

THE LARGE-SCALE ISOLATION OF PROTOPLASTS
FROM IMMATURE TOMATO FRUIT

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It has long been realised that the study of isolated protoplasts offers a potentially new approach to problems of plant cell biology. For this potential to be fully realised, intact protoplasts must be produced, free from contamination by cell debris, on a scale sufficiently large to enable biochemical and physiological experiments to be performed. This aim, though not impossible as has been suggested (1), is not easily achieved because the extreme fragility and physical characteristics of intact protoplasts prevent them from being handled by normal techniques such as centrifugation, filtration, or even pouring from one vessel to another.

Recently it was shown that plant protoplasts could be isolated in large numbers by treating the parenchymatous placental tissue of immature tomato fruit with commercially available pectinase enzymes in a plasmolysing medium (2). This communication will now describe the apparatus and techniques developed for the large-scale production of these protoplasts.

Because of the complex composition of the plant cell wall, the effect of limited enzymic treatment was to weaken, rather than completely dissolve, the treated walls. However the weakened walls of pectinase-treated, plasmolysed parenchymatous placental tissue of tomato could be teased apart so gently as to release protoplasts in enormous numbers compared with the few obtained by the more vigorous teasing necessary for untreated plasmolysed tissues (3-6).

To produce an uncontaminated preparation of protoplasts on any scale required the execution of three operations: (a) enzyme treatment; (b) the release of protoplasts from within the weakened

cell walls; and (c) separation of the intact protoplasts from cell debris.

MATERIALS AND METHODS

Enzyme treatment consisted of incubating the parenchymatous placental tissue of 3- to 5-week old tomato fruit with a 20 per cent (w/v) solution of Rohm and Haas Co., Philadelphia, Pectinol R-10 in 20 per cent (w/v) sucrose (after filtration to remove the diatomaceous earth filler) for 1½ to 2 hours at 27°. Seeds were eliminated either by dissection or by using seedless fruit which were obtained by spraying emasculated flowers with a solution of β -naphthoxyacetic acid.

On a small scale, protoplasts were released from small pieces of pectinase-treated tissue by teasing them in a drop of 0.05 M Tris buffer pH 7.2 containing 0.01 M MgSO₄ and 20 per cent (w/v) sucrose, using two mounted needles. If the suspending medium was held in the depression of a cavity slide, the cell debris would sink to the bottom of the depression whilst the intact protoplasts would float up to the surface where they could be examined microscopically.

It was found that the scale of the preparations could be increased by teasing pectinase-treated tissue between two pieces of card clothing (*i.e.* staples arranged in parallel rows and mounted on a suitable cloth base) instead of using mounted needles.¹ On an intermediate scale, using the tissue from 2 to 3 fruit, the apparatus shown in

¹ Card clothing of the following specifications: Fillet card clothing, 1½ inches wide, 7 ply c. c. 125/1033 Ground, was obtained from James Holdsworth & Bros. Ltd., South Brook Mills, Mirfield, England.

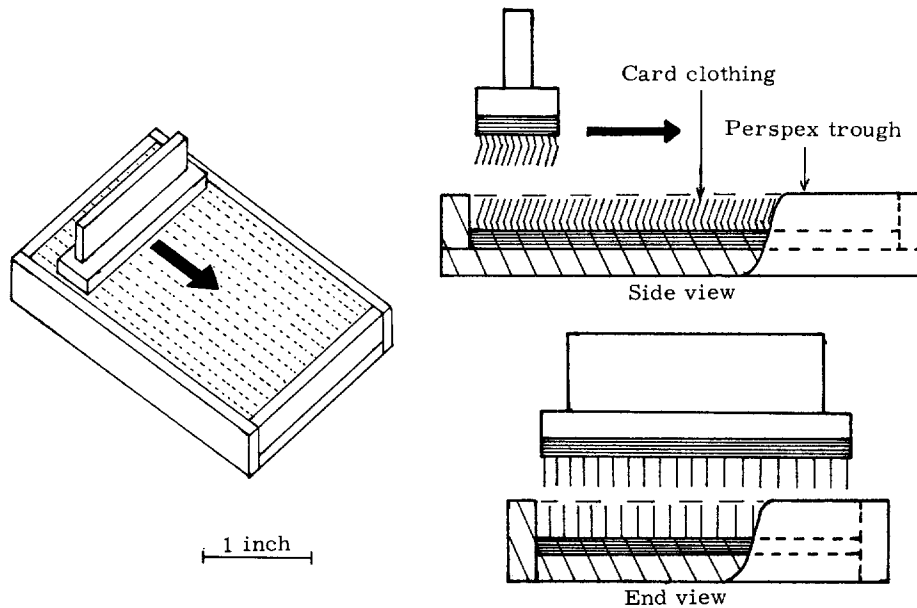


FIGURE 1 Apparatus for teasing pectinase-treated tissue between two pieces of card clothing.

Fig. 1 was used. One piece of card clothing covered the base of a shallow, rectangular Perspex trough with the parallel rows of metal staples running lengthwise. The second piece of card clothing, equal in width to the first but only $\frac{3}{4}$ inches long, was attached to a suitable handle and could be drawn along the trough by carefully interspersing the parallel rows of staples. The bends of the staples in the card clothing restricted the teasing motion to one direction only. The trough was partly filled with 0.05 M Tris buffer pH 7.2 containing 0.01 M MgSO_4 and 20 per cent (w/v) sucrose, and the pectinase-treated tissue was impaled on the staples at the starting end. The tissue was then teased by a few strokes with the second piece of card clothing, releasing protoplasts which became suspended in the plasmolyticum.

On a large scale, using the tissue from 10 to 20 fruit, the pectinase-treated tissue was teased by the apparatus shown in Fig. 2 which allowed an unlimited amount of tissue to be teased at one time. This apparatus consisted of a Perspex box, without top or bottom, to one inner surface of which was fixed a piece of card clothing. A second piece of card clothing, mounted on a wooden drum, could be made to rotate against the fixed piece, using a crank. A gap in the rotating card clothing

enabled pieces of pectinase-treated tissue to be fed from above between the rows of staples; as the drum was rotated both the released protoplasts and the cell debris were washed downwards by a spray of 0.05 M Tris buffer pH 7.2 containing 0.01 M MgSO_4 and 20 per cent (w/v) sucrose from a nozzle (G. Bray & Co., London) which gave a flat fan spray covering the full width of the drum.

The suspension of protoplasts and cell debris from the teasing apparatus was strained through two stainless steel wire gauges (18 mesh/inch, 26 standard wire gauges (S.W.G.), and 30 mesh/inch 28 S.W.G.) to remove larger pieces of cell debris, and fell into the vertical tube of the apparatus shown in Fig. 3. The density of this suspension in terms of the equivalent per cent (w/v) sucrose solution was determined by weighing a 1 ml sample and consulting prepared tables, and was usually found to vary between about 12 per cent and 9 per cent, depending on the amount of vacuolar juice released from the tissue during teasing. It was then possible to prepare a fresh solution of sucrose in 0.05 M Tris buffer pH 7.2 containing 0.01 M MgSO_4 whose density was such that it could be layered on top of the mixed suspension. This new medium was made to contain a sucrose density-gradient with a minimum of 6 per cent sucrose (*e.g.* from 12 per cent to 6 per

cent (w/v) sucrose, or from 9 per cent to 6 per cent (w/v) sucrose, depending on the density of the suspension). This medium was delivered from an apparatus of the sort described by Svensson (7) for producing chromatographic or electrophoretic density-gradients. The surface of this new medium was then brought to the top of the vertical tube by raising the piston-fit bung (coarse adjustment) and by introducing high density (40 per cent w/v) sucrose from a reservoir into the bottom of the tube (fine adjustment). The apparatus and its contents were then allowed to stand for about 1 hour, during which the protoplasts floated upwards from the mixed suspension into the fresh medium, and the sucrose density-gradient pre-

displaced upwards, and resulted in the uppermost layer (which now contained the protoplasts) overflowing into the polythene gutter (see Fig. 3). This clean suspension of protoplasts was immediately run off into whatever apparatus was appropriate for the following experiment; *e.g.*, into centrifuge tubes.

DISCUSSION

All the techniques used for the isolation of protoplasts initially produced a small proportion of vacuoles as well, but, whereas protoplasts were stable for many hours, vacuoles were less stable and the methods became selective for protoplasts after about 20 minutes.

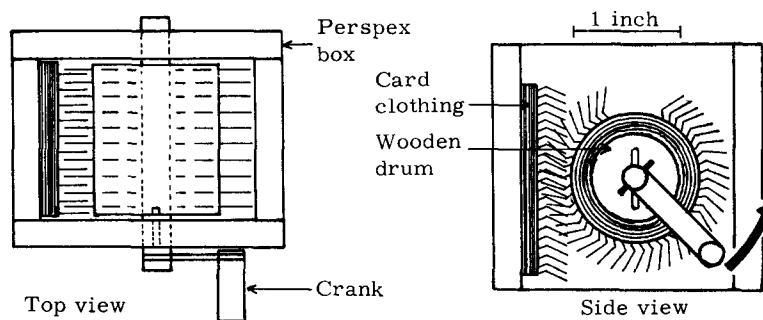


FIGURE 2 Apparatus for the large-scale teasing of pectinase-treated tissue, allowing an unlimited amount of tissue to be teased at one time.

vented this upward movement of protoplasts from forming convection currents. The course of this flotation was followed by direct observation through a horizontally mounted binocular microscope.

It was found that satisfactory upward flotation occurred at all concentrations of sucrose from 20 per cent (w/v) to a minimum of 6 per cent (w/v) in 0.05 M Tris buffer pH 7.2 containing 0.01 M $MgSO_4$. This latter solution was about isopycnic with protoplasts; below 5 per cent (w/v) sucrose in the Tris- $MgSO_4$ medium, osmotic instability of the protoplasts would have been incurred.

When the upward flotation was judged to be complete, the new suspension of protoplasts in fresh medium was finally separated from the old suspension of pectinase and cell debris. This was achieved by introducing more high density (40 per cent w/v) sucrose at the bottom of the vertical tube, which caused the contents of the tube to be

It was found that the pectinase treatment described above would only release protoplasts from the very thin-walled, large, isodiametric cells of the highly vacuolated region of parenchymatous placental tissue (*cf.* reference 8). The thicker walls of the elongated cells of the more densely protoplasmic region were much more resistant to enzyme attack; thus the method was selective and produced a more or less uniform population of protoplasts from one kind of tissue. Protoplasts obtained by this method have been shown to accumulate neutral red in their vacuoles and to respond to the presence of growth substances such as indol-3-yl-acetic acid (2, 9).

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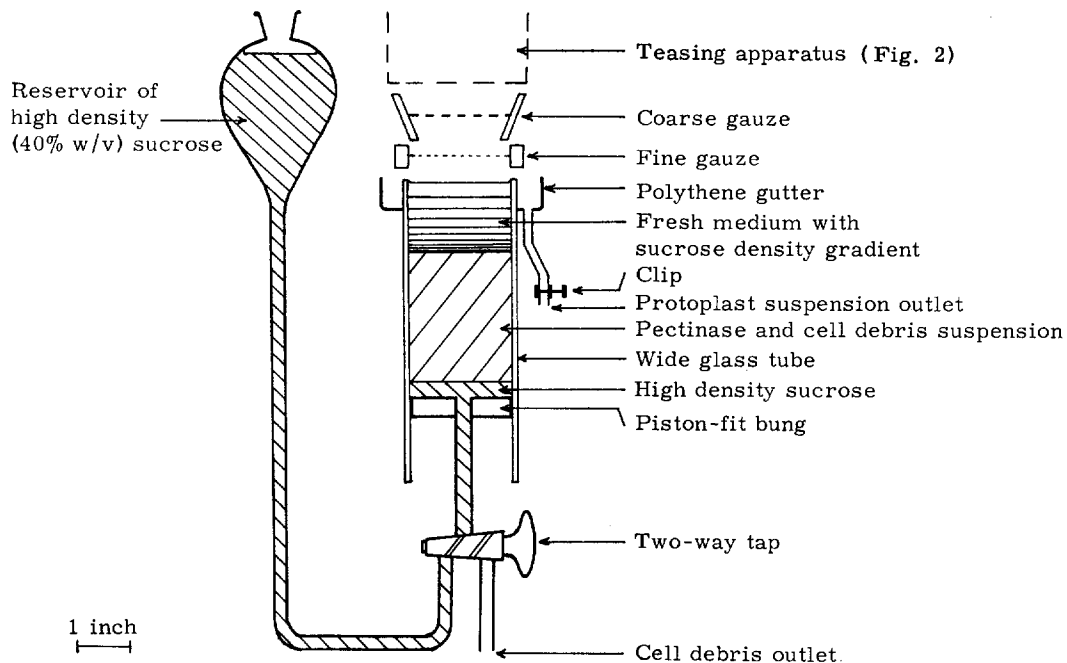


FIGURE 3 Apparatus for the large-scale separation of protoplasts from cell debris by flotation.

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