A Method for the Preparation of Plant Protoplasts

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A simple procedure for the preparation of plant cell protoplasts is described. The leaf is first treated with pectinase under conditions that do not liberate cells; cellulase is then used to release protoplasts. The yields are comparable to those obtained by methods that entail the prior preparation of free cells. The method is of general application but is particularly useful with tissues that cannot tolerate the shaking needed to liberate intact cells.

The preparation of protoplasts from leaf mesophyll usually requires the use of several enzymes, typically pectinase and cellulase. Only in a few favourable cases is a single enzyme preparation sufficient. The enzymes are used either simultaneously[1] or sequentially[2]. The simultaneous application of several crude enzymes has the great advantage of simplicity, but for several reasons is not as satisfactory as the sequential procedure. Both cells and protoplasts are exposed throughout to all the toxic substances which may be present in the mixture; the efficiencies of the enzymes, particularly cellulase, are often reduced under these conditions so that higher concentrations or much longer periods of treatment are required[1,2]. When large numbers of protoplasts are required, for example in work with viruses, long periods of digestion are undesirable because of the difficulties in avoiding infections by microorganisms; the rich enzyme solutions encourage any stray organisms to proliferate rapidly.

The sequential procedure[2] has been used with great success for preparing protoplasts from tobacco leaf mesophyll. It consists of two steps.

1. The leaf material is shaken with pectinase solution at 25 °C to release intact mesophyll cells.

2. The free cells are collected and shaken gently with cellulase solution at 35 °C to convert them to protoplasts.

When this method was applied to leaves of pea (*Pisum sativum*, cv. Chemin Long), the initial shaking with pectinase to release cells caused so much damage that the subsequent treatment with cellulase produced almost no live protoplasts. Reduction of the violence of shaking to prevent damage resulted in negliglible yields of free cells. An extremely simple modification of the procedure was therefore devised which has proved very successful in many cases where the original method was too traumatic. It has also been used successfully in the preparation of tobacco mesophyll protoplasts when it had the advantage that it required fewer manipulations. Unlike the sequential procedure in its original form[2] the new method cannot be used selectively to prepare the protoplasts from palisade cells; instead it gives a mixture of protoplasts derived from both spongy and palisade tissues.

The enzyme preparations were used sequentially but no attempt was made to produce free cells. The precise conditions, for example the osmolarity of the solutions, pH and concentrations of enzymes were determined experimentally for each tissue. The procedure for pea leaf was typical, however, and was as follows.

1. Expanded leaves were taken from 4 weeks old plants, the lower epidermis was removed and the leaves were cut into 2 cm squares and placed in a solution of 1% Macerozyme (All Japan Biochemicals Ltd.) in 0.7 M sorbitol at pH 5.4 (1-2 g tissue per 50 ml enzyme solution).

2. After infiltration *in vacuo* the mixture was agitated either by rotation on a wheel at 15 rpm or by shaking with a reciprocating motion at 50 strokes/min with a 5 cm stroke for 1 hour at 25 °C. Very few cells were released.

3. The Macerozyme solution was then decanted and replaced by 50 ml of a 2% solution of Onozuka SS (All Japan Biochemicals Ltd.) in 0.7 M sorbitol at pH 5.2. The mixture was shaken with a reciprocating motion at 50 strokes/min with a 5 cm stroke at 35 °C. After 1.5-2 hours large numbers of protoplasts virtually free from walled cells were released.

4. The mixture was filtered through surgical gauze and the protoplasts were collected and washed by centrifugation.

Table I shows the effects of different concentrations of the enzymes on the yield of pea protoplasts. The results were similar to those obtained by the method that involved the preparation of intact cells[2].

Table I. The influence of enzyme concentration on the release of protoplasts from pea leaves.

Macerozyme [%] (1 hour at 25 °C)	Onozuka [%] (2 hours at 35 °C)	Yield of protoplasts [$\cdot 10^{-3}$/ml]
0.0	2.0	0
0.25	2.0	40
0.50	2.0	100
1.0	2.0	150
2.0	2.0	120
1.0	0.0	0
1.0	0.5	44
1.0	1.0	60
1.0	2.0	100
1.0	4.0	100

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