Inulinase Immobilization on Macroporous Anion-Exchange Resins by Different Methods

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Inulinase was immobilized on AV-16-GS macroporous anion-exchange resin by physical and chemical methods. Binding of the enzyme to the carrier by the studied methods led to a shift of catalysis optimal temperature towards higher values and to extension of the range of optimum pH values. Our modification of glutaraldehyde method of inulinase immobilization increased catalytic activity of the preparation in comparison with the common glutaraldehyde method.

Key Words: *inulinase; immobilization; adsorption; glutaraldehyde method; modified glutaraldehyde method*

Inulinases of different origin cleave inulin and other fructose-containing polymers to fructose by clearing the glycoside bonds. Inulinase (2,1-β-D-fructane-fructanohydrolase, EC 3.2.1.7) is highly prevalent in higher plants and microorganisms. This enzyme can be used for isolation of fructose from raw plants: Jerusalen artichoke (*Helianthus tuberosus*), dahlia (*dahlia*), elecampane(*Inula helenium*), chicory(*Cichorium*), dandelion (*Taraxacum*), and burdock (*Arctia*).

At present, fructose is derived from starch by the enzymatic method consisting of several stages catalyzed by α-amylase, glucoamylase, and glucose isomerase. This process yields 45% fructose syrup. The use of inulinase for cleavage of inulin-containing raw material results in a single-stage production of 95% fructose syrup. In addition, inulinase functions under milder conditions compared to acid hydrolysis requiring high concentrations of hydrogen ions, high temperature, and use of special acid-resistant equipment.

Immobilization transfers the enzyme from homogenous soluble catalysts into heterogeneous ones with all their technological advantages. This approach improves the resistance of protein preparations to denaturing environmental conditions and provides the possibility for repeated use of the biocatalysts in pharmaceutical and food industry.

Inulinase was successfully bound to porous glass and bentonite and was incorporated in alginate hydrogels [2]. The enzyme isolated from *Escherichia coli* was effectively immobilized on Duolite A 568 and Amberlite 94 S anion-exchange resins [4]. Inulinase incorporated in PAAG retains more than 45% its initial activity at 45°C [3]. It was proven that the use of pure cross-linked inulin and completely methylated inulin (not inulinase substrates) as a carrier for inulinase immobilization is ineffective because of low activity of the resultant biocatalysis [1]. Inulinase from *Kluyveromyces marxianus* was adsorbed on Streamline DEAE [5] anionite and on Duolite A 568 [7]. An amperometric bi-enzymatic biosensor for evaluation of inulin content in foodstaffs was created on the basis of immobilized fructose dehydrogenase and inulinase [6].

However, despite numerous enzymatic preparations immobilized on various carriers were described, the choice of polyelectrolyte and method of protein binding to the ion exchanger matrix remains largely empirical, and we therefore used different methods for inulinase immobilization on AV-16-GS anion exchange macroporous resin.

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MATERIALS AND METHODS

We used inulinase from *Aspergillus awamori* BKMF 2250. Inulin (Spofa) served as the substrate for enzymatic reaction.

Protein content was measured by the method of Lowry, catalytic activity was measured spectrophotometrically on a KFK-3 photoelectrocolorimeter. Inulinase activity was calculated by the formula:

$$A = \frac{a}{180\ bt},$$

where *A* is catalytic activity, U/ml; *a* is content of fructose, μmol; *b* is enzyme content in reaction mixture, mg/ml hydrolysate; *t* is duration of hydrolysis, min; and 180 is fructose molecular weight.

The quantity of inulinase catalyzing the formation of 1 μmol fructose within 1 min was taken as 1 U of enzyme activity.

For evaluation of activity of enzymatic preparation, inulin hydrolysis reaction was carried out with two bioreactors (control and experimental); the incubation medium was mixed with a magnetic stirrer.

Anion exchange AV-16-GS resin synthesized by polycondensation of polyethylene polyamine, epichlorohydrine, and pyridine served as the carrier. This polyelectrolyte was chosen because it passed sanitary and chemical tests and was allowed for use in food industry and for water purification.

The ionite was prepared for immobilization by conditioning and transition into the needed ion-exchange form. To this end, the studied specimens of polyelectrolyte were put into saturated NaCl solutions for 3-4 h (in order to prevent the cracking of the granules), after which the carrier was washed in distilled water. In order to remove mineral admixtures, ionite was treated with HCl in ascending concentrations (0.5, 1.0, 2.0, 3.0 mol/liter, 5 volumes per volume of the resin) until obtaining Fe-free solution. The ionite was then treated with HCl in descending concentrations and washed with distilled water to the neutral reaction of medium. After that the ionite was treated with sodium hydroxide solutions in ascending concentrations (0.10-0.25 mol/liter) and washed with distilled water. For more complete removal of admixtures, acid/alkaline treatment was repeated 3 times.

Adsorption immobilization (5 g carrier in 50 ml acetate buffer, pH 4.5) was carried out overnight at ambient temperature. The enzyme (5 ml) was added to ionite suspension and mixed in a flask on an electric mixer for 1.5 h at 25°C, after which the mixture was centrifuged for 5 min at 250*g*.

For covalent immobilization by the glutaraldehyde method, 2.5 g resin was left for 24 h at ambient temperature in 20% glutar dialdehyde solution (1:1) in a corked flask. The carrier was then washed with distilled water, enzyme solution (2.5 ml; pH 4.5) was added, and the mixture was incubated for 24 h in a closed vessel. Immobilized preparation obtained by adsorption and covalent immobilization was washed from protein in acetate buffer. Control was carried out with an SF-46 spectrophotometer at λ=280 nm.

For increasing catalytic activity of inulinase, a modified glutaraldehyde method was used. The experiment was carried out as follows: 45 ml 4% succinic anhydride solution in chloroform was added to 2 g anionite and boiled in a water bath for 4.5 h, after which the mixture was incubated for 16 h at ambient temperature. The resin was washed in warm chloroform and dried in the air, after which 10 ml thionyl chloride was added and the mixture was boiled for 30 min. Ethylene diamine (20 ml) was drip-dropped to the carrier (washed in toluene and dried) at 20°C; the mixture was left for 20 h and then washed 10 times with distilled water, 5 times with 3% ammonium solution, and again in water. Glutaraldehyde (10 ml, 2% solution) was added to ionite and mixed on a magnetic mixer for 3 h at 50°C. The resin was then separated from solution, washed in water, and incubated with the enzyme solution according to the above method.

The results were statistically processed using Statgraphics software. The significance of differences between the control and experimental values was evaluated using Student's *t* test.

RESULTS

Four-stage purification of inulinase from *Aspergillus awamori* including acetone precipitation, fractionation with ammonium sulfate, gel filtration on Sephadex G-25, and ion exchange chromatography on a column packed with DEAE cellulose yielded a preparation with 85-fold purity and 5% output.

Characteristics of enzymatic preparations obtained by inulinase immobilization on macroporous anion exchange AV-16-GS resin by the adsorption, glutar-

TABLE 1. Characteristics of Inulinase Preparations Immobilized on AV-16-GS by Different Methods

Method of immobilization	Characteristics of preparation	
	protein content, mg/g carrier	catalytic activity of immobilized inulinase, % of free enzyme
Adsorption	9	30
Glutaraldehyde	11	60
Modified glutaraldehyde	14	85

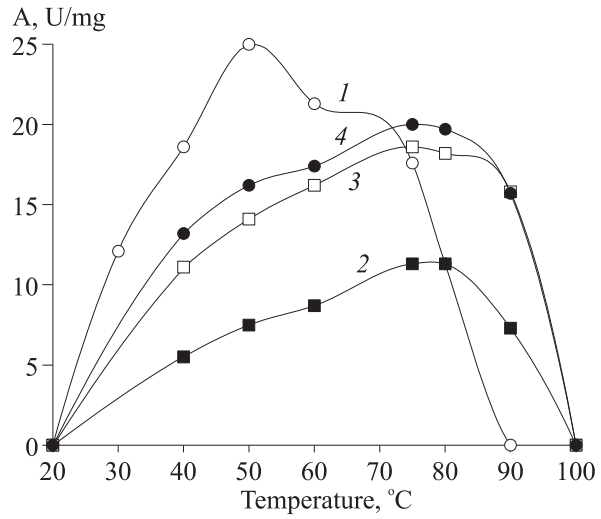

Fig. 1. Relationship between catalytic activity (A) of inulinase (free and immobilized on AV-16-GS) and temperature. Here and in Fig. 2: *1*) activity of free inulinase; activity of inulinase immobilized by: *2*) adsorption method; *3*) glutaraldehyde method; *4*) modified glutaraldehyde method.

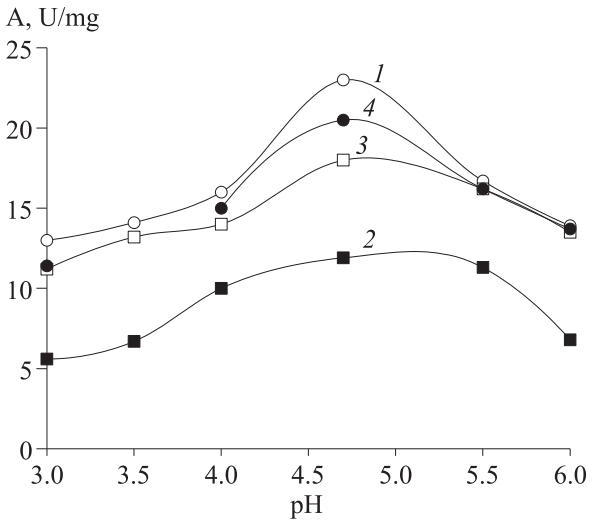

Fig. 2. Relationship between catalytic activity (A) of inulinase (free and immobilized on AV-16-GS) and medium pH.

aldehyde, and modified glutaraldehyde methods and results of studies of the relationship between catalytic activities of the resultant samples and temperature and pH values are presented in Table 1.

Temperature optimum for inulinase functioning bound to the carrier by the adsorption method is 70°C *vs.* 50°C for free enzyme (Fig. 1). Immobilized preparation retains 90% its maximum activity at 80°C, *vs.* just 10% for the free preparation.

After covalent immobilization of inulinase on AV-16-GS ionite by the glutaraldehyde and modified glutaraldehyde methods, the samples exhibited maximum activity at 75°C, retaining 70% of their catalytic capacity even at 90°C.

The pH optimum of 4.7 virtually did not change during physical and chemical binding of inulinase to the carrier matrix; the only change was a wider range of hydrogen ion concentrations at which the rate of inulin hydrolysis was maximum (Fig. 2).

The results of our laboratory studies indicate that despite difficulties of the modified glutaraldehyde method of inulinase immobilization on AV-16-GS ionite, it seems to be most promising for industry, because the resultant enzymatic preparations retain up to 85% catalytic capacity of the free enzyme at the optimum temperature of functioning of 75°C.

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