Immobilization of α-Chymotrypsin in Thermally Reversible Isopropylacrylamide-Hydroxyethylmethacrylate Copolymer Gel

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ABSTRACT: In this study, α-chymotrypsin was immobilized via physical entrapment within thermally reversible poly(isopropylacrylamide-*co*-hydroxyethylmethacrylate), poly(NIPAM-*co*-HEMA) copolymer gels. The thermoresponsive gel matrices in cylindrical geometry were prepared in an aqueous buffer medium by a redox polymerization method. The kinetic behavior of enzyme–gel cylinders was investigated in a batch reactor. The results indicated that the overall reaction rate was controlled by the substrate diffusion through the gel matrix. Due to the thermoresponsive character of the carrier gel, the maximum activity was achieved at 30°C with the enzyme–gel system while the free enzyme exhibited maximum activity at 40°C. © 1998 John Wiley & Sons, Inc. J Polym Sci A: Polym Chem **36:** 543–552, 1998

Keywords: isopropylacrylamide; hydroxyethylmethacrylate; chymotrypsin; thermoresponsive gels; enzyme immobilization

INTRODUCTION

In recent years, thermoresponsive behavior of poly(isopropylacrylamide), poly(NIPAM) has been extensively investigated by different researchers.[1–12] The kinetics of discontinuous volume phase transition was described by Matsuo and Tanaka.[13] Hirotsu determined the critical points of the volume phase transition in poly(NIPAM) gels.[14] The thermodynamics of the phase transition was also investigated by Hirotsu et al.[15]

On the other hand, poly(NIPAM)-based carriers have been proposed as a new alternative for the immobilization of enzymes and cells. Hoffman et al. achieved the immobilization of asparaginase and β-galactosidase within poly(isopropyl acrylamide)-based carriers.[16,17] The same group also studied cell immobilization within poly(isopropyl acrylamide) matrices for steroid conversion.[18] A saccharide sensitive phase transition was observed with a covalently crosslinked polymer network of *N*-isopropylacrylamide in which a lectin, concanavalin-A, was immobilized.[19] Recently, soluble and temperature-sensitive polymer–enzyme conjugates were prepared by the copolymerization of NIPAM with the reactive comonomers having succinimide functional groups.[20,21] Beta-D-glucosidase and alkaline phosphatase were covalently immobilized onto the responsive carrier via the ester functional end group. The carboxylated form of these conjugates was obtained by the same research group.[22] These conjugates flocculate and precipitate in the aqueous medium above the LCST and redissolve when cooled below that temperature.[20–22] Temperature responsive semitelechelic co-oligomers of NIPAM with *N,N*-dimethylacrylamide (DMAAm) or butylmethacrylate were synthesized in the carboxylated form using radical telomerization.[23] A thermoresponsive polymer–enzyme conjugate of lipase was obtained by covalent coupling via carboxyl end groups.[24,25] The produced conjugate exhibited its native enzyme ac-

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tivity below LCST, above which it precipitated and its catalytic function was shut off.[25]

In our recent studies, we produced thermally reversible poly(isopropyl acrylamide-*co*-hydroxyethylmethacrylate), poly(NIPAM-*co*-HEMA) copolymer gels. The synthesis and the volume phase transition behavior of these gels were studied previously.[26] In this study, poly(NIPAM-*co*-HEMA) gel was proposed as an alternative carrier for the physical immobilization of enzymes due to proper thermoresponsive and mechanical properties. α-Chymotrypsin was immobilized via physical entrapment within cylindrical gel matrices made of poly(NIPAM-*co*-HEMA). The kinetic behavior of enzyme–gel cylinders, the effects of reaction conditions on the activity and the stability of immobilized enzyme were investigated in batch fashion.

EXPERIMENTAL

Materials

N-Isopropylacrylamide (NIPAM, Aldrich Chem. Corp., Milwaukee, WI) was recrystallized from hexane–acetone solution. 2-Hydroxyethylmethacrylate (HEMA, Sigma Chemical Co., St. Louis, MO) was distilled under vacuum to remove the inhibitor. *N,N,N,N*-Methylenebisacrylamide (MBAM, BDH Chemicals Ltd., UK) was used as a crosslinker without further purification. In the preparation of gel matrix, potassium persulfate (KPS, BDH Chemicals Ltd.) and tetramethylethylenediamine (TEMED, Sigma Chemical Co.) were used as the initiator and the accelerator, respectively. Polyethylene glycol (PEG 4000, M_r: 4000, BDH Chemicals Ltd.) was used as a diluent. α-Chymotrypsin (CT, 52 μmol BTEE/mg protein-min, Sigma Chemical Co.) was immobilized without further purification. A synthetic substrate, Benzoyl-L-Tyrosine Ethyl Ester (BTEE, Sigma Chemical Co.) was utilized in the received form to measure free and immobilized α-chymotrypsin activities. All the enzyme, initiator and accelerator solutions were prepared in borate buffer, having a pH of 7.8.

Preparation of Enzyme–Gel Cylinders

The enzyme–gel matrices in the cylindrical form were prepared by a redox polymerization procedure. In this preparation, the NIPAM/HEMA mol ratio was fixed at 73.6/26.4. A typical synthesis procedure is exemplified below. NIPAM (100 mg), 0.04 mL HEMA, and 3.3 mg MBAM were dissolved within 0.7 mL of borate buffer (pH 7.8). PEG 4000 (100 mg) was then dissolved within the resulting solution. Initiator solution ((0.1 mL) including 50 mg KPS/mL and 0.4 mL of enzyme solution including 120 μg of α-chymotrypsin were added. The glass tube with the polymerization medium was placed in a thermostatic water bath (Fryka, Kalthetechnic, Germany) and kept at +4°C for 30 min for thermal equilibrium. Accelerator solution (0.1 mL) with 10% (v/v) TEMED was injected into the polymerization medium at +4°C. Then the polymerization mixture was equally divided into three glass tubes (3.2 mm in internal diameter and 85 mm in length) and the tubes were placed in a suitable sealed container. The container was purged with nitrogen for 10 min and the gelation was conducted at +4°C for 24 h in nitrogen atmosphere. At the end of this period, thermally reversible poly(NIPAM-*co*-HEMA) cylindrical gels (3.2 mm in diameter and 60 mm in length) including immobilized α-chymotrypsin were removed from the glass tubes by applying pressure and cutting into cylinders 20 mm in length. The cylindrical gel rods were washed extensively with cold borate buffer. The washed gels were collapsed in borate buffer medium at 35°C for about 2 h. Then the enzyme activity in the washing solution was checked. The enzyme–gel matrices were stored within borate buffer at +4°C until use. Two different enzyme loadings were used for the preparation of α-chymotrypsin containing poly(NIPAM-*co*-HEMA) gel matrices. Production conditions of gel matrices were defined in Table I.

Characterization of Enzyme–Gel Cylinders

The responsive behavior of copolymer gel matrix prepared with 73.6/26.4 NIPAM/HEMA mol ratio was investigated in our previous study.[26] The variation of equilibrium swelling degree of poly(NIPAM-*co*-HEMA) gel with the temperature was determined in an aqueous buffer medium (pH 7.0), by using a gravimetric procedure described elsewhere.[26] The equilibrium swelling ratio was defined as the weight ratio of water contained within the gel to dry polymer. The variation of equilibrium linear swelling ratio of the poly(NIPAM-*co*-HEMA) gel with the temperature was also determined in the same medium by using an optical system specially constructed for this purpose.[26] The linear equilibrium swelling ratio was defined as the ratio of gel diameter at any

Table I. The Production Conditions of Enzyme-Gel Matrices Used in the Systematical Experiments

Code	NIPAM (mg)	HEMA (mL)	MBAM (mg)	Buffer (mL)	KPS (mL)[a]	TEMED (mL)[b]	PEG (mg)	Enzyme (μg)[c]
EG1	100	0.04	3.3	0.7	0.1	0.1	100	120
EG2	100	0.04	3.3	0.7	0.1	0.1	100	600

[a] KPS concentration was 50 mg/ml.
[b] TEMED concentration was 10% by volume.
[c] added into the gelation medium within 0.4 ml of borate buffer.

temperature to the gel diameter at the production conditions. The gels in the form of long cylinders (3.2 mm in diameter and 60 mm in length in the production conditions) were used in these experiments.

The ethanol content of aqueous hydrolysis medium was directly effective on the solubility of BTEE, the hydrolysis rate, and the activity of free α-chymotrypsin. For this reason, the equilibrium swelling ratios of poly(NIPAM-*co*-HEMA) gel cylinders prepared with 73.6/26.4 NIPAM/HEMA mol ratio, were determined in ethanol–water solutions having different ethanol contents. The ethanol concentration of the aqueous medium was changed between 0–100% (by volume). The equilibrium swelling ratio measurements were performed at three constant temperatures (i.e., 15, 25, and 36°C).

Enzymatic Activity Experiments

Activity experiments were performed in the cylindrical batch reactors placed in a shaking water bath equipped with a temperature control system. Borate buffer–ethanol solution (20 mL), having a pH of 7.8, was utilized as the aqueous reaction medium. Nine cylindrical gel rods (3.2 mm in diameter and 20 mm in length in the production conditions) were used in each run and the same gel rods were used in all batch runs. The total gel volume was about 1.5 mL (i.e., in the production conditions of gels) and this amount was kept constant in all runs. The initial substrate concentration, the ethanol concentration and the temperature were changed either in the presence of free enzyme or the enzyme–gel matrices by fixing the shaking rate to 250 cpm. After each activity experiment, the gel rods were washed with 200 mL fresh borate buffer (pH 7.8) by shaking the medium at 25°C for 1 h to remove any residual substrate or product within the gel matrix. The gel rods were stored in the refrigerator at +4°C within 100 mL of fresh borate buffer during the period between the activity experiments. One batch run with the enzyme–gel matrices was performed per day and each run was continued for 4 h.

Determination of Enzyme Activity

The activity of free or immobilized enzyme was determined by monitoring the BTEE conversion by using a spectrophotometric method proposed by Bailey et al.[27] The apparent BTEE hydrolysis rates were calculated based on the following equation[28]:

$$R = S_o[(dA/dt)/(A_f - A_o)] \qquad (1)$$

where R is the reaction rate, S_o is the initial BTEE concentration, A_o and A_f are the absorbances of the medium at 258 nm, before the addition of enzyme (i.e., zero reaction time) and at the complete conversion of substrate, respectively. dA/dt is the derivative of the absorbance with respect to time during the initial course of reaction. This value was determined as the slope in the equation of the straight line obtained by applying a least square algorithm on the absorbance–time data.

RESULTS AND DISCUSSION

Definition of the Carrier Matrix

In this study, poly(NIPAM-*co*-HEMA) gels were selected as the carrier matrix for physical immobilization of α-chymotrypsin. Poly(NIPAM) is one of the well-known thermoresponsive carriers used for immobilization studies for enzymes and cells.[16–25] Thermal cycling is usually applied with the these gels for either "on-off" control of the immobilized agent activity or to enchance the immobilized system performance.[16,18] In this

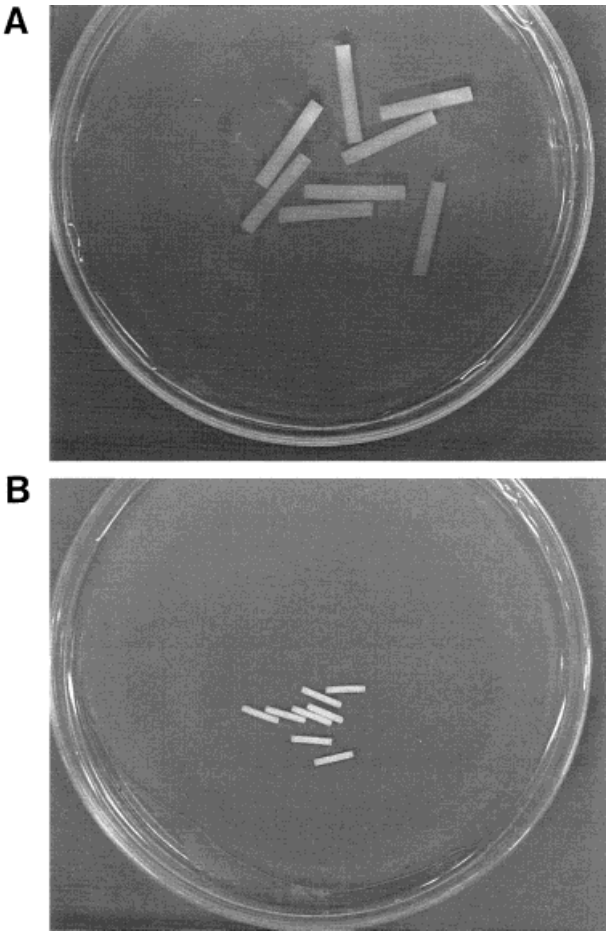

Figure 1. The photographs of poly(NIPAM-*co*-HEMA) gel cylinders at (A) +4°C and (B) 37°C. The magnification is the same for both photographs.

method, reswelling and deswelling of the gel matrix were induced by cycling of the medium temperature. The conventional poly(NIPAM) gels produced in the block form may exhibit some problems in these applications. One problem is the coexistence of swollen and shrunken phases in a shrunken cylindrical poly(NIPAM) gel in an alcohol–water solution.[14] The formation of water pockets within the gel structure depending upon the skin formation on the gel surface especially above the LCST may be considered as another phenomenon affecting on the homogeneity of the gel structure.[18] On the other hand, bending of long cylinders and formation of cracks on the gel surface during the shrinking process are the other mechanical problems that may appear with the conventional poly(NIPAM) gels.

In this study, the copolymerization of NIPAM with HEMA in the presence of PEG 4000 as a diluent caused some improvements in the mechanical properties. The coexistence of shrunken and swollen phases and the formation of water pockets during the shrinking period were not observed with the NIPAM–HEMA copolymer gels. Due to the controlled thermosensitivity of NIPAM–HEMA gel, the mechanical problems (i.e., crack formation or disintegrations in the gel structure) did not appear with the prepared enzyme–gel matrices during the immobilization experiments. α-Chymotrypsin containing poly(NIPAM-*co*-HEMA) gel matrices including 73.6% NIPAM and 26.4% HEMA (i.e., mol %) were produced by a redox copolymerization method.[26] Photographs of poly(NIPAM-*co*-HEMA) gel cylinders at +4 and 37°C are shown in Figure 1. As seen here, there was a significant difference in the gel volume due to the thermoresponsivity of the carrier matrix. It should be noted that the shrinking was achieved without forming any deformation in the cylindrical geometry and by keeping the homogeneity of gel structure [Fig. 1(B)].

Determination of Enzyme Loading

In the selection of lowest enzyme loading to the gels, a set of activity runs were performed in a batch reactor with a series of α-chymotrypsin immobilized poly(NIPAM-*co*-HEMA) gels prepared by changing the enzyme loading between 15–600 μg. The prepared enzyme–gel matrices having 1.5 mL of total volume in the production conditions were tested within 20 mL of reaction volume at 25°C with the initial subtrate concentration of 240 μM. The results are given in Table II. As seen

Table II. The Activities of α-Chymotrypsin Immobilized Poly(NIPAM-*co*-HEMA) Gels Prepared with Different Enzyme Loadings

Enzyme Type	Enzyme Loading (μg)[a]	Activity (μM BTEE/min)[b]
Free	5[c]	8.6
Immobilized[d]	15	0.26
Immobilized[d]	30	0.84
Immobilized	120	1.40
Immobilized	600	2.37

[a] The amount of enzyme loaded into 1.5 mL of gel volume in the production conditions.
[b] Activity test conditions; 240 μM BTEE, pH: 7.8, 25°C, 1.5 mL of total gel volume within 20 mL of reaction volume.
[c] The amount of free enzyme used within 20 mL of reaction volume.
[d] Gel preparation conditions are the same with Table I except the amount of enzyme loaded into the gel was different.

here, 15 and 30 μg of enzyme loadings provided reasonably lower activities relative to that achieved by using 5 μg of free enzyme in the same reaction volume. These activities were far from adequate because the reaction period for complete conversion of substrate was too long. On the other hand, more appreciable hydrolysis rates were achieved with the enzyme–gel matrices prepared with 120 and 600 μg enzyme loadings. The gels with these enzyme loadings also provided reproducible, stable, and accurately measurable activity values with the repeated runs. Therefore, although the amount of enzyme used in the immobilization was reasonably higher relative to the amount used in the free case, the gel matrices prepared with 120 and 600 μg enzyme loadings were preferred in the systematical batch runs to determine the effects of process conditions on the hydrolysis rate of BTEE.

To determine the enzyme leakage from the selected gels, the prepared gel matrices were first washed +4°C for 24 h within 50 mL of borate buffer by stirring the medium at 250 rpm. Then they were transferred into another 50 mL of fresh borate buffer and collapsed in this medium at 35°C for 2 h. The total enzymatic activity in the washing solutions were determined separately by using 240 μM of initial BTEE concentration; 3.8 and 1.6% of initially loaded enzymatic activities were found in the washing solutions for EG1 and EG2 gel matrices, respectively. A significant enzyme leakage (i.e., >0.1% of initially loaded activity into the gel) from the prepared gel matrices was not observed in the repeated washings (i.e., equilibrium swelling at +4°C and shrinking at 35°C for 2 h). These results indicated that an appreciable leakage was only detected in the first washing and that enzyme leakage was not a problem during the succesive use of enzyme–gel matrices.

Effect of Initial Substrate Concentration

The effect of initial substrate concentration on the hydrolysis rate was investigated for both free α-chymotrypsin and α-chymotrypsin immobilized poly(NIPAM-*co*-HEMA) gel cylinders. The batch reactor experiments with free enzyme were performed by changing the initial concentration of BTEE between 15–400 μM at 25°C in 20 mL of reaction volume having a pH of 7.8. An aqueous solution including 95% borate buffer and 5% ethanol (by volume) was used as the hydrolysis medium. For accurate determination of kinetic parameters of free enzyme, the enzyme concentration was fixed to 0.25 μg/mL by using 5 μg enzyme within 20 mL of reaction volume. The plateau region was obtained after 160 μM of initial BTEE concentration in the graph indicating the variation of BTEE hydrolysis rate with the initial BTEE concentration. The Lineweaver–Burk plot of the free α-chymotrypsin–BTEE system is given in Figure 2. By applying the least-square estimation on the Lineweaver–Burk plot, K_m and r_m values were estimated as 63 μM and 11.0 μM BTEE/min, respectively. The estimated value of r_m was also expressed as 44.0 μmol BTEE/mg CT-min based on the amount of free enzyme.

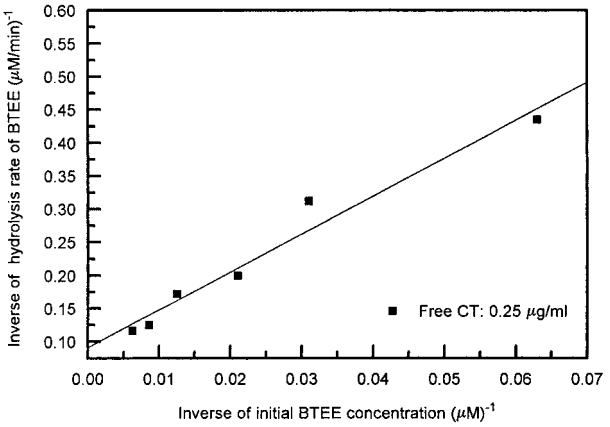

Figure 2. The Lineweaver–Burk plot of the free α-chymotrypsin–BTEE system.

To investigate the variation of activity of the enzyme–gel system with the initial substrate concentration, the initial concentration of BTEE was changed between 160–880 μM. The gel matrices prepared with 120 and 600 μg of enzyme loadings (i.e., coded as EG1 and EG2) and having 1.5 mL of gel volume were used in these experiments. The other hydrolysis conditions were the same with the those used in the free enzyme experiments. It should be noted that the maximum value of initial substrate concentration was limited by the solubility of BTEE in the reaction medium. The precipitation of BTEE in the hydrolysis medium (95% borate buffer and 5% ethanol) was observed with 1200 μM of BTEE concentration. Therefore, a sufficiently lower value than the saturation concentration of BTEE was selected as the maximum value of initial substrate concentration (i.e., roughly 900 μM) to prevent the precipitation of BTEE. The variation of BTEE hydrolysis rate (r, μM BTEE/min) with the initial BTEE concentration in the presence of enzyme–gel matrices pre-

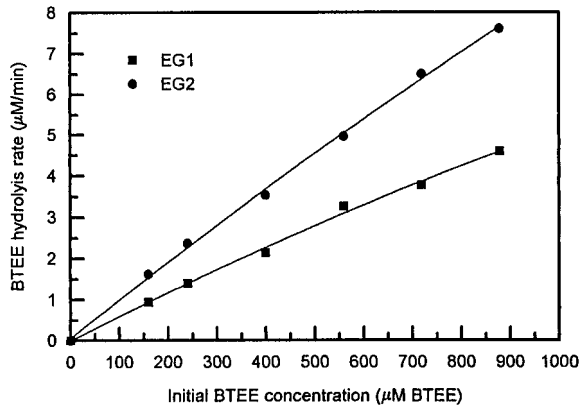

Figure 3. The variation of the BTEE hydrolysis rate with the initial BTEE concentration in the presence of enzyme–gel matrices prepared with different enzyme loadings.

pared with different enzyme loadings is given in Figure 3. As seen here, slightly higher BTEE hydrolysis rates could be obtained with the enzyme–gel matrix prepared with reasonably higher enzyme loading. To explain this behavior, BTEE hydrolysis rate was defined based on the mass of enzyme loaded to the gel matrix (r', μmol BTEE/mg CT-min). The variation of r' with the initial BTEE concentration is presented in Figure 4. An opposite tendency relative to Figure 3 was obtained here and the activity based on the mass of loaded enzyme decreased with the increasing enzyme loading. This behavior may be explained by the dominant effect of substrate diffusion on the overall hydrolysis rate. In the case of higher enzyme loading, more substrate is consumed in the closer parts to the outer surface of the cylindrical matrix because the number of available enzyme molecules increased. Therefore, the substrate concentration decreases more sharply with the decreasing radial distance in the gel matrix. This provides a higher concentration gradient value at the surface of cylindrical gel involving an increase in the substrate diffusion rate (i.e., apparent hydrolysis rate given by μM BTEE/min) according to the Fick's first law. The tendency in Figure 3 could be explained by this conclusion. On the other hand, due to the dominant effect of substrate diffusion, the increase occured in the apparent hydrolysis rate was not proportional to the enzyme content of the gel matrix. For this reason the hydrolysis rate defined based on loaded enzyme (i.e., r': μmol BTEE/mg CT-min) decreased with the increasing enzyme loading (i.e., Fig. 4).

Effect of Ethanol/Water Ratio

One of the important parameters controlling the BTEE hydrolysis rate is the ethanol/water ratio of the reaction medium. The water content of hydrolysis medium is an effective factor on the activity of α-chymotrypsin.[29,30] On the other hand, ethanol should be included in the hydrolysis medium due to the limited solubility of BTEE in water and the solubility of BTEE within the reaction medium increases with the increasing ethanol content. To test the effect of ethanol concentration on the activity of free enzyme, ethanol concentration was changed between 5–50% (by volume) by fixing the initial BTEE concentration to 240 μM. The batch runs were performed at 25°C, within 20 mL of reaction volume at a constant pH of 7.8. The free enzyme concentration was 0.25 μg/mL. The variation of BTEE hydrolysis rate with the volume fraction of ethanol is given in Figure 5. As seen here, BTEE consumption rate significantly decreased with the increasing ethanol concentration in the hydrolysis medium. Similar results were also reported in the literature for the ester synthesis with α-chymotrypsin from *N*-acetyl-L-tyrosine and ethanol, and the increase in the reaction rate was explained by the increase in the amount of hydrated enzyme.[29,30]

On the other hand, the carrier (i.e., poly(NIPAM-*co*-HEMA) copolymer) is also responsive against the ethanol concentration in the hydrolysis medium. The variation of equilibrium degree of swelling of poly(NIPAM-*co*-HEMA) copolymer with ethanol concentration in water–

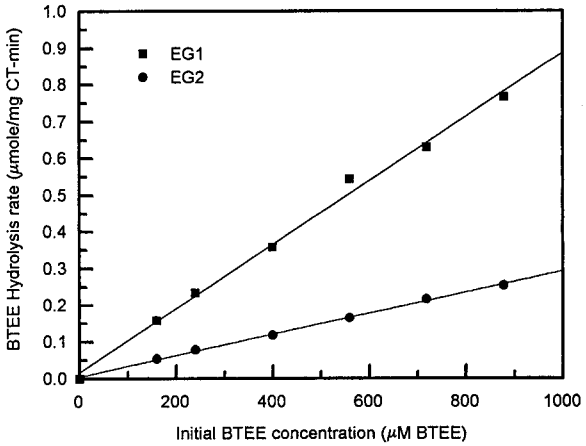

Figure 4. The variation of the BTEE hydrolysis rate based on the loaded enzyme with the initial BTEE concentration in the presence of enzyme–gel matrices prepared with different enzyme loadings.

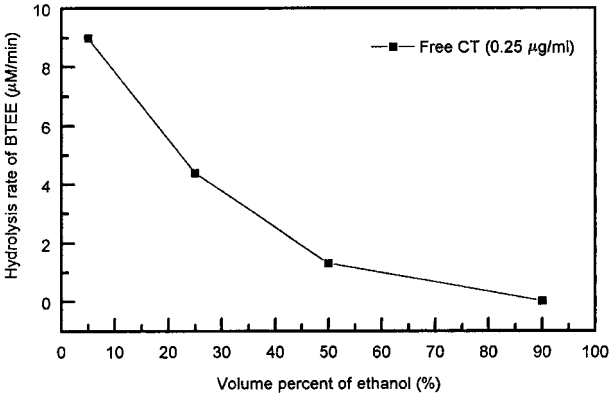

Figure 5. The variation of the BTEE hydrolysis rate with the ethanol concentration in the presence of free enzyme.

ethanol solution is given in Figure 6. It is known that the lower critical solution temperature (LCST) of poly NIPAM gel in water is around 33–34°C.[4,13–15] Here, two of the tried temperatures (i.e., 15 and 25°C) were below the LCST of poly(NIPAM) gel in water. At these temperatures, the copolymer was in the fully shruken state between 30 and 40% of ethanol concentration and in the swollen state both in pure water and ethanol. However, the swelling response of copolymer gel against ethanol concentration was slightly different at 36°C, which was a temperature slightly above the LCST of poly(NIPAM) in water.

To observe the total effect of ethanol/water ratio on the activity of CT immobilized poly(NIPAM-*co*-HEMA) gels, different reaction media having a pH of 7.8 were prepared by changing the ethanol concentration at three levels (i.e., 5, 25, and 50%, by volume). The variation of activity of enzyme–gel matrices with the initial BTEE concentration was investigated in these reaction media. The enzyme–gel matrices prepared with the 120 and 600 μg of CT loading were utilized in these runs performed at 25°C with a 20 mL of batch volume. The variation of BTEE hydrolysis rate with the initial BTEE concentration within the reaction media having different ethanol concentrations is given in Figure 7. As seen here, BTEE hydrolysis rates in the reaction medium having 25% ethanol were lower than those achieved in the medium including 5% of ethanol. This result may be explained as follows: first, the activity of α-chymotrypsin decreased with the increasing ethanol concentration of the reaction medium, as it was shown by the free enzyme experiments (Fig. 5). Second, the gel matrix collapsed when the ethanol concentration increased from 5 to 25% (Fig. 6), which in turn, caused an increase in the internal mass transfer resistance against the BTTE diffusion through the gel matrix. Therefore, the apparent BTEE hydrolysis rate decreased in the presence of 25% of ethanol due to these two reasons. On the other hand, no significant BTEE consumption was detected in the medium having 50% of ethanol, and the BTEE hydrolysis rates in this medium were not included in Figure 7.

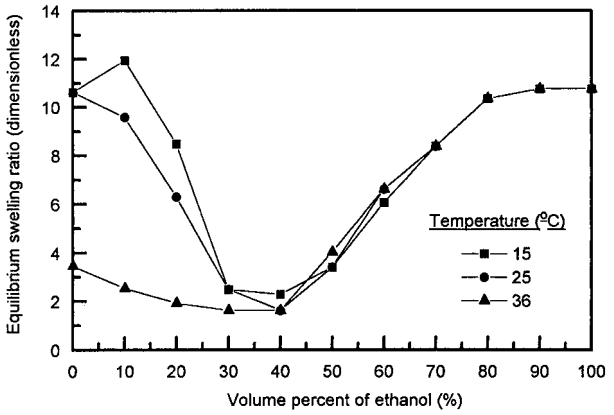

Figure 6. The variation of the equilibrium degree of swelling of poly(NIPAM-*co*-HEMA) gel with the ethanol concentration in water–ethanol solution.

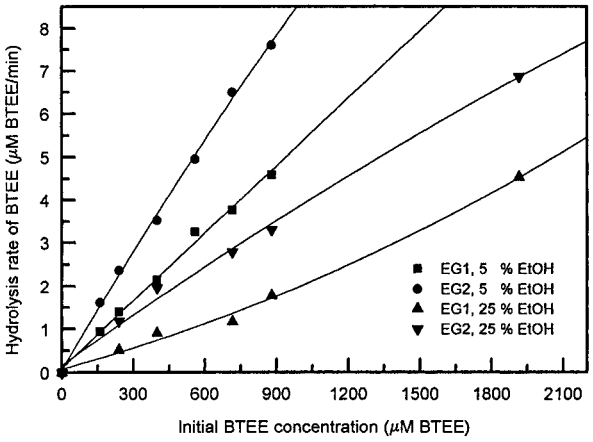

Figure 7. The variation of the BTEE hydrolysis rate with the initial BTEE concentration in the presence of enzyme–gel matrices within the reaction media having different ethanol concentrations.

Effect of Temperature

The effect of temperature on the activity of free enzyme is given in Figure 8. In this group of exper-

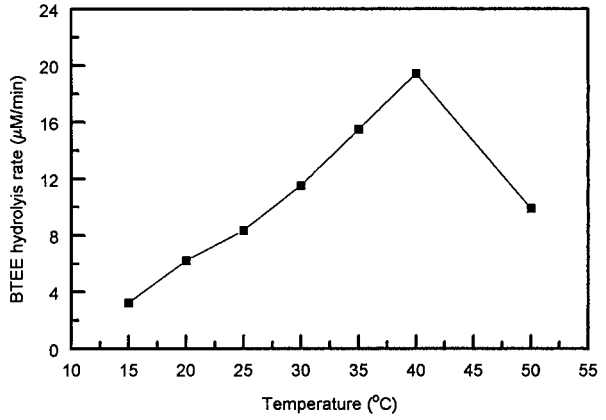

Figure 8. The effect of temperature on the activity of free enzyme.

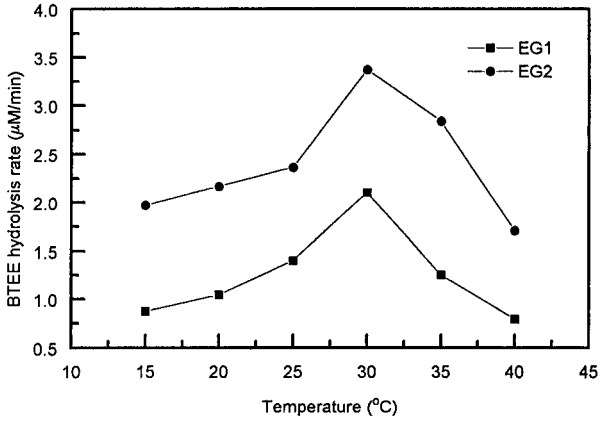

Figure 10. The effect of temperature on the activity of enzyme–gel matrices prepared with different enzyme loadings.

iments, BTEE and enzyme concentrations were fixed to 240 μM and 0.25 μg/mL, respectively. The temperature of the hydrolysis medium (20 mL) was changed between 15–50°C. As seen in Figure 8, the maximum activity was observed at 40°C for free enzyme.

On the other hand, the carrier gel is also responsive to temperature changes. The variation of equilibrium swelling ratio of the poly(NIPAM-*co*-HEMA) gel matrix with the medium temperature is given in Figure 9. Note that the swelling ratio at each temperature was determined both, gravimetrically and by measuring the diameter of cylinders (i.e., linear swelling ratio). As seen in this figure, the gravimetrically determined swelling ratio decreased from 12 to 1 while the linear one was changing from 1.1 to 0.5 by increasing medium temperature from +4 to 45°C. This behavior was slightly different than the conventional behavior of poly(NIPAM) gels in which the equilibrium linear swelling ratio exhibited a sharp and usually discontinuous decrease at around 33–34°C (i.e., at LCST of the gel).[4,13–15] In the case of the poly(NIPAM-*co*-HEMA) gel, the transition from fully swollen state to the shrunken one occurred continously and the linear swelling ratio decreased linearly by the increasing temperature in the range of 20–35°C. The continuous decrease in the equilibrium linear swelling ratio of copolymer may be attributed to the existence of HEMA (i.e., a nonresponsive comonomer) within the gel matrix.

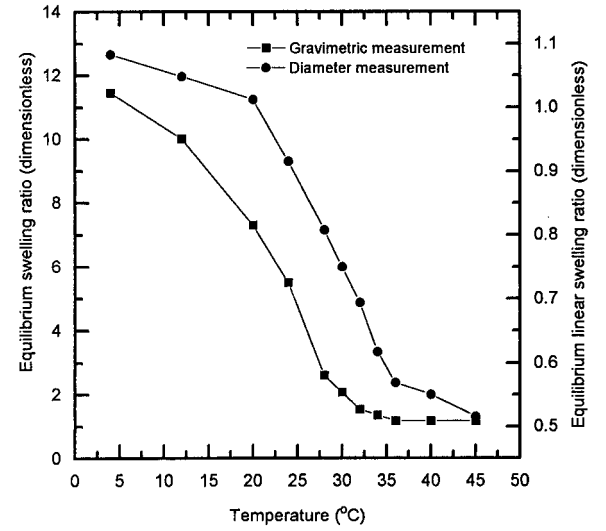

Figure 9. The variation of the equilibrium swelling ratio of the poly(NIPAM-*co*-HEMA) gel matrix with the medium temperature.

After definition of the behaviors of free enzyme and carrier matrix against the temperature, the effect of temperature on the activity of CT immobilized poly(NIPAM-*co*-HEMA) gel matrices was investigated. In this set, the medium temperature was changed between 15–40°C. The initial concentration of BTEE was fixed to 240 μM. The batch runs were performed within 20 mL of 95% borate buffer/5% ethanol medium having a pH of 7.8. The effect of temperature on the activity of enzyme–gel matrices prepared with 120 and 600 μg CT loading is given in Figure 10. As seen in this figure, the maximum activity was observed at 30°C for both of the gels. The activity of enzyme–gel matrices increased with the increasing temperature in the range of 15–30°C and a reasonably sharp decrease in the activity was observed at temperatures higher than 30°C. Before

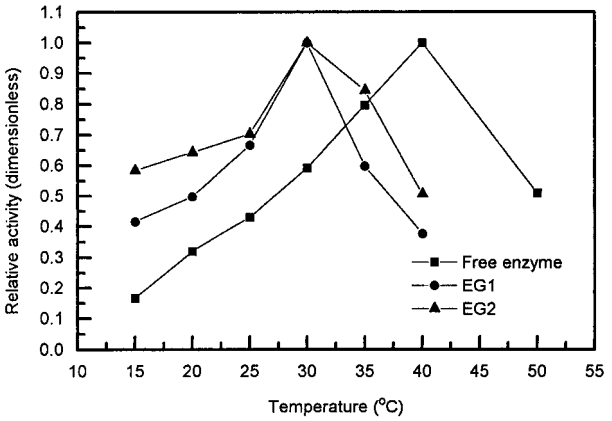

Figure 11. The variation of relative activities of the free enzyme and enzyme–gel matrix with the temperature.

explanation of this tendency, the variation of free enzyme activity with the temperature should be evaluated together with the response of poly(NIPAM-*co*-HEMA) gel against temperature for the definition of enzyme–gel system behavior. At lower temperatures, the gel was in the swollen state and the micropores within the gel were relatively open. Therefore, the effective diffusion coefficient of substrate was higher due to lower internal mass transfer resistance. On the other hand, the available surface area for substrate diffusion was also higher due to the swollen gel volume. These factors involved higher substrate diffusion rate through the gel matrix according to Fick's law, while the activity of immobilized enzyme was lower. The relative activites of free enzyme and the enzyme–gel matrices were plotted against the temperature in Figure 11 to show this effect clearly. The relative activity was defined as the ratio of apparent BTEE hydrolysis rate at any temperature to the maximum hydrolysis rate. At low temperatures, higher relative activities were achieved with the enzyme–gel system than that of free enzyme because the increase in the enzymatic activity by the increasing temperature was partly compensated by the decrease in the diffusion rate of substrate through the gel matrix. As seen in Figure 9, the gel was in the fully shrunken state after 30°C. Therefore, above this temperature, the internal mass transfer resistance for subtrate diffusion reached the maximum value involving a decrease in the effective diffusion coefficient of substrate. On the other hand, the surface area of gel for substrate diffusion also decreased significantly due to the shrinking of gel volume. Both of these factors were possibly more dominant than the increase in the enzymatic activity between 30 and 40°C and then prevented the increase in the apparent reaction rate in this temperature range. Then, the maximum apparent hydrolysis rate with the enzyme–gel system was achieved at 30°C, in contrast to the maximum observed at 40°C with the free enzyme. Note that a similar explanation was also proposed for the thermoresponsivity of β-galactosidase immobilized and thermally reversible poly(NIPAM-*co*-Acrylamide) gel microspheres.[17]

Stability of the Enzyme–Gel System

To test the storage and reaction stability of the enzyme–gel matrices, CT immobilized poly(NIPAM-*co*-HEMA) gels prepared with two different enzyme loadings (i.e., 120 and 600 μg) were utilized. In this set, the storage and reaction stability was monitored by the same batch runs. The en-

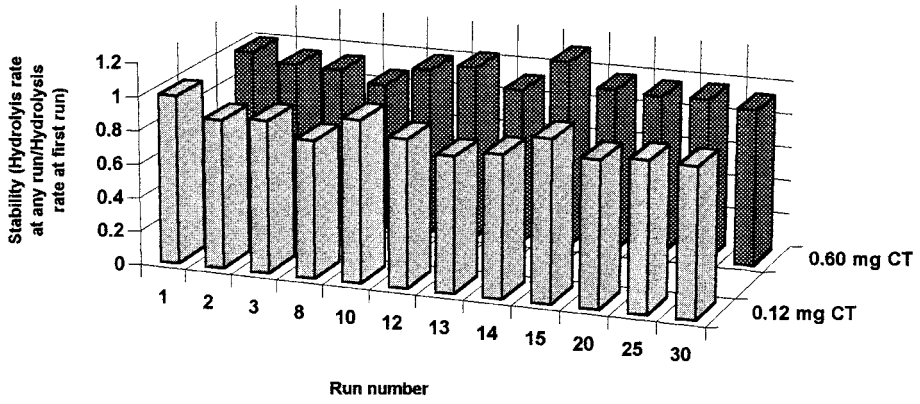

Figure 12. The variation of the BTEE consumption rate with the run number for the enzyme–gel matrices prepared with different enzyme loadings.

zyme–gel matrices were tested for 2 months by performing one batch run per 48 h. Each run was continued for 6 h and the enzyme–gel matrices were contracted at 35°C for 15 min after each run. The gel matrices within fresh buffer at a pH of 7.8 were stored in the refrigerator at +4°C between the runs. The batch runs were performed at 25°C within 20 mL of reaction volume having a pH of 7.8 with an initial BTEE concentration of 240 μM. The variation of the BTEE hydrolysis rate with the run number for the enzyme–gel matrices prepared with two different enzyme loadings is given in Figure 12. In this figure, the stability was defined as the ratio of initial BTEE hydrolysis rate in any run to that observed in the first run with the produced enzyme–gel matrix. As seen here, both of the CT immobilized poly(NIPAM-*co*-HEMA) gels were stable and no significant decrease in the activities were observed within 2 months.

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