

Protoplasts: Preparation from Higher Plants

Abstract. Protoplasts can be readily prepared from higher-plant tissue with a concentrated *Myrothecium cellulase* preparation. An ionic osmoticum produced a higher yield and more viable protoplasts than did the nonionic mannitol osmoticum. Addition of polyuronidases or of ethylenediaminetetraacetate did not increase the yield. Protoplasts could be prepared from root, leaf, and callus tissue as well as from coleoptiles.

Higher-plant protoplasts (plant cells whose walls have been removed) are a unique tool for use in physiological experiments. Cocking's work (1) and ours (2) demonstrate that the walls can be removed from at least some cell types by sufficiently powerful enzymes. A method has been described (2) for preparing *Myrothecium cellulase* that is six times more powerful, on a dry weight basis, than any *Aspergillus*, *Trichoderma*, or *Helix pomatia* cellulase preparation yet available. We now report an improved osmoticum for isolation and maintenance of the protoplasts, and extension of the list of tissues from which protoplasts have been prepared.

Protoplasts were prepared in conical digestion tubes containing 100 μ l of 2 percent *Myrothecium cellulase* enzyme in 0.5 molal mannitol or a mixture of salts (Table 1) at an equivalent osmotic concentration; a buffer system proved unnecessary. Small pieces of two peeled segments of *Avena* coleoptile were added to each tube in a manner reported (2), and the tubes were incubated in the dark at 25°C for 1.3 hours. Digestion was terminated by

washing twice with the appropriate osmoticum (diluting the enzyme 100-fold), and 10- μ l portions were cautiously transferred to microscope slides. The number of protoplasts was recorded on a hand tally while the slide was scanned. Table 1 compares the numbers of protoplasts released in the various osmotica, and also indicates, as a measure of viability, the percentage of protoplasts in each case that exhibited organized cyclosis (based on careful inspection of 30 to 50 representative protoplasts). The amount of debris present in each portion was rated arbitrarily between 0 and 3; most debris consisted of particles of denatured cytoplasm, sometimes accompanied by bits of cell wall. High values for debris indicated that digestion of cell walls had been satisfactory, but that the protoplasts were poorly stabilized and that many had burst. The first group of experiments (Table 1) shows that in all respects the ionic osmoticum was better than the mannitol over a wide range of relative concentrations of CaCl_2 and KCl ; the second and third groups also show that Na^+ could not be satisfactorily substituted for the K^+ , nor Mg^{++} for the Ca^{++} . To keep the external ions at a more physiological level, the optimum system would probably be a small concentration of K^+ and Ca^{++} in conjunction with mannitol, a combination that releases protoplasts about as well as the strictly ionic osmoticum.

Other osmotica have proved unsatisfactory. Compared with mannitol, only 72 percent as many protoplasts were released in 0.5 molal glycylglycine, and only 40 percent of those released exhibited cyclosis. Carbowax 1500 precipitated part of the cellulase enzyme mixture; sucrose both inhibited the re-

Table 2. Tissues tested for release of protoplasts by *Myrothecium* enzyme.

Tissue	Release (~ %)
<i>Suitable</i>	
<i>Avena</i> coleoptile	17
<i>Avena</i> primary leaf	20
<i>Avena</i> root tips	5
<i>Convolvulus</i> callus	40
Lettuce callus	30
<i>Unsuitable</i>	
<i>Acer pseudoplatanus</i> culture	0
<i>Avena</i> mesocotyl	0.001
<i>Coleus</i> petiole	.001
<i>Pisum</i> : green leaf, etiolated stem	.002
Potato tuber	0

lease of protoplasts and caused them to float, thereby increasing handling difficulties.

Under the best of conditions, removal of cell walls from viable cells is difficult. The maximum yield of intact protoplasts released from coleoptile segments amounts to only 17 percent of the cells present. Because cellulase solutions, although capable of digesting native cellulose, are rapidly inactivated by it (3), inordinately great concentrations of cellulase are essential. In the presence of such concentrations, even the walls of vascular tissues are partially degraded, as evidenced by the free helices or rings of lignified secondary wall residues often found in the protoplast preparations (Fig. 1).

Higher-plant protoplasts, because of their 1000-fold greater volume, cannot be centrifuged, washed, and pipetted like bacterial protoplasts without considerable breakage, but some can be maintained if handled carefully. *Avena* coleoptile protoplasts, prepared aseptically in 0.10 molal CaCl_2 and 0.14 molal KCl and plated on osmotically adjusted agar, can be removed to microscope slides and photographed the next day (Fig. 1; bright-field illumination). Such protoplasts exhibit a nucleus suspended in the center of the large central vacuole by numerous branching transvacuolar strands, along which mitochondria and spherosomes can be seen moving briskly; plastids, when present, are usually grouped around the nucleus. Viable coleoptile protoplasts (such as in Fig. 1) can be found even 14 days after plating on solid agar medium, although by that time all protoplasts are considerably enlarged and many have burst.

Some of the preparations contain small spheres less than 20 μ in diameter; these are partial protoplasts (Fig. 1) and can arise in either of two ways: (i) during the release of proto-

Table 1. Yield and viability of *Avena* protoplasts in various osmotica. Absolute yields of cells (from 10- μ l portions) from each treatment: group 1, >80; group 2, >250; group 3, >130. "Relative yield" is relative to the yield in mannitol. Numbers in parentheses are numbers of experiments.

Osmoticum (molal)	Relative yield	Streaming (%)	Debris rating (0-3)
<i>Effectiveness of ionic mixtures</i>			
0.5 Mannitol	1.00 (6)	32 (3)	1.8 (6)
.28 KCl	1.00 (2)	0 (1)	0.2 (2)
.019 CaCl_2 + 0.25 KCl	2.63 (6)	72 (3)	.3 (6)
.10 CaCl_2 + 0.14 KCl	1.75 (6)	80 (3)	.7 (6)
.175 CaCl_2 + 0.028 KCl	2.83 (6)	75 (3)	.6 (6)
.19 CaCl_2	1.32 (2)	63 (1)	.2 (2)
<i>Monovalent cation requirement</i>			
0.5 Mannitol	1.00 (8)	9 (8)	1.7 (8)
.10 CaCl_2 + 0.14 KCl	1.74 (8)	33 (8)	0.8 (8)
.10 CaCl_2 + 0.14 NaCl	0.93 (8)	28 (8)	.8 (8)
<i>Divalent cation requirement</i>			
.10 CaCl_2 + 0.14 KCl	1.74 (4)	29 (4)	0.6 (4)
.10 MgCl_2 + 0.14 KCl	1.01 (4)	29 (4)	.3 (4)

plasts some cells plasmolyze into more than one unit, thus releasing small protoplasts; (ii) once released, some protoplasts have been observed to rupture, release cell contents, and then heal to form smaller protoplasts with normal cyclosis. These partial protoplasts, usually few in number, generally disappear within 2 days on agar.

We attempted to determine whether the number of protoplasts released from peeled *Avena* coleoptiles by *Myrothecium* enzyme could be increased by addition of other substances. A pectin esterase preparation (Pectinol 42-E concentrate) and a polygalacturonase preparation (Pectinol 41-P concentrate; Rohm and Haas), at concentrations of 0.5 percent in the cellulase digestion mixture with mannitol, both inhibited protoplast release by more than 50 percent. In the presence of mannitol, CaCl_2 or MgCl_2 at 0.01M and EDTA (ethylenediaminetetraacetate) at concentrations greater than $5 \times 10^{-2}M$ also strongly inhibited release. The reason for this inhibition by calcium, as contrasted with its promotive effect when alone or with KCl (Table 1), is not clear, but the fact is well established. Concentrations of EDTA below $5 \times 10^{-2}M$ had little effect, although $10^{-5}M$ caused a small but significant increase in the number of protoplasts released.

Protoplasts prepared in the standard way were exposed to each of the above additions for 1 hour before de-

termination of the percentage survival. None of the additions caused significant bursting, nor did they, in separate experiments, affect the action of cellulase on carboxymethyl cellulose. These inhibitions, therefore, resulted from effects upon the wall-removal process itself, and not from bursting of the protoplasts after they had been released. The unexpected inhibition by high concentrations of EDTA suggests that EDTA probably does not loosen the middle lamella of coleoptile cells, since loosening would result in exposure of more cells to cellulase attack. EDTA does assist in the release of single cells from some tissues (4).

Several tissues of physiological interest have been surveyed for ability to release viable protoplasts when treated with *Myrothecium* cellulase in 0.5 molal mannitol (Table 2). Both leaf and root tissue of oat seedlings release protoplasts in good yield; a region 2 to 4 mm behind the root tip released protoplasts as well as the tip itself, although in neither case was viability (indicated by incidence of cyclosis and general phase-contrast appearance) as good as that of the coleoptile protoplasts. For the standard preparation, 5-mm subapical segments of 20-mm coleoptiles were used; protoplasts were released readily from segments of larger coleoptiles, but only in smaller yield from coleoptiles 10-mm long, largely because the epidermis of the short coleoptiles could not be removed cleanly. The fact that *Convolvulus* callus has exhibited colony formation from single cells is evidence that the individual cells retain the ability to grow and divide (5). Cocking (1) has found that various tissues of the tomato plant also release protoplasts by similar procedures.

Among the unsuitable tissues, only the *Acer* and potato-tuber cells showed signs of significant wall digestion. The *Acer* cells released no protoplasts when exposed to the cellulase for 2 hours, but, a few minutes after their return to distilled water, a few cells squirted their contents out of presumed holes in the weakened wall, allowing the cell wall to contract with a jerk. When potato tissue was placed in cellulase, digestion of some wall occurred, but the protoplasts apparently ruptured, leaving only starch grains visible.

To prepare protoplasts, the methods and tissues must be carefully matched. Obviously no one method is suitable for all cells. Although the addition, to the basic cellulase digestion mixture,

of pectinase or hemicellulase did not enhance release from coleoptile tissue, it may release protoplasts from some of the tissues we have listed as unsuitable; small amounts of these enzymes are, of course, already present in the *Myrothecium* enzyme mixture. In addition to steric factors and recalcitrant wall materials, one is also faced with the presence in many plants of cellulase inhibitors (6), which may be a limiting factor in the enzymic removal of cell walls around living plant protoplasts.

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References and Notes

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Gamma-A Cold Agglutinin: Importance of Disulfide Bonds in Activity and Structure

Abstract. Cleavage of disulfide bonds caused by mild reduction with mercaptoethanol produces a reversible loss of cold agglutinin activity and antigen-binding ability in a gamma-A polymer cold agglutinin. Return of agglutination and antigen-binding ability occur only in those molecules that have not been depolymerized by reduction. Loss of activity becomes irreversible if reduction is followed immediately by alkylation.

The difficulty of isolating γA antibodies has limited the opportunities to examine the role of disulfide bonds in both biological activity and structure of these antibodies. Cold agglutination of red blood cells, observed in the serum of a patient with reticulum cell sarcoma (1), was found to be caused by a γA antibody with a

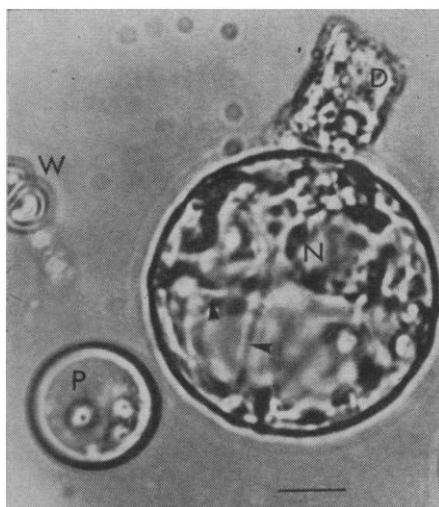


Fig. 1. An *Avena* coleoptile protoplast. After enzymic digestion of cell walls, the protoplasts were placed on 0.6 percent agar for 20 hours before transfer to a microscope slide for photography. N, nucleus; W, rings of secondary walls of digested vascular elements; D, debris from a disintegrated protoplast; P, partial protoplast (see text). The darts indicate transvascular strands; the bar is 10 μ .