Protoplasma (1988) 143: 165–169

# An Extrinsic Marker for Higher Plant Cell Walls

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Received September 14, 1987
Accepted December 10, 1987

## Summary

Cytochrome c was ionically bound to cell walls as a useful extrinsic marker. The marker binds rapidly, can readily be removed by salt treatment, and can be quantitated by conventional spectrophotometric procedures. The use of the marker allows each step of a cell wall purification to be monitored qualitatively and, furthermore, it provides a rapid, simple quantitative method to determine the yield of purified cell walls once isolated from the cell.

*Keywords:* Purification; Yield; Cell walls; Extrinsic marker; Cytochrome c.

*Abbreviations:* pI Isoelectric pH; EDTA Ethylenediaminetetra-acetic acid; kD kiloDaltons; Cyt c Cytochrome c; HEPES N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES 2(N-Morpholino)ethanesulfonic acid.

## 1. Introduction

The plant cell wall is considered to be a complex, dynamic, extracellular matrix which is composed of complex polysaccharides, structural protein, and enzymes. To study the function or biological activity of cell wall components, a rapid purification method for cell walls is required and a method for determining yield is desired. The quantification of cell walls has been difficult because of the lack of an easily assayable marker (HARRIS 1983). To find a positive marker, one approach has been to look for a unique intrinsic or endogenous enzyme which is specifically associated with cell walls. In all cases, however, the cell wall-associated enzymes have a soluble counterpart found in the cytoplasm and so far, selective inhibition has not provided a distinction between isozymes (NAGAHASHI and SEIBLES 1986). A second approach would be to extrinsically label the cell wall and monitor the marker during purification. Cytochrome c appears to be an ideal externally applied marker because it is small in size (12 kD), can readily diffuse through the wall matrix, and it is a basic protein (pI = 10.6) which reversibly binds to the negative charges in the wall.

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## 2. Materials and Methods

### 2.1. Plant Material

Corn seeds (*Zea mays* L., WF9 X MO17) were germinated and harvested as described previously (NAGAHASHI *et al.* 1986). The cortex (16–18 g fr wt) was physically removed from the stele and used as a source of primary cell walls. Red delicious apples and Russet potatoes were purchased at a local market. Storage tissue was first peeled, diced (50 g fr wt), and ground with a mortar and pestle. All tissues were initially homogenized at 4 °C in either 1 mM $Na_2S_2O_5$ plus 5 mM 2-mercaptoethanol and 2 mg/ml cytochrome c or standard homogenization medium as described in the text.

### 2.2. Cell Wall Isolation and Purification

For all tissue types, the subsequent cell disruption was done with a Parr $N_2$ bomb followed by sonication (NAGAHASHI and SEIBLES 1986). The crude homogenates were sequentially centrifuged at 1000 **g** for 5 minutes (crude cell walls), 6000 **g** for 20 minutes (crude mitochondria), and 80,000 **g** for 35 minutes (crude microsomes). The pelleted fractions were washed in cold water to remove unbound cytochrome c and particulate-bound cytochrome c was determined as described below. Cell walls were further purified by washing in water, trapping the walls on a cheesecloth sieve (40 × 38 threads/in$^2$), and sifting through the contaminants. Cell walls isolated and purified by these procedures were shown to be free of morphologically identifiable contaminants and were free of biochemical markers for cyptoplasmic components (NAGAHASHI and SEIBLES 1986).

*2.3. Quantitative Assays*

The cell wall-bound cytochrome c was solubilized by layering a 1 ml aliquot of cell wall suspension over 1 ml of 3 M NaCl. Initially, the cell walls floated on top of the salt and after 15 to 20 minutes they sedimented to the bottom leaving the solubilized cytochrome c behind. After salt extraction, the solubilized cytochrome c was reduced with crystals of sodium dithionite and read immediately at 550 nm in a Beckman model 35 spectrophotometer. The recovery of solubilized cytochrome c was greater than 98% of the original bound protein. For membrane fractions, 0.02% triton X-100 was included in the salt treatment and extracted membranes were repelleted at 100,000 **g**. The extinction coefficient of reduced cytochrome c was not changed by the presence of 1.5 M NaCl ($E_{1cm}^{1mM} = 27.2$). Estimation of protein was performed by a modified LOWRY (MARKWELL *et al.* 1978) and phosphatidyl choline was determined as described previously (DITTMER and WELLS 1969).

## 3. Results and Discussion

*3.1. Recovery and Enrichment of the Extrinsic Marker*

After complete cell disruption in medium containing cytochrome c, three particulate fractions were collected by differential centrifugation (Table 1). Although most of the cytochrome c (97%) was bound to the crude cell wall fraction (1000 **g** pellet) of potatoes and apples, considerable binding to non-cell wall components was observed for corn tissue (Table 1). The crude cell walls were purified by washing in water, trapping the walls on a cheesecloth sieve, and sifting through the membrane vesicles, cytoplasmic fragments, and large organelles. Since starch grains did not bind cytochrome c they were easy to distinguish from the red cell walls (Fig. 1). To effectively rid cell walls of starch grains, potato tuber cell walls were washed and trapped twelve times on a single layer of cheesecloth (40 × 38 threads/in$^2$). For apple tissue, four layers of this cheesecloth were used and the washing step was repeated four times. The recovery of cell walls after washing was 88% for tuber cell walls and 82% for apple cell walls as judged by the recovery of bound cytochrome c in the purified cell walls compared to the crude 1000 **g** pellet. These results indicate an overall recovery of 85% for tuber cell walls and 79% for apple cell walls from the original cellular homogenates. Calculations were based on the assumption that the amount of membrane bound cytochrome c in the 1000 **g** pellet was negligible and could be ignored. The assumption was justified by our previous report (NAGAHASHI and SEIBLES 1986) which showed the 1000 **g** fraction only contained less than 3% of the total membrane phospholipid. The total amount of membrane-associated cytochrome c contributing to the crude cell wall fraction was estimated to be less than 0.1%. The slightly lower yield of apple cell walls during the washing procedure was attributed to larger loss of small fragments which passed through the sieve as well as a loss of wall fragments entrapped in the multiple layers of cheesecloth. To rapidly remove starch grains from potato tuber cell walls, a coarse sieve (24 × 24 threads/in$^2$) can be used. After 5 washings, starch was effectively removed but the loss of cell walls through the coarse sieve was greater (54% recovery of cell walls).

While providing a method for quantitating percent recovery of cell walls, from crude preparations, the cytochrome c marker was not useful for estimation of purity (Table 2). Purity of isolated cell walls, as estimated by bound cytochrome c, depended on how the enrichment was expressed. For example, when enrichment of the marker was based on mg cytochrome c bound/mg protein, potato tuber cell walls only showed a 2.3-fold increase over the 1000 **g** pellet. If enrichment

Table 1. *The distribution of exogenously bound cytochrome c to particulate fractions isolated from plant tissues.* Tissues were suspended in 1 mM $Na_2S_2O_5$, 5 mM 2-mercaptoethanol, and 2 mg/ml cytochrome c and then ground with a mortar and pestle, extruded from a $N_2$bomb, and sonicated. The cellular homogenates were sequentially centrifuged as indicated. The pelleted fractions were washed in cold water to remove unbound cytochrome c and particulate-bound cytochrome c was determined. The 1000 **g** pellet from apples contained 45.4 mg cytochrome c while the 1000 **g** pellet from potatoes contained 96.1 mg cytochrome c. The percentage was based on the total particulate-bound cytochrome c. ± Range is the deviation from the mean for 3 experiments with corn and potatoes and 2 experiments with apple tissue. For corn tissue, (root cortex or coleoptiles), considerable amounts of non cell wall material (20%) also bound cytochrome c and pelleted with cell walls at low speed centrifugation. This contaminant was discovered when the 1000 **g** pellet was washed on the sieve and pinkish red particulate matter passed through. Although, the contaminant was readily distinguished from cell walls and easily removed by sieving, it was difficult to quantitate recovery of corn cell walls from the crude homogenate

| Crude particulate fraction | Potato tuber homogenate | Apple fruit homogenate | Corn root homogenate |
|---|---|---|---|
| | | % Cytochrome c Bound | |
| Cell walls (1000 **g**; 5 minutes) | 97.3 (± 0.3) | 96.5 (± 0.9) | 94.1 (± 2.0) |
| Mitochondria (6000 **g**; 20 minutes) | 1.1 (± 0.1) | 1.3 (± 0.6) | 5.0 (± 2.0) |
| Microsomes (80,000 **g**; 35 minutes) | 1.6 (± 0.2) | 2.3 (± 0.2) | 0.9 (± 0.2) |

Fig. 1. Photomicrograph of crude cell walls isolated from potato tubers in the presence of exogenously added cytochrome c. The crude cell wall fraction shows lack of staining by starch bodies. Some starch bodies appear to be slightly stained however, this is due to the stained overlapping cell wall. Pictures were taken with a Leitz Orthoplan light microscope. × 150

was based on mg cytochrome c/nmole phospholipid, then a 24-fold enrichment was observed. In storage tissue such as potato tubers and apple fruit, the major cell wall contaminant was starch and its removal did not increase either ratio. When enrichment was expressed as mg cyt c/mg dry wt, a 14-fold enrichment was observed because starch makes up most of the weight of a 1000 **g** pellet. In this regard, expression of enrichment of any marker for cell walls may not necessarily indicate the purity of the cell wall fraction. The purity of cell walls used in this study was evaluated previously (NAGAHASHI and SEIBLES 1986) by morphological examination in conjunction with biochemical analysis of cytoplasmic associated markers. This approach still appears to be the best method for evaluating purity of isolated cell wall preparations (DRACUP *et al.* 1984, GOLDBERG 1985). Cell walls used in this study contained no visible organelles or membrane vesicles at the ultrastructural level and were free of cytoplasmic biochemical markers.

### *3.2. Binding-dissociation of Extrinsic Markers*

Because cytochrome c binding was greatest under acidic conditions and the binding was considerably reduced at high ionic strength the marker cannot be applied in homogenization mediums normally used in plant cell fractionation studies (Fig. 2). Standard homogenization medium can be used when cytochrome c is applied to aliquots of various particulate fractions *after* they have been isolated and resuspended in water or low molarity buffer. When the marker was applied in this manner, the distribution of cytochrome c was virtually identical to the results in Table 1.

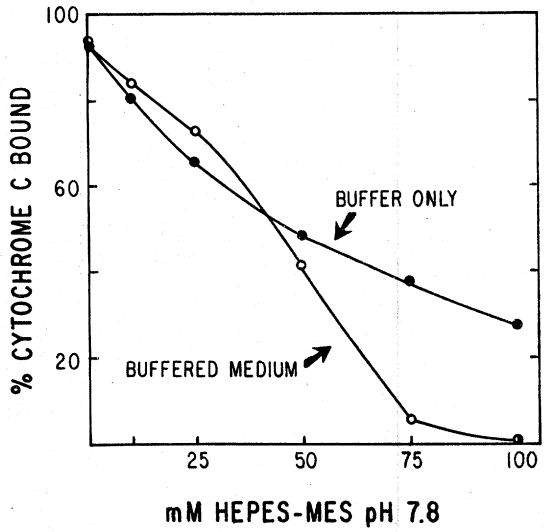


Fig. 2. The effects of homogenization medium on the binding of cytochrome c to purified potato tuber (Russet) cell walls. Cell walls were purified as described previously. As the ionic strength of the buffer at pH 7.8 is increased, the binding of exogenously added cytochrome c decreases. When a standard homogenization medium (0.3 M sucrose, 1 mM $Na_2S_2O_5$, 5 mM 2-mercaptoethanol, 5 mM NaEDTA and 0.1 M HEPES-MES pH 7.8) is used at full strength, minimum binding is achieved. As the standard homogenization medium is diluted to give the final buffer concentrations as indicated, the binding in buffer or buffered homogenization medium is very similiar at low molarity buffer (less than 50 mM). Bound cytochrome c was solubilized and quantitated as described in the text

Other attempts to use histochemical stains (congo red, ruthenium red, toluidine blue O) for cell walls as extrinsic labels met with limited success. Stains were difficult to quantitate because they were not readily removed from cell walls, they have broad absorbance

Table 2. *Enrichment of an extrinsic cell wall marker (cytochrome c) in a potato tuber preparation.* Crude cell walls (1000 **g** pellet) and purified cell walls were first isolated and aliquots were used to determine bound cytochrome c, total protein, dry weight, and total phospholipid content

| | Total bound Cyt c (mg) | Total protein (mg) | Total dry weight (mg) | Total phosphatidyl choline (nmoles) |
|---|---|---|---|---|
| Crude cell walls | 308.0 | 12.67 | 5667.2 | 425.4 |
| Purified cell walls | 282.2 | 5.16 | 380.0 | 16.7 |
| | mg cyt c/ mg protein | mg cyt c/ mg dry wt | mg cyt c/ nmoles PC | |
| Crude cell walls | 24.31 | 0.054 | 0.72 | |
| Purified cell walls | 54.69 | 0.743 | 16.90 | |

spectras, absorbance was pH dependent, and some are carcinogenic (congo red). Although toluidine blue O was removed from potato tuber cell walls after multiple washes in 3.0 M NaCl, the stain was not completely removed from apple or corn root cell walls. Cytochrome c as an extrinsic marker has many advantages over cell wall stains in that, 1) it reversibly binds to the cell wall for easy quantitation, 2) it is nontoxic, 3) the extinction coefficient is not affected by pH (MARGOLIASH and FROHWIRT 1959) or the presence of salt, 4) it has a narrow absorbance spectrum and, 5) it provides a visible monitor for cell walls during any aqueous purification procedure, which uses a filtration, differential centrifugation, or density gradient centrifugation step.

## Acknowledgements

We thank BEVERLY MALEEFF for the excellent photomicrographs and ROBERT MOREAU for phospholipid analysis.

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Protoplasma (1988) 143: 170–175

# Perinuclear Organization in the Heterotrich Ciliate *Stentor coeruleus*

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Received October 12, 1987
Accepted November 25, 1987

## Summary

Different fixation techniques were employed to obtain satisfactory fixation of the endoplasm of *Stentor coeruleus* for ultrastructural investigations. The nuclei of *S. coeruleus* are surrounded by a flattened fenestrated cisterna. The space between the nuclear envelope and the cisterna (=perinuclear space) is continuous with the cytoplasm via channels. The envelopes of both, micronucleus and macronucleus, are connected to the fenestrated cisterna by filamentous material. This organization accounts for the close association between micronucleus and macronucleus in *Stentor coeruleus*. The fenestrated cisterna is compared to similar structures occurring in other organisms, and its possible function is discussed.

*Keywords:* Ciliate; *Stentor;* Nuclei; Fenestrated cisterna.

*Abbreviations:* fC fenestrated cisterna; FV food vacuole; km km fibers; MaNu macronucleus; MiNu micronucleus; My myonome; NE nuclear envelope; PC perinuclear cisterna; PfC pore of fenestrated cisterna; PS perinuclear cytoplasmic space; EGTA ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid); GA glutaraldehyde; HEPES N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PEG polyethylene glycol; PIPES piperazine-N,N′-bis[2-ethanesulfonic acid]; PTA phosphotungstic acid.

## 1. Introduction

The heterotrich ciliate *Stentor coeruleus* has a macronucleus shaped as a string of nodes (=moniliform) and several small micronuclei which are situated in macronuclear depressions (TARTAR 1961, SKARLATO 1982). Both types of nuclei have been studied in detail by light and electron microscopy (PAULIN and BROOKS 1975, PELVAT and EMERY 1982, SKARLATO 1977, TARTAR 1961) and their changes during the life-cycle have been recorded (PAULIN and SKARLATO 1979, RAIKHEL *et al.* 1981, SKARLATO 1979, 1982). Several authors have mentioned that the micronuclei of *S. coeruleus,* like in some other species of the genus (NILSSON 1986), are attached to the macronucleus (*e.g.,* SKARLATO 1982), but the structural base for this attachment has not been described. This may be due to the poor preservation of the cytoplasm and the membranous systems around the nuclei of this large organism, as it is difficult to fix satisfactorly for electron microscopy.

In this ultrastructural study, two modified methods of fixation of *S. coeruleus* are presented. These differ in their effect on the contrast and density of cytoplasmic organelles, but result in a better preservation of the endoplasm of *Stentor* than the commonly used method of HUANG and PITELKA (1973). It is shown that the nuclei of *S. coeruleus* are surrounded by an additional membrane system which appears to be involved in attaching the micronuclear envelope to that of the macronucleus.

## 2. Material and Methods

*Stentor coeruleus* has been collected in a pond (Kuhlake) in Berlin. Clones were obtained by the method of TARTAR (1961), cultured in "Eau de Volvic" (french mineral water) and fed with *Chlorogonium.* For electron microscopy, starved cells were concentrated and washed with fresh culture medium by careful centrifugation, and then transferred in microscopical dishes. Preparation and fixation were as follows:

A. Conventional method (HUANG and PITELKA 1973):

1. Pretreatment (15–20 minutes) with relaxation medium (10 mM EGTA, 50 mM Tris buffer, 3 mM magnesium sulfate, 7.5 mM ammonium chloride, 10 mM phosphate buffer, pH 7.1). Prefixation (1–2 minutes) with 12.5% glutaraldehyde (GA) and 1% phosphotungs-

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