α-Galactosidase in immobilized cells of *Citrulus vulgaris* L.

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Summary

Cells of suspension culture *Citrullus vulgaris* cv. "Samara" were permeabilized by Tween 80 and immobilized by glutaraldehyde. The highest α-galactosidase 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 with 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 glycosidases that catalyse the hydrolysis of aryl and alkyl glycosides are involved in a wide variety of biological processes. In the last decades, several methods for determining and immobilization of these enzymes have been developed. Many matrices from synthetic polymers or biological materials have been used for the immobilization of cells and enzymes [1-3].

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is an exoglycosidase removing terminal galactose residues from the galactosacharides and carbohydrate moiety of glycoproteins.

α-Galactosidase 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 galactooligosacharides (stachyose, verbascose, ajugose) in soybean meal into food and feed materials [4].

Microorganisms are the preferred sources of α-galactosidase [5]. Although melibiase is generally present also in plants, this source has not been used previously.

In this paper, the enzymatic hydrolysis of the terminal α-galactosidic leakage of glycosides (raffinose, stachyose) by free, as well as by glutaraldehyde immobilized cells of *Citrullus vulgaris* cv. Samara was studied.

MATERIAL AND METHODS

Tissue cultures

Long-term tissue cultures and cell suspension were derived from watermelon seedlings *Citrullus vulgaris* cv. "Samara" as was previously described [3].

Cell permeabilization

Cell suspensions were harvested during exponential growth phase (day 12 of the culture cycle), then filtered through a nylon cloth, and 15 g of fresh mass were 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 l of distilled water and then with 2 l of 0.15 M NaCl solution.

Immobilization

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

Fresh and dry mass determination

Fresh and dry mass of cell suspensions was determined gravimetrically. For determination of dry mass, the samples were dried to constant weight at 105°C.

Storage stability

Stability of α-galactosidase during storage was monitored in the following experiments. The immobilized cells were stored at 4°C in 0.15 M NaCl supplied with following compounds: a - chloramphenicol 50 mg/l, b - chlortetracycline hydrochloride (CLCTC) 50 mg/l, c - (l-methyl-dodecyl)-dimethylamine-4-oxide (ATDNO) 100 mg/l [6]. These experiments were repeated at least three times.

Glucose utilization

The immobilized cells and cell suspensions were exposed to initial glucose concentration 200 mg/l in the cultivation medium devoid of sucrose. Concentration of glucose was determined by the method of Trinder [7].

Enzyme assay

The enzyme assay was performed by the modified method of Kim et al. [1] using p-nitrophenyl-α-D-galactopyranoside (αPNG) as substrate. The reaction mixture contained 0.1 g of wet cells and 0.5 mg αPNG in 2 ml McIlvaine 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 rpm) and the reaction was stopped by addition 2 ml of 1 M Na_2CO_3. Substrate conversion was calculated basing on the decrease in substrate concentration following a three-hour incubation. The nitrophenol released was determined spectrophotometrically at 420 nm. The cells were separated from the reaction mixture, dried and the enzyme activity was calculated for 1 g of dry mass.

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 Doumas et al. [8], using bovine serum albumin as a standard.

Cell viability

This was determined by the method of Dixon [9] with 2,3,5-triphenyl-tetrazolium chloride (TTC), fluoresceindiacetate and oxygen electrode, respectively.

RESULTS AND DISCUSSION

Cells immobilized by cell entrapment are cultivated in a similar way as cell suspension cultures [10]. After cell immobilization in glutaraldehyde some plasmoly-

sis occurs, as well as aggregation of cells. According to respiration rate and vital staining (fluorescein or 2,3,5-triphenyltetrazolium chloride) cells immobilized by glutaraldehyde were not viable. Also glucose was utilised only by cell in suspension, but not by immobilised ones (Figure 1).

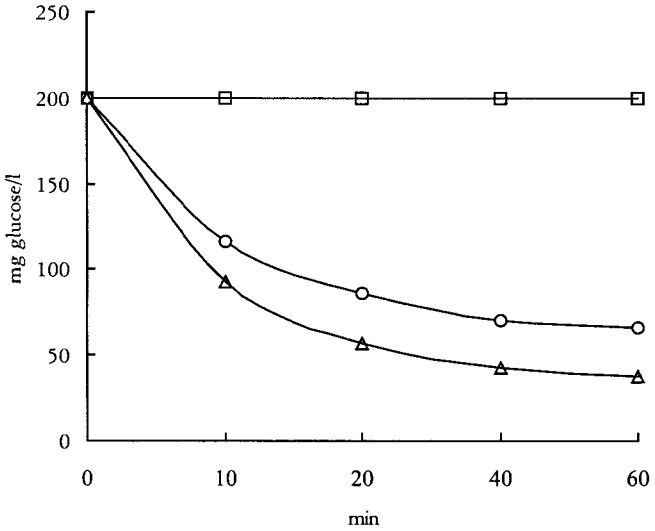

Fig. 1. Time course of glucose utilization by cells immobilized with glutaraldehyde (*squares*), alginate (*triangles*) and by cell in suspension (*circles*).

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

Table 1.

α-galactosidase activity in cell suspension and in immobilized cells of watermelon.

cells	proteins $\left(\frac{mg}{g\ dry\ mass}\right)$	activity $\left(\frac{nkat}{g\ dry\ mass}\right)$	specific activity $\left(\frac{nkat}{mg\ protein}\right)$
suspension	27.3+0.17	5.2+0.13	0.19
permeabilized	9.6+0.14	4.2+0.12	0.43
immobilized	9.5+0.14	4.0+0.11	0.42

Sucrose is probably the most widely used carbon source in plant tissue cultures. After inversion of sucrose, glucose and fructose were present in the media in roughly equal amounts during the first few days, but the cells did not consume fructose until glucose was present [11]. The cells immobilized in alginate gels uti-

lized glucose, while the glutaraldehyde crosslinked cells did not (Figure 1).

Similar properties as in the case of α-galactosidase immobilised in watermelon cells were reported for β-galactosidase isolated from winter rape [12], poppy, ginseng and gherkin [13].

α-Galactosidase in the immobilised cells of watermelon had a pH optimum at 5.4 like the viable cells in suspension (Figure 2). Enzyme hydrolysis of p-nitrophenyl-α-D-galactopyranoside was linear within 3.5 h reaching 65-70% of substrate conversion, and then practically stopped. The temperature optimum of the studied enzyme activity in immobilized cells and in the cells in suspension was at 60 °C (Figure 3).

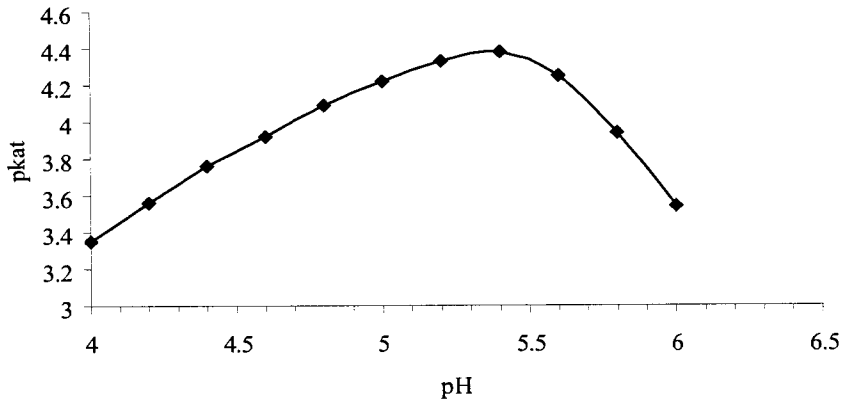

Fig. 2. pH optimum of α-galactosidase in immobilized cells of watermelon.

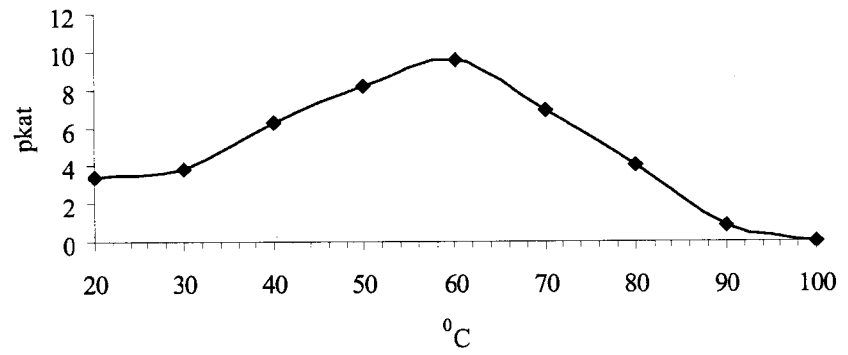

Fig. 3. Effect of temperature on activity of α-galactosidase in immobilized cells of watermelon.

Partially purified enzyme preparations of α-galactosidase from gherkin and poppy seedlings were inhibited by galactose and glucose in a moderate way. A similar inhibitory effect was observed in immobilised cells, too [14].

The inhibitory effect of 0.1-0.5 mM p-chlormercuribenzoic acid on α-galactosidase can be eliminated with 5-10 mM cysteine, dithiothreitol or 2-mercaptoethanol [3]. These results indicate that SH-groups are essential for the enzyme activities of both α-galactosidase and β-galactosidase [13, 14].

As illustrated in Table 2, the activity of the enzyme in watermelon cells immobilized by glutaraldehyde (in 0.15 M NaCl with all preservatives tested) during six-month storage is still relatively high. The same phenomenon - an increase of α- and β-galactosidase activity during storage – was observed in the immobilized cells of poppy and ginseng [13, 14]. 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 α-galactosidase in the immobilized watermelon cells on storage.

	original activity in suspension culture (%)				
preservation agent	0 months	1 month	2 months	3 months	6 months
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 biotransformational potential of glutaraldehyde immobilized cells. Immobilization of the cells makes enzyme isolation unnecessary, whereas the specific enzyme activity of biocatalysts remains quite high [13, 14]. The cells immobilized by glutaraldehyde (by cross linking) compared with the cells immobilized by entrapment in beads (alginate, carrageenan or other matrices) bring some important advantages. It encourages product release, prevents cell aggregation, protects cells from shear stress, gives good cell-to-cell contact, and it preserves the activity of multifunctional systems). Glutaraldehyde is therefore successfully used for immobilization of many plant cells [14].

The chemical synthesis of many compounds usually involves time-consuming multistep reactions that require stereo- and regioselective control. Biotransformations (using immobilized cells or biocatalysts) not only provide an alternative and efficient solution to these synthetic problems, but also offer environmentally clean technologies that profit from very mild reaction conditions [10].

α-Galactosidase and other hydrolases can be perspectively applied in biotrans-

formation processes of compounds widely used in the pharmaceutical industry, as well as in the food industry; their application in structure studies of these compounds is another possible field of their practical use [15, 16, 17].

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α-GALAKTOZYDAZA W IMMOBILIZOWANYCH KOMÓRKACH *CITRULUS VULGARIS* L.

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Streszczenie

Za pomocą Tween 80 wywołano przepuszczalność komórek kultury *Citrullus vulgaris* odmiany Samara, po czym immobilizowano je za pomocą glutaraldehydu. Najwyższą aktywność α-galaktozydazy zaobserwowano przy pH 5.4 w temperaturze 60°C. Przez 3,5 godziny prowadzono liniową hydrolizę substratu, co doprowadziło do konwersji substratu w wysokości 65-70%. Komórki cechowały się wysoką aktywnością enzymów, a stabilność podczas dłuższego utrzymywania ich w stanie immobilizacji wykazała odpowiednie właściwości fizyczno-mechaniczne (fizyczną wytrzymałość na siły zniekształcające oraz łatwe oddzielenie produktu od biokatalizatorów).

Słowa kluczowe: przepuszczalność komórek, glutaraldehyd, Tween 80, arbuz.