

Effects of Permeabilization on the Biotransformation of Phenylalanine by Immobilized Tobacco Cell Cultures

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Immobilized tobacco cells excreted only a small proportion of their main secondary metabolite caffeoylputrescine into the culture medium. Experiments designed to release more of the compound by permeabilization caused the loss of caffeoylputrescine, probably by oxidative reactions. Moreover, rather mild treatments with permeabilizing agents (*e.g.* *n*-propanol) resulted in severe growth inhibition. The ability of permeabilized cells to form caffeoylputrescine and other hydroxycinnamoyl conjugates from phenylalanine decreased considerably even when such cells were still able to metabolize phenylalanine into various ethyl acetate extractable compounds (*e.g.* hydroxycinnamic acids and acetophenones). The formation of new biotransformation products suggests that permeabilized cells could be used as a tool for testing the enzymatic capabilities of a cell culture.

Introduction

Immobilized plant cells have attracted attention as suitable tools for the production of interesting secondary metabolites [1–5]. Arguments for the use of immobilized rather than freely suspended cells resulted from problems associated with fermentation of the latter [2, 6]. Immobilization of plant cells should reduce the shear hydrodynamic stress and could provide distinctly higher volumetric productivities [2]. Moreover, the low specific productivity of many suspension culture systems was thought to be improved by immobilization due to better cell-to-cell interactions [5, 7]. On the other hand, an absolute requirement for the use of immobilized plant cells was that the desired products were released into the medium. However, it was soon evident that metabolites usually stored in the cells were also accumulated within the entrapped cells [8]. Thus, immobilization

seemed to be restricted to systems where the desired product was spontaneously released into the culture medium [9–12]. Consequently a number of attempts have been made to permeabilize cells with chemical agents [13–17]. The usefulness of chemical agents for the permeabilization of immobilized plant cells must be questioned since toxic concentrations are usually needed for distinct product release [8]. Destruction of cell compartmentation seemed to be the consequence of all chemical permeabilizations [18]. We would like to substantiate the latter assumption by our studies on the production of hydroxycinnamoylputrescines (HCA-Put) by freely suspended and immobilized tobacco cells [19].

Materials and Methods

Plant material: Selection, maintenance and characteristics of the tobacco cell line TX4 have been described ([19, 20] and ref. cited therein).

Immobilization and permeabilization: Cells were harvested by filtration. 30 g fresh mass was suspended in 100 ml 3% alginate and then dropped into a 50 mM solution of CaCl₂. 4 g beads contained *ca.* 1 g cells. The beads were treated with permeabilizing agents by adding the indicated amount of the agent to the culture medium (v/v) for the time period indicated. After washing 6 g beads were weighed into 20 ml of 1:4 or 1:8 diluted growth medium (MS (21) +

Abbreviations: HCA, hydroxycinnamic acid; CAF, caffeic acid; HCA-Put, hydroxycinnamoylputrescine; Phe, phenylalanine.

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2 μ M 2,4-dichlorophenoxyacetic acid) and cultured in 50 ml flasks on a gyratory shaker.

Analyses of feeding experiments: The compounds ([U- 14 C]phenylalanine and [1,4- 14 C]putrescine) were added filter-sterilized to the cultures. The beads were extracted twice with 70% MeOH at 70 °C and squeezed. The MeOH extracts and the medium were separated into an ethyl acetate and a water phase. A part of the ethyl acetate extract was silylated with MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) and analyzed by GC-MS as described recently [22]. Ethyl acetate extracts were also analyzed by TLC (toluene:ethyl formate:acetic acid 5:4:1; benzene:methanol:acetic acid 45:8:3) and HPLC (conditions: Nucleosil C₁₈, 5 μ , 250 \times 4 mm; solvent A: 1.5% H₃PO₄, solvent B: 1.5% H₃PO₄, 20% CH₃COOH, 25% CH₃CN; linear gradient from A to B within 40 min, 1 ml/min, detection 280/320 nm). Distribution of radioactivity was measured directly on TLC plates with a Berthold Silena NIM multi-channel scanner or by scintillation counting of HPLC eluates or of scraped TLC zones. The water extracts were analyzed by the recently described HPLC systems for HCA-Puts [23] or the above HPLC-system which was used for HCA-glucose esters, HCA-Puts and the free HCA acids.

Identification of the metabolites: Co-chromatography, MS- and NMR spectroscopy revealed, beside the HCA-Puts, the following compounds, derived from Phe, in cells treated with *n*-propanol: 1- β -O-glucose esters of *p*-coumaric acid (1 H NMR spectrum, HPLC co-chromatography, alkaline hydrolysis and TLC chromatography), ferulic- and caffeic acid (HPLC co-chromatography and hydrolysis [24]); free *p*-coumaric, ferulic and caffeic acid (GC-MS of

MSTFA derivatives, TLC and HPLC co-chromatography); compounds which were not always formed by permeabilized cells such as coniferyl alcohol or various hydroxyacetophenones were identified by GC-MS and comparison with synthetic material.

Results

Effect of "permeabilization" on the fate of caffeoylputrescine

For studying the fate of the main secondary metabolite Caf-Put, TX4 cells were fed with labelled Phe as tracer. Uptake of Phe from the medium was quite similar in freely suspended and immobilized cells (Table I). Generally between 30–50% of exogenously added Phe was incorporated into the residue of untreated cells, mostly into protein. Up to 80% of extractable radioactivity of fed Phe was converted into HCA-Puts, *e.g.* Caf-Put. HCA glucose esters were only found when higher levels of Phe were given in addition to the labelled tracer. When the cells were permeabilized, the medium, cells and beads became dark. Radioactivity in the medium increased up to 30% of that of the initially added label. However, the efflux of radioactivity was not as high as one would have expected based on the loss of extractable radioactivity from the cells (Table I). Thus the "permeabilization" mainly caused incorporation of HCA-Puts into the residue (Table I). Similar results were observed when other permeabilizing agents such as DMSO (5–30%) or chitosan (up to 5%) were used. Water immiscible solvents such as chloroform (Table I) or diethyl ether resulted in the highest loss of soluble radioactivity. In summary, whenever increased radioactivity in the medium in-

Table I. Distribution of radioactivity in free and immobilized cells 24 h after being treated with permeabilizing agent for 30 min. The incorporation into the residue (protein, phenolics, binding to alginate) was calculated from the sum of radioactivity in the medium and the cell extract.

Treatment	% of Radioactivity added in the					
	Medium		Cell Extract		Residue	
	Free	Immob.	Free	Immob.	Free	Immob.
Control	6	7	50	49	44	44
1% <i>n</i> -Propanol	10	7	47	50	43	43
2.5% <i>n</i> -Propanol	20	15	17	10	63	75
5% <i>n</i> -Propanol	14	16	2	8	84	76
Chloroform-saturated H ₂ O	26	14	2	3	72	83

Table II. Percentage of phenolics and cinnamoylputrescines released into the culture medium of immobilized “permeabilized” cells 24 h after the treatment. Cells contained 2 mg caffeoylputrescine or 200 *E*₇₂₅-units phenolics/flask ± 10% at the beginning of the experiment when the cells had been transferred back to the culture medium after a 30 min period in the permeabilizing medium.

Treatment	% of Metabolites released into the medium	
	Phenolics	Cinnamoylputrescines
Control	9.2	8.1
1% <i>n</i> -Propanol	12.5	10.3
2.5% <i>n</i> -Propanol	17.8	3.1
5% <i>n</i> -Propanol	20.2	1.3
10% <i>n</i> -Propanol	26.8	1.5

dicated permeabilization, a much greater loss of extractable radioactivity from the cells was noted.

When the content of total phenolics in the medium was measured by the Folin test [19], the increase

noted correlated well with the release of radioactivity. However, the levels of the main phenolic Caf-Put as determined by HPLC, decreased greatly (Table II). Most of the HCA-Puts were evidently oxidized

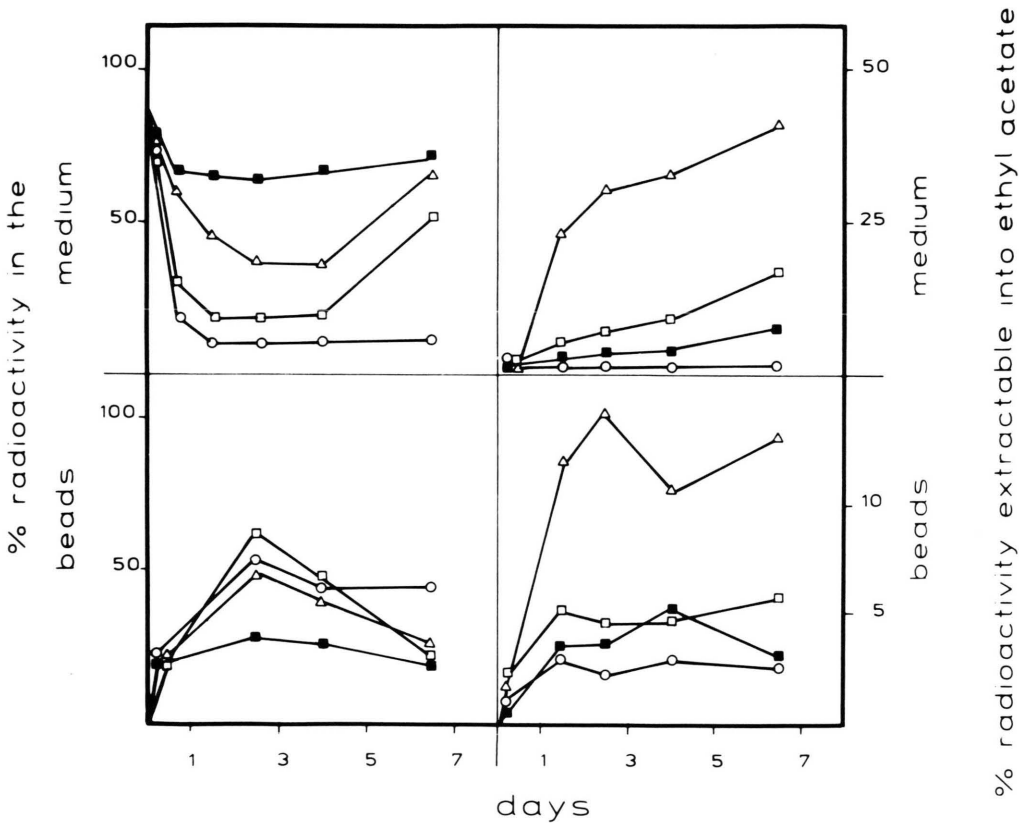


Fig. 1. Time courses of the distribution of radioactivity in the medium and the beads when 1 μ Ci L-[U-¹⁴C]phenylalanine (2 mg) was added to immobilized cells permeabilized with various concentrations of *n*-propanol for 1 h. The curves on the left give the proportion of added label in the medium and the beads. The curves on the right indicate the proportion of total radioactivity extractable into ethyl acetate. (○) = untreated beads, (□) = 0.5%, (△) = 2.5%, (■) = 5% *n*-propanol treatment. Two flasks were harvested on the days indicated.

and incorporated into the residue and only a smaller amount was released as phenolics of unknown structure into the medium.

Biosynthetic potential of permeabilized cells

The polymerization of the HCA-Puts indicated that several enzymes had retained their activity in these "permeabilized" cells. It was of interest to see whether the permeabilized cells might perform at least a few of the reactions along the biosynthetic pathway to the HCA-Puts. DMSO, chitosan, acetone, chloroform and diethyl ether were tested as permeabilizing agents in addition to *n*-propanol. *n*-Propanol treatment was found to result in better biotransformation capabilities than the other agents and it was thus used for the following experiments.

Fig. 1 indicates a strongly reduced uptake of Phe with increasing concentrations of *n*-propanol. Ex-

traction of the medium showed the accumulation of ethyl acetate soluble compounds in addition to Phe. In the 2.5% *n*-propanol treatment, 38% of the added radioactivity was found to comprise metabolites of Phe. The main component of the extract containing the highest level of ethyl acetate extractable radioactivity was free *p*-coumaric acid, a compound which did not accumulate in untreated cells. Free caffeic and ferulic acid were also detected in lower amounts in several extracts. In the medium of 0.5 and 1% *n*-propanol-treated cells, 4-hydroxy-, 3-methoxy-4-hydroxy- and 3,5-dimethoxy-4-hydroxyacetophenone were found to be the main labelled constituents of the ethyl acetate extracts. The GC spectra of the ethyl acetate extracts of the cells and the media for a given time and treatment compared well, and the radioactivity of the ethyl acetate extractable compounds in the cells reflected roughly the ratio bead

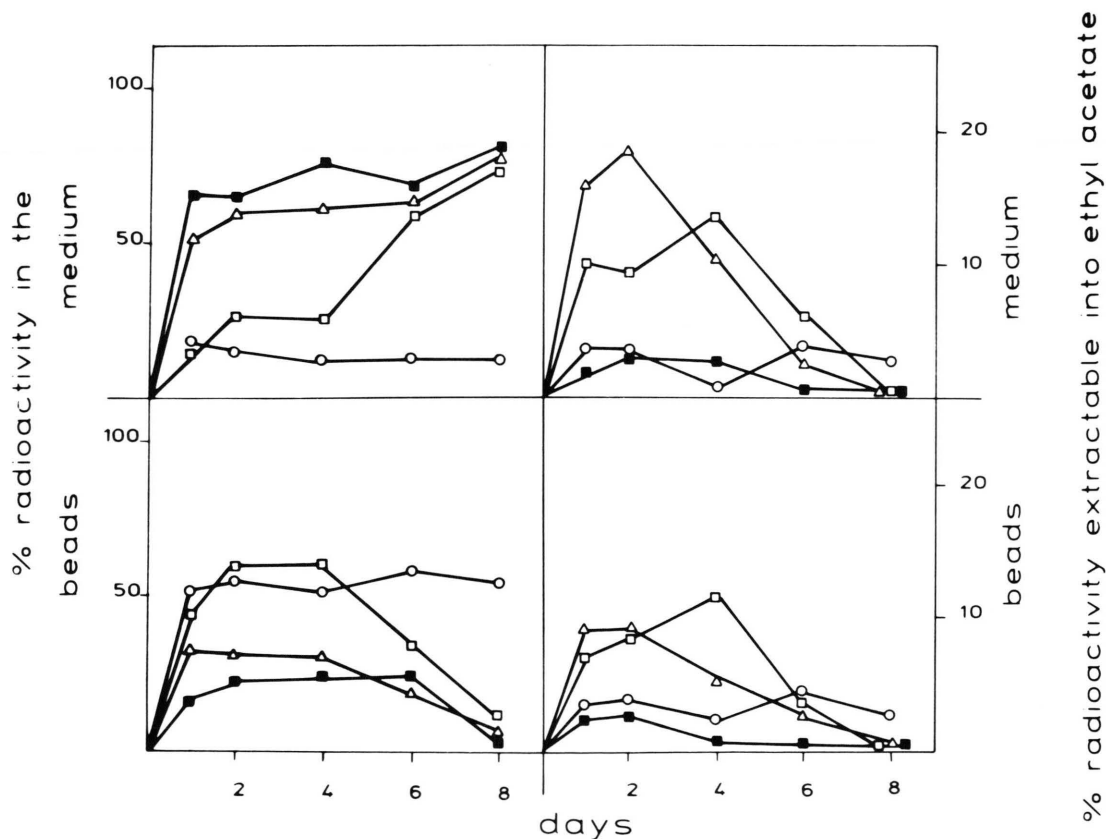


Fig. 2. Distribution of radioactivity in the medium and the beads when labelled phenylalanine (Fig. 1) was added to immobilized cells for 24 h at various times after the 1 h permeabilization procedure. The values on day 4, for example, denote that feeding began at 72 h after the permeabilization. The curves on the left give the proportion of added label in the medium and the beads. The curves on the right present the proportion of total radioactivity extractable with ethyl acetate. (○) = untreated cells, (□) = 1%, (△) = 2.5%, (■) = 5% *n*-propanol treatment.

volume:medium. Evidently the free HCAs could not be stored in the cells and were freely distributed between the medium and the cells.

In the above experiment the accumulation of ethyl acetate extractable compounds was followed over a period of 7 days when Phe was added 1 h after the permeabilization procedure. For determining how long treated cells are able to metabolize Phe, biotransformation capacity per 24 h was tested at various times after the permeabilization (Fig. 2). Cells treated with 2.5% *n*-propanol metabolized phenylalanine most efficiently into ethyl acetate soluble compounds on the first two days after treatment and had nearly lost this ability after 7 days (Fig. 2). The milder treatments (Fig. 2) appeared to result in less damage, as shown by the extended ability for biotransformation and by the conjugation of a portion of the formed HCAs to give water-soluble compounds. GC comparisons of the ethyl acetate extracts of the media derived from the immobilized cells treated in various ways showed that the range of compounds produced diminished with the severity of the disturbance caused by the treatment with *n*-propanol. Thus, cells treated with 2.5% *n*-propanol formed all three HCAs, during the 2 days after the treatment, mainly *p*-coumaric acid on the next 2 days and no cinnamic acids after 7 days.

Since in untreated cells nearly all the extractable metabolites of Phe were water-soluble compounds (Fig. 2), the water phases of the ethyl acetate extractions were also analyzed. Water-soluble radioactivity in the medium was almost always accounted for by unchanged phenylalanine, which indicated that treated and untreated cells which were able to form water-soluble HCA conjugates did store these compounds mainly in the cells. Only traces of known HCA-Puts or glucose esters were detected in the medium. The cell extracts of untreated or mildly treated beads contained a variety of HCA derivatives. Besides the natural HCA-Puts constituents, there were *p*-coumaroyl-, caffeoyl-, feruloylglucose esters which were identified by co-chromatography and alkaline hydrolysis. With increasing concentrations of the permeabilizing agent, or with ageing of the cells after the permeabilization, the formation of all HCA conjugates was disrupted or ceased. Thus the radioactivity found in the water phases of cells treated with 2.5 or 5% *n*-propanol was accounted for mainly by unchanged Phe. Hence, the main reason for the appearance of labelled ethyl acetate extractable com-

pounds seems to be the fact that some reactions of the general phenylpropanoid pathway were still active in cells whose capability for the conjugation of the HCAs was already inhibited. The formation of HCA-Puts was also not found when Phe and Put were fed together to permeabilized cells.

Discussion

The results of this paper demonstrate that immobilization does not necessarily cause increased release of stored compounds compared to freely suspended cells [8]. Our efforts to enhance the efflux of Caf-Put from viable cells by chemical permeabilization failed. Even weakly treated cells (with 0.5% *n*-propanol for 30–120 min causing no increased efflux) did not show any growth after several weeks of culture while free cells grew out of untreated beads after a few days. Beside the growth inhibition and the loss in viability, destruction of the desired key product was noted under all conditions of permeabilization. The latter observation may be best explained by the assumption that chemical permeabilization destroyed compartmentation of the cells [18]. In intact cells Caf-Put was safely stored in the vacuoles, separated from enzymes which could react with this compound. In chemically treated cells the compartmentation was disrupted and consequently Caf-Put came in contact with enzymes it never encountered in the intact cell. Thus permeabilization may lead to reactions which never occur in the fully compartmentalized cell. The presence of the *o*-dihydroxyl group in Caf-Put was most likely the site of attack by ubiquitous enzymes such as phenolases and peroxidases. The darkening of the beads and the incorporation of Caf-Put in the residue provided strong evidence that such oxidizing reactions were responsible for its loss in treated cells. As similar reactions and polymerizations may occur with many constituents such compounds must be regarded as unsuitable for any permeabilization procedure. On the other hand, one could also envisage that with other structures enzymatic modifications may lead to new compounds not found in the intact cell.

In consequence, chemical permeabilization does not seem to represent a way of enhancing product yields in the medium of viable immobilized cells [8, 18]. Thus the search for variant lines releasing their products into the medium [25] or for cultural conditions triggering the efflux of products [26] seem to be superior approaches for establishing culture systems

where the desired product can be harvested from the medium. A potential value of permeabilized cells may, however, be seen in a different field. The demonstration of further and altered metabolism of cell constituents and intermediates in permeabilized cells suggests that such cells may be of special interest for initial evaluations of the enzyme reactions that might be possible with such a culture. In our case, the formation of novel biotransformation products derived from Phe was not only due to the accumulation of known or expected intermediates of a disrupted biosynthetic pathway but also due to further unpredictable reactions of such intermediates. Of special interest was the accumulation of acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone) in cells treated with 0.5% *n*-propanol. This compound has only been detected in tobacco cells during infection with *Agrobacterium tumefaciens* [27]. Thus, initially, the bacterium may cause a membrane permeabilization and

disruption of compartmentation of the attacked cells analogous to the chemical treatment. In some experiments the main-labelled compound in ethyl acetate extracts was the lignin precursor coniferyl alcohol. However, the occurrence of cinnamic acid derived ethyl acetate extractable metabolites was not consistent and may depend upon the physiological state of the cells and the extent of permeabilization. Thus further research with permeabilized cells should analyze the impact of the cells' physiological state on the biotransformation capability and should look for techniques to stabilize the enzyme reactions.

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