# MELIBIASE IN IMMOBILIZED CELLS OF WATERMELON

**J. Stano,[1] E. Tokhtaeva,[2] K. Micieta,[3] M. Fulmekova,[4] M. Varadinova,[5] V. Foltan,[1] and M. Duricek[5]**

UDC 547.965

*Cells of suspension culture* Citrullus vulgaris cv. *"Samara" were permeabilized by Tween 80 and immobilized by glutaraldehyde. The highest melibiase activity was at pH 5.4 and 60°C. The hydrolysis of substrate was linear for 3.5 h, reaching 65–70% conversion of the substrate. The cells, characterized by high enzyme activity and stability in long-term storage, showed convenient physico-mechanical properties (physical protection from shear forces and easy separation of product from biocatalysts).*

**Key words**: cell permeabilization, glutaraldehyde, Tween 80, watermelon.

Plant cells were first immobilized by Brodelius et al. 1979 [1]. In the last decades several methods for fixation of biocatalysts have been developed. Enzymes, living or nonliving microorganisms and animal and plant cells, as well as combined systems, have been bound within or to carrier materials [2–5]. Immobilization of cells or enzymes represents an effective way to obtain highly efficient enzyme catalysts important for biotransformation processes [6]. Many matrices from synthetic polymers or biological materials (e.g., agar, agarose, kappa-carragneenan, collagen, chitosan, polyacrylamide, polyurethane, cellulose) have been used for the immobilization of cells [7, 8]. The spontaneous adhesion or covalent binding of cells to the surface of insoluble carriers was also examined [9, 10]. Recently, polyvinylalcohol and glutaraldehyde [11, 12] or Tween 80 and glutaraldehyde [13] have been used for cell immobilization.

Melibiase ($\alpha$-D-galactoside galactohydrolase EC 3.2.1.22) $\alpha$-galactosidase is an exoglycosidase removing terminal galactose residues from the galactosaccharides and carbohydrate moiety of glycoproteins. Melibiase is of particular interest in view of its biotechnological applications. The enzyme has been employed for the hydrolysis of raffinose to aid in the crystallization of sucrose. The studied enzyme is also used to convert the galactooligosaccharides (stachyose, verbascose, ajugose) in soybean meal to food and feed materials [14].

Microrgamisms are the preferred sources of melibiase [15]. Although melibiase is generally present also in plants, this source has not been used previously. In this paper, the enzymatic hydrolysis of the terminal $\alpha$-galactosidic linkage of glycosides (raffinose, stachyose) by free, as well as glutaraldehyde-immobilized, cells of *Citrullus vulgaris cv*. "Samara" was studied.

Cells immmobilized by cell entrapment are cultivated in a similar way as cell suspension cultures [7, 8, 16].

The microscopic observation of watermelon cells immobilized with glutaraldehyde showed small morphological changes in comparison with cells in suspension. A thinning of the cell walls after permeabilization by Tween 80 was observed. Important was also the appearance of cell plasmolysis and a small aggregation of cells occurring after immobilization. According to the respiration rate and vital staining (fluorescein or 2,3,5-triphenyltetrazolium chloride), cells immobilized by glutaraldehyde were not viable. Also glucose was utilized only by cells in suspension, but not by immobilized ones (Fig. 1).

The permeabilization of the studied cells by Tween 80 led to a decrease in proteins while the enzyme activity showed a moderate decrease, thereby increasing the specific activity. By glutaraldehyde crosslinking a moderate fall in the enzyme activity has been found (Table 1).

1) Garden of Medicinal Plants, Faculty of Pharmacy, Comenius University, Odbojarov 10, 83232 Bratislava 3, Slovak Republic, e-mail: korenova@fpharm.uniba.sk; 2) Department of Biophysics, National University of Uzbekistan, Vuzgorodok, 700174 Tashkent, Uzbekistan Republic; 3) Department of Botany, Faculty of Natural Sciences, Comenius University, Rehakova 39, 81101 Bratislava 1, Slovak Republic; 4) Department of Organization and Management in Pharmacy, Faculty of Pharmacy, Comenius University, Odbojarov 10, 83232 Bratislava 3, Slovak Republic; 5) Faculty of Pharmacy, Comenius University, Odbojarov 10, 83232 Bratislava 3, Slovak Republic. Published in Khimiya Prirodnykh Soedinenii, No. 1, pp. 53-55, January-February, 2005. Original article submitted April 7, 2003.

0009-3130/05/4101-0065 

TABLE 1. Melibiasese Activity in Cells Suspension and in Immobilized Cells of Watermelon

| Cells | Protein | Activity | Specific activity |
|---|---|---|---|
| | (mg/g dry mass) | (nkat/g dry mass) | (nkat/mg protein) |
| Suspension | 27.3±0.98 | 5.2±0.29 | 0.19 |
| Permeabilized | 9.6±0.60 | 4.2±0.22 | 0.43 |
| Immobilized | 9.5±0.63 | 4.0±0.22 | 0.42 |

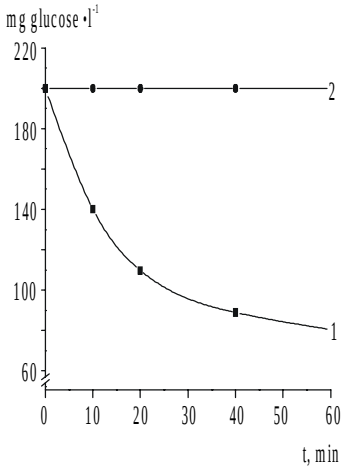


Fig. 1

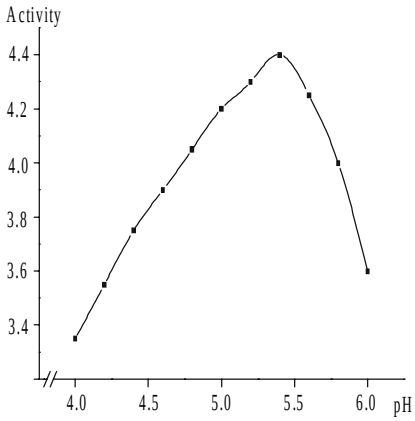


Fig. 2

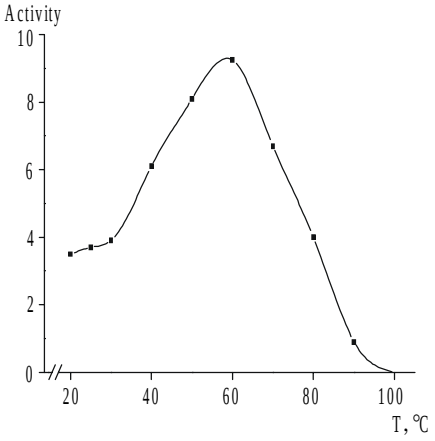


Fig. 3

Fig. 1. Time course of glucose utilization by cells immobilized with glutaraldehyde (1) and by cells in suspension (2).
Fig. 2. pH optimum of melibiase in immobilized cells of watermelon. Activity (nkat/g dry mass).
Fig. 3. Effect of temperature on activity of melibiase in immobilized cells of watermelon. Activity (nkat/g dry mass).

Sucrose is probably the most widely used carbon source in plant tissue cultures. After inversion of sucrose, glucose and fructose are present in the media in roughly equal amounts during the first few days, but the cells did not consume fructose until glucose is present. The cells immobilized in alginate gels utilized glucose while the glutaraldehyde crosslinked cells did not (Fig. 1) [17].

Similar properties as in the case of melibiase immobilized in watermelon cells were reported for $\beta$-galactosidase isolated from winter rape [18], poppy, ginseng, and gherkin [19].

Melibiase in immobilized cells of watermelon had a pH optimum at 5.4 like the viable cells in suspension (Fig. 2). Enzyme hydrolysis of *p*-nitrophenyl-$\alpha$-D-galactopyranoside was linear within 3.5 h, reaches 65–70% of substrate conversion, then practically stops. The temperature optimum of the enzyme activity in immobilized cells and in the cells in suspension was at 60°C, indicating a relatively high degree of temperature stability of this hydrolase (Fig. 3).

Partially purified enzyme preparations of melibiase from gherkin and poppy seedlings were inhibited by galactose and glucose in a moderate way [20]. A similar inhibitory effect was observed in immobilized cells, too.

The inhibitory effect of 0.1–0.5 mM *p*-chlormercuribenzoic acid on melibiase can be eliminated with 5–10 mM cysteine, dithiothreitol, or 2-mercaptoethanol [21]. These results indicate that SH-groups are essential for the enzyme activities of both melibiase and $\beta$-galactosidase [20].

As illustrated in Table 2, the activity of the enzyme in watermelon cells immobilized by glutaraldehyde (in 0.15 M NaCI with all the preservatives tested) during 6 months storage is still relatively high. The same phenomenon (an increase of $\alpha$- and $\beta$-galactosidase activity during storage) was observed in the immobilized cells of poppy and ginseng [19–21]. The observed increase in the activity on storage remains unclear. It might be due to a gradual dissociation of inhibitory compounds originally interacting with the enzyme. The tested preservatives do not influence the enzyme activity.

TABLE 2. Stability of Melibiase in the Immobilized Watermelon Cells on Storage

| Conservance | Original activity in suspension culture, % | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 6 |
| | month | | | | |
| None | 65 | - | - | - | - |
| CLCTC (50 mg/l) | 65 | 67 | 69 | 76 | 87 |
| ATDNO (100 mg/l) | 65 | 68 | 70 | 77 | 89 |
| Chloramphenicol (50 mg/l) | 63 | 67 | 69 | 77 | 90 |
| Sodium azide (200 mg/l) | 63 | 66 | 69 | 76 | 89 |
| Frozen in 0.15 M NaCl | 64 | 68 | 71 | 78 | 92 |

CLCTC, chlortetracycline hydrochloride; ATDNO, (1-methyldodecyl)-dimethylamine-4-oxide; original activity = enzyme activity (100%) in cell suspension without immobilization.

The immobilization costs are very low, and no special equipment is needed. Aeration, agitation, and the kind of cultivation medium have no influence on the biotransformational potential of glutaraldehyde immobilized cells. Immobilization of the cells makes enzyme isolation unnecessary, whereas the specific enzyme activity of biocatalysts remains quite high [9]. The cells immobilized by glutaraldehyde (by crosslinking) compared with the cells immobilized by entrapment in beads (alginate, carrageenan, or other matrices) bring some important advantages.

Melibiase and other glycosidases can potentially be applied in biotransformation processes in the pharmaceutical industry as well as in the food industry to obtain important compounds; their application in structure studies of these compounds is another possible field of their practical use [22–26].

## EXPERIMENTAL

**Tissue Cultures.** Long-term tissue cultures and cell suspensions were derived from watermelon seedlings *Citrullus vulgaris cv.* "Samara" as was previously described [19].

**Cell Permeabilization.** Cell suspensions were filtered through a nylon cloth, and 15 g of fresh mass was suspended in 50 ml of 5% Tween-80 in 0.15 M NaCl solution. Permeabilization proceeded for 3 h under moderate stirring at 20°C. The cells were filtered off and washed first with 3 liters of distilled water and then with 2 liters of 0.15 M NaCl solution.

**Immobilization.** The permeabilized cells were immediately suspended in 50 ml of 0.15 M NaCl solution, with slow addition of 5 ml of 25% glutaraldehyde under mild stirring at room temperature for 2 h. The immobilized cells were washed with 2 liters of distilled water and 2 liters of 0.15 M NaCI solution and separated by filtration.

**Fresh and Dry Mass Determination.** The fresh and dry mass of cell suspensions were determined gravimetrically. For determination of dry mass, samples were dried to constant weight at 105°C.

**Storage Stability.** The stability of melibiase during storage was monitored in the following experiments. The immobilized cells were stored at 4°C in 0.15 M NaCI supplied with the following compounds: a) chloramphenicol 50 mg/liter, b) chlortetracycline hydrochloride (CLCTC) 50 mg/liter, c) (l-methyl-dodecyl)-dimethylamine-4-oxide (ATDNO) 100 mg/liter [27]. These experiments were repeated at least three times.

**Glucose Utilization.** The immobilized cells and cell suspensions were exposed to an initial glucose concentration 200 mg/liter in a cultivation medium [28, 29] devoid of sucrose. The concentration of glucose was determined by the method of Trinder [30].

**Enzyme Assay.** The enzyme assay was performed by the modified method of Kim et al. [31] using *p*-nitrophenyl-$\alpha$-D-galactopyranoside ($\alpha$PNG) as substrate. The reaction mixture contained 0.1 g of wet cells and 0.5 mg $\alpha$PNG in 2 ml McIlwaine buffer, pH 5.4. The control contained boiled cells. Both mixtures were kept for 20 min to 5 h at 30°C on a rotary shaker (80 r.p.m.) and the reaction was stopped by addition 2 ml of 1 M $Na_2CO_3$. The substrate conversion was calculated based on the decrease in substrate concentration following 3 h incubation. The nitrophenol released was determined spectrophotometrically at 420 nm. The cells were separated from the reaction mixture, and dried, and the enzyme activity was calculated for 1 g of dry mass [21].

The determination of enzyme activity was repeated at least five times and the enzyme activity is expressed in katals. Protein content was determined by the method of Bradford [32] using bovine serum albumin as a standard.

**Cell Viability.** This was determined by the method of Dixon [33] with 2,3,5-triphenyltetrazolium chloride (TTC) or fluorescein diacetate and oxygen electrode, respectively.


## ACKNOWLEDGMENT

This work was partially supported by the Grant Agency VEGA (Bratislava) grant No. 1/0023/03. We are grateful to Prof. P. Kovacs (Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Bratislava) for proofreading this article.


## REFERENCES


1. P. Brodelius, B. Deus, K. Mosbach, and M. H. Zenk, *FEBS Lett*., **103**, 93 (1979).
2. J. Klein and F. Wanner, *Appl. Biochem. Bioeng*., **4**, 18 (1983).
3. A. J. Rosevear, *J. Chem. Technol. Biotechnol*., **34B**, 127 (1984).
4. A. C. Hulst and J. Tramper, *Enzyme Microbiol. Technol*., **11**, 546 (1989).
5. M. Forster, *Enzyme Microbiol. Technol*., **16**, 777 (1994).
6. A. M. Klibanov, *Science*, **219**, 722 (1983).
7. J. Tampion and M. D. Tampion, *Immobilized Cells. Principles and Applications*, Cambridge University Press, Cambridge (1987).
8. A. C. Hulst and J. Tramper, *Enzyme Microbiol. Technol*., **11**, 546 (1989).
9. J. M. S. Cabral, M. M. Cadete, J. M. Novais, and J. P. Cardoso, *Ann. N.Y. Acad. Sci*., **434**, 483 (1984).
10. P. Parascandola and V. Scardi, *O. Appl. Microbiol. Biotechnol*., **26**, 507 (1987).
11. P. Hasal, V. Vojtisek, A. Cejkova, P. Kleczek, and O. Kofronova, *Enzyme Microbiol. Technol*., **11**, 546 (1992).
12. K. Y. A. Wu and K. D. Wisecarver, *Biotechnol. Bioeng*., **39**, 447 (1992).
13. J. Stano, P. Nemec, K. Weissova, P. Kovacs, D. Kakoniova, and D. Liskova, *Phytochemistry*, **38**, 859 (1995).
14. A. M. Kachurin, K. N. Neustroev, A. M. Golubev, and F. M. Ibatullin, *Biokhimiya*, **58**, 550 (1993).
15. R. Kaneko, I. Kusakabe, Y. Sakai, and K. Murakami, *Agric. Biol. Chem.*, **54**, 237 (1990).
16. T. Furuya, T. Yoshikawa, and M. Taira, *Phytochemistry*, **23**, 999 (1984).
17. R. Hamilton, H. Pedersen, and C. K. Chin, *Biotechnol. Bioeng. Symp*., **14**, 383 (1984).
18. T. Sawicka and A. Kacperska, *J. Plant Physiol*., **145**, 357 (1995).
19. J. Stano, P. Nemec, L. Bezakova, D. Kakoniova, P. Kovacs, K. Neubert, and D. Liskova, *Acta Biochim. Pol*., **45**, 621 (1998).
20. D. Budik, *Diploma Thesis*, Faculty of Pharmacy, Comenius University, Bratislava, 1992, 42 p.
21. J. Stano, P. Nemec, D. Kakoniova, P. Kovacs, K. Neubert, and D. Liskova, *Biologia*, **50**, 279 (1995).
22. P. Mucaji, D. Grancai, M. Nagy, S. Czigleova, M. Budesinsky, and K. Ubik, *Czech. Slov. Pharm.*, **6**, 274 (2001).
23. L. Bilisics, D. Liskova, M. Kubackova, O. Auxova, and D. Kakoniova, *Biologia*, **49**, 911 (1994).
24. K. Fukase, T. Yasukochi, Y. Suda, M. Yosida, and S. Kusumoto, *Tetrahedron Lett*., **37**, 6763 (1996).
25. E. Bedir, I. A. Khan, and L. A. Walker, *Pharmazie*, **57**, 491 (2002).
26. I. Mahmond, F. A. Moharram, M. S. A. Marzouk, M. W. Linsheid, and M. I. Saleh, *Pharmazie*, **57**, 494 (2002).
27. F. Devinsky, D. Mlynarcik, I. Lacko, and L. Krasnec, *Pharmazie*, **34**, 574 (1979).
28. T. Murashige and F. Skoog, *Physiol. Plant*., **15**, 473 (1962).
29. C. L. Brown and R. H. Lawrence, *Forest. Science*, **14**, 62 (1968).
30. P. Trinder, *Ann. Clin. Biochem.*, **6**, 24 (1969).
31. W. D. Kim, O. Kobayashi, S. Kaneko, Y. Sakakibara, G. G. Park, I. Kusakabe, H. Tanaka, and H. Kobayashi, *Phytochemistry*, **61**, 621 (2002).
32. M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
33. R. A. Dixon, *Plant cell culture. - A practical Approach. IRL Press*, Oxford, Washington DC, (1991).