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STUDY OF IMMOBILIZED AND EXTRACELLULAR INVERTASE OF LEMON BALM

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*Cell suspensions of lemon balm (*Melissa officinalis *L.) were permeabilized by Tween 20, Tween 80, ethanol, hexadecyltrimethylammonium bromide, and hexadecylpyridinium chloride, and immobilized by glutaraldehyde. The invertase pH optimum was 4.5 at temperature 50°C. The hydrolysis of substrate was linear for 4 h, reaching 60% conversion. The cells had high invertase activity and good stability, and in long-term storage they showed good physicomechanical properties. The culture medium (without cells) was used for the identification and determination of extracellular enzyme activity. Intracellular activity was estimated from the cell suspension. For the lemon balm cell suspension, the intracellular activity accounted for 83.7% of the total activity, and the extracellular one for 12.7%. The intracellular specific activity is 4.2 times higher. Our method permits the rapid, simple, and specific identification and determination of plant invertase.*

Key words: identification, determination, immobilization, invertase.

Glycosidases are involved in several important biological processes such as digestion, biosynthesis of glycoproteins, and catabolism of glycoconjugates. Immobilization techniques have a great impact on technology nowadays.

The quality of human nutrition is, besides other nutrients, dependent on the quality, quantity, structure, and physicochemical properties of sugars, peptides, and other compounds in food. Biotransformation of this compound is necessary in some biotechnological processes [1, 2]. The determination of sugar and glycosidases plays an important role in many fields of basic and applied research [1, 3].

Invertase (β-D-fructofuranosidase, EC 3.2.1.26), also called sucrase, catalyses the hydrolysis of sucrose to glucose and fructose [4]. The enzymes are important industrial products with applications in the production of non-crystallizable mixtures of glucose and fructose (invert sugars), enabling production of fructose-containing preparations and soft-centered chocolates [3–5].

The development of new techniques of immobilization, identification, and determination of biocatalysts is tightly connected with the progress of biotechnological processes. Because of the fact that the cell wall slows down the transport of many compounds from and into the cell, we were interested in exploring possibilities of the permeabilization of the cell wall. We assume that immobilized cells or biocatalysts of plant origin could play, in biotechnological processes, a similar role as representatives of various microorganisms.

In this paper we turned our attention to the study of the effect of permeabilization on enzymatic hydrolysis of sucrose using immobilized cells and distribution of intra- and extracellular invertase in lemon balm cells. Sucrose was used as substrate for the study of invertase activity.

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TABLE 1. Protein Content and Invertase Activity in 14 Day Old *Melissa officinalis* L. in Permeabilized Cell Suspensions

Cells	Activity, μkat/g dry mass	Specific activity, μkat/mg protein
	Suspension (protein 26.3 mg/g dry mass)	
	2.94±0.08	0.112
	Permeabilized (protein 9.6 mg/g dry mass)	
0.1% HTAB	3.26±0.06	0.339
0.1% HPCH	3.28±0.07	0.341
5% Tween 20	3.12±0.08	0.325
5% Tween 80	3.18±0.07	0.331
30% ethanol	2.71±0.08	0.282
50% ethanol	2.76±0.07	0.288
	Immobilized (protein 9.7 mg/g dry mass)	
0.1% HTAB	2.58±0.09	0.266
0.1% HPCH	2.63±0.09	0.271
5% Tween 20	2.38±0.07	0.245
5% Tween 80	2.46±0.07	0.255
30% ethanol	2.17±0.06	0.224
50% ethanol	2.09±0.07	0.215

The values are expressed as means of five replicates (n = 5) ± SDE.

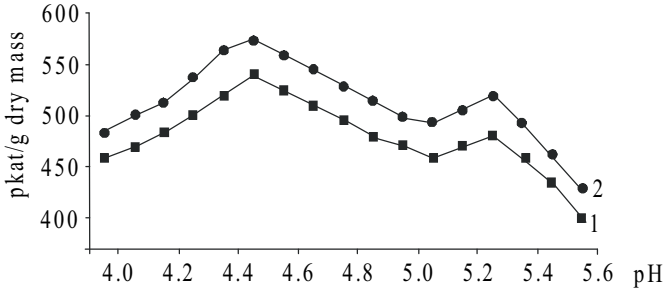

Fig. 1. pH optimum in cell suspension and in cells of *Melissa officinalis* immobilized by glutaraldehyde. *1* – Cell suspension; *2* – immobiblized cells.

Glutaraldehyde immobilized lemon balm cells showed little morphological changes in comparison with cells in suspension. A little cell plasmolysis, cell wall thinning, and some aggregation of the cells occurred during immobilization. It was observed that the cells immobilized by glutaraldehyde did not utilize glucose and were not viable, as they do not show respiratory activity and were not stained with fluorescein or 2,3,5-triphenyltetrazolium chloride (TTC).

The permeabilization of the studied cells by Tween 20, Tween 80, ethanol, hexadecylpyridinium chloride (HPCH), and hexadecyltrimethylammonium bromide (HTAB) led to the leakage or degradation of proteins, while the enzyme activity increased moderately with the exception of samples treated by ethanol, and the specific activity increased in all samples tested. Permeabilized cells were cross-linked with glutaraldehyde. In glutaraldehyde cross-linking, only a small fall in the enzyme activity compared to the samples before cross-linking was found (Table 1).

In the case of aminopeptidase activities, the cells cross-linked by glutaraldehyde show a large decrease in enzyme activity. Glutaraldehyde may cross-link the enzyme center (amino groups), subsequently decreasing its activity. All results indicate that amino groups are not essential for invertase activity [6, 7]. The immobilized cells, like viable cells, had invertase pH optimum at 4.5, and a minor peak of activity appeared at pH 5.3, too (Fig. 1). Enzyme hydrolysis of sucrose was linear for 4 h, reaching 60% of substrate conversion, then practically stops. The temperature of the studied enzyme in immobilized cells was 45°C and in a cell of suspension culture was 50°C.

TABLE 2. Effect of Glucose, Fructose, and Galactose on Invertase Activity of Cell Suspension and Immobilized Cells of Lemon Balm, %

Concentration, mM	Original activity, %					
	Glucose		Fructose		Galactose	
	A	B	A	B	A	B
0	100	100	100	100	100	100
1	75	76	64	65	157	154
5	74	75	56	57	154	150
10	68	70	55	56	146	143
20	65	65	52	54	140	136

A - cell suspension; B - immobilized cells.

TABLE 3. Stability of Invertase in Immobilized Lemon Balm Cells on Storage

Stabilization	Original activity, %				
	0 month	1 month	2 month	3 month	6 month
None	100	-	-	-	-
CLCTC (50 mg/L)	89	88	85	83	79
ATDNO (100 mg/L)	89	88	85	83	79
Chloramphenicol (50 mg/L)	89	87	84	83	78
Sodium azide (200 mg/L)	89	87	84	81	77
Frozen in 0.15 M NaCl	90	84	82	80	75

CLCTC - chlortetracycline hydrochloride; ATDNO - (1-methyldodecyl)dimethylamine *N*-oxide.
Original activity = enzyme activity (100%) in cell suspension without immobilization.

A relatively high degree of temperature stability of α-galactosidase activity was also observed in immobilized *Amsonia* cells [8]. The activity of immobilized enzyme was inhibited by glucose and fructose and activated by galactose in a moderate way (Table 2). As illustrated in Table 3, the activity of the enzyme in lemon balm cells immobilized with glutaraldehyde (in 0.15 M NaCl with all preservatives tested) during 6 months storage is still relatively high. The α-galactosidase activity of *Amsonia* cells immobilized by glutaraldehyde in the some way as lemon balm cells after 6 months storage was a little higher than that of invertase [8].

The distribution of intracellular and extracellular enzyme activity is shown in the Table 4.

The data indicate 83.7% intracellular and 12.7% extracellular distribution of the enzyme activity tested. The intracellular specific activity is 4.2 times higher. The distribution of intra- and extracellular activity of aminopeptidase and invertase is very similar [9]. It is very interesting that the activity of extracellullar galactosidases is 3-4-fold higher than the activity of aminopeptidase and invertase [8, 9].

The immobilization of isolated biocatalysts and cells are techniques widely used in biotechnologies. Immobilization techniques have had a great impact on technology [10–12].

Immobilized cells (cells enclosed in hydrogels) are cultivated in a similar way as cell suspension cultures [12, 13].

Sucrose is probably the most widely used carbon source in plant tissue cultures. Its utilization is followed by a rapid initial inversion and sequential phases of glucose and fructose consumption. Glucose and fructose are present in the media in roughly equal amounts after the first few days of inoculation, but the cells do not consume fructose until glucose is present [14]. In contrast to cells immobilized in alginate gels [15, 16], the glutaraldehyde-crosslinked cells did not utilize glucose.

TABLE 4. Invertase Activity in Cell Culture and Cell Culture Medium of 14 Day Old Lemon Balm

Fraction	Volume, mL	Protein, mg/g fresh mass	Activity, nkat/g fresh mass	Specific activity, nkat/mg protein
Intracellular activity (Homogenate of isolated cells)	2	1.3±0.16	116±0.38	89.2
Extracellular activity (Culture medium without cells)*	8	0.8±0.17	17±0.18	21.3

*Corresponding to the amount of isolated cells.

Properties similar to invertase immobilized in lemon balm were reported for invertase isolated from other plants [17]. A minor peak of acid invertase was observed also for enzyme isolated from microorganisms [5]. In general, higher plants contain a family of invertase, which can be discriminated into three types of enzymes, namely vacuolar, cell wall, and cytosolic [18] ones. Invertase with acidic pH optima is localized either in the vacuole or in the cell wall. The former is a soluble protein, which has two or more isoenzymes. Soluble acid invertase plays important biological functions related to sucrose metabolism and would presumably hydrolyze sucrose to supply hexose for cell growth and development [19, 20]. Perhaps the presence of different isoenzymes is needed to achieve different goals, which may be regulated differentially [21].

By cell wall permeabilization of yeasts, a very significant increase of phenylalanine ammonialyase (PAL) activity was observed [22]. By cell wall permeabilization of the cell suspension culture a noticeable increase of invertase activity was not observed (Table 1).

The inhibitory effect of *p*-chloromercuribenzoic acid in 0.1– 0.5 mM concentration can be eliminated with 5–10 mM 2-mercaptoethanol, 5–10 mM dithiothreitol, and 5–10 mM cysteine, indicating that –SH groups are essential for enzyme activity [23, 24]. The activity of partially purified enzyme preparations of invertase from gherkin and poppy seedlings was inhibited by glucose and fructose and activated by galactose in a moderate way [23, 24]; a similar effect of galactose was observed with immobilized cells too (Table 2). Isla et al. [25] found that fructose is a competitive, and glucose a non-competitive, inhibitor of invertase.

The immobilized cells as well as viable cells had an apparent K_m for invertase of 4.6 mM. Similar properties were reported for invertase isolated from rice (K_m = 6.6 mM [25]), poppy (K_m = 5 mM [23]), maize scutellum (2.9 mM), and *Schizophyllum communae* (K_m = 4.8 mM [26]).

As illustrated in Table 3, the activity of the studied enzyme in lemon balm cells immobilized by glutaraldehyde (in 0.15 M NaCl with all preservatives tested) during 6 months storage is still relatively high.

It is known that immobilization of plant cells (by entrapment in beds) compared with a free-cell suspension brings some important advantages [27]. It encourages product release, prevents cell aggregation, protects the cell from shear stress, gives good cell-to-cell contact, and preserves the activity of multifunctional systems. The formation of cell aggregates in cell suspensions and the degree of differentiation are important from the biotechnological point of view. In cells immobilized by cross-linking with bifunctional reagents (e.g., glutaraldehyde), tyrosine-decarboxylase, DOPA-decarboxylase, and α- and β-galactosidase [28, 29] still reach high values.

The immobilization of many plant cells by glutaraldehyde and their storage in 0.15 M NaCl with all the preservatives tested seems to be a very convenient method for long-term preservation of different catalysts [28, 29]. Contrary to our results with proteases, cross-linking with bifunctional reagent (glutaraldehyde) is not a suitable method for their immobilization. Glutaraldehyde may cross-link the active center of an enzyme and subsequently decrease its activity [6]. Thus, as an alternative, immobilization by alginate, pectate, or other hydrogels indicates that this classical method is more appropriate for several enzymes [30, 31] than cross-linking by means of glutaraldehyde [7].

The production of extracellular glycosidases [29, 32] as well as other enzymes [13, 33] of plant or other origin might be of some importance for biotechnological application in food and pharmaceutical research and in industry [34, 35]. These enzymes are generally present in plants. Until now they have not been used in biotechnological processes [32, 36].

The immobilization costs are very low and no special equipment is needed. Immobilization of whole cells makes enzyme isolation unnecessary, whereas the enzyme activity remains high. Both the reaction kinetic parameters and the physico-mechanical properties of the biocatalysts are fully comparable with those of biocatalysts prepared by immobilization of soluble enzyme on insoluble carriers [37]. Biotransformations not only provide an alternative and efficient solution of many synthetic problems, but also offer environmentally clean technologies that profit from very mild reaction conditions [38].

In this way immobilized sacharase and other glycosidases can be perspectively applied in biotransformation processes pharmaceutically, as well as in the food industry to produce important compounds, and their application in structure studies of these compounds is another possible field of their practical use [32, 39–41].

EXPERIMENTAL

General abbreviations: d.m., dry mass; kat, katal; HTAB, hexadecyltrimethylammonium bromide; HPCH, hexadecylpyridinium chloride; ATDNO, (1-methyldodecyl)-dimethylamine N-oxide; CLCTC, chlortetracycline hydrochloride; TTC, 2,3,5-triphenyltetrazolium chloride.

Callus and Cell Suspension Cultures. Long-term callus cultures were derived from stems of *Melissa officinalis* L. and continuously subcultured every two weeks on *Murashige-Skoog* [42] medium. For suspension induction the callus culture (2 g) was inoculated into liquid Phillip-Collins [43] medium, and cell suspension cultures were grown on the rotatory shaker (110 r.p.m.) in 250 mL flasks, containing 100 mL medium at 27° C under 16 h light period (45–60 $\mu M/m^2 s$). The suspension was subcultured every two weeks.

Cell Permeabilization and Immobilization by Glutaraldehyde. Cell suspensions were filtered through a nylon cloth, and 10 g of fresh mass was suspended in 50 mL of 0.15 M NaCl with 5% Tween 20, 5% Tween 80, 30% ethanol, 50% ethanol, 0.1% hexadecyltrimethylammonium bromide (HTAB), and 0.1% hexadecylpyridinium chloride (HPCH). Permeabilization was carried out for 3 h with moderate stirring at 20° C. The cells were filtered off and washed with 2 L distilled water and 3 L of 0.15 M NaCl solution with slow addition of 5 mL 25% glutaraldehyde under mild stirring at room temperature for 3 h. The immobilized cells were then separated by filtration and washed with 2.5 L of distilled water and 3 L of 0.15 M NaCl solution.

Determination of Fresh and Dry Mass. Fresh and dry mass of cell suspensions was determined gravimetrically. The samples were dried to a constant weight at 105° C.

Glucose Utilization. The immobilized cells and cell suspensions were exposed to an initial glucose concentration of 250 mg/L in cultivation media [11, 44] without sucrose. The concentration of glucose was determined by the method of Trinder [45].

Cell Viability. Cell viability was determined by the method of Dixon [46] with 2,3,5-triphenyltetrazolium chloride (TTC), fluorescein diacetate, and oxygen electrode.

Influence of Temperature and Some Sugars on Enzyme Activity. The influence of temperature was tested from 20°C to 100°C. The effect of glucose, galactose, and fructose on the activity of invertase in suspension culture and immobilized cells was tested at concentrations 1, 5, 10, and 20 mM.

Storage Stability. The stability of invertase during storage was monitored in the following experiments. The immobilized cells were stored at 4° C in 0.15 M NaCl supplied with the following compounds: a) chloramphenicol 50 mg/L; b) chlortetracycline hydrochloride (CLCTC) 50 mg/L; c) (1-methyldodecyl)-dimethylamine N-oxide (ATDNO) 100 mg/L [47], and in 0.15 M NaCl at –10°C. These experiments were repeated at least three times.

Identification and Determination of Intra- and Extracellular Invertase. Using saccharose as substrate we identified and determined the intra- and extracellular activity of invertase. Cell suspension cultures were used to determine the intracellular enzyme activity. The cells (10 g) were filtered and washed twice with 1.5 L of distilled water. Soluble proteins were extracted by grinding the cells in a precooled mortar using a ratio of 1:2 (g/mL) of cells and McIlvaine buffer pH 4.5 at 4° C. The homogenate was filtered through two layers of nylon cloth and centrifuged at 15000 g at 4°C. For determination of extracellular enzyme activity the cultivation medium (without cells) was used after centrifugation (15000 g, 10 min at 4° C).

Enzyme Assay. Enzyme activity was determined by the modified method of Rubio et al. [4] using saccharose as the substrate. The reaction mixture contained a suitable amount of enzyme (0.2 – 0.4 mL) and 0.4 mM saccharose in McIlvaine buffer pH 4.5, in a final volume of 2 mL. Enzyme activity was determined at 30°C for 30 – 60 min. The control contained temperature-inactivated enzyme (100° C, 10 min). The enzyme activity is expressed in katals. Proteins were determined by the method of Doumas et al. [48] using bovine serum albumin as standard. The glucose content released by the enzyme was determined by the method of Trinder [45].

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