0273-2289/88/1901—0001$02.00

Effect of Temperature Cycling on the Activity and Productivity of Immobilized β-Galactosidase in a Thermally Reversible Hydrogel Bead Reactor

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ABSTRACT

The enzyme β-galactosidase has been immobilized within thermally reversible hydrogel beads that exhibit LCST (lower critical solution temperature) behavior. The hydrogel beads containing the immobilized enzymes swell and expand below the LCST and deswell and shrink above the LCST. This behavior is reversible. The enzyme was physically entrapped in a crosslinked hydrogel of a copolymer of *N*-isopropylacrylamide (NIPAAm) and acrylamide (AAm), and formed as beads in an inverse suspension polymerization. The beads were placed in a packed bed column reactor which was operated in a continuous, single pass mode, either isothermally at 30 or 35°C, or with temperature cycling between 30 and 35°C. The thermal cycling significantly enhanced overall reactor enzyme activity relative to isothermal operation at either the higher or lower temperature. It is postulated that mass transfer rates within the hydrogel beads are greatly enhanced by the movement of water in and out of the beads during the expansion or collapse of the polymer chain network as temperature is cycled.

Index Entries: β-Galactosidase; enzyme immobilization; poly-*N*-isopropylacrylamide; lower critical solution temperature; thermally reversible hydrogels; inverse suspension polymerization; temperature cycling in a packed bed reactor.

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INTRODUCTION

Enzymes are often immobilized on or within solid supports in order to stabilize them and lengthen their useful lifetimes (*1–5*). Many polymeric matrices for immobilized enzymes are hydrophilic in order to be compatible with these immobilized species (*5,6*). Such polymeric matrices normally do not exhibit any significant changes in their physical or chemical properties when subjected to the mild variations in environmental factors, such as temperature, pH, or ionic strength of an immobilized enzyme process. The main effect of such environmental changes is on the activity and stability of the enzyme immobilized within these matrices. The most serious operational problem in an immobilized enzyme reactor is the increasing mass transfer resistance within the hydrogel matrices (*8*).

Certain hydrophilic polymers, such as poly-*N*-alkylacrylamides show unique thermal reversibility properties, precipitating out of aqueous solution above a critical temperature (called the lower critical solution temperature or LCST), and redissolving below this temperature (*9–11*). When crosslinked, such polymers form hydrogels that collapse and deswell above, and then reverse and reswell below their LCST (*12,13*). We have previously immobilized asparaginase, an enzyme used in leukemia treatment, in polyNIPAAm/AAm copolymer LCST hydrogels (*14,15*). We found that the enzyme activity was shut off above the LCST, but was regained when the temperature was lowered below the LCST. This effect was reversible. This "on/off" characteristic of such enzyme-hydrogels is caused by changes in the pore structure within the hydrogel as temperature is raised or lowered. Pore sizes and their interconnections and the relative fraction of water that is "free" water in the gel will all change significantly as the gel is shrunk or swelled and so can significantly affect the diffusion rates of substrate in and product out of the gel (*8,16*). Figure 1 shows this schematically. Thus, in this study we have immobilized β-galactosidase in a poly(NIPAAm) copolymer gel in order to test the hypothesis that cyclic swelling and deswelling would enhance overall enzyme activity. Such continuous "breathing" of the immobilized enzyme bead could also reduce the possibility of product inhibition of the enzyme. We selected β-galactosidase as a model enzyme both for its easy assay and also for its industrial importance (*17,18*). We selected a polyNIPAAm/AAm copolymer gel composition having an LCST ca. 37–40°C (*14*). The effect of temperature cycling on the activity of β-galactosidase immobilized within this gel was then studied in a continuous, single pass, packed bed reactor.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (Eastman Kodak) was recrystallized with hexane. Acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulfate,

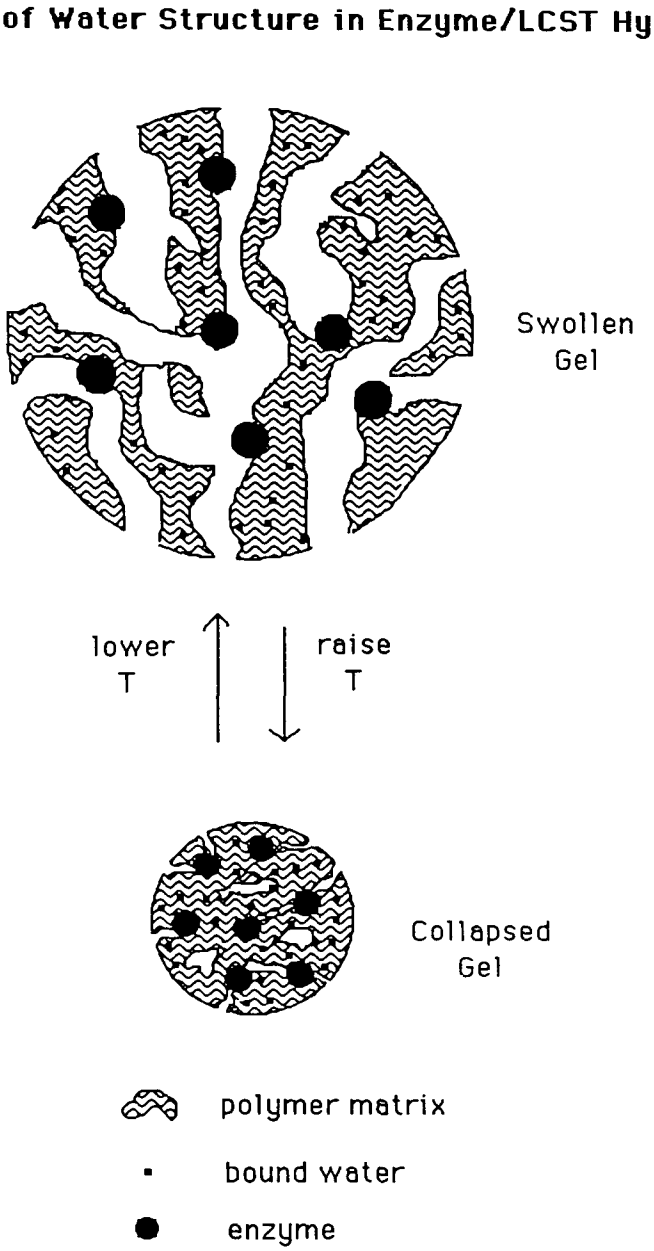

Fig. 1. Schematic diagram of the water and pore structure in a swollen and collapsed thermally reversible (LCST) hydrogel containing an immobilized enzyme.

N,N,N'N'-tetramethylenediamine, β-galactosidase (from *E.coli*), O-nitrophenol β-D-galactopyranoside, and mercaptoethanol were obtained from Sigma. Pluric surfactant L-81 was from BASF Wyandotte Corp. and paraffin oil (Saybolt viscosity, 180–190) was from American Laboratories. All other chemicals were reagent grade.

Preparation of the Hydrogel Beads Containing Entrapped Enzyme

β-Galactosidase was entrapped during an inverse suspension polymerization of the hydrogel using paraffin oil as the continuous phase and Pluronic L-81 as the surfactant. 7.18 g of NIPAAm and 0.5 g of AAm (10% mole ratio to NIPAAm monomer) with 0.32 g of N,N'-methylene-bis-acrylamide (4% weight ratio to total monomer) as a crosslinker were added into 38 mL of 0.1 *M* sodium phosphate buffer, pH 7.4 and dissolved. Nitrogen was bubbled to remove the dissolved oxygen. 50 mg of ammonium persulfate and 1,000 U of β-galactosidase (1.8 mg of protein) were dissolved in 2 mL of buffer and added to the monomer solution. The enzyme–monomer solution was immediately poured into 400 mL of paraffin oil containing 1 mL of Pluronic L-81, which was previously purged with nitrogen to remove residual oxygen. After confirming that aqueous droplets had formed in the oil phase, 0.5 mL of *N,N,N',N'*-tetramethylenediamine (TEMED) was injected into the continuous organic phase to initiate the polymerization. Agitation speed was 500 rpm in a 500 mL resin kettle that was immersed in an icewater bath. Nitrogen was supplied continuously above the surface of the paraffin oil phase to avoid disturbing the beads with nitrogen bubbles. The polymerization was performed at low temperature to dissipate the heat of polymerization and also to avoid any temperature rise above the LCST. After 1 h, the mixture was transferred to a 1 L separatory funnel and excess 0.1 *M* sodium phosphate buffer, pH 7.4 was added to separate out the immobilized enzyme beads, which had a density greater than water and which settled into the aqueous phase. The immobilized enzyme beads obtained by this phase separation were washed several times with 0.1 *M* phosphate buffer, freeze-dried and stored in the freezer (−20°C). The sizes of the immobilized enzyme beads ranged from 200–400 μm in the swollen state.

Assay of the Immobilized Enzyme Activity

1.15 m*M* of O-nitrophenol β-D-galactopyranoside (ONPG) containing two activators for enzyme, 0.01 *M* β-mercaptoethanol, and 0.01 *M* magnesium chloride (6 H_2O) was used as a substrate. Absorbance at 410 nm was used to calculate the degree of conversion (exit product concentration/initial substrate concentration) and activity. The extinction coefficient of hydrolyzed ONPG was 3.5 mmolar at pH 7.4 in sodium phosphate buffer.

Preparation and Operation of the Packed Bed Enzyme Reactor

Two grams of the freeze-dried beads containing 2.0 g of immobilized β-galactosidase were swollen in 0.1 *M* sodium phosphate buffer, pH 7.4 at 4°C overnight and then degassed. The swollen immobilized enzyme beads were packed in a water-jacked glass column (1.27 cm internal diameter and 20 cm length). Flow rate was controlled with a peristaltic pump. Eluant was collected in test tubes with a fraction collector (LKB) in 2 min intervals. Buffer was used to dilute the high concentration of product in the eluant for a more accurate absorbance value. Conversion and activity were calculated based on the absorbance at 410 nm for each fraction.

Temperature cycling was carried out using our own program on an Apple II computer interfaced with a heater and a solenoid valve for cooling tap water. The operation was either isothermal at 30 or 35°C or cycled every 10 min at 1°C/min between 30 and 35°C. A schematic diagram of the packed bed reactor system is shown in Fig. 2.

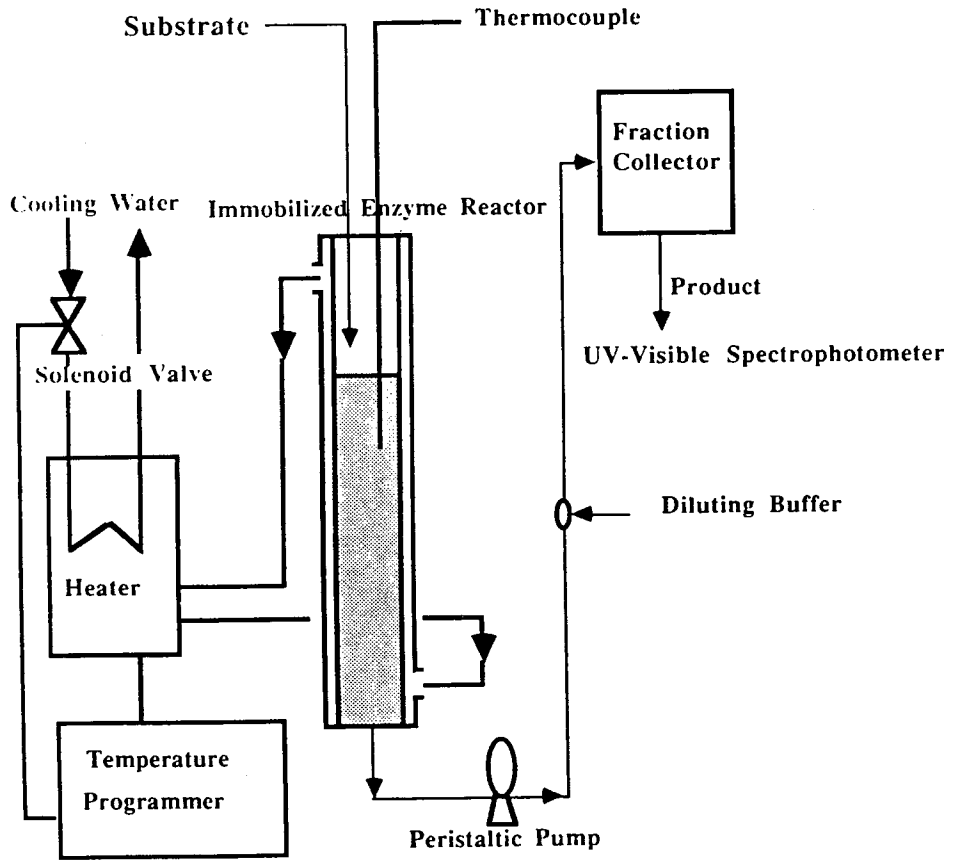

Fig. 2. Schematic diagram of the packed bed reactor system used in this study.

RESULTS AND DISCUSSION

Conversion in the immobilized β-galactosidase bead reactor system is compared for thermal cycling between 30 and 35°C vs isothermal operations at 30 and 35°C in Fig. 3. It can be seen that the conversion cycles with the temperature cycling, and is at all times higher than isothermal operation at either 30 or 35°C. Conversion appears to maximize at 35°C and minimize at 30°C during the thermal cycling operation. During the heating process, the deswelling of the hydrogel squeezes out the product and any remaining substrate from inside the hydrogel matrix leading to the measured increase in conversion, while in the cooling process the swelling and expansion of the hydrogel bead draws in the substrate along with any product in the surrounding fluid. Thus, the conversions shown in Fig. 3 are not the true conversions, but "apparent" conversions that do not take into account the product that remains inside the gel bead matrices.

Although the maximum activity peaks appear at 10 min intervals, corresponding to the temperature cycle of 10 min, two split peaks are

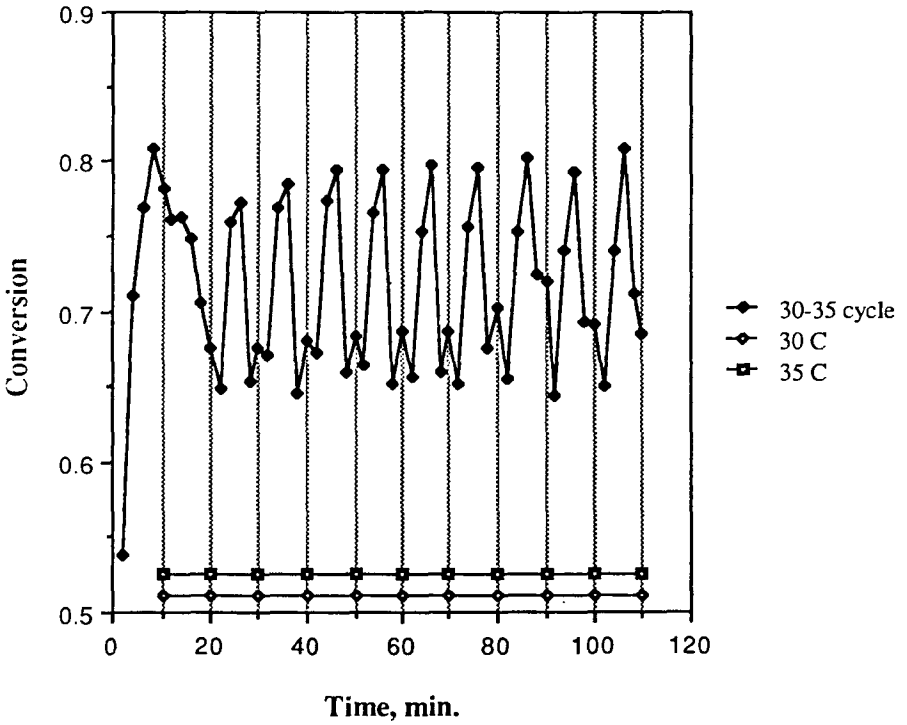

Fig. 3. Conversion as a function of time in the packed bed reactor operated isothermally at 30 and 35°C or cycled between 30 and 35°C. Conversion is the ratio of outlet product (molar) concentration to inlet substrate (molar) concentration.

sometimes observed around the minimum. This may be owing to a "burst effect" in the LCST hydrogel beads. As the gel begins to reheat after reaching 30°C, a collapsing, densified polymer skin will form around the surface of the beads. This may increase the hydrostatic pressure within the bead, and if this collapsing skin is weak in places then the aqueous pore fluid within the gel may be released in bursts, since fissures form in this skin. Eventually this densified polymer skin will coalesce, and the rate of release of product will be slowed down, causing the subsequent drop in conversion seen shortly thereafter. We have seen similar burst effects followed by slow release of vitamin B12 and methylene blue from such LCST gels (*13,19*). It appears that this burst effect may disappear as the number of temperature cycles increase, possibly because of the relaxation of the crosslinked structure of the hydrogel permitting a more uniform skin to form upon heating of the gel.

The activity of the hydrogel immobilized enzyme is no doubt influenced by both kinetic and diffusional factors. All of these factors together comprise the "microenvironment" of the enzyme. Our data suggest that below 30°C, in the swollen state, activity is controlled by enzyme kinetics, and above 35°C, in the deswollen state, it is controlled by diffusion. In the intermediate region between 30 and 35°C, there is a maximum in activity owing to the optimum combination of these opposing effects. The increase in conversion during the thermal cycling may also be related to reduced product inhibition of the enzyme, since the average local product concentration in the vicinity of the enzyme should be lower than in the case of isothermal operation.

The LCST of the gel we have used is 37 to 40°C. Thus, at 30°C the gel is significantly more swollen than at 35°C, and because of the greater volume and lower tortuosity of the pore structure, substrate and product diffusion rates in and out of the gel should both be higher at 30 than at 35°C. On the other hand, the intrinsic diffusivities of these species will be higher at 35°C. Furthermore, the intrinsic enzyme turnover rate should be higher at 35 than at 30°C. The enzyme is also at a higher effective concentration in the gel at 35°C owing to the reduced volume of the deswollen gel. These various opposing factors tend almost to balance, with the increase in reaction kinetics at isothermal operation at 35°C being more important than the decrease in diffusion rates, and this leads to the somewhat higher conversion at 35°C relative to 30°C, for isothermal operation (Fig. 3). If the immobilized enzyme gel were isothermally operated closer to the LCST (37–40°C), then the overall conversion would be expected to drop sharply relative to 30°C or even 35°C, because of the greater collapse of the gel. The thermal cycling operation, on the other hand, takes advantage of both the enhanced enzyme kinetics at 35°C, and then enhanced mass transfer rates occurring during the cyclic swelling and deswelling. We have also derived the activity and productivity of the packed bead reactor from the conversion data, and one can see that all

of those performance characteristics are higher for the thermal cycling operation (Table 1).

It should be noted that the pore size cutoff of the particular gel used was determined in preliminary experiments to be much below the molecular weight of the β-galactosidase (590,000). Thus, it is unlikely that any enzyme could leach out of the gel. On the other hand, the average pore size was well above the molecular weights of the substrate and product being used in this experiment. It is also clear that the shrinking and swelling of the gel do not harm the enzyme, since the enzyme activity appears to be completely reversible with each cycle for the 2 h period studied.

In conclusion, we have been able to demonstrate that the average conversion, the activity, and the productivity, are all increased by thermally cycling an immobilized catalyst such as an enzyme within an LCST hydrogel. In order to maximize the activity of such an immobilized enzyme in an LCST hydrogel, the heating and cooling rates and the temperature cycling range, should be matched with the particular enzyme and hydrogel composition. Ideally, the temperature cycling should be carried out as rapidly as possible (as long as the polymer chains are able to respond to the rate of change), and the temperature range should be selected to optimize the opposing factors of enzyme kinetics and diffusion. The LCST hydrogel may be molecularly engineered by selecting and polymerizing a wide range of copolymer compositions, or by utilizing other existing polymers or modified polymers that exhibit this behavior. The crosslinker concentration in the copolymerization is also an important variable, since it can control the pore strcture of the hydrogel. Acidic or basic monomers may also be incorporated to provide pH sensitivity in addition to the thermal reversibility of the immobilized enzyme-gel system.

We consider such "catalytic hydrogels" to have wide potential application not only in immobilized bioreactors, but also in industrial catalytic reactors, where one desires to avoid catalyst poisons which may be excluded from the gel matrix (*20*). Reactants (or drugs) may also be "delivered" to a reaction environment from such gels by varying temperature in periodic, cyclic pulses (*20,21*).

Table 1
Comparison of Reactor Performance for Isothermal vs Temperature Cycling Operation for a Packed Bed with Reactor Immobilized β-galactosidase in a Thermally Reversible Hydrogel[a]

	Conversion	Activity	Productivity
30°C Isothermal	0.511	373.1	36.24
35°C Isothermal	0.526	441.6	50.47
30–35°C Temp. cycling	0.716 (avg.)	522.9	54.90

[a]Conversion is the ratio of outlet product (molar) concentration to inlet substrate (molar) concentration. Activity is defined as μmole product/min-g dry immobilized enzyme and productivity is μmole product/min-g dry immobilized enzyme-ml of reactor volume.

ACKNOWLEDGMENTS

The authors would like to acknowledge the generous support of the Johnson & Johnson Company and the Washington Technology Center at the University of Washington. We also wish to thank James Nightingale for his assistance in designing the temperature cycling program.

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