiose bearing a displaceable
5 substituent at the C1 position with a partially purified cellulolytic enzyme contained in micelles.
2. The method of claim 1 wherein the displaceable
10 substituent is a halogen atom, methyl, allyl, trifluoroethyl, or acetyl.
3. The method of claim 2 wherein the halogen is
15 fluorine.
4. The method of claim 1 wherein said cellobiose is β -
D-cellobiosyl fluoride.
20
5. The method of claim 1 wherein said enzyme is an endoglucanase with transglycosylation activity, said endoglucanase catalyzing glycoside hydrolysis with
25 retention of the stereoisomeric configuration about the anomeric carbon.
6. The method of claim 1 wherein said enzyme is a
30 retaining type endoglucanase selected from the group consisting of a 38 kDa endoglucanase isolated from *Trichoderma viride*, Endoglucanase I from *Trichoderma reesei*, Endoglucanase II from *Trichoderma reesei*, and Endoglucanase from *Chrysosporium lignorum*.

35

- 71 -

7. The method of claim 1 wherein the cellulolytic enzyme is further defined as a fraction of *Trichoderma viride* cellulase prepared by a procedure comprising salting out, gel permeation chromatography, preparative
5 isoelectric focusing, and ion exchange chromatography.

8. The method of claim 1 wherein said enzyme exhibits a specific activity of cellulose synthesis of from 0.1491
10 to 3.8814 Units/mg in a 5:1 ratio of acetonitrile to sodium acetate buffer at room temperature.

9. The method of claim 1 wherein said enzyme fraction
15 is a single band of 38 kilodaltons when subjected to SDS-PAGE.

10. The method of claim 1 wherein the micelles comprise
20 substrate dissolved in an aqueous buffer and an organic solvent.

11. The method of claim 10 wherein the organic solvent
25 is acetonitrile, 1,4-dioxane, nitromethane, or isooctane.

12. The method of claim 10 wherein the organic solvent
is acetonitrile.

30

13. The method of claim 1 wherein the micelles are formed in an organic solvent/aqueous buffer with a ratio between 5:1 and 2:1.

35

- 72 -

14. The method of claim 1 wherein the micelles are formed in a 5:1 acetonitrile:aqueous buffer ratio.

5 15. The method of claim 1 wherein the micelles are formed in an organic solvent/aqueous buffer ratio of 2:1.

16. The method of claim 1, further comprising
10 terminating the cellulose synthesis with cellobiose.

17. A method of synthesizing cellulose I comprising contacting an isolated terminal complex with UDP-glucose
15 under conditions effective to produce cellulose I, said terminal complex comprising a cellulose synthase, and said terminal complex retaining its cellulose I synthesis activity.

20

18. The method of claim 17 wherein said terminal complex is isolated from a cotton plant cell.

25 19. A method of synthesizing cellulose I, the method comprising the steps of:

obtaining a plasma membrane enriched fraction from
cotton fibers;

30

mixing said plasma membrane enriched fraction with
an equal volume of solubilization buffer, said
buffer comprising 0.1% digitonin to obtain a
mixture;

35

- 73 -

layering said mixture on a 30% (w/v) glycerol cushion, centrifuging and collecting the supernatant to form a solubilized enzyme fraction; and

5

contacting said solubilized enzyme fraction with UDP-glucose in a reaction mixture.

10 20. The method of claim 19, wherein said plasma membrane enriched fraction is obtained by:

grinding cotton fibers to a fine powder in liquid nitrogen and extracting said powder with cold
15 extraction buffer comprising 50 mM
3-[N-morpholino]-propanesulfonic acid (Mops),
pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a
combination of protease inhibitors (0.5 mM
PMSF, 10 μ M leupeptin, 0.1 mM
20 N-a-p-tosyl-L-lysine chloromethyl ketone, and
0.1 mM L-1-tosylamide-2-phenyl-ethyl
chloromethyl ketone) to obtain membranes; and

resuspending said membranes in resuspension buffer
25 comprising 50 mM Mops, pH 7.5 and 0.25 M
sucrose.

21. The method of claim 19 wherein said solubilization
30 buffer is further defined as comprising 50 mM Mops, pH
7.5, and 0.25 M sucrose.

22. The method of claim 19 wherein said centrifugation
35 is at 100,000g for 1 hour at 4°C.

- 74 -

23. The method of claim 19 wherein said reaction mix is further defined as comprising 10 mM bis-trispropane-Hepes buffer (pH 7.6), 20 mM CB, 8 mM MgCl₂, 1mM CaCl₂, 100 μ M cyclic 3':5'-GMP), 0.05% digitonin, 3 mM NaN₃, and 1 mM UDP-Glc.

24. A method of solubilizing a terminal complex, said terminal complex retaining its cellulose I synthesis activity, the method comprising the steps of:

grinding cotton fibers to a fine powder in liquid nitrogen and extracting said powder with cold extraction buffer comprising 50 mM 3-[N-morpholino]-propanesulfonic acid (Mops), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and a combination of protease inhibitors (0.5 mM PMSF, 10 μ M leupeptin, 0.1 mM N-a-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) to obtain membranes; and resuspending said membranes in resuspension buffer comprising 50 mM Mops, pH 7.5 and 0.25 M sucrose.

25. A composition comprising cellulose I obtained by the method of any of claims 1-23.

26. The composition of claim 25 wherein said cellulose I consists essentially of β -glucan sheets comprising no more than two layers of glucan chains in thickness.

- 75 -

27. A micelle comprising acetonitrile, aqueous buffer, cellobiose having a displaceable substituent and an enzyme in accordance with claims 5-9.

5

28. The micelle of claim 27 wherein the aqueous buffer is sodium acetate buffer.

10 29. The micelle of claim 27 obtainable by agitating a mixture of acetonitrile and sodium acetate buffer at a ratio of 5:1.

15 30. The micelle of claim 27 obtainable by agitating a mixture of acetonitrile and sodium acetate buffer at a ratio of 2:1.

FIGURE 1B



FIGURE 1A

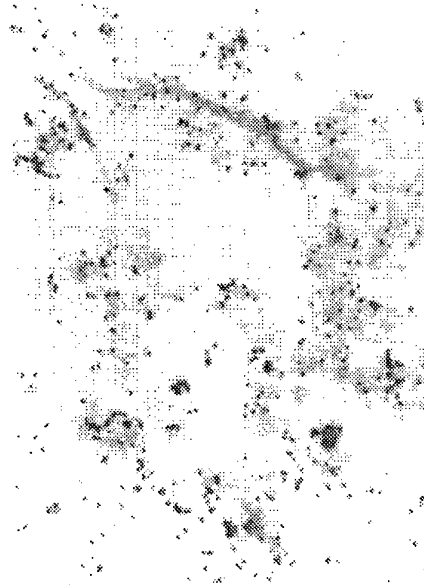


FIGURE 1D

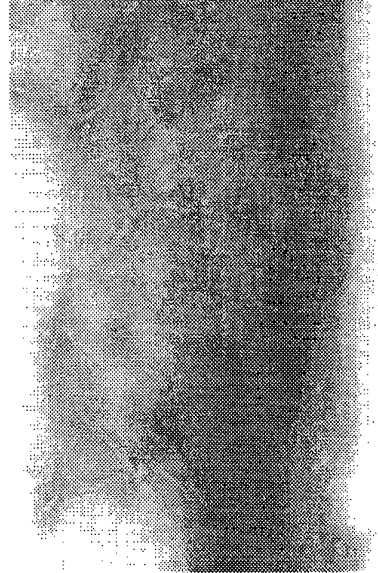


FIGURE 1C



FIGURE 2A

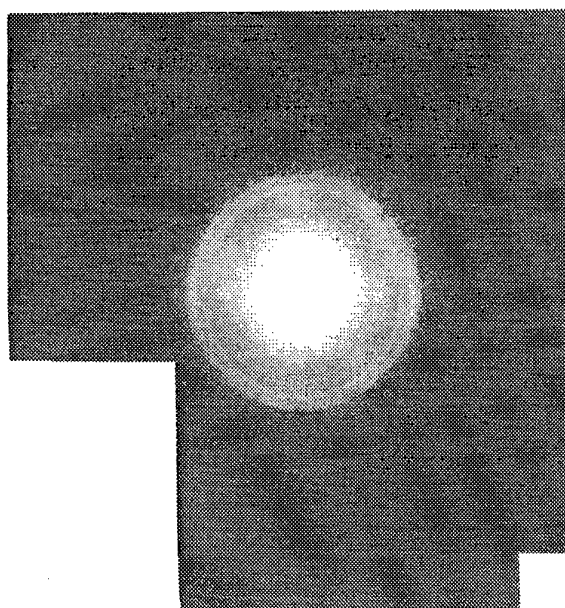
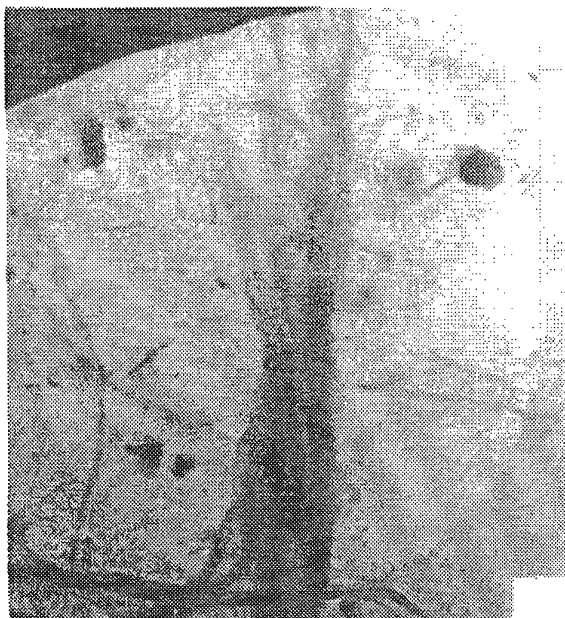


FIGURE 2B

FIGURE 2C



FIGURE 2D

4/10

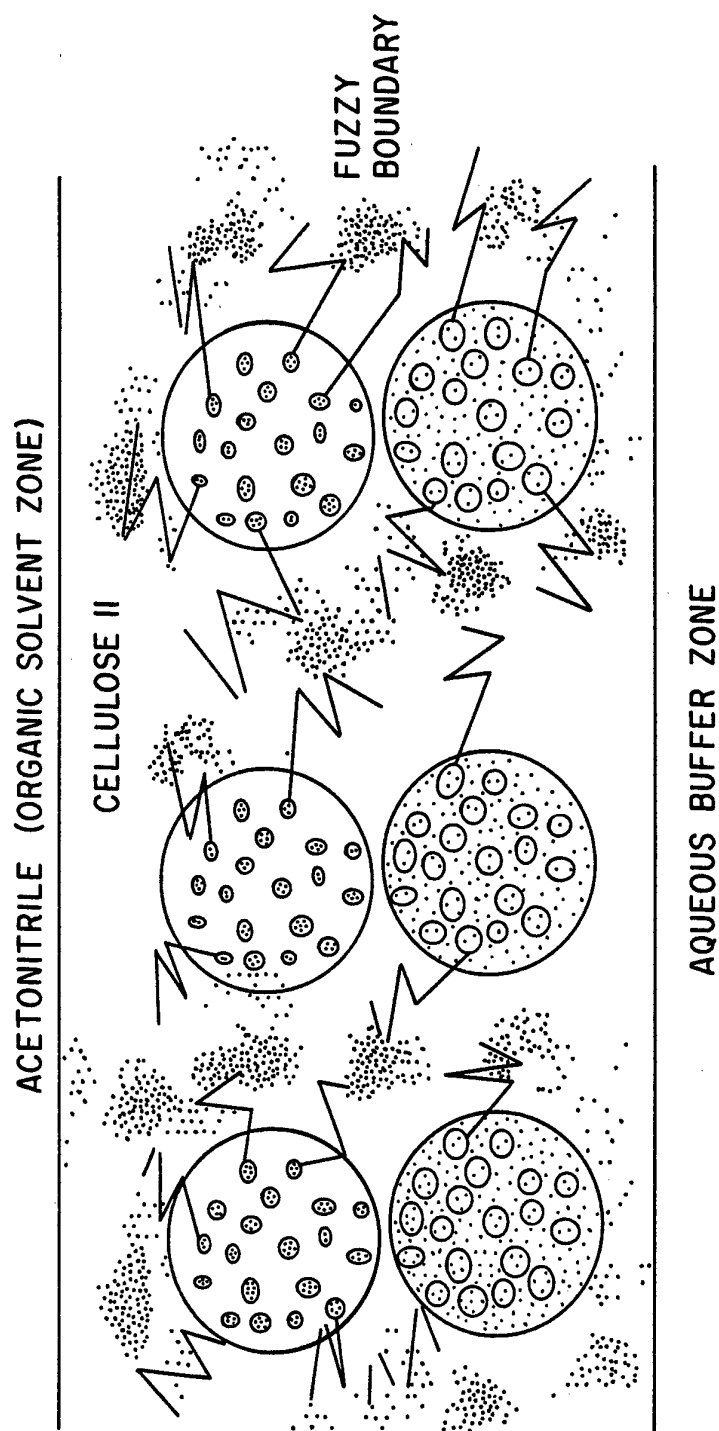
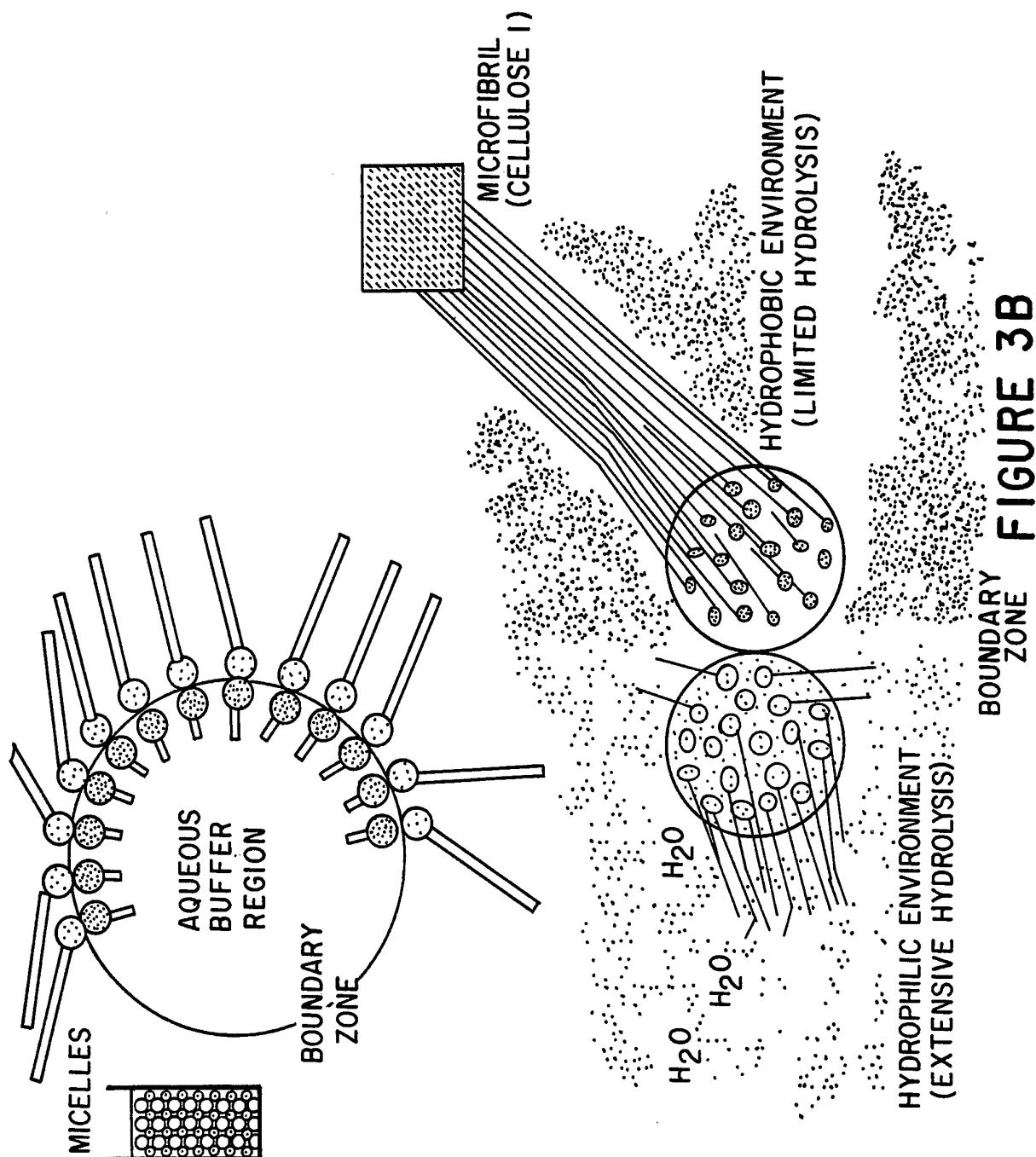


FIGURE 3A

5/10



6/10

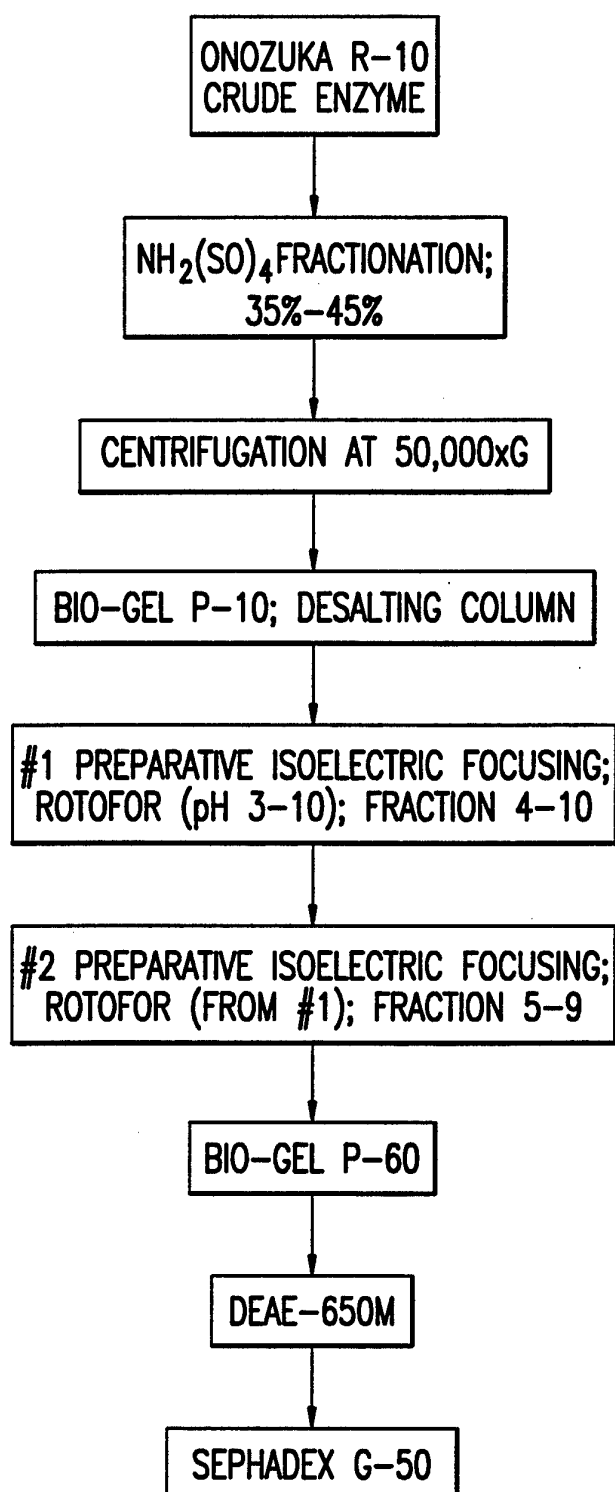


FIGURE 4

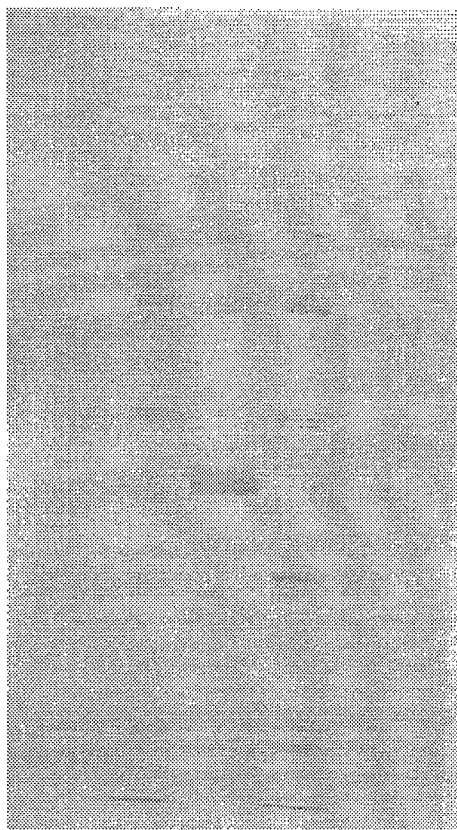


FIGURE 5

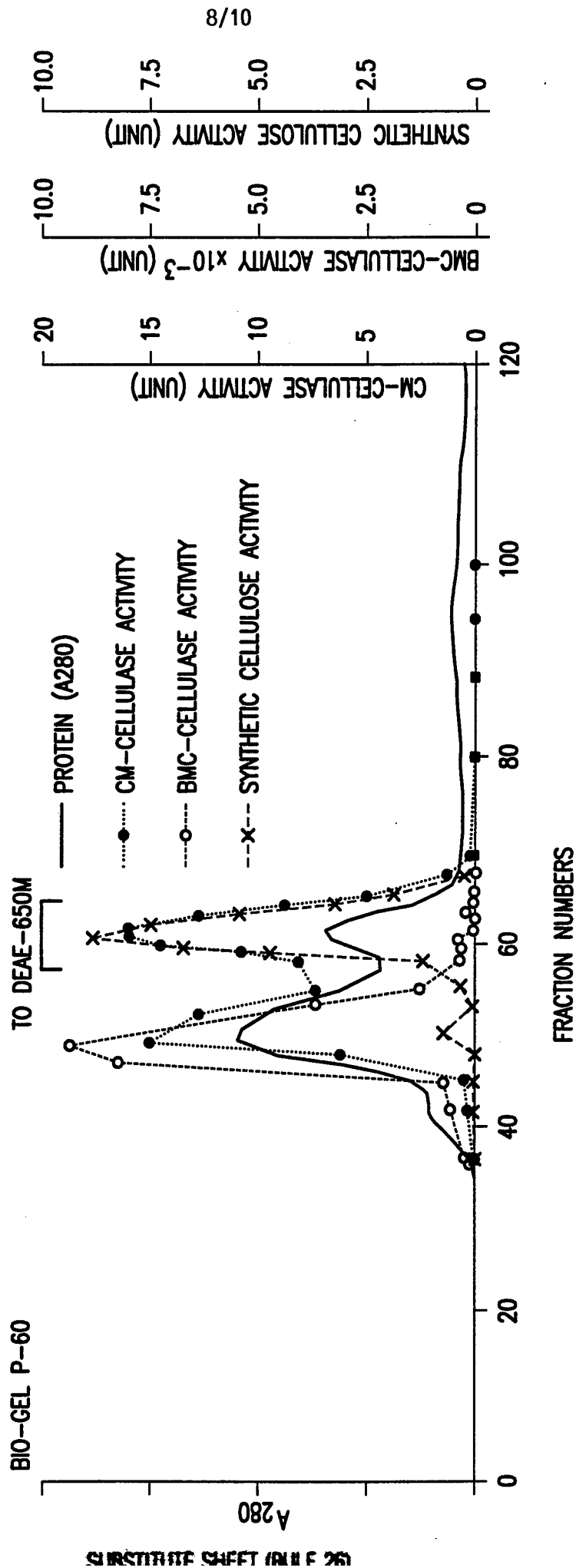


FIGURE 6

9/10

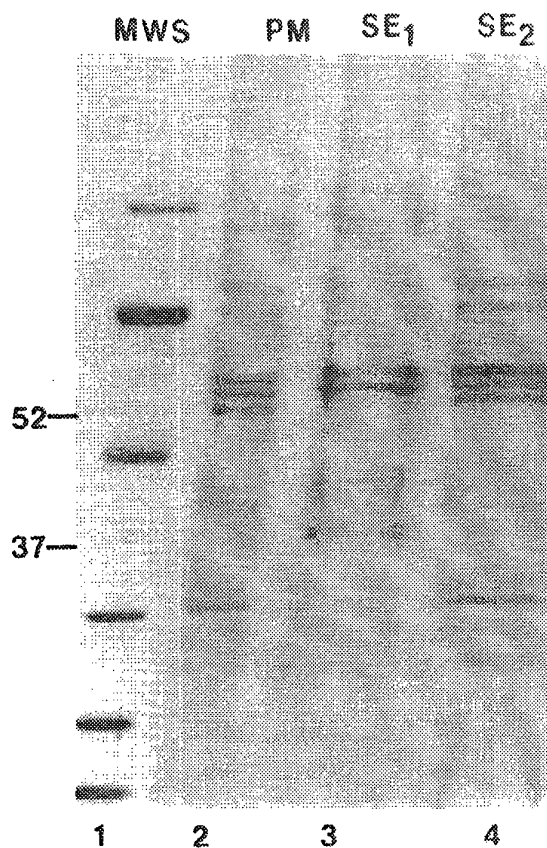


FIGURE 7A

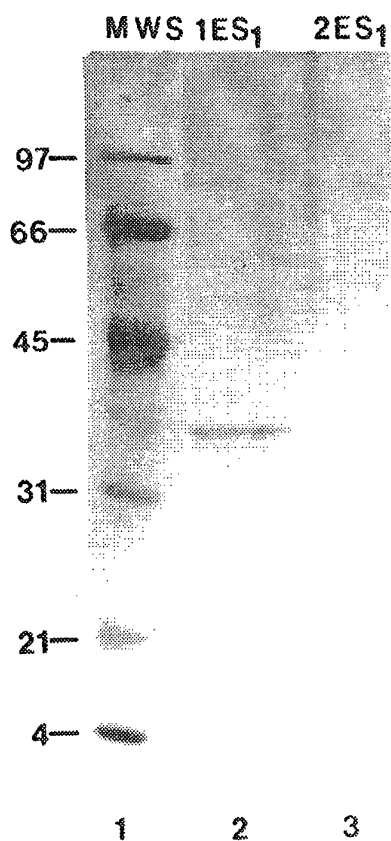


FIGURE 7B

10/10

FIGURE 8A

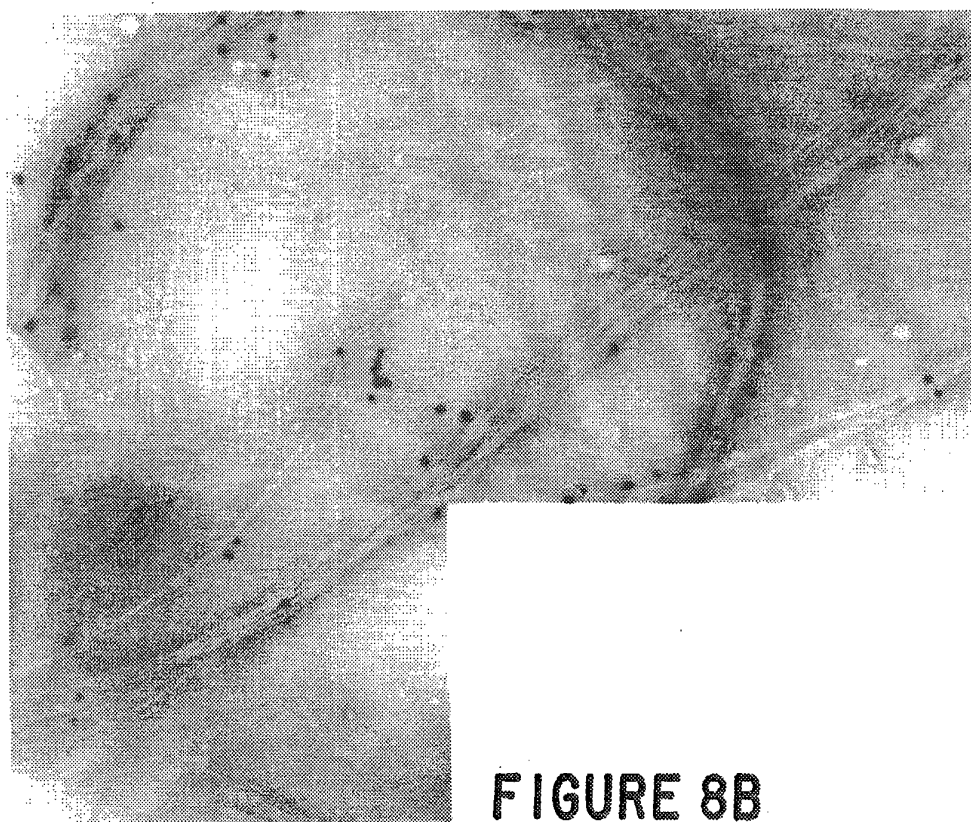
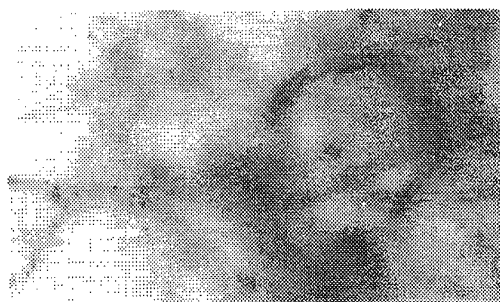


FIGURE 8B

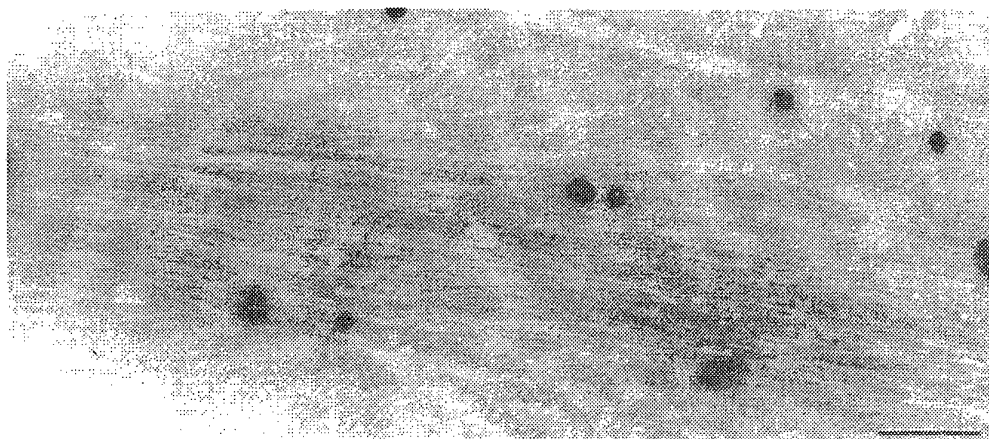


FIGURE 8C