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Permeabilization of Immobilized Plant Cells, Resulting in Release of Intracellularly Stored Products with Preserved Cell Viability

Peter Brodelius[1], and Kjell Nilsson

Pure and Applied Biochemistry, University of Lund, Chemical Center, P.O. Box 740, S-22007 Lund, Sweden

Summary. Plant cells, entrapped in agarose or alginate, were permeabilized in a new procedure that retain cell viability, permitting the possibility of reusing biomass after release of intracellular products. Dimethylsulfoxide (DMSO) was used to make the cells permeable. The activity of isocitrate dehydrogenase, an intracellular enzyme, was used as an indicator of plasma membrane permeability. Cells from three plant species require different concentrations of DMSO for complete permeabilization (i.e., for maximal isocitrate dehydrogenase activity). Cells of *Catharanthus roseus* permeabilized with up to 5% DMSO remained viable, and released 85–90% of the intracellularly stored products (ajmalicine isomers). In model production systems, *C. roseus* cells entrapped in agarose or alginate beads were intermittently permeabilized for release of products in a cyclic process. An increase in product yield was observed for each cycle because of increase in biomass (cell growth) within the beads.

Introduction

The production of commercially significant biochemicals by submerged cultures of plant cells is a technique of great biotechnological potential (Barz et al. 1977; Staba 1980). A wide variety of secondary products, many of which are of commercial interest, have been isolated from plant tissue cultures (Zenk 1978; Kurz and Constabel 1979). A major problem in producing such substances in a biotechnological process is, however, the low yield of the target substance. Although selected cell lines, i.e., high-producing clones, may be used to increase the yield (Ogino et al. 1978; Deus and Zenk 1982; Yamamoto et al. 1982), the generation of biomass, which is slow and expensive, limits at present the applicability of plant cell cultures on a commercial basis. Thus, it appears to be of importance to utilize the biomass over an extended period of time. We have been investigating the feasibility of using immobilized plant cells for this purpose (Brodelius et al. 1980; Brodelius and Mosbach 1982; Brodelius 1983) and have observed a production phase that is longer for immobilized plant cells than for freely suspended cells.

Products of commercial interest are often intracellular and so we have focused on methods to release products without affecting the viability and biosynthetic capacity of the immobilized cells. In an initial study (Felix et al. 1981) on the permeabilization of immobilized plant cells, we used relatively harsh methods to make cells permeable that resulted in cell death. In this paper we report on a method for reversible permeabilization of immobilized plant cells, that means render cells permeable for the release of intracellular products without destroying cell viability and permitting the reuse of biomass after product isolation. This method may be of fundamental importance for the development of an economically feasible process for the production of biochemicals with cultivated plant cells.

Materials and Methods

Chemicals. Agarose (type VII), NADP, and D,L-isocitric acid (trisodium salt) were obtained from Sigma (St. Louis, MO). Sodium alginate (Manucol DH) was from Alginate Industries (Girven, UK). Tryptamine and DC-Alufolien (Kieselgel 60 F_{254}) were obtained from Merck (Darmstadt, FRG) and (2-^{14}C)-tryptamine from NEN (Boston, MA). Ajmalicine was purchased from Roth (Karlsruhe, FRG). Secologanin was kindly provided by Dr. C. R. Hutchinson, University of Wisconsin, Madison, WI. All

Offprint requests to: P. Brodelius

1 *Present address:* Institute of Biotechnology, Swiss Federal Institute of Technology, Hönggerberg, CH-8093 Zürich, Switzerland

other chemicals and biochemicals were of analytical grade and were purchased from commercial sources.

Cultivation of Cells. Plant cells were cultivated at 26° C in suspensions on a rotary shaker at 100 rpm in the following media: *Catharanthus roseus:* LS-medium (Linsmaier and Skoog 1965) supplemented with 2,4-dichlorophenoxyacetic acid (2.2 mg/l) and naphtaleneacetic acid (1.86 mg/l), pH 6.0 (medium A). *Daucus carota* Ca68 (this cell line was kindly supplied by Dr. I. A. Veliky, National Research Council Canada, Ottawa): 71v-medium (Veliky and Rose 1973) supplemented with 2,4-dichlorophenoxyacetic acid (1.5 mg/l), indoleacetic acid (1.0 mg/l), naphthaleneacetic acid (0.1 mg/l) and kinetin (0.25 mg/l), pH 5.0 (medium B). *Datura innoxia:* SH-medium (Schenk and Hildebrand 1972) supplemented with 2,4-dichlorophenoxyacetic acid (0.5 mg/l), kinetin (0.1 mg/l) and p-chlorophenoxyacetic acid (2.0 mg/l), pH 5.8 (medium C).

Immobilization of Plant Cells. Cells of *C. roseus, D. carota,* and *D. innoxia* were immobilized by entrapment in agarose according to a newly developed procedure (Nilsson et al. 1982). The cells (2.0 g wet weight) were mixed with agarose (8 g of a 5% w/v solution) at 40° C. The suspension was subsequently poured into soy oil (50 ml) also kept at 40° C under continuous stirring. When agarose droplets of appropriate size (diameter approximately 1 mm) had formed, the whole mixture was cooled on an ice bath until the agarose solidified. The beads containing the immobilized cells were collected and washed.

Cells of *C. roseus* were also immobilized by entrapment in calcium alginate as previously described (Brodelius et al. 1979). The cells (15 g wet weight) were mixed with sodium alginate (35 g of a 5% w/v solution) and the suspension dripped into medium A containing 50 mM $CaCl_2$ (200 ml). After 30 min the beads were collected and washed with medium A containing 5 mM $CaCl_2$.

Permeabilization Procedure. Freely suspended or immobilized cells were permeabilized by incubation in medium A containing various concentrations of DMSO on a rotary shaker (100 rpm) for 30 min. A 10 : 1 ratio of medium to beads was used unless otherwise stated.

Biosynthetic Studies. The synthesis of ajmalicine isomers from tryptamine and secologanin by agarose- and alginate-entrapped cells of *C. roseus* with intermittant product release was studied over a period of 12–14 days (see Figs. 6 and 7). The growth phase was carried out by incubating the immobilized cells (2.0 g agarose or 5.0 g alginate beads) in medium A (10 ml). Before transfer to the production phase, the beads were washed (1 × 10 ml) with an "alkaloid-producing" medium (Zenk et al. 1977) modified by obmitting the hormones (medium D). The beads were then transferred to medium D (5 ml) containing ^{14}C-labelled tryptamine (0.5 mM) (350,000 dpm/μmol) and secologanin (0.5 mM). After 3 days the beads were collected and washed with medium D (2 × 10 ml) and then placed in medium D (10 ml) containing 5% (v/v DMSO for 30 min. The DMSO-containing medium was then removed, and the beads were washed with medium D (2 × 10 ml) and transferred to medium A (10 ml). The growth/production cycle was then repeated with subsequent permeabilization. The media collected were combined, adjusted to pH 10 and extracted with methylene chloride (3 × 10 ml). The extracts were analyzed for radiolabelled ajmalicine by TLC-chromatography (Brodelius and Nilsson 1980).

Analytical Procedures. Isocitrate dehydrogenase activity within immobilized permeabilized plant cells was determined as has been described (Felix et al. 1981).

Cell growth was determined by following the increase in dry weight as a function of incubation time. Samples were collected on preweighed dry filter papers and dried at 60° C until constant weight.

The integrity of the plasma membrane was studied by the fluorecein diacetate staining technique (Widholm 1972). The cells (0.2 g wet weight) were incubated in medium A (5 ml) containing fluorecein diacetate (0.1 mg/ml) for 30 min and then observed in a fluorescence microscope.

Results and Discussion

Monitoring of Permeability

Various enzyme activities within plant cells can be employed for monitoring the permeability of the plasma membrane. Enzymes requiring nucleotide coenzymes such as NADP, ATP, or CoA are convenient to use for this purpose. These coenzymes cannot penetrate an intact plasma membrane and thus no enzyme activity is expressed unless the plasma membrane of the cell has been made permeable. In our initial studies (Felix et al. 1981) on the permeabilization of immobilized plant cells, we investigated a number of enzymes, including hexokinase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme, and citrate synthase, and found essentially no enzyme activity unless the cells were permeabilized.

In the present study we have used isocitrate dehydrogenase (ICDH) to monitor the permeability of the plasma membrane. The immobilized cell preparations were incubated in a solution containing the substrate with constant stirring. The supernatant was recirculated with a peristaltic pump through a flow cuvette in a spectrophotometer and the absorbance at 340 nm was recorded continuously. The observed changes in NADPH concentration over time are illustrated in Fig. 1 for untreated and DMSO-treated (5%) cells of *C. roseus.* Untreated cells show a relatively low ICDH-activity, while cells treated with DMSO for 30 min express a high activity. This indicates that DMSO has rendered the cells permeable to isocitrate and NADP(H), allowing the direct assay of an intracellular enzyme.

Effect of DMSO Concentration on Permeability and Viability

A wide variety of substances can be employed for the permeabilization of immobilized plant cells (Felix et al. 1981). DMSO was selected as the permeabilizing agent in this study since treatment with this substance results in preparations with relatively high enzyme activity expressed (Felix et al. 1981, Felix and Mosbach 1982). Furthermore, DMSO is widely used

to pretreat cells (both plant and animal) before freezing (cryopreservation) in order to lower the detrimental effect of ice crystals (Dougall and Wetherell 1974; Bajaj 1976). From cryopreservation experiments it can be concluded that the cells remain viable after treatment with DMSO.

In order to determine the optimal time for permeabilization, immobilized cells of *C. roseus* were treated with 5% DMSO for various periods of time with subsequent assay of ICDH activity. The immobilized cells appeared to be fully permeabilized (maximal ICDH-activity reached) after treatment for 5 min as shown in Fig. 2. The immobilized cell preparations were, however, usually treated for 30 min in order to assure maximum permeabilization. In this context it should be pointed out that increasing the concentration of DMSO from 5 to 10% does not result in any further increase in expressed ICDH-activity (c.f. Fig. 3).

The effect of DMSO concentration on the permability of the plasma membrane of various immobilized plant species was investigated. Figure 3 shows that the sensitivity of the plasma membrane to DMSO differs considerably for the three species studied. Cells of *C. roseus* appear to be completely permeabilized after treatment with 5% DMSO, while cells of *D. carota* and *D. innoxia* require around 10% and 25% DMSO, respectively, for complete permeabilization. These differences may be due to differences in the morphology of the cell aggregates or in the composition of the plasma membranes and/or cell walls. The effect of DMSO on the plasma membrane of *Catharanthus roseus* was also studied by staining with fluorecein diacetate. Few cells (less than 10%) appeared to have an intact plasma membrane after treatment with 5% DMSO. Further studies are in progress to determine how large a fraction of the cell population is actually affected (permeabilized) by the DMSO treatment and whether a single permeabilized cell can recover and divide.

The lowest concentration of DMSO required for full permeabilization can easily be estimated from experiments of this type. It is of importance to determine this concentration, i.e., the mildest conditions for maximal effect, since it can be expected that the viability of the treated cells is highly influenced by the DMSO concentration used. To verify this, suspensions of *C. roseus* cells were treated with various concentrations of DMSO, washed free of the DMSO and incubated in growth medium (medium A). The increase in dry weight as a function of incubation time is shown in Fig. 4. The cells treated with 2%DMSO grow at approximately the same rate as untreated cells. On the other hand, the cells treated with 5 or 10% DMSO show a lag phase of approximately 2 and 8 days, respectively, before growth occurs. *Catharanthus* cells can apparently tolerate treatment with up to 10% DMSO for 30 min without loss of viability, and it appears possible to permeabilize the plant cells while preserving viablity. Whether the long lag phase observed for the cells treated with 10% DMSO is due to survival of only a few cells or to slow recovery of the treated cells has not been determined. It may, however, be pointed

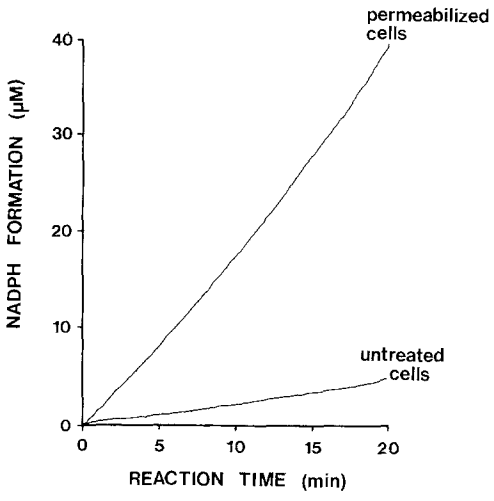

Fig. 1. Time course of the isocitrate dehydrogenase reaction in agarose-entrapped cells of *C. roseus*. The reaction mixture (5 ml substrate solution plus 1 g of wet beads) was monitored continuosly at 340 nm by pumping the supernatant through a flow cell at a rate of 5 ml/min. No reaction could be observed without isocitrate (result not shown)

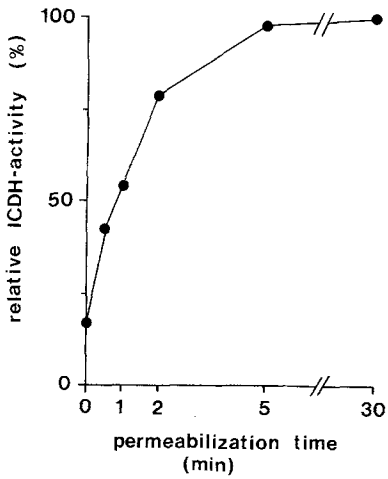

Fig. 2. Relative isocitrate dehydrogenase (ICDH) activity as a function of permeabilization time. 100% ICDH activity is defined as the maximal amount of enzyme activity observed. Agarose-entrapped cells of *C. roseus* (1 g of wet beads) were treated with 5% DMSO in medium A (10 ml) for the times indicated and then washed rapidly and assayed as described in the legend to Fig. 1

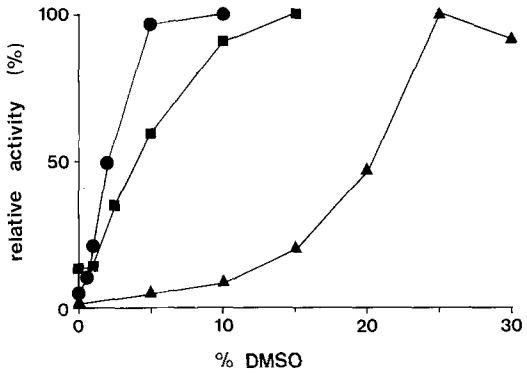

Fig. 3. Relative isocitrate dehydrogenase activity as a function of DMSO concentration. 100% ICDH activity is defined as the maximal amount of enzyme activity observed for each cell preparation. Beads (1 g wet weight) were treated with the DMSO concentrations indicated in medium A (10 ml) for 30 min then washed and assayed as described in the legend to Fig. 1. (−●−) *C. roseus*, (−■−) *D. carota*, (−▲−) *D. innoxia*

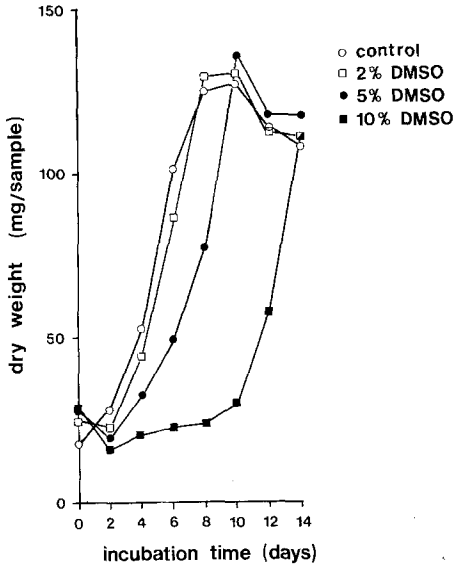

Fig. 4. Dry weight of various preparations of freely suspended cells of *C. roseus* as a function of incubation time. The cells were treated with the DMSO concentration indicated for 30 min before inoculation into growth medium (medium A)

out that a relatively high cell density is normally required for the survival of plant cells in suspension after transfer to a fresh medium because the medium has to be conditioned by the cells, which argues for metabolic activity of a relatively large number of cells also after treatment with 10% DMSO.

The approach for establishing the minimal concentration of DMSO required for maximal effect as outlined above may also find applications in cryopreservation experiments to increase the survival rate of preserved cells. It appears that the concentration

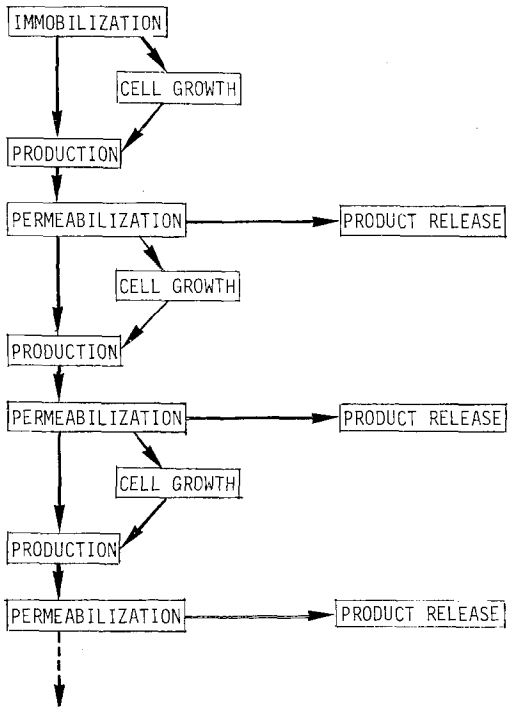

Fig. 5. Schematic diagram of a process for the production of intracellular plant biochemicals using intermittant product release from immobilized plant cells

of DMSO used in such experiments has not been optimized. In one study, it was concluded that for growth of carrot cells after freezing and thawing, 5 or 10% DMSO was superior to 0 or 15% DMSO (Dougall and Wetherell 1974). These results are in agreement with our findings that 10% DMSO permeabilizes carrot cells (Fig. 3).

Product Release

The synthesis of the indole alkaloid ajmalicine from distant precursors was used as a model production system for plant biochemicals. Agarose-entrapped cells of *C. roseus* were incubated in a medium containing two precursors – tryptamine (labelled with ^{14}C) and secologanin – and after 3 days of incubation, the cells were analyzed for ajmalicine isomers. One sample of immobilized plant cells was made permeable to ajmalicine with a 5% DMSO treatment in medium D and a parallel sample was extracted with methanol in a Soxhlet apparatus for total ajmalicine. In the DMSO treated cells, 85–90% of the total intracellular ajmalicine was released due to permeabilization of the entrapped cells. Quantitative release of products is thus possible under conditions where the viability of the immobilized cells is not affected to any great extent.

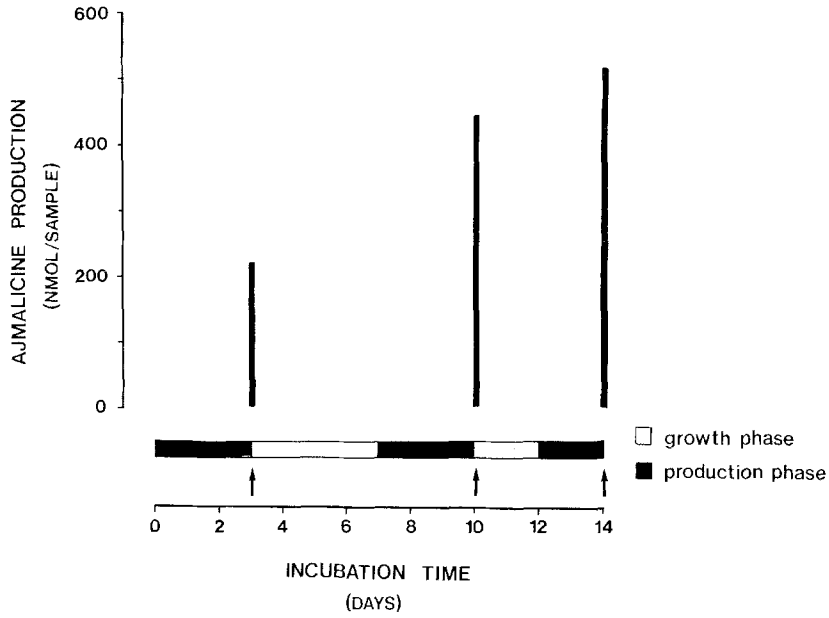

Fig. 6. Ajmalicine production by agarose-entrapped cells of *C. roseus*. The cells were intermittantly permeabilized *(arrows)* as described in the methods section

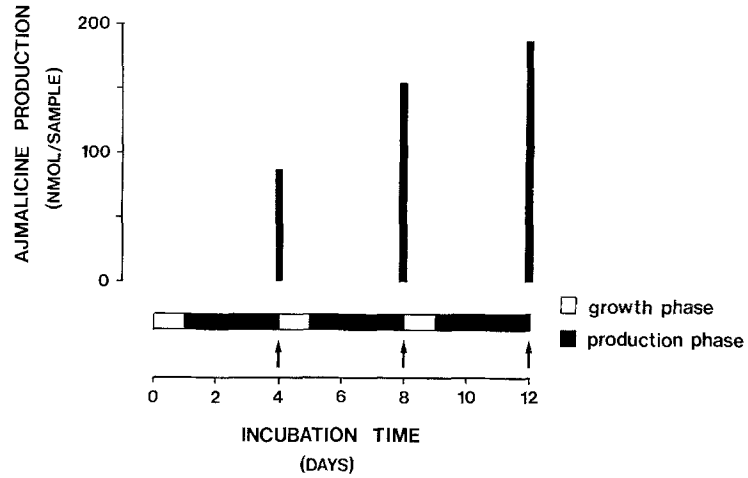

Fig. 7. Ajmalicine production by alginate-entrapped cells of *C. roseus*. The cells were intermittantly permeabilized *(arrows)* as described in the methods section

Intermittant Release of Products: A Model Production System for Plant Cell Biochemicals

The results of the experiments discussed above suggest, in principle, that it would be possible to intermittantly release intracellular products by permeabilization of the immobilized cells as schematically illustrated in Fig. 5. The cycle, including an optional growth phase, a production phase and a permeabilization procedure, may be repeated several times.

We have carried out some model experiments according to the procedure outlined in Fig. 5. The synthesis of ajmalicine isomers from tryptamine and secologanin was studied with immobilized cells of *C. roseus*. The results have been summarized in Figs. 6 and 7. Cells entrapped in agarose (Fig. 6) or calcium alginate (Fig. 7) could intermittantly be treated with DMSO to release the produced ajmalicine isomers. No significant difference was observed between agarose- and alginate-entrapped cells in these biosynthetic studies. A hormone-free medium was used in the production phase in order to limit growth. The increase in product yield observed in both experiments was most likely due to the increase of biomass due to the growth of cells within the polymer beads. Growth occured not only in the growth medium (medium A) but also to some extent in the production medium (medium D). For a steady state production optimization of the cycle has to be carried out. At present such studies are being carried out in our laboratory. The extension to de novo synthesis of secondary products from a simple carbon source with subsequent release by permeabilization is also in progress.

Conclusions

The results presented in this report clearly demonstrate that it is possible to release products stored within immobilized cells by a permeabilization procedure that does not affect the viability and biosynthetic capacity of the cells. A continuous process based on intermittant release of products has been developed that can be of fundamental importance for the future development of the production of biochemicals with cultivated plant cells since the biomass can be reutilized in such a process.

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