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ENHANCED STABILITY OF ENZYMES IN PERMEABILIZED AND IMMOBILIZED CELLS

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ABSTRACT

Plant cells obtained from suspension cultures of *Catharanthus roseus* were immobilized in agarose beads or in a polyurethane matrix. Permeabilization of these cells with dimethylsulfoxide allowed measurement of the enzymes isocitrate dehydrogenase (primary metabolism) and cathenamine reductase (secondary metabolism). Enhanced storage and operational stability was achieved by treating the cells with hardening agent glutardialdehyde in combination with hexamethylenediamine.

INTRODUCTION

As shown previously (Felix et al., 1981) cells of the plant *Catharanthus roseus* obtained from suspension cultures can be permeabilized by treatment with various agents. The cells became permeable to exogenous substrates retaining the activity of the enzymes isocitrate dehydrogenase and cathenamine reductase, which are marker enzymes for primary

and secondary metabolism, respectively. (For a review on various permeabilization techniques see Felix, 1982, and for a review on permeabilized plant cells, Brodelius and Mosbach, 1982). In this context it was demonstrated that externally added NADPH increased the transformation of the indole alkaloid cathenamine to ajmalicine isomers. For some applications, notably biotransformation of high-value compounds, the use of permeabilized cells in the immobilized state may turn out to be an interesting "intermediate" alternative to the use of isolated enzymes/coenzymes on the one hand and intact cells on the other. This will particularly be the case when dealing with systems where inward- and outward-diffusion of the compounds to be converted is hindered for intact cells and where the alternative approach using isolated enzymes/coenzymes is too laborous or leads to unstable systems. Recent studies demonstrating that coenzymes bound to water-soluble polymers can interact with enzymes present in permeabilized cells point to the application of such systems also in a flow-through fashion (Chand and Mosbach, unpublished).

Although the stability of the two enzymes studied (Felix et al., 1981) proved to be quite high we attempted to enhance the stability further using different immobilization techniques as well as chemical hardening.

Recently Murata et al. (1980) tested toluene-treated *E.coli* immobilized in carrageenan for the production of glutathione. They found an enhanced operational stability of immobilized, permeabilized cells after treatment with the hardening agent, glutardialdehyde in the presence of hexamethylenediamine. We made analogous experiments with these plant cells as well as testing new supports: gelatin foam structure (Larreta Garde et al., 1981) and polyurethane (Klein and Kluge, 1981).

MATERIALS AND METHODS

Culture conditions. Cellsuspension cultures of the plant *Catharanthus roseus* were obtained as described (Felix et al.,1981).

Permeabilization procedures. 4 g (2g wet cells + 2g carrier) cells immobilized in agarose (or alginate or carrageenan or gelatin) or cells embedded in hypol (20 ml) were placed in columns and permeabilized by pumping 20 ml 10% DMSO solution (Felix et al. 1981) through the columns at a rate of 3 ml/min for 30 min at 22°C.

Immobilization procedures. 5 g plant cells (wet weight) were entrapped in 5 g 5% agarose or 5 g 5% alginate or 5 g 3% carrageenan as described earlier (Brodelius and Nilsson, 1980). A crosslinked gelatin foam structure was obtained following the procedure by Larreta Garde et al. (1981) mixing 0.5 ml potassium phosphate buffer (pH 6.8), 1g of plant cells (ww), 2ml of 10% gelatin and 1.5% glutardialdehyde solution. The foam block obtained was fragmented (pieces approx. 2x2mm size) to allow flow through the column (1.6cm x 9.95 cm). A hypol 3000 foam was obtained by rigorously mixing 10g wet cells (no further addition of water) with 3g of hypol 3000 (a polyurethane from W.R.Grace, Lexington, Mass., 02173, USA) followed by quickly filling the mixture into a column (1.6cm x 9.95cm). During polymerization the volume increased to 35ml. The tops had to be cut off as the surface of the foam is not porous (Klein and Kluge, 1981).

Measurement of enzyme activities. Isocitrate dehydrogenase was measured by placing 4 g of immobilized, permeabilized cells in a small column and allowing 10 ml 50 mM potassium phosphate buffer (pH 7.5), 4 mM D,L-isocitric acid, 1 mM $NADP^+$ and 1.5 mM $MgCl_2$ to recycle. The progress of the reaction,leading to NADPH formation, was monitored at 340nm by pumping the reaction mixture (10ml) through a flow cell at 3 ml/min. Cells immobilized in hypol were assayed analogously allowing the same reaction mixture (10 ml) to pass through the column (1.6cm x 9.95cm). Cathenamine reductase was assayed by pumping 5ml of a buffer solution containing cathenamine, isocitrate and NADPH (Felix et al., 1981) through the same column (4 g agarose beads or 20ml hypol). The reaction was monitored by testing the probes with HPLC.

Hardening procedure. 10g wet cells or 10g cells embedded in agarose were suspended in 60ml hexamethylenediamine solution for 10 min followed by addition of 5ml of 12.5% glutardialdehyde (30min) as described by Murata et al. (1980). It is worth pointing out that the wet weight of the free cells dropped from 5g to 2g during the hardening process. This loss was compensated for by the addition of water prior to immobilization.

Operational stability. The reaction was followed by recycling 10ml of the reaction mixture including 10% DMSO through the columns at a speed of 3ml/min . After 30 min the flow rate was reduced to 0.24 ml/min. Pumping continued for 47½ hours after which fresh reaction mixture was added. DMSO was always included to prevent infections. This schedule was followed for 14 days, then fresh medium was added less frequently and under non-recycling conditions.

RESULTS AND DISCUSSION

Of the different immobilization techniques applied, only cells entrapped in agarose or hypol 3000 (a polyurethane) showed both isocitrate dehydrogenase and cathenamine reductase activities (Table 1). In contrast, cells embedded in alginate or carrageenan or in a cross-linked gelatin foam structure were not active (results not shown). In case of alginate or carrageenan this may be caused by the charged support hindering the entry of $NADP^+$ into the beads (and cells). Immobilization in hypol support lead to highly active preparations both with regard to isocitrate dehydrogenase and cathenamine reductase activity. As this entrapment method is rapid, taking only about five minutes, it represents an interesting alternative.

Table 1 : Comparison of different immobilization techniques

Immobilization method a)	activity of isocitrate dehydrogenase b)	activity of cathenamine reductase c)
agarose (+intact cells)	5	92
agarose/DMSO	100	100
HMDA+GA/agarose/DMSO	88	10
agarose/HMDA+GA/DMSO	86	7
DMSO/HMDA+GA/agarose	0	n.d.
hypol 3000/ DMSO	74	59
HMDA+GA/hypol 3000/DMSO	36	6

a) The sequence in the preparation varied, one example : immobilization in agarose / hardening with hexamethylenediamine (HMDA) and glutardialdehyde (GA) / permeabilization with DMSO.

b) 100 corresponds to 1.71 mmoles NADPH formed per liter, per gram wet cells and per minute.

c) 100 corresponds to 1.54 µmoles ajmalicine isomers (ajmalicine, 19-epiajmalicine, tetrahydroalstonine) formed per liter, per gram wet cells and per hour.

To enhance stability of enzymes in permeabilized, immobilized cells, a hardening procedure was applied. Similar activities of the enzyme isocitrate dehydrogenase in cells embedded in agarose were obtained whether the cells are hardened with hexamethylenediamine (HMDA) and glutardialdehyde (GA) or not. The combination of hardening and immobilization in hypol 3000 was less favorable. This may be in part compensated by the increased stability. Cathenamine reductase was found to be active for at least five days of operation, when both immobilization and hardening were used (data not shown). If permeabilized cells were treated with hardening agent, no activity at all was detected (Table 1). In this case, GA can freely enter the cells and seems to block isocitrate dehydrogenase. If intact cells are treated with HMDA/GA, the

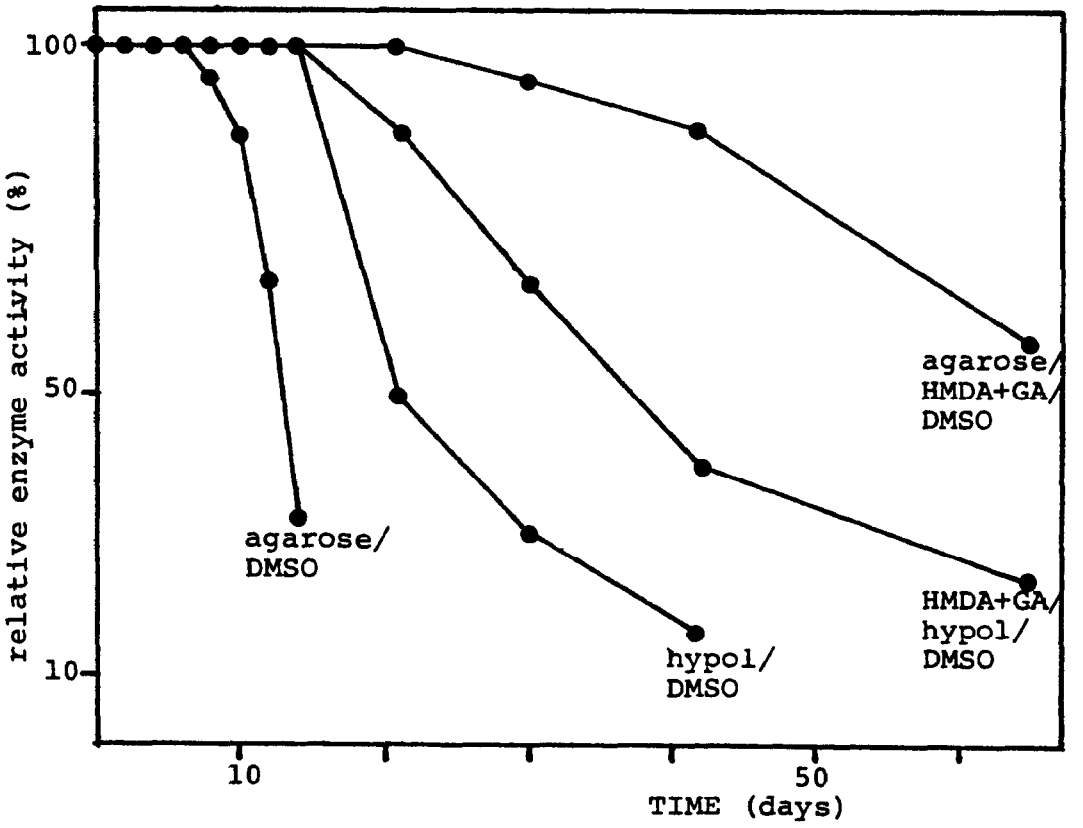

Fig.1. Operational stability of immobilized plant cell columns at 22°C (tested enzyme: isocitrate dehydrogenase)

cells become partially permeable to $NADP^+$ without subsequent DMSO treatment (44% activity). One can assume that crosslinking of the membrane proteins occurs, which changes membrane fluidity.Subsequent treatment with DMSO leads to delipidation of the membrane without the escape of intracellular proteins (compare with Aragón et al.,1980).

In earlier experiments (Felix et al., 1981) it was noted that stirring or shaking was the cause for the decrease of enzyme activities in permeabilized and immobilized cells over several days. Therefore cells embedded in agarose were handled with much care. This and the hardening of cells with HMDA/GA lead to a drastically increased operational stability of isocitrate dehydrogenase in cells embedded in agarose (Fig.1). The half-life could be increased from 4 days (Felix et al., 1981) to 72 days. The immobilization in hypol 3000 leads itself to a highly stable preparation. An additional hardening also increases the stability.

As isocitrate dehydrogenase is used only as a model enzyme, other interesting enzymes will be tested as the results in Fig. 1 look very promising.

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