

Isolation and culture of anucleate protoplasts from cotton fiber; assessment of viability and analysis of regenerated wall polymers

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Abstract. Procedures were developed for the isolation and culture of an anucleate protoplast system from cotton fibers actively undergoing secondary wall synthesis. Because the fibers at this stage are elongated single cells ($30\ \mu\text{m} \times 1\text{--}2\ \text{cm}$), most of the cellular vesicles released in the process of isolation are anucleate. After purification, the protoplast population was nuclei-free. When transferred to culture medium, the anucleate protoplasts (cytoplasts) synthesized starch, hydrolyzed fluorescein diacetate for up to 9 days and formed cell wall material for at least 7 days. The composition of the regenerated cell walls was dependent upon the substrate supplied in the medium: β -1,3-linked glucans were predominantly synthesized when 1 mM UDP [^{14}C] glucose was supplied; β -1,4-linked glucans were predominantly synthesized when 1 mM [^{14}C]-glucose was supplied. Thus the composition of the regenerated cell walls formed by the anucleate protoplasts was similar to the secondary cell wall synthesized by intact cotton fibers under the same culture conditions.

Introduction

In cotton (*Gossypium hirsutum* L.) fiber cells, the change from primary to secondary cell wall synthesis occurs ca. 18 days postanthesis in greenhouse-grown bolls and 14 days postanthesis in ovules grown in vitro [24]. At this stage in development, the fiber cells extend ca. 4 cm for boll-grown fibers and 2 cm for fibers grown in culture. A protoplast system from pre-fiber ovule epidermal cells isolated during primary cell wall synthesis on the day of anthesis has been developed [16]. A similar system to study precursor utilization during active secondary wall synthesis was desired; however, because of the elongated nature of the fiber cells at the onset of secondary wall synthesis, intact, nucleated protoplasts could not be obtained. Many anucleate protoplast-like vesicles could be isolated from this stage and the possibility of using these 'cytoplasts' (or 'subprotoplasts') in studies concerning wall metabolism was explored.

Anucleate plant cell systems have been developed for use in somatic cell hybridization as carriers of cytoplasmic traits, such as male sterility [1, 6, 23]. Anucleate algal cells of *Micrasterias* and *Acetabularia* have been shown

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to be valuable in physiological studies concerning the role of the nucleus on cellular activities in development [5] and anucleate cells of higher plants have been used in studies concerning nuclear influence on chloroplast metabolism [29].

Anucleate cells of higher plants have not been used in cell wall metabolism studies previously. Anucleated yeast protoplasts were reported to be unable to synthesize the fibrillar β -1,3-glucan components of the wall [22]. However, if it is assumed that the glucan synthetases present in a cell at the time of isolation have a low turn over, then cell wall glucan biosynthesis in anucleated cells should continue until the substrate is exhausted or the synthetases are no longer active. In order to evaluate the usefulness of the cotton fiber cytoplasts in this respect, we had to determine the functional life-time, and synthetic capabilities of the anucleate cell fragments. In this paper we report the isolation and culture of cotton fiber cytoplasts, as well as the data regarding viability and cell wall regeneration of the system and the analysis of the regenerated wall material.

Materials and methods

Fiber cytoplasts can be isolated from fiber cells of cotton ovules grown *in vitro* at any time after the fibers have initiated elongation. For our studies concerning secondary wall metabolism, the fibers of ovules grown in culture for 15 to 17 days [24] were used. Attempts to isolate cytoplasts from fibers of greenhouse-grown bolls have been unsuccessful, due to rupture of the plasma membrane upon separation from the cell wall during plasmolysis. This occurred to a much lesser extent with culture-grown fibers.

Enzyme mixture

Fifty ml of enzyme solution was prepared according to Gould *et al.*, [16], with the substitution of 28 000 units (0.7 g/50 ml) "CEL," purified cellulase (Worthington/Cooper Biomed) for Cellulysin, and the addition of 0.1 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The concentrated enzyme mixture (1100 mOs/kg, or 0.9 M mannitol equivalent), was diluted with culture medium, which adhered to the ovule fibers, to yield a final solution of 740 mOs/kg, or the equivalent of 0.6 M mannitol equivalents.

Isolation and culture of anucleate protoplasts

Ovules of *Gossypium hirsutum* L. (Acala SJ-2) were harvested on the day of anthesis and cultured according to procedures of Beasley and Ting [3] with the following modifications [mg/l:], indole acetic acid (IAA), 3.5; Kinetin, 0.004; gibberellic acid (GA), 0.18; H_3BO_4 , 31.00.

The contents of 7 to 9 flasks of cultured ovules (ca. 20 ovules/flask) were pooled, leaving enough culture medium to cover the ovules. The submerged

tissue was placed under reduced pressure (aspirator driven) for 10 min in order to wet the fiber walls. Excess medium was drained from the ovules. Twenty-five ml of the enzyme mixture was added immediately, and 25 ml added 15 to 20 min later to lessen the severity of the osmotic change. The tissue was incubated on a gyrotory shaker at 60 rpm. After 2 to 3 h, when most of the fiber cells had become detached from the ovules, the ovules were removed from the digestion mixture. The detached fiber cells were further incubated for 2 h. Ten ml of 40% ficoll solution (w/v) in 0.1 M CaCl_2 and 0.17 M sorbitol was gradually added to the incubation mixture [final ficoll concentration was ca. 4.5%]. The suspension was passed through a stainless steel mesh (1.0 mm² pore size) and centrifuged 1000 × g for 30 min. Cytoplasts collected in the top ml of the solution; fiber debris, protoplasts containing nuclei, and broken protoplasts collected in the pellet. Three percent ficoll in 0.1 M CaCl_2 /0.17 M sorbitol was layered directly on top of the enzyme suspension and the subprotoplasts allowed to float into the less dense layer (10 to 15 min). The upper layer containing the cytoplasts was transferred to a sterile 15 ml conical centrifuge tube and centrifuged 1000 × g for 10 min. Cytoplasts collected at the top of the solution. Culture medium was layered directly on top of the gradient and the cytoplasts allowed to float into the layer (10 to 15 min). This layer was removed and the floatation process repeated until most of the cytoplasts had been transferred from the 3% ficoll layer to culture medium. Cytoplasts were diluted to a final density of 2×10^5 cytoplasts/ml and cultured according to Gould et al. [16]. Glucose and/or UDPglucose were supplied, 1 mM; [¹⁴C]glucose and/or UDP[¹⁴C]glucose (ICN) were supplied as described for each experiment.

Viability and cell wall formation were determined as previously described [16]. The presence of nuclei was determined using a 0.0005% solution of 6-diamidino-2-phenylindole (DAPI, Sigma) fluorescence.

Isolation of newly synthesized polymers

Cytoplasts were harvested after 7 d of culture. Cultures were pooled and diluted once with water and centrifuged 1000g, 10 min. The pellet was washed 3 times with water and extracted in one of two ways: (a) extraction with hot water, chloroform/methanol and acetic/nitric reagent (A/N) was followed as outlined previously [11, 16]; (b) for analyses of cell wall composition, cultures were pooled, pelleted and extracted with water as described above and separated by filtration through a millipore 0.2 μ filter into a 'hot water soluble' fraction and a pellet. Prior to acetylation and periodation analysis, the cellular pellet was treated with α-amylase to remove starch [21].

Analysis of neutral sugars

The cell wall fraction was hydrolysed in 2 N trifluoroacetic acid (TFA), and neutral sugars were reduced, acetylated, and analyzed as previously described [16].

of the fluorescent moiety by the fiber cytoplasts continued for up to 9 d of culture. After this time, however, the fluorescence of the medium was observed, indicating loss of membrane integrity. After one week, ca. 35% of the total label incorporated from [^{14}C]glucose or UDP[^{14}C]glucose was released by α -amylase from the hot water insoluble pellet of cytoplasts indicating a continued synthesis of starch. Calcofluor staining of cytoplasts was apparent within 24 h, fluorescence encompassed ca. 10% of the cytoplasts. After one week of culture, ca. 90% of the cytoplasts showed fluorescence with Calcofluor indicating continued synthesis of cell wall material. Cell wall regeneration was further investigated.

Time course of cell wall regeneration

Incorporation of the label from UDP[^{14}C]glucose into hot water, chloroform/methanol, and total glucan (A/N soluble plus A/N insoluble) fraction was followed over a period of 7 d (Figure 2). A significant increase of label in

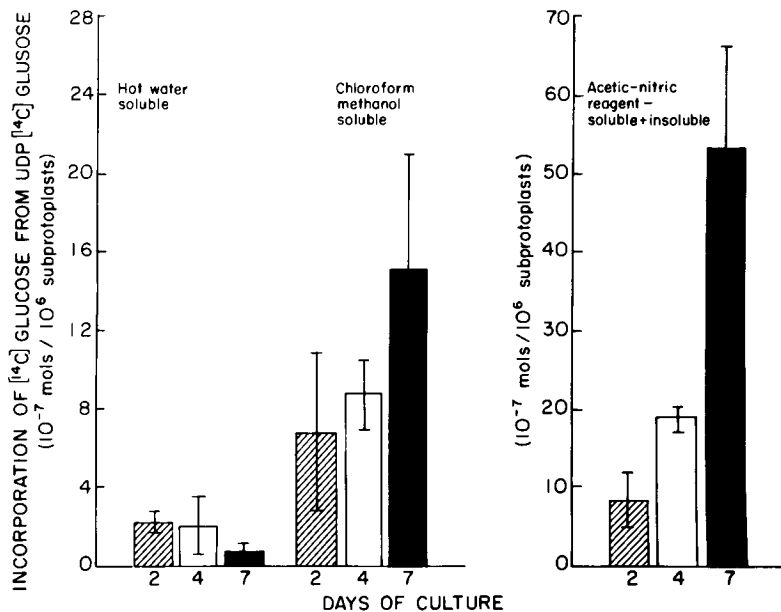


Figure 2. Time course of label incorporation. Incorporation of radioactivity from UDP- ^{14}C]glucose into the hot water soluble, chloroform/methanol soluble and cell wall fractions of the subprotoplasts after 2, 4, and 7 days of culture. Cell wall fraction is defined as A/N reagent soluble plus A/N reagent insoluble components which contain the glucans and other polymeric carbohydrates of the regenerated cell walls. Cytoplasts were cultured in medium containing UDP[^{14}C]glucose (61.4 kBq/nmol), unlabeled 1 mM UDPglucose, and 1 mM glucose for the time indicated.

the glucan fraction indicated cell wall biosynthesis through this period. Also, a large proportion of label ($\sim 30\%$), was associated with the lipid (chloroform/methanol soluble) fraction of the cytoplasts.

Neutral sugar analysis of cell wall components

The distribution of label into the neutral sugar components of the TFA hydrolyzed polysaccharides synthesized after culture in UDP[^{14}C]glucose for 7 d is presented in Figure 3. The analysis profile showed that the majority of label was incorporated into glucose containing polymers. Other labeled sugars found in the polymeric material were arabinose, xylose, mannose and galactose.

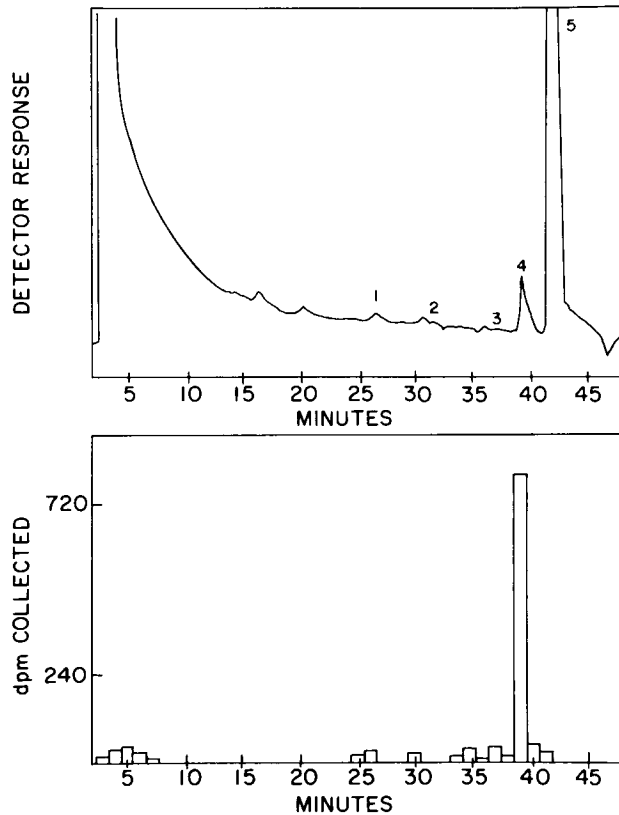


Figure 3. GC-analysis of the neutral sugar components and distribution of label in the cell wall regenerated by the anucleate cells. Cytoplasts were cultured for 7 days in medium containing UDP[^{14}C]glucose (61.4 kBq/nmol), unlabeled 1 mM UDPglucose, and 1 mM glucose. 1 = arabinose; 2 = xylose; 3 = mannose; 4 = glucose; 5 = myo-inositol standard.

Linkage analysis

Chromatography of the periodation products from cell wall material synthesized by cytoplasts cultured on medium containing UDP[¹⁴C]glucose, yielded 86% labeled glucose and 4.8% labeled glycerol and erythritol. This suggests a preponderance of β -1,3-linked glucans and that only small amounts of β -1,4-linked glucans were present.

Methylation and subsequent GC/MS analysis of the cell wall polymers synthesized by subprotoplasts cultured with [¹⁴C]glucose showed β -1,4-linked glucose and terminal glucose units (Figure 4). Because the material had been treated with α -amylase, the remaining 1,4-linked material is assumed to be in the β configuration.

Discussion

Isolation

Elongated cells, such as palisade cells and cells from suspension culture, have been frequently used as starting material to obtain cytoplasts [1, 6, 23]. This technique is supplemented with high speed centrifugation, which forcibly removes nuclei from the protoplasts [23]. The procedure reported here utilizes these approaches. However, because of the large surface to volume ratio of the elongated fiber cells, the initial ratio of cytoplasts containing nuclei to those without nuclei, can be 1:100 prior to purification. Following centrifugation, the preparation was completely free of protoplasts containing nuclei.

Viability

The cytoplasts displayed some continued metabolic functioning for up to 9 d. Fluorescein uptake and retention by the cytoplasts as well as incorporation of the [¹⁴C]glucose into cell wall glucans between day 4 and 7 after start of culture suggests that the wall synthesizing machinery was still functioning for some time after the 4th d of culture.

Utilization of UDPglucose

Vesiculation of the fiber cell occurs, *in situ*, within the fiber cell wall and therefore, all cytoplasts formed are 'right-side out.' Vesicles prepared from plasma membrane fractions can form 'inside out' as well as 'right-side out'. Determination of the percentage of right-side out vesicles in such a preparation is a problem faced by workers in the field [18]. The question of sidedness is important in both the plasma membrane vesicle and cytoplast systems because of the unknown mode of uptake or utilization of molecules such as UDPglucose when supplied to the outer face of the plasma membrane. Hydrolysis of UDPglucose by phosphatases released by membrane vesicles

and cytoplasts is expected. Because of extra-cellular hydrolysis of UDP[^{14}C]-glucose and liberation of the [^{14}C]glucose moiety, it was anticipated that label would be taken up and utilized by the cytoplasts in the same manner as [^{14}C]glucose. However, our preparations incorporated the label from UDP[^{14}C]glucose differently than the label from [^{14}C]glucose (Figure 3 and 4). Furthermore, incorporation of label into the A/N insoluble fraction from UDP[^{14}C]glucose (1 mM, supplied in the culture medium) was not diminished when cytoplasts were simultaneously cultured in varying concentrations of unlabeled glucose (up to 1 mM) in experiments designed to test competition of UDPglucose and glucose [15]. The data suggests that UDPglucose was utilized differently than glucose by the cytoplasts and the difference could not be attributed to hydrolysis of the molecule.

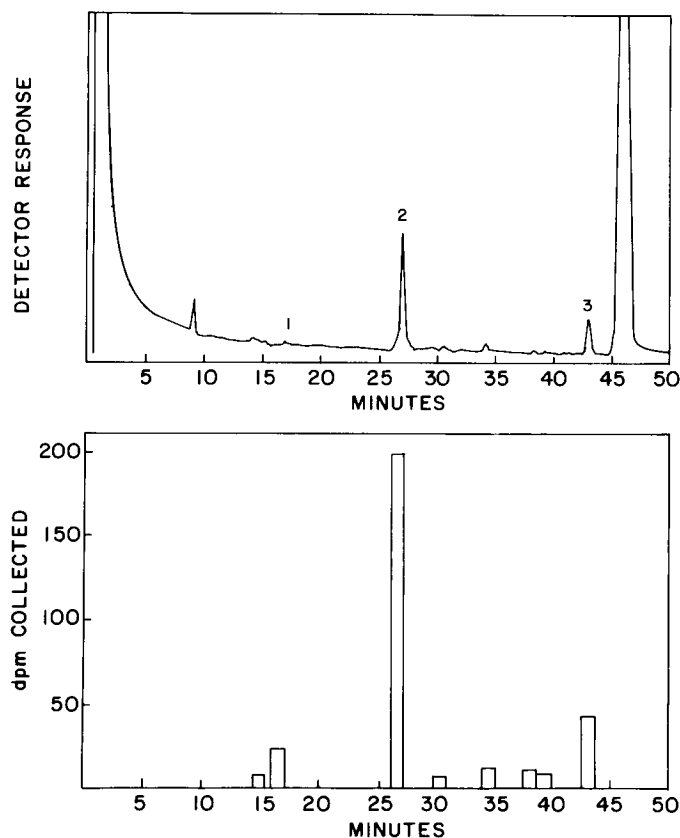


Figure 4. GC-analysis of sugar linkage in synthesized cell wall polymers and representation of the distribution of label in permethylation analysis. Subprotoplasts were cultured for 7 days in medium containing [^{14}C]glucose (61.4 kBq/nmol) and unlabeled 1 mM glucose. 1, equivalent to terminal glucose; 2, equivalent to 1,4-linked glucose; 3, equivalent to multicross-linked hexoses which may be caused by under-methylation of cellulose.

These results are comparable with results obtained using intact cotton fibers. In short term (20 min) labelling experiments, label from UDP[¹⁴C]-glucose, supplied in the incubation medium of cotton ovules, was incorporated into A/N insoluble material at a much greater rate than from [¹⁴C]-glucose [12, 13]. An explanation for these data is that UDPglucose may be utilized at the outer surface of the cell by the glucan synthetase complexes which are thought to span the plasma membrane [12].

Although UDPglucose is the presumed precursor of cellulose, this has been difficult to prove [10]. In all systems utilized from higher plants, which include intact cotton fibers [11, 20, 24] protoplasts [16, 21] and plasma membrane preparations [8, 9], the predominant products of UDPglucose have been β -1,3-linked glucose polymers rather than the β -1,4-linked glucose polymer. The reasons for this are unknown, but may reflect a response to wounding [7] and to changes in the concentration of Ca⁺⁺ on both sides of the plasma membrane [10].

Because of these difficulties, a cell free cellulose synthesizing system, which would demonstrate precursor and cofactor requirements, has not been developed from higher plants. A recent study reported high rates of β -1,4-glucan, defined as 24% KOH insoluble product, synthesized from UDP[¹⁴C]-glucose by plasma membrane preparations derived from *Phaseolus aureus* [8]. Later analysis of the product revealed a predominance of β -1,3-linked glucan synthesized from UDPglucose [9], which corroborates our findings.

Incorporation of label into a lipid fraction

A high proportion of label retained by the cellular pellet (30%) was found in the chloroform/methanol extract of fiber cytoplasts cultured with 1 mM UDP[¹⁴C]glucose. Approximately 0.4 to 8% of label was incorporated into this fraction by pre-fiber protoplasts cultured with 1 mM [¹⁴C]glucose [16] and 12% by intact fiber cells cultured with 1 mM UDP[¹⁴C]glucose. When intact fibers were cultured with decreased, 5 μ M UDP[¹⁴C]glucose, however, incorporation into the chloroform/methanol fraction was as high as 80% [13]. This lipid-rich fraction may contain intermediates involved in complex polysaccharide synthesis [14] and lipid intermediates are thought to be involved in glucose translocation through membranes [19]. The reason for the differences in the amount of label incorporation into the lipid fractions of the pre-fiber protoplasts and the anucleate fiber protoplasts are unknown. One possibility is that the differences in incorporation reflect developmental differences in metabolism due to differences in developmental stage at isolation. In this view, the anucleate fiber protoplasts continue a metabolism similar to that of intact fiber cells. A more likely possibility is that because of the finite nature of the anucleate fiber protoplasts, a buildup of label occurs in products with more stable synthases, as activities of other enzymes, such as the β -1, 3- and β -1,4-glucanases, decay.

Characterization of the regenerated cell wall

In preliminary experiments, [^{14}C]glucose incorporation into wall polymers by fiber cytoplasts, an average of 29% of the incorporated label was recovered as A/N insoluble material [15] in contrast to ca. 7.1% incorporated into this fraction by pre-fiber protoplasts cultured under identical conditions [16]. Acetic-nitric reagent insolubility is a characteristic of high molecular weight cellulose [27]. When UDP[^{14}C]glucose was supplied, 8 to 10% of the labeled moiety was incorporated into the A/N insoluble fraction by fiber cytoplasts.

Based on sugar and linkage analysis, the polymers formed by cytoplasts in culture with 1 mM [^{14}C]glucose were β -1,4-linked glucans. The polymers synthesized by fiber cytoplasts cultured in 1 mM UDP[^{14}C]glucose were predominantly β -1,3-linked glucans, and a smaller amount of β -1,4-linked glucans. These data are comparable with results reported using intact cotton fibers incubated with 1 mM UDP[^{14}C]glucose and 1 mM [^{14}C]glucose during active secondary wall synthesis [13]. The fiber cytoplasts, isolated during active secondary wall synthesis, incorporated label from precursors in a manner similar to that of intact fiber cells during the same stage of wall synthesis.

Comparison of regenerated wall material to that of other protoplast systems

Plant protoplasts typically synthesized walls containing low amounts of cellulose regardless of the source or developmental stage of the original tissue. Micrographs of the surface of tobacco and other protoplasts during early stages of cell wall regeneration reveal dense networks of fibrillar wall material, which appear to be cellulosic, surrounding the protoplasts [28]. Analysis of cell walls regenerated by plant protoplasts, however, indicate that the walls contain a cellulose content between 0 and 8% [2, 4, 21, 25, 26]. Tobacco protoplasts isolated from leaf mesophyll cells containing secondary wall material, regenerated cell walls containing 5% cellulose [4]. Protoplasts of *Vinca* regenerated cell walls composed of β -1,3- and β -1,4-linked non-cellulosic glucans and differed in composition from the wall material of the original cell suspension culture [26].

A question raised by these and similar studies involving cell wall regeneration by plant protoplasts is why the cell walls regenerated by protoplasts are different in composition from the cell walls of the original tissue? The hemicellulose and extension content in callus cell walls can be influenced by the growth regulators in the culture medium [17]; however, the influence of the growth regulator composition of the medium on the regeneration of cell walls in plant protoplasts is unknown. Because protoplasts, isolated from tissue which has synthesized secondary cell wall material, regenerate cell walls of a different composition [4], suggests that changes in metabolism occur in protoplasts either during the isolation process or during culture. It is also

possible that the cell wall regenerated by a protoplast is more comparable with the phragmoplast in formation as well as in composition, than with any other specific cell wall type [N. Carpita, personal communication]. On the other hand, cell wall regeneration by protoplasts may be a wound response since the composition of the regenerated wall is usually high in β -1,3-linked glucans, comparable with callose [4], and synthesis of β -1,3-linked glucans as a response to wounding has been reported for suspension-cultured soybean cells [7].

In the cotton fiber cytoplasm system, the absence of a nucleus imposes a limited metabolic life-time on the cell wall synthesizing complexes; in spite of this, β -1,4-linked glucose polymers can be synthesized from glucose. The enrichment of β -1,4-linked glucose polymers in the wall material synthesized by cytoplasts when compared to those of other protoplast systems may be due to the anucleate nature of the system which prevents a shift in wall metabolism from occurring and/or to isolation of the cells during a period of intense secondary wall synthesis.

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