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Immobilization of Invertase onto a Copolymer of Methacrylamide and N,N′-Methylene-*bis*(acrylamide)

Scientific Note

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INTRODUCTION

Primary considerations for the support applied to enzyme immobilization are hydrophilic characteristics, high surface area, high porosity, mechanical and chemical stability, and surface chemical variety (*1*). Base materials available for enzyme immobilization include natural polymers, synthetic polymers (such as maleic anhydride-based polymers, methacrylate-based polymer, and styrene-based polymers), and inorganic silicas (such as silica gel and glass beads). The main disadvantage of a natural polymer is that it is easily contaminated biologically and hydrolyzed during the operating process. Silica-based materials, though having good mechanical properties, cannot remain resistant at the higher pH. The advantages of synthetic polymers are their inertness to microorganism attack, the ease of preparing various polymers with different degrees of porosity, and their chemical composition for a particular enzyme and application.

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Acrylamide is a hydrophilic monomer and its polymer possesses amide groups in the side chains that can be activated by several methods for chemical coupling of enzymes and proteins. Owing to their swelling property in aqueous solutions, polyacrylamide matrices have been largely used as an entrapment technique for enzymes. As an insoluble carrier for covalent immobilization of enzyme, the polyacrylamide is commonly copolymerized with a crosslinking agent (*2*). In this work, methacrylamide was used as the monomer in preparing a crosslinked methacrylamide-based polymer support for enzyme immobilization. Invertase has been immobilized covalently on various carriers such as porous glass (*3,4*), activated clay (*5*), corn stover (*6,7*), bead cellulose, glycidylmethacrylate (*8*), styrene-divinyl benzene copolymer (*8,9*), granular chicken bone (*10*), and magnetic silica (*11*), but immobilization of invertase by chemical coupling on methacrylamide-based polymer has not been previously described.

MATERIALS AND METHODS

Preparation of Polymer Support

Beads of copolymerized methacrylamide and N,N′-methylene-*bis*-acrylamide were prepared by means of a W/O type suspension polymerization. An aqueous solution consisting of DMF (26.6 mL), water (53.4 mL), methacrylamide (12 g), methylene-*bis*-acrylamide (8 g), and ammonium persulfate (1.6 g) was prepared in an ice-bath. Oil phase consisting of 1,2 dichlorethan (200 mL) and cellulose acetate-butyrate (6 g) was added to a flask installed with a reflux condenser and a mechanical stirrer. When stirring under nitrogen was begun, aqueous solution was added to the flask. Polymerization was carried out for 6 h at 50°C. The copolymerized beads were washed five times with acetone and then washed for 2 d by Soxhelt extraction method through the use of methanol. The physical properties of the polymer beads were measured by the Accelerated Surface Area and Porosimetry System (ASAP 2000, Micromeritics Instruments).

Modification of Polymer Support

The copolymerized beads were modified by the following methods:

$$P\text{—}CONH_2 \xrightarrow[90°C]{NH_2(CH_2)_2NH_2} P\text{—}CONH(CH_2)_2NH_2 + NH_3 \quad (1)$$

$$P\text{—}CONH_2 \xrightarrow{OCl^-,\ OH^-} P\text{—}NH_2 + CO_3^{=} \quad (2)$$

$$P\text{—}CONH_2 \xrightarrow[60°C]{OH^-} P\text{—}COO^- + NH_3 \quad (3)$$

In the first reaction (amination), beads and ethylene diamine were mixed and reacted at 90°C for 20 h or longer. The modified beads were then washed with distilled water and acetone, alternatively. In the second reaction (Hofmann degradation), beads and sodium hypochlorite were mixed by stirring for 2 h. Sodium hydroxide was then added. The mixture was finally washed with distilled water. In the third reaction (alkaline hydrolysis), the beads were partially modified with 0.2*N* sodium carbonate or sodium hydroxide to convert the amide groups into carboxyl groups. The zeta potential of the modified beads was measured by Zetasizer 3 (Malvern Instruments, UK). In all the measurements of zeta potential, beads were dispersed in a 0.02*M* phosphate buffer at pH 7.

Immobilization of Invertase

To 1 g of modified beads containing surface amino groups (after amination or Hofmann degradation) or nonmodified beads was added 40 mL of an aqueous 5% (v/v) solution of glutaraldehyde (pH 7). The beads were agitated for 12 h (16 h in the case of nonmodified beads) at 35–40°C. Invertase (Sigma Chemical Co., St. Louis, MO, Grade VII) solution was added to the activated beads and incubated for 10 h (16 h in the case of nonmodified beads) at room temperature. The buffer used was acetate (pH 5).

Determination of Invertase Activity

The invertase activity of the beads was determined by measuring the reduced sugar production caused by the hydrolysis of 0.8*M* sucrose at pH 5 and 30°C. When the activity of soluble invertase was assayed, the sucrose concentration was 0.15*M*. All the assay solutions were constantly agitated at 500 rpm. The amount of reduced sugar produced was determined by the DNS method. One unit of activity was determined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of sucrose/min under the conditions of the assay.

RESULTS AND DISCUSSION

Bead Properties

The prepared copolymer beads were sieved into several groups that have properties shown in Table 1. It was observed that the beads with particle size between 100 and 1000 μm are almost identical in their specific surface area, pore diameter, and pore volume. The fraction of particles that were >100 μm or <1000 μm in size is more than 90% of the total polymer beads prepared by the method of the W/O type suspension polymerization. These beads had a specific surface area of about 100 m^2/g for enzyme immobilization, which is comparable to that of a typical commercialized support Spherosil silica (120 m^2/g) (*11*).

Table 1
Physical Properties of the Prepared Copolymeric Beads

Particle diameter, μm	Surface area, m^2/g	Pore diameter, Å	Pore Volume, cm^3/g
100–250	103.6	147	0.381
250–420	104.2	154	0.401
420–590	94.1	160	0.402
590–1000	103.1	153	0.394

Influence of Modification Method on Invertase Immobilization

The activities of the immobilized invertases coupled to the copolymer beads modified by amination and Hofmann degradation were determined as 6896 U/g-bead and 579 U/g-bead, respectively. A bound activity of 10.2 U/g-bead was obtained from the nonmodified beads and no activity was detected from the immobilized invertase prepared from the beads modified by alkaline hydrolysis. All the immobilized invertase activities were assayed against 0.8*M* sucrose at 30°C and pH 5. According to these data, the most effective method was found to be the amination of the polymer beads with ethylene diamine, followed by the covalent coupling of invertase via Schiff's base derivatization through the usage of glutaraldehyde. The crosslinked polymer in the modification with sodium hypochlorite followed by sodium hydroxide, which is well-known as the Hofmann degradation of amide groups to amino groups, could be partially hydrolyzed owing to the high chemical reactivity of the sodium hypochlorite. The free amino groups of modified copolymer were then crosslinked again when they were incubated with glutaraldehyde. The amount of available amino groups was thus reduced and led to decrease immobilized activity in comparison with the immobilized invertase obtained from the support after amination. The nonmodified beads were coupled with only a small amount of enzymes when glutaraldehyde was directly used, because glutaraldehyde has very low reactivity toward amide groups. In the procedure of alkaline hydrolysis, the beads were treated with 0.2*N* sodium carbonate or sodium hydroxide at 60°C to reduce the amide groups to carboxyl groups. After activation by 10% thionyl chloride, the carboxylated beads were coupled with invertase in an alkaline condition at room temperature. The high pH condition might not be conducive to invertase. Therefore, no activity was detected from this kind of invertase conjugates. It is clear that the carboxylated beads should be activated by another method before immobilization of enzymes that cannot retain their activity at a higher pH value.

Effects of Reaction Time on Modification

The copolymerized beads proved to be a very efficient support for chemical modifications on the tract of enzyme immobilization. The time

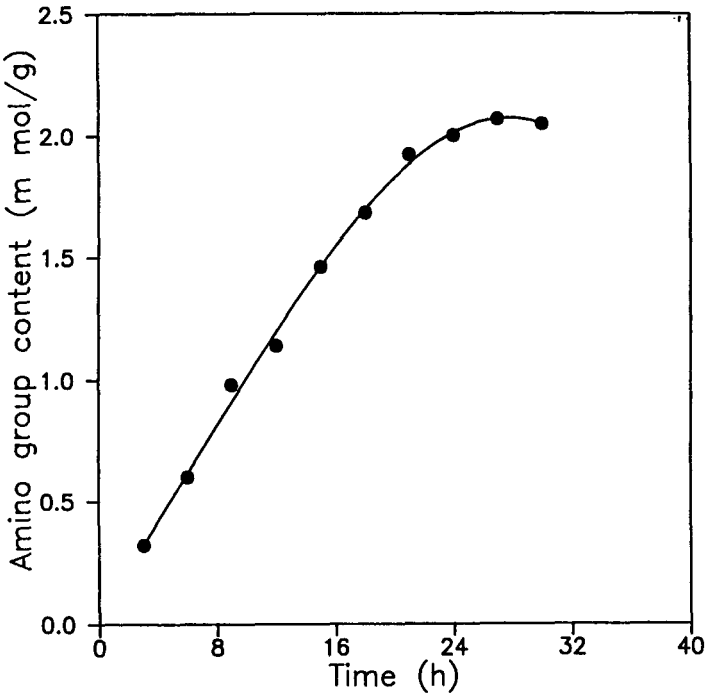

Fig. 1. Effect of amination time on the amino group content of polymer beads. The amination temperature was 90°C.

course of amination by anhydrous ethylene diamine at 90°C is shown in Fig. 1. The maximum amino density was approx 2 mmol g^{-1} as the amination time reached 20 h or more. While in the presence of a base, the amide groups of the copolymer were hydrolyzed; the formation of carboxylated derivatives as a function of time is presented in Fig. 2. Negatively charged carboxyl groups were characterized by the zeta potential. As shown in Fig. 2, the zeta potential of the beads gradually decreased from −2.4 to −33.5 mV when they were treated with 0.2*N* sodium carbonate at 60°C for 90 min. The zeta potential stayed at about −30 mV for a longer time of alkaline hydrolysis.

Properties of Immobilized Enzyme via Ethylene Diamine Modification

The immobilized invertase was very stable and retained its activity after a 1-mo preservation. The pH dependence of the activity of soluble invertase and immobilized invertase obtained from the copolymerized beads (particle size 250–420 μm) modified with ethylene diamine was studied at 30°C and the same ionic strength in the pH range from 2–8. The results are presented in Fig. 3, which shows that the pH optimum for catalytic activity of immobilized invertase was slightly shifted to the acidic region. As the zeta potential of the copolymer beads (−2.4 mV) became

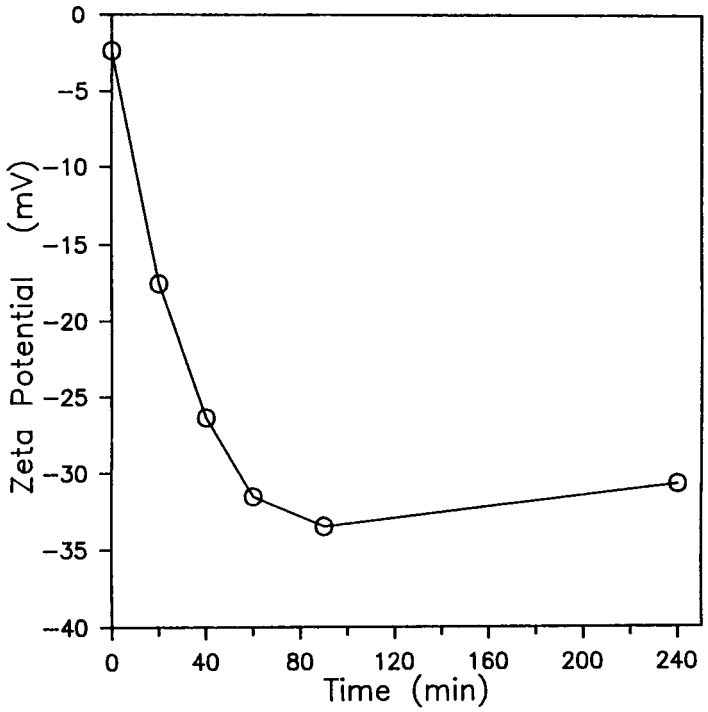

Fig. 2. Effect of alkaline hydrolysis time on the zeta potential of modified beads. The hydrolysis temperature was 60°C.

positive (14.1 mV) when they were modified by ethylene diamine and incubated with glutaraldehyde, the immobilized invertase showed higher relative activity than native invertase at low pH value was described in Fig. 3.

Temperature-dependence of the activity of soluble and immobilized invertase was studied in the temperature range from 30–80°C at pH 5. The results are presented in Fig. 4. It was found that the apparent optimum temperature for the catalytic activity of immobilized invertase (60°C) was about 10°C higher than that of the soluble enzyme (50°C). The difference was owing to higher stability of the immobilized enzyme. At 70°C, the activity of the immobilized invertase retained 60% of its maximum value, but the soluble enzyme retained only 15%. It is clear that immobilization made invertase more stable at higher temperatures.

The effect of substrate concentration on the activity was also investigated at 30°C and pH 5. The results are listed in Table 2. The sucrose hydrolysis with both soluble and immobilized invertases was described very well by the Michaelis-Menten model up to sucrose concentrations of 0.2*M* (soluble enzyme) and 1.0*M* (immobilized enzyme). Kinetics parameters for soluble and immobilized enzymes as reported in Table 2 were thus

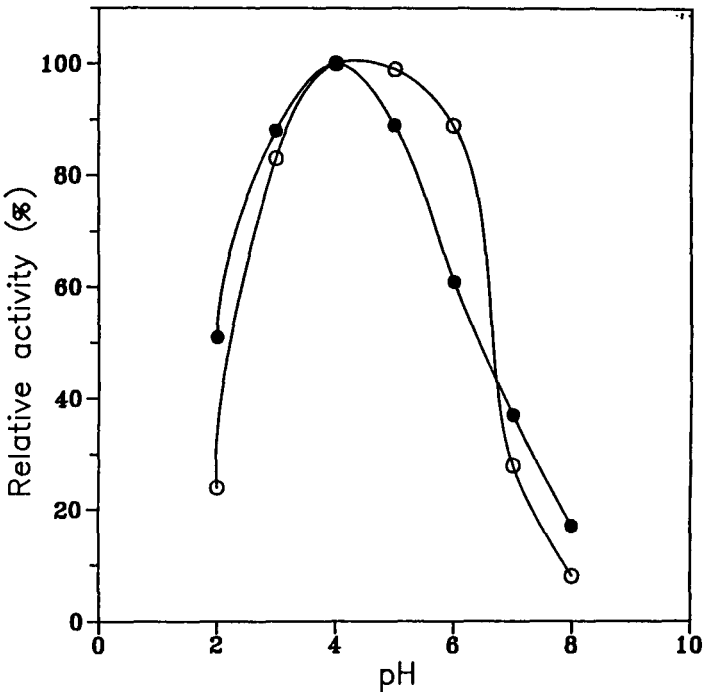

Fig. 3. Effect of pH on the activity of soluble and immobilized invertase. Experiments were carried out at 30°C: ○, soluble invertase; ● immobilized invertase. With both soluble and immobilized enzyme the maximum activity measured (soluble enzyme, 380 U g^{-1} solid; immobilized enzyme, 7780 U g^{-1} support) was taken as 100%.

determined from the initial velocity data with initial sucrose concentrations up to 0.2*M* and 1.0*M*, respectively. In order to reduce the effect of external diffusion, all experiments were carried out at an agitation speed of 500 rpm. It was found that the K_m value of the immobilized invertase was 10 times that of the soluble invertase. As the beads for the preparation of this invertase conjugate had an average pore size of about 150 Å and were larger in particle size (250–420 μm), internal diffusional effects might be mainly responsible for the increase in Michaelis constant of the immobilized enzyme. The Michaelis constant for immobilized invertase was thus regarded as an apparent value. The ratio of the V_{max} value of the immobilized invertase to that of the soluble invertase suggested that about 20 mg enzymes were immobilized into 1 g of support, if the loss of activity was not taken into account. In this study, we chose 30°C and pH 5, the optimal growth condition of *Zymomonas mobilis*, as the condition of interest. This immobilized invertase has been added into the fermentation of sucrose by *Z. mobilis* in order to enhance the ethanol production yield. Experimental results on this aspect will be published elsewhere.

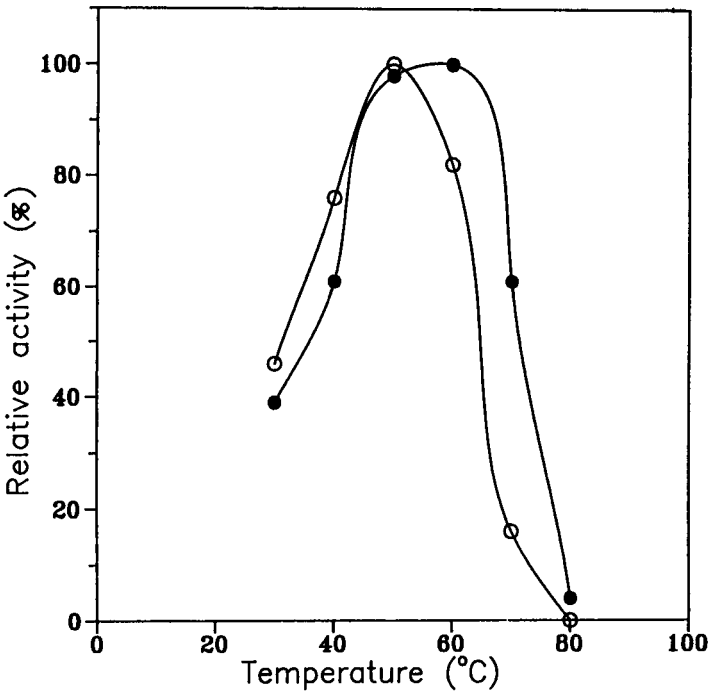

Fig. 4. Effect of temperature on the activity of soluble and immobilized invertase. Experiments were carried out at pH 5: ○, soluble invertase; ●, immobilized invertase. With both soluble and immobilized enzyme the maximum activity measured (soluble enzyme, 810 U g^{-1} solid; immobilized enzyme, 17650 U g^{-1} support) was taken as 100%.

Table 2
Kinetics of Soluble and Immobilized Invertase[a]

	K_m	V_{max}
Soluble enzyme	0.024*M*	425 μmol/min·mg-solid
Immobilized enzyme	0.24*M*	8715 μmol/min·g-bead

[a]Determined at 30°C and pH 5.

Comparison of Invertase Immobilization in the Literature

The stable invertase conjugate obtained from amination of the copolymer beads were superior in catalytic activity than that from magnetic silica support and other synthetic polymer supports, as shown in Table 3. As this kind of comparison was not based on the same quality of invertase used for immobilization, the data reproduced in Table 3 are more for the sake of literature survey. The immobilized invertase obtained in this work achieved a very high activity, 17650 U (g support)$^{-1}$ against 0.8*M* of sucrose at 60°C and pH 5, as well as possessing good physical properties of the copolymer. The copolymer prepared in this work for enzyme immobilization possessed a matrix structure with various possibilities of modification and different degrees of functionalization.

Table 3
Comparison of Invertase Immobilization by Covalent Binding

Carrier	Activity U/g-support	Assay condition	Invertase Used	Ref.
Magnetic silica	469	55°C, pH 4.5 0.292*M* sucrose	Sigma, Grade VII from baker's yeast	*11*
Poly(tetraethylene pentaminomethylstyrene)	3050	25°C, pH 4.5 0.146*M* sucrose	VEB Aromatic Leipzig from baker's yeast	*9*
Poultry bond activated by			Sigma, from *C. utiliis*	*10*
acry-azide	38	37°C, pH 5		
glutaraldehyde	4.4	0.5*M* sucrose		
carbodiimide	1.0			
Glycidyl methacrylate	333		not reported	*8*
PS-DVB	334			
Cellulose	205			
Corn stover	36000	40°C, pH 4.5	Sigma, Grade VI	*7*
Porous silica	1400	0.4*M* sucrose	from baker's yeast	
Methacrylamide-N,N′-methylene-*bis*(acrylamide) copolymer	17650	60°C, pH 5 0.8*M* sucrose	Sigma, Grade VII from baker's yeast	this work

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