Protoplasts were first released from leaf segments plasmolyzed in KNO₃ (Klerker, 1892). A few cells were cut without apparent damage to the protoplasts, and intact protoplasts were released by controlled deplasmolysis. Kuster (1909) used an aqueous solution of Ca(NO₃)₂ to produce subprotoplasts. In another study (Michel, 1937), leaf tissue was pretreated with KNO₃, then mechanically cut and protoplasts released using a viscous solution of sucrose. Protoplasts can be mechanically released only from cells in which a high degree of plasmolysis occurs followed by subsequent separation from the cell wall. Mechanical release, however, is slow, with recovery of relatively few intact protoplasts.

Large quantities of intact protoplasts are released using cell wall-degrading enzymes (Power and Cocking, 1969; Takebe et al., 1968). A major concern in the use of this method, however, is the potential damage to the physical structure and physiological activity of the protoplasts. Pilet (1972, 1973), using a few protoplasts mechanically released from root cells of Allium cepa, found that cell wall-degrading enzymes reduced transaminase activity. Research efforts to identify other potentially harmful effects have lagged, however, because of the inability to nonenzymatically release the large quantities of intact protoplasts needed for such studies.

Although intact protoplasts can be released enzymatically from somatic cells of most plant tissues, release from pollen grains presents special problems. At the immature tetrad stage, cell walls are only partially susceptible to enzymatic degradation (Bhojwani and Cocking, 1972; Deka et al., 1977), and release requires as long as 30 hr (Redenbaugh et al., 1980). The cell wall of mature pollen, however, contains sporopollenin, one of the most chemically inert natural plant constituents known (Gheradini and Healey, 1969) and one that is totally resistant to all of the commonly used cell wall-degrading enzymes. Baldi et al. (1985) released pollen protoplasts using a polysaccharide solvent, but the procedure required a very high temperature (75°C) that reduced viability.

This study describes a unique, rapid process for the nonenzymatic release of intact viable protoplasts from mature pollen, and shows microscopic observations on physical alterations to the surface of nonenzymatically released protoplasts by a mixture of enzymes.

**Materials and Methods**

Plants of ‘Monroe’ bean were grown in commercial planting medium (Fisons Sunshine mixture, Fisons Western, Vancouver, Canada) in 4.5-liter clay pots in a greenhouse at 25/21°C (day/night), with a coincident photoperiod of 14/10 hr (day/night). Freshly opened flower buds were collected between 0730 h and 0930 h. Within 5 min of collection, pollen from each flower was shaken into the well of a 0.4 × 1.2-cm microcavity slide, followed immediately by the addition of two drops of an experimental protoplasm-release solution that contained various concentrations and combinations of NaCl, KCl, CaCl₂, and H₃BO₃ in glass-distilled water. Slides were then placed in closed chambers at 25°C and 98% RH. Osmotic stabilizers, sucrose (0.03 to 1.2 M), mannitol (0.03 to 1.2 M), and sorbitol (0.03 to 1.0 M), were included along with the protoplast-release solutions to evaluate their influence on both quantity and extent of release and on prolonged stability of the released protoplasts. The pH of the release solutions was adjusted between 5.0 and 9.0 using 1.0 N HCl or 1.0 N KOH. In other trials, the same solutions were solidified with 0.5% or 1.0% agar. Pollen was shaken onto the surface of agar medium in a 9.0 × 1.0-cm petri dish and covered with a drop of phloxine/methyl green dye (Owczarzak, 1952).

The total number of pollen grains from which protoplasts were released and the number of those totally released from the cell wall were determined microscopically 5 min after pollen application. The extent of protoplasm release was recorded as: 1) total, the number of pollen grains showing protoplasm release (partial plus free); and 2) free (Fig. 1B), protoplasts totally removed from the cell wall. Figure 2 shows several intact protoplasts from one treatment.

Each treatment mean included pollen from a minimum of 12 flowers picked at random, replicated three times, and repeated six times during a period of 30 days. At least 500 pollen grains were evaluated from each flower. All data were statistically evaluated by analysis of variance and treatment means were compared by LSD at P = 0.05 (Snedecor, 1956).

Viability of released protoplasts was determined using methylene blue (Vairo, 1961) and fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970).

To investigate the possible physical alteration of pollen pro-
Fig. 1. (A) Bean pollen protoplasm partially released after 55 sec in 0.03 M NaCl + 1.0 M sucrose, pH 6.5. Bar = 5 µm. (B) Same bean pollen protoplasm totally free from the cell wall after 60 sec.

To release protoplasts by cell wall-degrading enzymes, pollen from each of 20 freshly opened bean flowers was shaken into the wells of two microcavity slides and covered with two drops of protoplast-release solution containing 0.03 M NaCl, 300 mg CaCl₂/liter, 100 mg H₃BO₃/liter, and 0.29 M sucrose at pH 6.5. After 5 min, a drop of protoplast-release solution containing 1% (w/v) Rhozyme HP-150 (Rohm and Haas, Philadelphia), 1% (w/v) cellulase R-10 and 1% (w/v) Macerozyme R-10 (both from Kinki Yokut Biochemical, Nishinomiya, Japan) was added to one slide and one drop of the protoplast-release solution without enzymes was added to another slide. After 15 min at 25°C, pollen from each slide was washed with the same protoplast-release solution into separate 3-ml centrifuge tubes and centrifuged (4 min in a Sorvall GLC-1 with a relative centrifugal force of 45 × g). The wash solution was poured off, and the washing procedure was repeated. A drop of solution containing washed protoplasts from enzyme-free or enzyme-treated solutions was frozen in N₂ slush and transferred under vacuum using a Hexland CT 1000A Cryo unit (East Challow, Oxfordshire, England) for viewing in a Hitachi S-530 scanning electron microscope.

Results and Discussion

In nature, pollen hydrates within seconds of contacting the stigma. Water absorbed by the pollen from the stigma exerts a pressure on the interior of the cell wall. Because the outer layer covering the pore of the pollen is weaker than the cell wall, it ruptures, and the protoplasm protrudes through the pore initiating pollen tube growth. We believed that if this naturally occurring process could be greatly accelerated, the entire protoplasm could be removed from within the cell wall in seconds.

Bean pollen hydrated rapidly in glass-distilled water, but most protoplasts (i.e., 80%) ruptured (Table 1). In 0.01 M NaCl solution, intact protoplasts were released from almost all pollen grains; however, of those released only \( \approx 75\% \) were totally free from the cell wall. With 0.02 to 0.06 M NaCl, a little more than 90% of the protoplasts released were totally free from the cell wall. At a salt concentration of 0.08 M, the percentage of pollen grains that released totally free intact protoplasts had decreased to 80%, and at 0.10 M none of the protoplasts released were totally free from the cell wall. Potassium chloride solutions of the same molarity as NaCl gave similar results in protoplasm release (Table 1). Even very low concentrations (<0.005 M) of supplemental

| Protoplast release | Salt concentration (M) | Intact protoplasts released (no./100 pollen grains) |
|--------------------|-------------------------|-----------------------------------------------------|
|                    | NaCl                    | Free      | 0.01 | 0.02 | 0.04 | 0.06 | 0.08 | 0.10 |
| Total              | 20                      | 91       | 90   | 91   | 92   | 75   | 50   |      |
| Free\*             | 20                      | 68       | 85   | 85   | 84   | 60   | 0    |      |
| LSD\(_{0.05}\) NS  | 6                       | 6        | 6    | 6    | 6    | 7    |      |      |
| KCl                | 20                      | 89       | 88   | 87   | 85   | 70   | 45   |      |
| Total              | 20                      | 66       | 85   | 82   | 79   | 55   | 0    |      |
| Free\*             | 20                      | 66       | 85   | 82   | 79   | 55   | 0    |      |
| LSD\(_{0.05}\) NS  | 6                       | 6        | 6    | 6    | 6    | 6    |      |      |

Protoplasts totally free from the cell wall.

Table 1. Influence of salt concentration at pH 6.0 on release of bean pollen protoplasts.
calcium chloride totally prevented the release of protoplasts (data not shown).

In a 0.03-m NaCl solution at pH 5.0, many protoplasts burst. Of those still intact, 38% were totally free from the cell wall (Table 2). Few protoplasts ruptured, and essentially all of the protoplasts released were totally free from the cell wall as pH was increased from 6.0 through 9.0. In a 0.03-m NaCl solution at pH 6.0, protoplasm release commenced between 30 and 45 sec after contact with the solution. Release was essentially completed within 5 min.

Total number of pollen that released intact protoplasts in agar-solidified salt media (Table 3) was similar to that obtained in liquid (Table 1) media at the same concentration. On 0.5% agar, the number of pollen grains releasing totally free intact protoplasts was significantly increased with each increment increase at pH 6.0, protoplasm release commenced between 30 and 45 sec after contact with the solution. Release was essentially completed within 5 min.

Table 2. Influence of pH on the release of bean pollen protoplasts in a 0.03-m NaCl solution.

| Protoplast release | 5.0 | 6.0 | 7.0 | 8.0 | 9.0 |
|--------------------|-----|-----|-----|-----|-----|
| Total              | 65  | 95  | 88  | 89  | 83  |
| Free              | 25  | 90  | 84  | 85  | 80  |
| LSD0.05           |    | NS  | NS  | NS  | NS  |

*Protoplasts totally free from the cell wall.

Table 3. Influence of salt concentration on release of bean pollen protoplasts in agar-solidified medium at pH 6.0.

| NaCl (m) | 0.005 | 0.01 | 0.02 | 0.04 |
|----------|-------|------|------|------|
| Protoplast release | Agar (%) | 0.5 | 1.0 | 0.5 | 1.0 | 0.5 | 1.0 |
| Total    | 85    | 80   | 81   | 87   | 82   | 86   | 79   | 81   |
| Free     | 25    | 5    | 45   | 7    | 60   | 18   | 68   | 16   |
| LSD0.05  | 6     | 7    | 7    | 6    | 7    | 7    | 6    | 6    |

*Protoplasts totally free from the cell wall.

Table 4. Influence of supplemental CaCl2 and H2BO3 on the release of bean pollen protoplasts in a 0.03-m NaCl solution at pH 6.0.

| Protoplast release | H2BO3 (m) | 0.5 | 100 | 200 | 0.0 | 50 | 100 | 100 | 100 |
|--------------------|-----------|-----|-----|-----|-----|----|-----|-----|-----|
| Intact protoplasts released (no./100 pollen grains) | Total | 86 | 85 | 90 | 86 | 85 | 90 | 85 | 88 |
| Free              | 82       | 81 | 63 | 60 | 70 | 30 | 73 | 85 | 30 |
| LSD0.05           | 6        | 6  | 6  | 6  | 7  | 7  | 6  | 7  | 7  |

*Protoplasts totally removed from the cell wall.

Table 5. Effect of sucrose concentration on the release of bean pollen protoplasts in a 0.03-m NaCl solution at pH 6.0.

| Protoplast release | Sucrose (m) | 0.03 | 0.09 | 0.15 | 0.29 | 0.44 | 0.58 | 0.73 | 0.88 | 1.17 |
|--------------------|-------------|------|------|------|------|------|------|------|------|------|
| Intact protoplasts released (no./100 pollen grains) | Total | 80 | 90 | 92 | 90 | 88 | 80 | 75 | 76 | 75 |
| Free              | 78 | 87 | 88 | 86 | 85 | 75 | 50 | 41 | 25 | 11 |
| LSD0.05           | 7 | 7 | 7 | 7 | 7 | 6 | 6 | 6 | 6 | 6 |

*Protoplasts totally released from cell wall.

reaction of pollen to release of totally free intact protoplasts was similar to that of pollen given only supplemental CaCl2, at the same concentration.

Sucrose, in the absence of NaCl, did not effect a release of protoplasts (data not shown). However, addition of 0.03 to 0.29 m sucrose to a 0.03-m NaCl solution increased the total number of pollen-releasing protoplasts and the number of totally free protoplasts (Table 5). Addition of 0.44-m sucrose decreased both the total number of pollen releasing intact protoplasts and the number of totally free protoplasts. The number of pollen releasing intact protoplasts then remained the same with additions of 0.58 to 1.17 m sucrose. The percentage of pollen grains releasing totally free intact protoplasts, however, was significantly decreased with each successive increment increase of sucrose from 0.58 through 1.17 m.

Although sugars and sugar alcohols are preferred protoplasm stabilizers, salts can also be used (Meyer, 1974). However, protoplasts take up salt, and prolonged exposure to salt can be injurious. With the process described in this study, however, protoplasm release was so rapid (<5 min) that potential salt damage would be minimized.

Mannitol concentrations at 0.3 to 15.0 m and sorbitol at 0.3 to 1.0 m in dilute salt solutions did not alter the total number of pollen grains releasing intact protoplasts or the viability of protoplasts when compared to similar dilute salt solutions without sorbitol or mannitol (data not shown).

At least 80% of the protoplasts released in 0.02 to 0.06-m NaCl solutions retained their viability for at least 16 hr, as determined by methylene blue (Vairo, 1961) and by FDA (Heslop-Harrison and Heslop-Harrison, 1970).

Although only totally free protoplasts were evaluated in the study, partially free protoplasts (Fig. 1A) are of special interest, since they are less fragile and leave less cellular debris to interfere with microscopic examination. When sufficient protoplasm surface is exposed, they could prove useful for fusion and for transfer of genetic material by microinjection or electroporation. Although not shown in any of the tables, partials make up a large portion of the total protoplasts released. The number of partials produced and the amount of protoplasm release can be regulated by controlling the concentration of CaCl2, H2BO3,
and sucrose used in the dilute salt solution and by controlling agar concentration.

All nonenzymatically released protoplasts, both totally free and partially free, remained intact. When nonenzymatically released protoplasts were incubated with cell wall-degrading enzymes, few (<5%) remained intact.

Exposure of nonenzymatically released protoplasts (Fig. 3A) to cell wall-degrading enzymes resulted in a severe disintegration of the protoplasm surface (Fig. 3B). The surface of a protoplast contains large quantities of carbohydrate constituents (Williamson et al., 1976), and their loss through enzymatic degradation could be detrimental to the function and longevity of the protoplasm and could influence in vitro plant regeneration. Cell wall-degrading enzymes also release wall-bound enzymes such as peroxidase and catalase (Vairo, 1961; Strand et al., 1976), which could alter physiological processes associated with the protoplasm.

This study demonstrates for the first time that large quantities of viable intact protoplasts can be rapidly released from mature bean pollen without the use of cell wall-degrading enzymes. These results suggest the intriguing possibility that plants can be regenerated from mature pollen protoplasts. This procedure for nonenzymatically released protoplasts could be used for genetic manipulation through somatic hybridization and genetic transformation.

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