

Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products

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Received November 3, 1987/Revised version received March 3, 1988 – Communicated by C. Harms

SUMMARY

Plant cell suspension cultures producing secondary metabolites have been permeabilized for product release by electroporation. The two cell cultures studied, i.e. *Thalictrum rugosum* and *Chenopodium rubrum*, require about 5 and 10 kV cm⁻¹, respectively, for complete permeabilization (release of all the intracellularly stored product). The number of electrical pulses and capacitance used had a relatively limited effect on product release while the viability of the cells was strongly influenced by the latter. Conditions for complete product release resulted in total loss of viability of the cells after treatment. The release of product from immobilized cells was also achieved by electroporation. Cells entrapped in alginate required less voltage for permeabilization than free or agarose entrapped cells.

INTRODUCTION

The first commercial processes based on plant cell cultures for the production of phytochemicals have recently been introduced^{1, 2}. These processes are operated on a relatively small scale but they have shown that the commercial utilization of plant cell cultures is feasible. However, at present a number of limitations hamper the wider use of this technology on an industrial scale³.

We have been involved in the last few years in studies on the possible utilization of immobilized plant cells⁴. An inherent advantage of such preparations is the possible continuous operation of a process. Cultivated plant cells most often store secondary products intracellularly in the vacuoles. Therefore, attempts are now being made to develop procedures for the induced release of such products. Various chemical agents have been used for the permeabilization of plant cell membranes^{5, 6}. Recently, the use of ultrasound for product release from cultivated plant cells was reported⁷.

Electroporation has become widely used to improve the transfer of genes into plant protoplasts⁸. The plasma membrane of the protoplasts is made permeable by electric pulses of appropriate voltage resulting in an efficient uptake of foreign DNA. A protoplast population is a relatively homogeneous cell preparation and the optimal electroporation conditions are therefore readily determined. Plant cell suspension cultures, on the other hand, are morphologically heterogeneous and individual cells respond differently to the applied electric pulses. Moreover, electroporation for gene transfer is thought to require the permeabilization of only the plasma membrane. For the release of compounds stored in the vacuole, two membranes (i.e. both the plasma membrane and the tonoplast) must be permeabilized.

Here we report on the use of electroporation for the release of secondary metabolites from cultivated plant cells. The technique is illustrated by the release of the betacyanin from freely suspended cells of *Chenopodium rubrum* and the release of berberine from freely suspended, agarose-entrapped and alginate-entrapped cells of *Thalictrum rugosum*.

MATERIALS AND METHODS

Cell cultures

Cell suspension cultures of *T.rugosum* and *C.rubrum* were cultivated in MS medium as previously described⁹. These cell cultures were kindly supplied by Dr. J. Berlin, GBF, Braunschweig, FRG. Cells used in the experiments were in the early stationary phase. This was 9–10 days from the last subculture for cells of *T.rugosum*, and 14 days for *C.rubrum*.

Immobilization

Cells of *T.rugosum* were immobilized in agarose (type VII, Sigma, St. Louis) by mixing the polymer (4 % w/v; 9 g) with cells (1 g) at 37 °C. Beads (diameter 0.5–1.0 mm) were made from this polymer/cell suspension as described elsewhere⁹.

Cells were immobilized in 2 % (v/w) alginate (Protan HF, Protan AS, Drammen, Norway) as previously described⁹.

Electroporation

The electroporation experiments were carried out in a 0.35 or 1.0 ml chamber of a Dialog ElectroPoratorR (Dialog GmbH, Düsseldorf, FRG). In order to reduce sampling errors and simplify the manipulations, the cells were filtered through a metal screen (500 µm) and collected by centrifugation. They were then resuspended in fresh medium at a density of 10 % (v/v).

The electroporation chamber was filled with this suspension or with a suspension of beads containing immobilized cells and 3 or 10 pulses with an initial field strength of between 0.3 and 15 kV cm⁻¹ were applied. The resistance of the chamber containing this suspension was approximately 580 Ohms. The interval between the pulses was 10 sec. The capacitance was varied between 10 and 40 nF in some experiments in order to obtain exponential decay constants for the pulse of between 6 µsec and 23 µsec. A capacitance of 20 nF (12 µsec decay constant) was used unless otherwise stated.

Analysis

Cells were allowed to stand in the bathing medium for 10 minutes after the electroporation step, after which time, no more product release was seen. The cells were then removed by centrifugation and the amount of the berberine or betacyanin in the medium was determined. Berberine was quantitated by TLC as previously described¹⁰ and betacyanin was quantitated spectrophotometrically at 540 nm. The measurements from 3 samples were averaged for each data point in the figures. For berberine, the spread of these points was less than 10% each side of the mean, and for betacyanin, the spread was less than 5% each side of the mean. Relative release was determined as % of total intracellular product as measured by extraction with methanol which extracts 100% of the compounds. The betacyanin content of the *C.rubrum* cultures in stationary phase is 0.2–0.3% by dry weight, and the berberine content of the stationary *T.rugosum* cultures is 0.4–0.5% of dry weight.

Cell viability was estimated by plating the treated cells at different dilutions in growth medium solidified with 0.6 % w/v agarose. The

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number of colonies formed was determined after 4 weeks incubation in the dark at 27 °C.

RESULTS AND DISCUSSION

The release of secondary products from freely suspended cells as a function of voltage is shown in Figure 1. The two cell lines studied show a different response. *T.rugosum* is completely permeabilized at a voltage of 5 kV cm⁻¹ while in the range of 10 kV cm⁻¹ is required for the release of all the betacyanin from *C.rubrum* (equal to the release using solvent extraction). A possible explanation for this difference may be the fact that the latter cell line grows in larger aggregates than the former. It is interesting to note that the two curves are essentially parallel in the responsive region. Whole cells, as used in this study, require much higher voltages for permeabilization than protoplasts. For instance, the best results for permeabilization of soybean cell culture protoplasts were obtained with pulses of 0.4 kV cm⁻¹ after which 78% of the surviving protoplasts (around 15 % of the total number of protoplasts) had been permeabilized¹¹.

Figure 1 also shows the viability of *T.rugosum* as a function of voltage. The viability curve is essentially the reverse of the release curve. The viability of the cells was determined by a plating procedure and Figure 2 shows cells treated at 0.3 and 9.6 kV cm⁻¹, respectively. Microscopic examination of the cells following electroporation showed that there was extensive collapse of their structure, probably resulting from the disruption of the membrane system. The secondary products are distributed unevenly in the cells of a culture. All the cells in any particular aggregate either contain product or none do. There was no clear difference between cells with and without secondary products in their susceptibility to damage by electroporation. As expected, larger cells were permeabilized before smaller cells. From these experiments it appears that it is not possible to release secondary product efficiently without irreversible damage to the cells. However, extensive variation of the protocol in order to achieve release while retaining a high percentage of viable cells was not attempted.

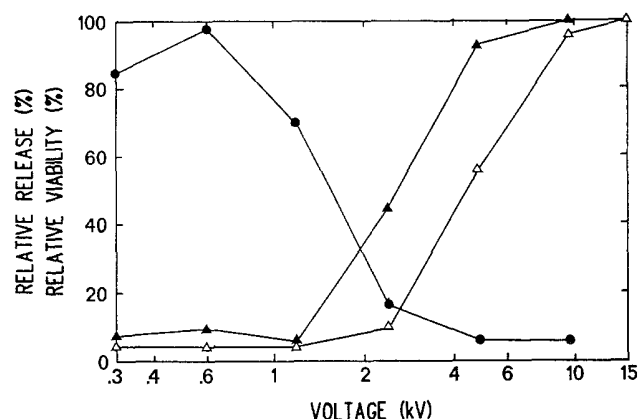


Figure 1. Release of secondary products and viability as a function of voltage applied during electroporation.

- (▲) Release of berberine from cells of *T.rugosum*.
- (△) Release of betacyanin from cells of *C.rubrum*.
- (●) Viability (colony formation) of *T.rugosum*.

The number of pulses, as far as was tested (3 or 10 pulses), used during the electroporation experiments did not significantly influence the release of product (data not shown). The experiments were therefore generally carried out using three pulses.

The effects of the capacitance (and therefore the exponential decay constant of the pulse) used in the electroporation experiments on product release and cell viability have been studied. The results are shown in Figure 3. The capacitance used also did not have a significant influence on the product release. Essentially the same amount of product was released at a fixed voltage at the capacitances tested. The cell viability was, on the other hand, affected by the capacitance at low voltages. A marked decrease in viability of *T.rugosum* cells was observed when the capacitance was increased from 10 to 40 nF, and therefore the pulse length increased fourfold (Figure 3). The amount of product released was

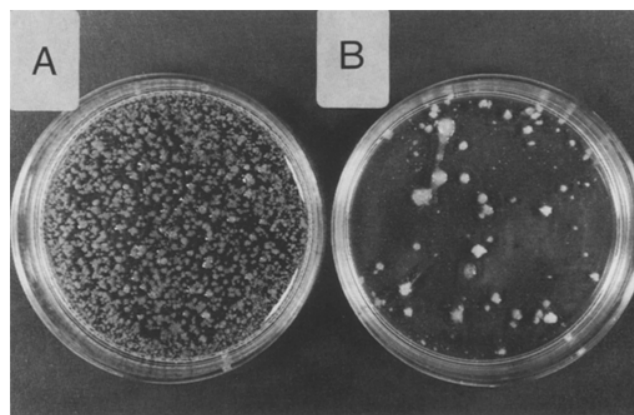


Figure 2. Plating of *T.rugosum* cells after electroporation treatment. (a) Cells treated at 0.3 kV cm⁻¹ (no significant loss of viability). (b) Cells treated at 9.6 kV cm⁻¹.

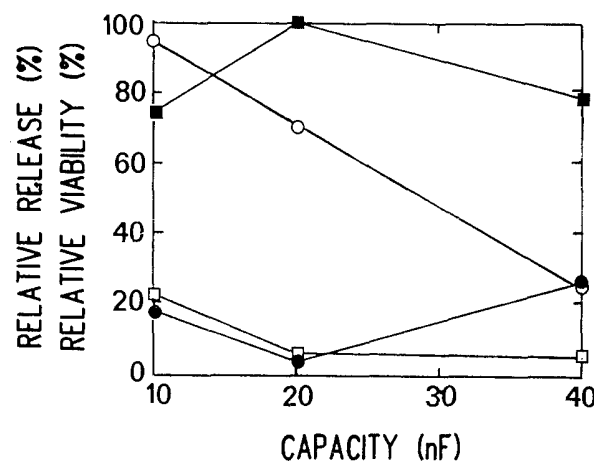


Figure 3. Release of berberine and viability of *T.rugosum* cells treated at different voltages as a function of the capacitance used during electroporation.

- Open symbols = viability; solid symbols = release.
- (● ○) Cells treated at 1.2 kV cm⁻¹.
- (■ □) Cells treated at 4.8 kV cm⁻¹.

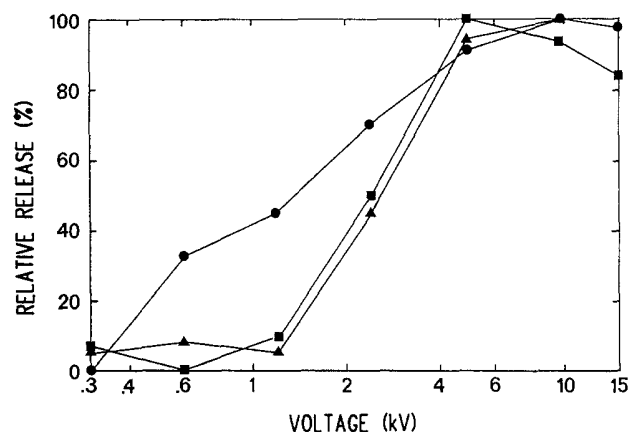


Figure 4. Relative release of berberine from different preparations of *T.rugosum* cells as function of voltage applied during electroporation.

- (▲) Freely suspended cells.
- (■) Agarose-entrapped cells.
- (●) Alginate-entrapped cells.

constant. Thus in these cases, electroporation can result in cell death under certain conditions without release of significant amounts of product from the vacuoles. This is particularly the case when long pulses are used.

The release of berberine from immobilized cells of *T. rugosum* after electroporation has been studied. The cells were immobilized in alginate or agarose gels³. The cells entrapped in agarose responded to electroporation in the same manner as freely suspended cells while cells entrapped in alginate showed a different pattern, as illustrated in Figure 4. For complete product release the same voltage (5 kV cm⁻¹) was required for the three different cell preparations. However, at 0.6 and 1.2 kV cm⁻¹ only the alginate-entrapped cells show significant release of product (around 30 and 50 %, respectively). We ascribe the differences observed for cells entrapped in the two polymers to the different ionic character of these polymers. Agarose is essentially uncharged while alginate is negatively charged and contains high concentrations of calcium ion. Thus, cells in alginate experience a higher voltage gradient because the polymer beads are transparent to, or may even concentrate the electric field.

Here we have reported on the use of electroporation for the permeabilization of cultivated plant cells resulting in the release of intracellularly stored products. The heterogeneity of such a culture makes it difficult to establish optimal conditions and the quantitative release of intracellularly stored products appear to result in non-viable cells. Permeabilization with chemicals (e.g. dimethylsulfoxide or Triton X-100) also lead to cell death^{9-12,14}, and probably lead to the solubilization of membrane and other cell components. This electroporation procedure should prove valuable in the downstream processing of plant cell cultures since an efficient extraction of product may be obtained under relatively mild conditions. Although this has not been measured, it appears that little solubilization of such membrane components occurs when using electroporation alone, although release of other small solutes must occur. In fact, it may be possible to use this process for the extraction of soluble proteins from cells. Furthermore, electroporation should be applicable to other cell types, e.g. bacterial, fungal and animal cells. With such cell types, which grow in essentially monodisperse suspensions, it may be possible to optimize the

electroporation conditions such that product release is achieved under conditions where cell survival is sufficient to allow repeat harvesting of products from continuous cultures.

ACKNOWLEDGMENTS

This work was supported in part by a grant to P.B. from the Swiss National Science Foundation (3.318-0.86).

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