Covalent immobilization of invertase on chemically activated poly(2-hydroxyethyl methacrylate) microbeads

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Properties of invertase immobilized on poly(2-hydroxyethyl methacrylate) microbeads activated by epichlorohydrin or cyanuric chloride were studied. After 20 repeated uses for 3 days, the activity of the immobilized enzyme was 92—93%.

Key words: covalent immobilization, activation, poly(2-hydroxyethyl methacrylate), invertase.

Immobilization of enzymes is very important for various applications, *e.g.*, in food industry, medicine, and pharmacology. Enzyme immobilization onto solid supports enables repeated use, provides significant reduction in the operation costs, facilitates easy separation, accelerates recovery, and increases the enzyme stability by protecting the active material from deactivation.[1,2]

Invertase catalyzes sucrose hydrolysis to glucose and fructose, which are used as sweeteners, because they do not crystallize. Various immobilization methods have been described, such as gel entrapment,[3,4] covalent attachment,[5,6] and adsorption.[7] Immobilization of invertase onto natural and synthetic polymers, *viz.*, calcium alginate,[8] polypyrrole/polytetrahydrofuran graft copolymer,[9,10] and polyethyleneimine,[11] was investigated.

In the present study, invertase was immobilized onto poly(2-hydroxyethyl methacrylate) (PHEMA) microbeads using epichlorohydrin (ECH) and cyanuric chloride (CC) as activating materials. The effect of immobilization on the enzyme activity, kinetic parameters, activity retention, and capability of repeated use of invertase was investigated.

Experimental

The following commercial reagents were used: invertase (β-fructofuranosidase, E.C. 3.2.1.26, grade V practical from bakers yeast) and sucrose (Sigma, USA); tricalcium phosphate (TCP) (Riedel-De Haën AG, Germany); epichlorohydrin, cyanuric chloride, benzoyl peroxide, polyvinyl alcohol, and ethylene glycol dimethacrylate (Merck AG, Germany); 2-hydroxyethyl methacrylate (Aldrich, USA). Other reagents were of reagent grade (Merck AG, Germany) and used as received.

Preparation of poly(2-hydroxyethyl methacrylate) microbeads. Microbeads of PHEMA were prepared by suspension polymerization. A solution of polyvinyl alcohol (0.325 g) and TCP (0.325 g) in distilled water (50 mL) was stirred for 1 h with a speed of 350 rpm at 70 °C under nitrogen, and then 2-hydroxyethyl methacrylate (10 mL), benzoyl peroxide (0.1 g), and ethylene glycol dimethacrylate (1.0 mL) as the cross-linker were added. The mixture was purged with nitrogen and stirred for 6 h at 70 °C with a speed of 350 rpm. The suspension was cooled to ~20 °C, and 37% HCl (1 mL) was added to dissolve TCP. The formed microbeads were separated, washed with distilled water to remove polyvinyl alcohol and excess acid, kept in distilled water for ~16 h, washed with ethanol and acetone, and dried *in vacuo* at ~20 °C.

Characterization of microbeads. Microbeads of PHEMA were distributed in chloroform. The average particle size and size distribution curves were obtained on a Mastersizer S particle size analyzer (Version 2.15, Malvern Instruments Ltd., Great Britain).

Activation with epichlorohydrin. Microbeads of PHEMA (0.1 g) were shaken with ECH (5 mL) for 4 h at 30 °C and kept for ~16 h at ~20 °C. The activated microbeads were separated and washed (3×5 mL) with phosphate buffer (40 mmol L^{-1}, pH 7.0).

Activation with cyanuric chloride. Microbeads of PHEMA (0.1 g) were treated with a solution of CC (2% wt/v in dioxane) as described above.

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Immobilization of invertase on microbeads. The activated microbeads (3.0 g) were added to the enzyme (0.04 g) dissolved in 100 mL of phosphate buffer. The mixture was stirred for 4 h at 30 °C. The microbeads were separated, and the unbound enzyme was removed by washing three times with phosphate buffer. The immobilized enzyme was kept at 4 °C until use.

Enzyme loading. The amount of the immobilized enzyme was determined by measuring the protein content in the solution before and after immobilization using the Lowry method.[12] A calibration curve was constructed using bovine serum albumin (1.0—10.0 mg mL^{-1}) as a standard.

Assay of invertase activity. The activity of free and immobilized invertase was determined by the Folin—Wu assay. According to this method, the substrate (1 mL of a $2.9 \cdot 10^{-2}$ *M* solution of sucrose) was added to the enzyme (0.2 mg of free enzyme or 0.1 g of immobilized enzyme) in 1.0 mL of phosphate buffer, and the mixture was incubated for 15 min at 30 °C. Then the mixture was cooled, and an alkaline copper tartrate (1 mL) was added to terminate the reaction. The mixture was placed in a boiling water bath until the brown color was obtained and then cooled down to ~20 °C. A solution of phosphomolybdic acid (1 mL) was added, the mixture was thoroughly vortexed, phosphate buffer (10.0 mL) was added, and the absorbance of the blue-colored complex was measured at 640 nm. The amount of glucose was calculated from the calibration curve and used in the calculation of enzyme activity. The extent of hydrolysis of sucrose was 8.0%. All measurements were carried out twice (relative error <1%). One unit of enzyme activity was defined as the amount of enzyme that catalyzes hydrolysis of 1 μmole of sucrose per min under these conditions.

Determination of K_M and V_{max} values. The Michaelis constant (K_M) and maximum rate (V_{max}) were determined by measuring the initial reaction rates using sucrose solutions in phosphate buffer ($1.46 \cdot 10^{-3}$—$1.46 \cdot 10^{-2}$ mol L^{-1}). The assay procedures were the same as described above.

Storage stability of the enzyme. The free and immobilized enzymes were kept at 4 °C. Storage stabilities were estimated by measuring the enzyme activity after certain time intervals.

Possibility of reuse. Samples of enzymes immobilized on activated PHEMA microbeads were used repeatedly 20 times within 3 days, and their activities were measured.

Thermal stability of the free and immobilized enzymes. Thermal stability of free and immobilized invertase was estimated by measuring the residual activity of the enzyme after incubation for 90 min at 60 and 70 °C in the presence of the substrate (1 mL of a $2.9 \cdot 10^{-2}$ *M* sucrose solution per 0.2 mg of the free enzyme or per 0.1 g of the immobilized enzyme). The enzyme activity was determined after 15-min intervals. The first-order inactivation rate constant (k_i) remained unchanged and was calculated from the following equation:

$$\ln A_t = \ln A_0 - k_i t, \quad (1)$$

where A_0 is the initial enzyme activity, and A_t is the enzyme activity after time t (min).

Results and Discussion

Properties of microbeads. The particle size distribution of the PHEMA microbeads is presented in Fig. 1.

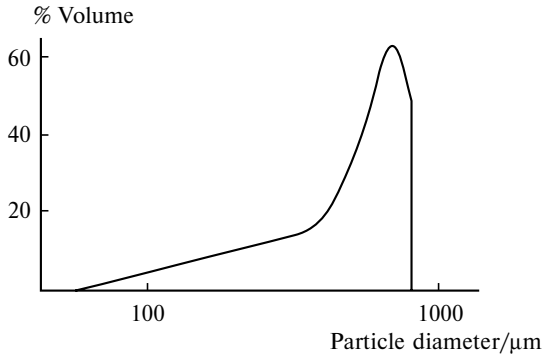

Fig. 1. Particle size distribution curve of the PHEMA microbeads.

Approximately 50% of the particles are between 500 and 1000 μm in diameter. The results of particle size distribution are given below (specific surface area 0.0344 $m^2 g^{-1}$).

Diameter of PHEMA	$D_{0.1}$	$D_{0.5}$	$D_{0.9}$	$D_{3.2}$	$D_{4.3}$
			μm		
Value	161.79	544.57	782.36	174.52	502.73

The following designations are used: $D_{0.1}$ means that the size of 10% particles is smaller than the indicated value; $D_{0.5}$ — the size of 50% particles is larger and that of 50% is smaller than the indicated one (this parameter is also named the mass median diameter); $D_{0.9}$ — the size of 90% particles is smaller than the indicated value; $D_{3.2}$ is the surface mean diameter; $D_{4.3}$ is the volume mean diameter.

The mass median diameter (MMD) and volume mean diameter (VMD) of the particles are 544.57 and 502.73 μm, respectively.

Enzyme loading. The amount of invertase covalently bound to the PHEMA microbeads was 10.4 and 9.87 mg (g of microbeads)$^{-1}$ for the activation by ECH and CC, respectively. The enzyme binding efficiency was 78 (ECH) and 74% (CC). The activity of the bound enzyme was 68 and 67%, respectively. According to available data,[5,13] the typical invertase content is 4.0—18.6 mg g^{-1} for immobilization on polymeric supports and dry gels.

Parameters affecting the enzyme activity. We studied effects of the pH value, temperature, and substrate concentration on the enzymatic reaction rate, as well as the enzyme stability and repeated use capability. The activities of free and immobilized invertase were calculated by measuring the absorbance of the solution at 640 nm as described above.

Effect of pH. The pH dependence of the activity of the free and immobilized enzymes was determined in the pH range of 3.0—8.0 at 30 °C. The results are shown in Fig. 2. The pH value for the maximum substrate conversion was found to be 4.5 for free invertase and 5.0 and 5.5 for invertase immobilized on the PHEMA microbeads

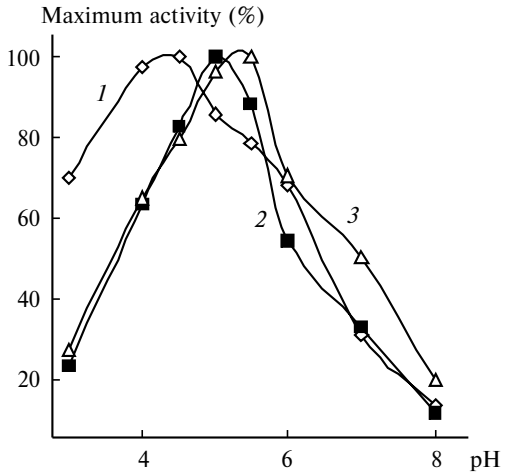

Fig. 2. Effect of pH on the activities of free invertase (*1*) and invertase immobilized on the PHEMA microbeads activated by ECH (*2*) and CC (*3*).

activated by ECH and CC, respectively. The optimum pH value of immobilized invertase was shifted by unity to the alkaline region. The change in the optimum pH value for the supported enzyme can be attributed to diffusional limitations and secondary interactions, because enzyme immobilization on the support affects its three-dimensional structure and arrangement of functional groups. As a result, each invertase molecule is stabilized due to attachment to the microbead surface in several points. According to published data, the optimum pH value for free invertase is 4.5 (see Refs 4, 6, and 8) or 5.0,[7] whereas for invertase immobilized on lactam-amide graft copolymers and a composite material of gel and cellulose acetate fiber they are 5.0 (see Ref. 7) and 5.5 (see Ref. 4), respectively. The optimum pH values found in the present study agree with those described previously.

Temperature effect. The temperature effect on the invertase activity is shown in Fig. 3. The optimum temperatures were 45 °C for free invertase and 55 and 60 °C for invertase immobilized on the PHEMA microbeads for ECH and CC activation, respectively. The increase in the optimum temperature was caused by changing the physical and chemical properties of the enzyme upon immobilization. Covalent bond formation with the support can also reduce the conformational flexibility of the enzyme and make it more thermally stable. Therefore, the diffusion of the substrate and formation of enzyme—substrate complexes can occur at higher temperatures. The optimum temperatures have been reported as 45 °C (see Refs 6 and 7) and 55 °C (see Ref. 5) for free invertase; 55 °C (see Refs 5 and 9), 60 °C (see Refs 9 and 10), and 65 °C (see Ref. 7) for invertase immobilized on different synthetic matrices (functionalized copolymers, conducting polypyrrole/polytetrahydrofuran graft copolymers, and others). The differences in the optimum temperature

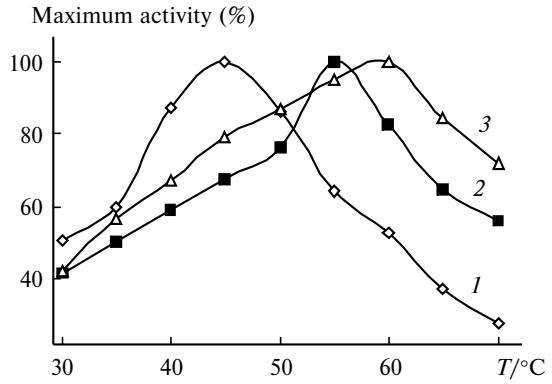

Fig. 3. Temperature effect on the activities of free invertase (*1*) and invertase immobilized on the PHEMA microbeads activated by ECH (*2*) and CC (*3*).

can be due to the matrix type and immobilization conditions.

Kinetic parameters. Kinetic parameters (K_M and V_{max}) for the free and immobilized enzyme were determined using sucrose as the substrate. The linear increase in the hydrolysis rate was observed with an increase in the sucrose concentration from $1.46 \cdot 10^{-3}$ to $1.46 \cdot 10^{-2}$ mol L^{-1}. Therefore, the kinetic parameters were determined from the plot of the initial hydrolysis rate *vs.* concentration in the Lineweaver—Burk coordinates (Fig. 4). The V_{max} values were calculated as $6.56 \cdot 10^{-2}$ mol L^{-1} min^{-1} for the free enzyme and $3.94 \cdot 10^{-2}$ and $3.56 \cdot 10^{-2}$ mol L^{-1} min^{-1} for the immobilized enzyme (for ECH and CC activation, respectively). Thus, an about twofold decrease in V_{max} is observed upon immobilization. The K_M values were $4.1 \cdot 10^{-3}$ mol L^{-1} for free invertase and $6.4 \cdot 10^{-3}$ and $6.6 \cdot 10^{-3}$ mol L^{-1} for immobilized invertase (for ECH

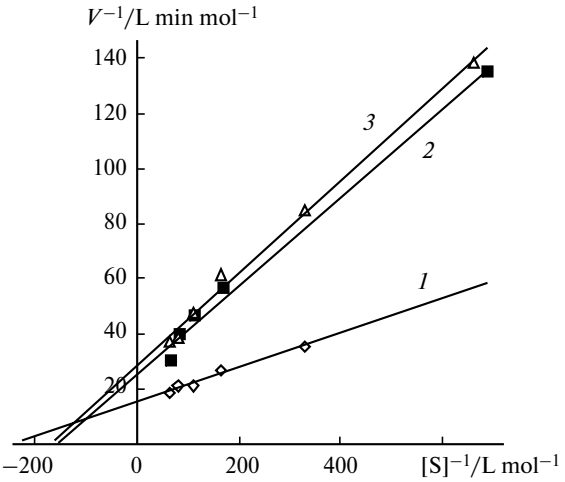

Fig. 4. Lineweaver—Burk plots for free invertase (*1*) and invertase immobilized on the PHEMA microbeads activated by ECH (*2*) and CC (*3*) (*V* is the hydrolysis rate, [S] is the substrate concentration).

and CC activation, respectively), indicating an about 1.5-fold increase in this parameter. The changes in V_{max} and K_M due to chemical binding of the enzyme to the support are quite regular, because the enzyme loses its three-dimensional conformation and, as a consequence, some activity. The environment of the enzyme on the solid support will be different than that in the bulk. This affects the diffusion and concentration of sucrose bound to the enzyme. Immobilization impedes the formation of an enzyme—substrate complex and decreases the reaction rate, changing V_{max} and K_M of the immobilized enzyme compared to those for the free enzyme.[6,14] For invertase immobilization on different supports, the K_M values range from $2.0 \cdot 10^{-3}$ to $7 \cdot 10^{-2}$ mol L^{-1}, and V_{max} vary from $1 \cdot 10^{-6}$ to $5 \cdot 10^{-2}$ mol L^{-1} min^{-1}.[4—6,9,10]

Storage stability. In solution, enzymes are unstable and denaturate on storage, resulting in the loss of activity. Enzymes immobilized on the solid phase are more stable. Storage stability was studied by measuring the enzyme activity at certain time intervals. The results are presented in Fig. 5. After one month the residual invertase activity was 76.9 and 78.8% of the initial value for the ECH- and CC-immobilized enzyme, respectively. For the free enzyme the residual activity is 35.7% after the same storage period. It has earlier been mentioned[6,7,15] that the residual activity of invertase immobilized on a lactam-amine graft copolymer, modified polyaniline, and microporous membranes of poly(2-hydroxyethyl methacrylate—glycidyl methacrylate was 80% of the initial activity. This should be expected: the attachment of the enzyme to the support increases its stability and decreases denaturation.

Possibility of reuse. Samples of the enzyme immobilized on microbeads were repeatedly used 20 times within 3 days; their relative activities are presented in Fig. 6. After 20-fold use, the activity of the enzyme was 92.2 and 93.4% of the initial value (activation by ECH and CC, respectively). Reproducibility of the results of immobilization gives important economical advantages for using the enzyme in industry and other practical applications

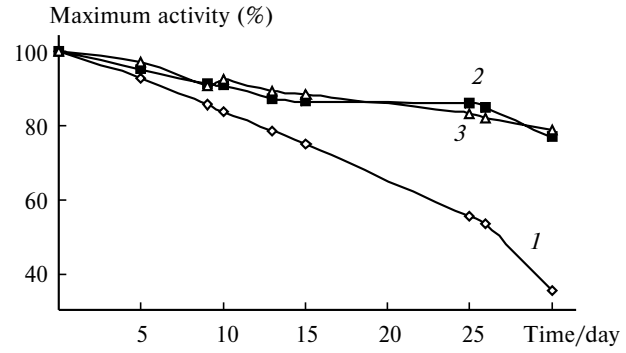

Fig. 5. Effect of storage on the activity of free invertase (*1*) and invertase immobilized on the PHEMA microbeads activated by ECH (*2*) and CC (*3*).

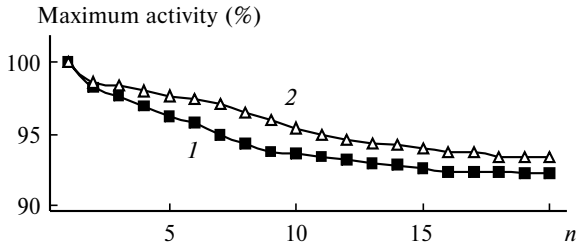

Fig. 6. Effect of reuse of immobilized invertase (*n*) on its activity upon ECH (*1*) and CC (*2*) activation.

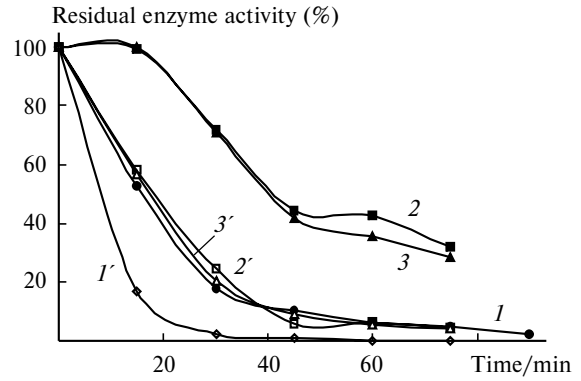

Fig. 7. Thermal inactivation of free invertase (*1*, *1′*) and invertase immobilized on the PHEMA microbeads activated by ECH (*2*, *2′*) and CC (*3*, *3′*) at 60 (*1—3*) and 70 °C (*1′—3′*).

(for instance, its applications as a biosensor or an enzyme reactor). In similar studies, the activity of invertase immobilized on synthetic polymeric matrices retained above 90% activity after 10—50 successive batch reactions, whereas only 55% initial activity held after cycling 10 times for invertase entrapped by calcium alginate.[4,5,15]

Thermal stability. The thermal stabilities of the free and immobilized enzymes were studied at 60 and 70 °C. The plots of retention of the initial activity *vs.* time for the free and immobilized enzymes are shown in Fig. 7. After 75 min of incubation at 60 °C, the residual activity was 28 and 33% of the initial activity for the CC- and ECH-immobilized enzyme, respectively. For the free enzyme the residual activity was 4% under the same conditions. At 70 °C the corresponding residual activities were 4, 4,

Table 1. Half-life values ($t_{1/2}$/min) and thermal inactivation constants (k_i/min^{-1}) for the free and immobilized enzymes

T /°C	Free enzyme		Immobilized enzyme			
			Activation by ECH		Activation by CC	
	$t_{1/2}$	k_i	$t_{1/2}$	k_i	$t_{1/2}$	k_i
60	16	$3.61 \cdot 10^{-2}$	41	$1.87 \cdot 10^{-2}$	41	$2.14 \cdot 10^{-2}$
70	9	$9.48 \cdot 10^{-2}$	18	$5.04 \cdot 10^{-2}$	17	$4.96 \cdot 10^{-2}$

and 0.04%. The half-life values ($t_{1/2}$) were calculated from the time plots of the residual activity at two different temperatures. The thermal inactivation constants (k_i) were calculated from the slope of time versus $\ln A_t$ plot and are given in Table 1.

This result demonstrates an increase in the thermal stability of invertase upon immobilization. Similar observations have earlier[15—17] been described for invertase covalently attached to various matrices.

Thus, high storage stability, reusability, and thermal stability are important advantages of immobilized invertase for various biotechnological and industrial applications.

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