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BRIEF COMMUNICATION

Activity of some aminopeptidases in immobilized cells of *Daucus carota*

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Abstract

Carrot cell suspension culture prior to immobilization by glutaraldehyde was permeabilised by *Tween 80*. The values of pH optimum for L-alanine aminopeptidase, L-proline iminopeptidase, and L-arginine aminopeptidase were 8.2, 7.4, and 7.9, respectively. The immobilized cells showed significantly lower aminopeptidase activity when compared to untreated cells. Alginate hydrogel was successfully used for immobilization of carrot cells retaining the activity of some aminopeptidases.

Additional key words: alginate, carrot, cell permeabilization, glutaraldehyde.

Plant cells were first immobilized by Brodelius *et al.* (1979) with alginate. Many matrices from synthetic polymers or biological materials have been used for the immobilization of cells. The application of glutaraldehyde for cell immobilization has been investigated (Hasal *et al.* 1992, Stano *et al.* 1995, 1997a,b, Hansen *et al.* 1998). Poppy cells immobilized in calcium alginate retained their biological activity for as long as 6 months. The cells performed the biotransformation of codeinone to codeine with higher biotransformation ratio than in cell suspension. This phenomenon is advantageous for the employment of immobilized plant cells in the biotransformational processes (Furuya *et al.* 1984, Berlin *et al.* 1989). The plant proteolytic enzymes play many roles in biochemical reactions such as peptide and protein degradation, postranslational protein modification, and in other processes as well (Stano *et al.* 1989, Heymann *et al.* 1990, Radlowski *et al.* 1994). Aminopeptidases (EC 3.4.11) were shown to be present in many plants (Radlowski *et al.* 1994, Stano *et al.* 1989, Benešová *et al.* 1974, Doi and Kawakami 1997).

In this paper the enzymatic hydrolysis of N-terminal peptidic linkage of synthetic substrates aminoacid-4-(phenylazo)-phenylamides, by free as well as glutaraldehyde or alginate immobilized carrot cells is described. Long-term tissue culture was derived from seedlings of *Daucus carota* L. and continuously subcultured every two weeks on Murashige-Skoog medium as was previously described by Blanáriková *et al.* (1996). Cell suspensions were filtered through a nylon cloth and 10 g of fresh mass was suspended in 40 cm^3 of 5 % *Tween 80* in 0.15 M NaCl solution. Permeabilization proceeded for 3 h under moderate stirring at 20 °C. The cells were separated by filtration, washed with 2 dm^3 of distilled water and 3 dm^3 of 0.15 M NaCl solution and separated again. The permeabilized cells were immediately resuspended in fixing solution (40 cm^3 of 0.15 M NaCl, 4 cm^3 of 25 % glutaraldehyde). The immobilized cells were then separated and washed (2 dm^3 of 0.15 M NaCl). For the immobilization by alginate cells were harvested by filtration. Cells (6 g of fresh mass) were resuspended in 2 % sodium alginate (20 cm^3)

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and then dropped into 50 mM $CaCl_2$ (100 cm^3). Created spherical gel particles were almost homogenous and 4 mm in diameter. The gel beds (4 g contained 1 g cells) with immobilized cells were collected and washed by the growth medium. The beds (5 g) were washed, then added to the of the growth medium (20 cm^3) and cultured in 100 cm^3 flasks on a rotatory shaker (1.25 rps) (Brodelius *et al.* 1979, Berlin *et al.* 1989, Shoichet *et al.* 1996). Fresh and dry mass of the cell suspensions was determined gravimetrically. For determination of dry mass, samples were dried to a constant mass at 105 °C.

As a parameter of cell viability the glucose utilization was determined. The immobilized cells and cell suspensions were exposed to an initial glucose concentration of 200 mg dm^{-3} in the cultivation medium (Blanáriková *et al.* 1996) without sucrose. The concentration of glucose was determined according to Trinder (1969). The cell viability was determined using 2,3,5-triphenyltetrazolium chloride (TTC), fluorescein diacetate, and oxygen electrode (Dixon 1991).

The enzyme activities of L-alanine aminopeptidase (L-Ala-AP), L-proline iminopeptidase (L-Pro-AP) and L-arginine aminopeotidase (L-Arg-AP) were determined by a modified method of Stano *et al.* (1989) using synthetic chromogenic substrates: L-alanine-4-(phenylazo)-phenylamide (L-Ala-PAP-amide), L-proline-4-(phenylazo)-phenylamide (L-Pro-PAP-amide), and L-arginine-4-(phenylazo)-phenylamide (L-Arg-PAP-amide). The reaction mixture contained of 1.7 cm^3 of Theorell-Stenhagen buffer (0.1 M H_3PO_4-NaOH) pH 8.2, 7.4, and 7.9, respectively, and 0.3 cm^3 substrate solutions in optimal concentrations: 2 mM L-Ala-PAP-amide, 2.5 mM L-Pro-PAPA-amide, or 1.6 mM L-Arg-PAP-amide, respectively. After preincubation (10 min at 30 °C) a suitable amounts of cells: 0.1 - 0.3 g of immobilized or native cells, respectively, was added. The control contained heat-treated (100 °C) cells. The mixtures were kept for 20 min at 30 °C on a rotatory shaker (1.25 rps), and the reaction was stopped by adding 0.5 cm^3 of 40 % TCA. The cells were separated from reaction mixture (Brodelius *et al.* 1979), dried and weighed. The concentration of the protonized 4-(phenylazo)-phenyl amine was determined spectro-photometrically at 500 nm. The enzyme activity is expressed in katals. Protein content was determined by method of Bradford (1976) using bovine serum albumin as the standard protein.

Aminopeptidases and dipeptidyl peptidase activities are widely distributed in various plants and cell suspensions (Benešová *et al.* 1974, Stano *et al.* 1989, 1994a,b). The cells immobilized by cross-linking with glutaraldehyde have retained high values of the tyrosine decarboxylase, DOPA-decarboxylase, α- and β-galacto-sidase and invertase activities for several months (Stano *et al.* 1995, 1996, 1997a,b, 1998, Poór *et al.* 1998a,b,).

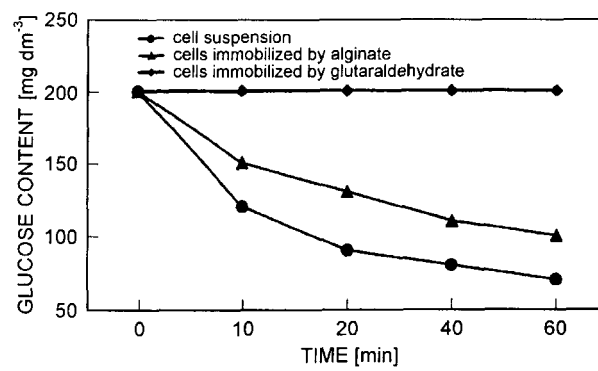

Fig. 1. Changes in glucose content in the medium.

Table 1. Activity of L-alanine aminopeptidase, L-proline iminopeptidase, and L-arginine aminopeptidase in cell suspension of glutaraldehyde and alginate immobilized cells of carrot. Prior to glutaraldehyde immobilisation the cells were permeabilized by *Tween 80*. The cells immobilised by alginate entrapment were not permeabilized.

	Protein [mg g^{-1} (d.m.)]	Activity [nmol g^{-1}(d.m.) s^{-1}] (L-Ala-Ap)	(L-Pro-AP)	(L-Arg-AP)	Specific activity [nmol g^{-1}(protein) s^{-1}] (L-Ala-Ap)	(L-Pro-AP)	(L-Arg-AP)
Suspension	36.7 ± 0.20	62.3 ± 0.21	51.4 ± 0.20	64.8 ± 0.20	1.84	1.19	1.49
Tween 80	14.3 ± 0.21	70.6 ± 0.20	62.1 ± 0.21	70.2 ± 0.21	6.13	4.32	5.32
Glutaraldehyde	14.2 ± 0.23	3.1 ± 0.21	2.1 ± 0.23	2.8 ± 0.23	0.26	0.14	0.20
Alginate	36.7 ± 0.20	24.3 ± 0.21	25.1 ± 0.23	25.2 ± 0.21	0.67	0.41	0.50

Immobilization with and without a soluble carrier was used in this study. Glutaraldehyde immobilized carrot cells showed some morphological changes in comparison with cells in suspension. According to respiration rate and vital staining, cells immobilized by glutaraldehyde were not viable. Glucose was utilised by cells in suspension and by alginate immobilized cells, but not by glutaraldehyde immobilized cells (Fig. 1). The *Tween 80* permeabilization of cells and their subsequent plasmolysis was due to thinning of the cell walls. The permeabilized cells showed loss of protein and a moderate increase in the aminopeptidase activity. The cells cross-linked by glutaraldehyde showed a high decrease in the enzyme activity (Table 1). Contrary to our results the immobilization of many plant cells by glutaraldehyde seems to be convenient method for long-term preservation

of different catalysts (Stano *et al.* 1995, 1998, Poór *et al.* 1998a). However, in the case of plant proteases, cross-linking with glutaraldehyde is not suitable method for their immobilization. Glutaraldehyde may crosslink the active centre of an enzyme and subsequently decrease its activity (Báleš *et al.* 1987). As an alternative, the plant cells immobilized by alginate have attracted attention (Furuya *et al.* 1984, Furusaki *et al.* 1988, Berlin *et al.* 1989). The immobilization of poppy cells by alginate indicates, that this classical method is more appropriate for several enzymes (Furuya *et al.* 1984) than cross-linking by glutaraldehyde (Table 1). The immobilization of cells by entrapment in beds facile, *e.g.*, continuous flow-through arrangement, improved separation of products from the biocatalysts, significant prolongation of the biocatalyst half-life, physical protection from shear forces, prevention of the cell aggregation, stimulation of secondary metabolite production, and preservation of the activity of multifunctional enzyme systems (Furuya *et al.* 1984, Berlin *et al.* 1989, Hulst *et al.* 1989, Shoichet *et al.* 1996). The immobilization of poppy cells by alginate seems to be suitable and convenient method for preservation of different catalysts (Furuya *et al.* 1984, Furusaki *et al.* 1988, Berlin *et al.* 1989).

Results of this study indicate that the immobilization by glutaraldehyde causes significant decrease of the activity of aminopeptidases and proteases *via* cross-linking. As a consequence of this the application of alginate for cell immobilization is more appropriate. The influence of immobilization on the enzyme activity of the cell needs further study.

References

Báleš, V., Gemeiner, P., Kuniak, L., Rexová-Benková, L., Vojtíšek, V., Zemek, L.: Enzýmové Inžinierstvo [Enzyme Engineering.] - Alfa, Bratislava 1987. [In Slovak.]

Benešová, M., Kovács. P., Senkpiel, K.: Aminopeptidase activity in poppy seedlings (*Papaver somniferum* L.) - Biochem. Physiol. Pflanz. **165**: 173-175, 1974.

Berlin, J., Martin, B., Nowak, J., White, L., Wray, V., Ctrack, D.: Effect of permeabilization on the biotransformation of phenylalanine by immobilized tobacco cell cultures. - Z. Naturforsch. **44c**: 249-254, 1989.

Blanáriková, V., Benešová, M., Šulková, A., Pšenák, M.: Callus culture of *Chelidonium majus* L.- Biológia (Bratislava) **51**: 76-77, 1996.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Brodelius, P., Deus, B., Mosbach, K., Zenk, M. H.: Immobilized plant cells for the production and transformation of natural products. - FEBS Lett. **103**: 93-97, 1979.

Dixon, R. A.: Isolation and maintenance of callus and cell suspension cultures. - In: Dixon, R.A. (ed.): Plant Cell Culture. A Practical Approach. Pp. 1-20. IRL Press, Oxford - Washington 1991.

Doi, H., Kawakami, M.: Purification of aminopeptidase from Australian classified barley flour. - Int. J. Biochem. cell. Biol. **29**: 345-352, 1997.

Furusaki, S., Nozawa, T., Isohara, T., Furuya, T.: Influence of substrate transport on the activity of immobilized *Papaver somniferum* cells. - Appl. Microbiol. Biotechnol. **29**: 437-441, 1988.

Furuya, T., Yoshikawa, T., Taira, M.: Biotransformation codeinone to codeine by immobilized cells of *Papaver somniferum* L. - Phytochemistry **32**: 999-1001, 1984.

Hansen, E.W., Olafsen, K., Klaveness, T.O., Kvernberg, P.O.: Probing the gelation of polyvinylalcohol-water-glutaraldehyde within a porous material by H n.m.r. - a preliminary investigation. - Polymer **39**: 1279-1287, 1998.

Hasal, P., Vojtíšek, V., Čejková, A., Kleczek, P., Kofroňová, O.: An immobilized whole yeast cell biocatalyst for enzymatic sucrose hydrolysis. - Enzyme Microbiol. Technol. **14**: 221-229, 1992.

Heymann, E., Erlansen - Albertsson, R., Mentlein, J., Naush, W., Sholz, G., Struckhoff, G., Yoshimoto, T.: Dipeptidyl-peptidase IV (DPP IV, EC 3.4.14.5) as an enzyme of the plasma membrane. - Beitr. Wirkstofforsch. **38**: 22-37, 1990.

Hulst, A.C., Tramper, J.: Immobilized plant cells. A literature survey. - Enzyme Microbiol. Technol. **11**: 546-558, 1989.

Poór, J., Stano, J., Tintemann, H., Mičieta, K., Andriamainty, F., Klimecký, A.: Activity of β-D-galactosidase in immobilized cells of tomato. - Bull. Food Res. **37**: 33-40, 1998a.

Poór, J., Stano, J., Čižmárik, J., Andriamainty. F., Borovkov, N.: Activity of α-D-galactosidase in immobilized cells of tomato. - Bull. Food Res. **37**: 127-134, 1998b.

Radlowski, M., Kalinowski, A., Kralikowski, Z., Bartowiak, S.: Protease activity from maize pollen. - Phytochemistry **35**: 24-32, 1994.

Shoichet, M.S., Li, R.H., White, M.L., Winn, S.R.: Stability of hydrogels used in cell encapsulation. An *in vitro* comparison of alginate and agarose. - Biotechnol. Bioeng. **50**: 374-381, 1996.

Stano, J., Kovács, P., Pšenák, M., Barth, A., Beneš, K.: Aminopeptidases isolated from seedlings of *Cucumis sativus* L. - Biológia (Bratislava) **44**: 321-328, 1989.

Stano, J., Kovács, P., Kákoniová, D., Lišková, D., Kirilova, N. V., Komov, V. P.: Activity of dipeptidyl peptidase IV in ginseng callus culture. - Biológia (Bratislava) **49**: 353-357, 1994a.

Stano, J., Nemec, P., Kovács, P.: Biochemical and histochemical studies studies of dipeptidyl peptidase IV and proline iminopeptidase in gherkin seedlings. - Histochem. J. **26**: 886-887, 1994b.

Stano, J., Nemec, P., Weissová, K., Kovács, P., Kákoniová, D., Lišková, D.: Decarboxylation of L-tyrosine and L-DOPA by immobilized cells of *Papaver somniferum*. - Phytochemistry **38**: 859-860, 1995.

Stano, J., Bezáková, L., Kovács, P., Kákoniová, D., Lišková, D.: α-D-Galactosidase in immobilized plant cells. - Pharmazie **51**: 245-247, 1996.

Stano, J., Bezáková, L., Kovács, P., Mičieta, K., Neubert, K., Menon, V.P., Andriamainty, F.H.: β-D-Galactosidase in immobilized cells of *Panax ginseng* (C.A.MEY). - Pharmazie **52**: 569-570, 1997a.

Stano, J., Nemec, P., Bezáková, L., Kovács, P., Kákoniová, D., Neubert, K., Lišková, D.: Invertase in immobilized cells of *Papaver somniferum* L. - Pharmazie **52**: 242-244, 1997b.

Stano, J., Nemec, P., Bezáková, L., Kákoniová, D., Kovács, P., Neubert, K., Lišková, D., Andriamainty, F., Mičieta, K.: β-D-Galactosidase in immobilized cells of gherkin - *Cucumis sativus* L. - Acta Biochim. Polon. **45**: 621-625, 1998.

Trinder, P.: Determination of blood glucose using an oxide-peroxidase systems with a non-carcinogenic chromogen.- Ann. clin. Biochem. **6**: 24-32, 1969.