Uniform Poly(isopropylacrylamide) Gel Beads for Immobilization of α-Chymotrypsin

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ABSTRACT: In this study, α-chymotrypsin was immobilized via physical entrapment within large, uniformly spherical, and thermally reversible poly(*N*-isopropylacrylamide) [poly(NIPAM)] beads. The gel beads were prepared in an aqueous dispersion medium by using Ca-alginate gel as the polymerization mold. In this preparation, potassium persulfate/tetramethylethylenediamine and sodium-alginate/calcium chloride were used as the redox initiator and the stabilizer systems, respectively. Thermoresponsive poly(NIPAM) gel beads 3 mm in size and including α-chymotrypsin were produced by the proposed procedure. The use of an aqueous bead-forming medium did not cause significant enzyme leakage during the preparation of enzyme-gel beads. Michaelis–Menten kinetics was used to define the behaviors of enzyme-gel beads prepared with different enzyme loadings. The Lineweaver–Burk plot indicated that the enzyme-gel system had a reasonably higher K_m value relative to that of free enzyme due to the internal mass transfer resistance against the substrate diffusion. The enzyme-gel system exhibited the maximum activity at 30°C due to the thermoresponsive character of the carrier matrix. However, the maximum activity with the free enzyme was observed at 40°C. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci **67:** 1127–1139, 1998

Key words: isopropylacrylamide; chymotrypsin; thermoresponsive gel; enzyme immobilization

INTRODUCTION

In recent years, there has been increasing interest in the use of thermoresponsive polymers as carriers in the immobilization of biologically active agents. Poly(*N*-isopropylacrylamide) [poly(NIPAM)] is one of the well-characterized members of the thermoresponsive polymer family.[1–15] The thermoresponsive behavior of poly(NIPAM) was extensively studied and defined by Tanaka et al.[1–9] Thermoresponsive gels have attracted much attention in different biotechnological applications as carrier matrices.[16–21] A saccharide-sensitive phase transition was observed with a covalently crosslinked polymer network of NIPAM in which a lectin, concanavalin-A, was immobilized.[16] Hoffman et al. performed a series of pioneer works on the use of crosslinked poly(NIPAM) gels in the immobilization of drugs, cells, and enzymes. Dong and Hoffman achieved the immobilization of asparaginase within crosslinked poly(isopropylacrylamide-*co*-acrylamide) membrane and found that immobilized asparaginase activity could be controlled by the swelling or deswelling of the gel matrix induced by cycling of the temperature.[18] β-Galactosidase was entrapped within copolymer beads of NIPAM and acrylamide (AAm) formed in an inverse suspension polymerization process.[19] The immobilized enzyme was tested both in batch mode and a packed bed reactor. The optimum temperature for the maximum activity of the immobilized enzyme was found to be lower in

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these two reactor types relative to that of free enzyme. Poly(NIPAM)-based gel matrix was also tried as a carrier in the immobilization of arthrobacter simplex, and the effect of temperature cycling on steroid conversion was investigated.[20]

In this study, α-chymotrypsin was immobilized via physical entrapment within large, uniformly spherical and thermally reversible poly(NIPAM) beads. The enzyme-gel beads were prepared in an aqueous dispersion medium by using Ca-alginate gel as the polymerization mold. The kinetic behavior of the enzyme-gel beads, the effect of temperature and the composition of hydrolysis medium on the relative activity, and the stability of enzyme-gel beads were investigated in batch fashion.

Table I Preparation Conditions of Thermoresponsive Enzyme-Gel Beads

Code	NIPAM (mg)	α-Chymotrypsin (μg)	Water in Disperse Phase (mL)
CT-NIPAM1	480	360	3.6
CT-NIPAM2	480	1800	3.6
CT-NIPAM3	160	120	1.2
CT-NIPAM4	160	600	1.2

Disperse phase: MBAM/NIPAM: 0.042 mg/mg, Na-alginat: 8.3 mg/mL, TEMED: 83 μL/mL.
Continuous phase: KPS: 10 mg/mL, $CaCl_2$: 30 mg/mL, Distilled deionized water: 40 mL.
Polymerization conditions: 20°C, 4 h, 250 rpm.

EXPERIMENTAL

Materials

NIPAM (Aldrich Chem. Co., Milwaukee, WI) was recrystallized from hexane-acetone solution before use. *N*,*N*,*N*,*N*-Methylenebisacrylamide (MBAM, BDH Chemicals Ltd., Poole, UK) was used as a crosslinker. In the preparation of gel beads, potassium persulfate (KPS, BDH Chemicals Ltd.) and tetramethylethylenediamine (TEMED, Sigma Chem. Co., St. Louis, Missouri) were selected as the initiator and the accelerator, respectively. Sodium alginate (medium viscosity, Sigma Chem. Co.) and calcium chloride (BDH Chemicals Ltd.) were used for the formation of alginate mold around the spherical gel beads. α-Chymotrypsin (CT, Sigma Chemical Co., 52 μmol BTEE/mg protein-min.) was used without further purification. The activity of α-chymotrypsin was determined by using a synthetic substrate, benzoyl-L-tyrosine ethyl ester (BTEE, Sigma Chemical Co.). Distilled-deionized water was used in all experiments.

Preparation of Enzyme-Gel Beads

The polymerization procedure developed by Park and Hoffman for the preparation of uniform poly(NIPAM) gel beads[22] was used with some modifications for the immobilization of α-chymotrypsin. The typical procedure for the preparation of α-chymotrypsin carrying gel beads is exemplified below. The continuous medium was prepared by dissolving 400 mg KPS and 1200 mg $CaCl_2$ in 40 mL of distilled-deionized water. The medium was purged with bubbling nitrogen for 1 h before the injection of the disperse phase including monomer, crosslinker, stabilizer, and enzyme. For the preparation of the disperse phase, 480 mg NIPAM, 20 mg MBAM, and 30 mg Na-alginate were dissolved within 2.6 mL cold distilled deionized water. One mL of enzyme solution including 0.36 mg α-chymotrypsin and 0.3 mL TEMED were added to the resulting solution. The disperse phase was dropped by a system including a syringe and a dosage pump into the continuous medium which was kept at 20°C and stirred magnetically with 250 rpm. The polymerization was conducted at 20°C with the same stirring rate for 4 h. The uniform and thermally reversible enzyme-gel beads 3 mm in size were obtained. The gel beads were filtered and washed extensively with cold distilled water and with cold borate buffer, respectively. The washed gel beads were collapsed in borate buffer medium (pH: 7.8) at 35°C for $\sim$ 30 min. The enzyme-gel beads were stored within the refrigerator at +4°C until use.

To determine the enzyme leakage during the preparation of gel beads, a 4 mL sample was taken from the continuous medium (40 mL) after separation of enzyme-gel beads and other polymeric impurities by filtration. The enzymatic activity of this sample was determined at 25°C with 159.5 μM of BTEE initial concentration in a batch having 20 mL of reaction volume at a pH of 7.8. The washed enzyme-gel beads were collapsed within 20 mL of borate buffer at 35°C for 30 min and the enzyme leakage in the shrinking medium was also determined by the same procedure.

The enzyme loading and the disperse phase/continuous medium volume ratio were changed in the preparation of thermoresponsive enzyme-gel beads. The preparation conditions are given

in Table I. As seen here, CT-NIPAM1 and CT-NIPAM2 were prepared by changing enzyme loading between 100 and 500 μg/mL disperse phases, with a constant disperse phase/continuous medium ratio of 3.6 : 40. In the preparation of CT-NIPAM3 and CT-NIPAM4, the enzyme loading into the disperse phase was changed in the same range but a lower disperse phase/continuous medium ratio (i.e., 1.2 : 40) was used. It was also possible to observe the effect of the disperse phase/continuous medium ratio at constant enzyme loading by comparing the activities of CT-NIPAM1 and CT-NIPAM3 or CT-NIPAM2 and CT-NIPAM4. The produced amounts of CT-NIPAM1 and CT-NIPAM2 were divided into three equal parts based on the gel weight after washing, and $\frac{1}{3}$ part was used in the activity experiments performed with the immobilized enzyme within 20 mL of reaction volume. However, all of the produced amounts of CT-NIPAM3 and CT-NIPAM4 were used in the activity experiments to achieve the same volume ratio of enzyme-gel beads to the reaction medium with CT-NIPAM1 and CT-NIPAM2.

Characterization of Enzyme-Gel Beads

Size and monodispersity of enzyme-gel beads were determined by optical microscopy. The surface morphology and the internal structure of the enzyme-gel beads were observed by a scanning electron microscope (JEOL, JEM 1200EX, Tokyo, Japan). For this purpose, the enzyme-gel beads were swollen to equilibrium in phosphate buffer solution at 15°C and subsequently freeze dried at −20°C. The freeze-dried beads were cut to obtain the cross-sectional view of the internal structure. The samples coated with a thin layer of gold ($\sim$ 100 Å) *in vacuo* were examined by the scanning electron microscope.

The variation of equilibrium swelling ratio of CT-loaded poly(NIPAM) gel beads with the temperature was studied in borate buffer solution having a pH of 7.8. The enzyme-gel beads coded as CT-NIPAM1 (prepared with 480 mg of NIPAM and 360 μg of CT, 3 mm in diameter in the production conditions) were equilibrated in borate buffer at +4°C for 24 h and the weight of swollen gel was measured after removing excess water by a filter paper. The medium temperature was raised and the gel beads were kept at the reached temperature for $\sim$ 6 h and the weight of gel was again recorded. The equilibrium swelling ratio measurements for CT-NIPAM1 gel beads were repeated at the various temperatures by following this procedure. The equilibrium swelling ratio of gel beads at any temperature was calculated by eq. (1), where Θ_V is the equilibrium swelling ratio of poly(NIPAM) gel beads at a particular temperature, M (g) is the swollen weight of gel beads at equilibrium, and M_o (g) is the dry weight of the gel beads.

$$\Theta_V = (M - M_o)/M_o \quad (1)$$

The variation of equilibrium linear swelling ratio of poly(NIPAM) gel beads with the medium temperature was also determined. The equilibrium diameter of gel beads was measured at any particular temperature in an optical system constructed for this purpose.[23] The equilibrium linear swelling ratio was calculated based on eq. (2).

$$\Theta_L = d/d_o \quad (2)$$

Where Θ_L is the equilibrium linear swelling ratio of poly(NIPAM) gel beads at a particular temperature, d (mm) is the diameter of gel beads at equilibrium, and d_o (mm) is the diameter of gel beads at the production conditions. In these experiments, five beads were followed and the mean of measured linear swelling ratios was calculated. The standard deviation of linear swelling ratio measurements with different particles was not higher than 5%.

The dynamic response of enzyme-gel beads was determined in a separate group of experiments. In order to follow the dynamic shrinking behavior, a step input having a magnitude of +36°C was applied by transferring the enzyme-gel beads (CT-NIPAM1) equilibrated in borate buffer at +4°C into another buffer solution kept at 40°C and at the same pH (i.e., 7.8). Then, the weight of gel beads was determined at designated times. The dynamic swelling behavior was monitored by applying a step input having the same magnitude on the medium temperature, but in the reverse direction (−36°C). In this experiment, the enzyme-gel beads equilibrated at 40°C were transferred into another buffer solution at +4°C.

In a separate group of experiments, the variation of equilibrium swelling ratio of poly(NIPAM) gel beads with the ethanol concentration was studied. In these experiments, the equilibrium swelling ratio of CT-NIPAM1 was determined in ethanol–water solutions by changing the ethanol

concentration between 0 and 100% (by volume). The equilibrium swelling ratio measurements in water–ethanol media were performed at three different temperatures (i.e., 15, 25, and 36°C).

Enzyme Activity

In the enzymatic activity experiments, the hydrolysis of synthetic substrate (i.e., BTEE) into benzoyl-L-tyrosine (BT) by free or immobilized CT was followed. All experiments were performed by using a batch system including a borate buffer medium having a pH of 7.8. The activities of free or immobilized enzyme were determined by monitoring the conversion of synthetic substrate (i.e., BTEE) according to the spectrophotometric method proposed by Clark and Bailey.[24] The absorbance of reaction medium was measured in a UV spectrophotometer (Hitachi Corp., Tokyo, Japan) at a wavelength of 258 nm. The apparent reaction rates were calculated based on the following expression.[24,25]

$$R = S_o[(dA/dt)/(A_f - A_o)] \qquad (3)$$

Where R is the hydrolysis rate of BTEE, S_o is the initial BTEE concentration, and A_o and A_f are the absorbances of the reaction medium before the addition of enzyme (i.e., zero reaction time) and at the complete conversion of substrate, respectively. dA/dt is the derivative of absorbance with respect to time during the initial course of reaction. dA/dt values were calculated by applying linear regression on the absorbance-time data obtained for the initial stage of reaction in which the absorbance of the reaction medium changed linearly with the time.

RESULTS AND DISCUSSION

Characterization of Enzyme-Gel Beads

α-Chymotrypsin containing poly(NIPAM) gel beads 3 mm in size were produced in the monodisperse form by using Ca-alginate gel as the polymerization mold. The photographs of uniform gel beads at +4 and 37°C are given in Figure 1. As seen here, the enzyme-gel beads were responsive to the temperature change and significantly collapsed with the increasing temperature. The variation of equilibrium swelling ratio of enzyme-gel beads with the medium temperature in borate buffer medium was determined both by gravimetric measurements and by monitoring the change of diameter of gel beads (i.e., linear equilibrium swelling ratio). The variation of equilibrium swelling ratio of enzyme-gel beads with the temperature is given in Figure 2. Here, the equilibrium swelling behavior of the gel sample coded as CT-NIPAM1 was exemplified, since there was no significant change in the observed tendencies with the changing enzyme loading and disperse phase/continuous medium ratio. As seen in Figure 2, the gravimetrically determined equilibrium swelling ratio decreased from 37.5 to 3 while the linear one was changing from 1.1 to 0.45 by increasing the medium temperature from +4 to 40°C. The lower critical solution temperature (LCST) values ranging between 33 and 34°C were reported for the neutral poly(NIPAM) gels depending upon the gelation method.[3,4,8,11] The observed transition range for the linear swelling ratio of enzyme-gel beads was slightly wider relative to the neutral poly(NIPAM) gels usually exhibiting a discontinuous volume transition at a certain temperature value between 33 and 34°C. The

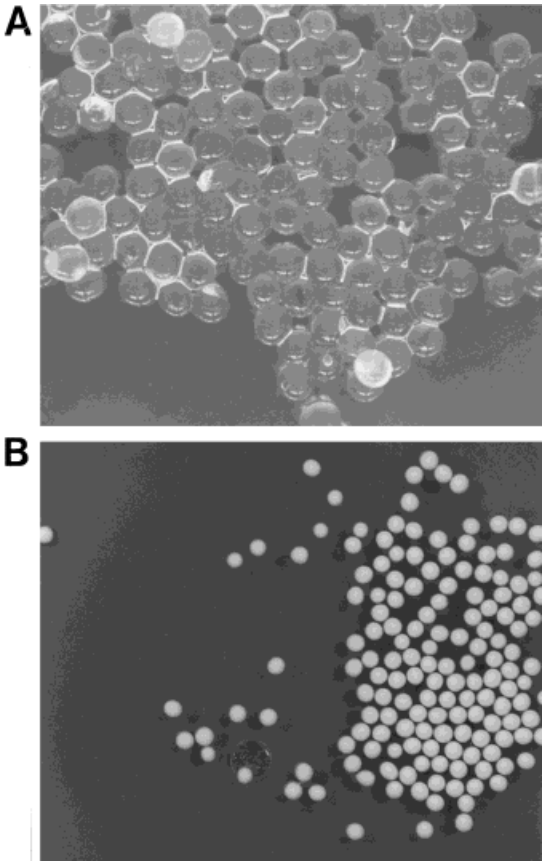

Figure 1 The photographs of thermoresponsive enzyme-gel beads, (A) +4°C, (B) 37°C.

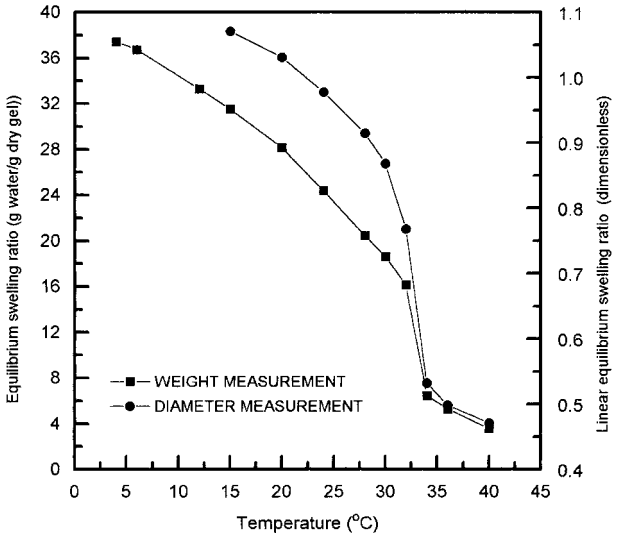

Figure 2 The variation of equilibrium swelling ratio of the enzyme-gel beads with the temperature.

continuous transition in the linear swelling ratio of enzyme-gel beads with the temperature may be attributed to the presence of Ca-alginate gel mold within the carrier structure. The alginate part of gel beads was not removed through the interpenetrating network to prevent the denaturation of α-chymotrypsin during the removal operation which could be performed by EDTA solution.

In order to observe the dynamic shrinking behavior of enzyme-gel beads, a step input was applied by changing the medium temperature from +4°C to 40°C. The variation of swelling ratio with the time under the applied step input is given in Figure 3. As seen here, the shrinking process was very fast in the first five minutes. The dynamic swelling behavior was monitored by applying the step input having the same magnitude on the medium temperature, but in the reverse direction. The variation of swelling ratio by time during the dynamic swelling period is also given in Figure 3. As seen here, the swelling process was faster within the first 180 min and after this period, the swelling rate gradually decreased with the time. On the other hand, the comparison of curves in Figure 3 indicated that the swelling response of enzyme-gel beads was reasonably slower relative to the shrinking.

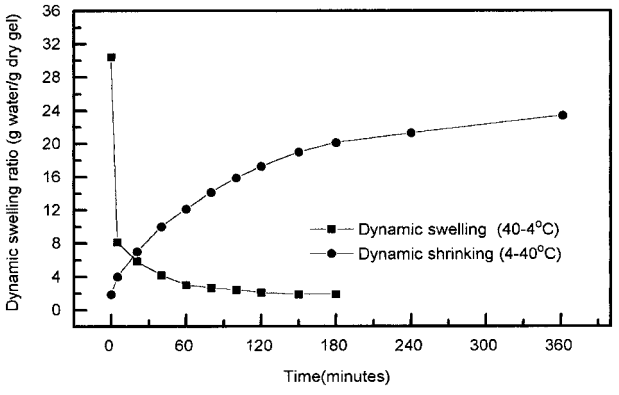

Figure 3 The dynamic swelling and shrinking behaviors of thermoresponsive enzyme-gel beads.

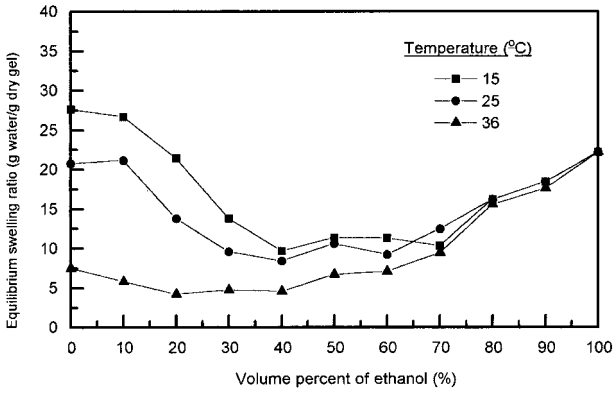

Figure 4 The variation of equilibrium swelling ratio of enzyme-gel beads with the ethanol concentration.

As reported in the literature, neutral poly(NIPAM) gels are also responsive against the alcohol concentration in the aqueous medium.[11] On the other hand, the presence of ethanol is necessary for the hydrolysis of BTEE by α-chymotrypsin since the solubility of BTEE in the reaction medium markedly increases with the increasing ethanol concentration. Therefore, ethanol concentration in the aqueous medium was changed between 0 and 100% (by volume) to observe the sensitivity of enzyme-gel beads against one of the components of BTEE hydrolysis medium. The variation of equilibrium swelling ratio of enzyme-gel beads with the ethanol concentration is given in Figure 4. The observed tendency was very similar to the reported behavior of neutral poly(NIPAM) gel within the methanol–water mixture.[11] As seen here, the swelling response against the ethanol in the temperature slightly above the LCST of enzyme-gel beads (i.e., 36°C) was reasonably different from those observed at the temperatures lower than LCST (i.e., 15 and 25°C). The enzyme-gel beads were in the swollen form both in pure water and pure ethanol and the equilibrium swelling ratio exhibited a minimum between 40 and 60% of ethanol concentration at 15 and 25°C. However, at 36°C the enzyme gel beads were in the shrunken state within pure water and the liq-

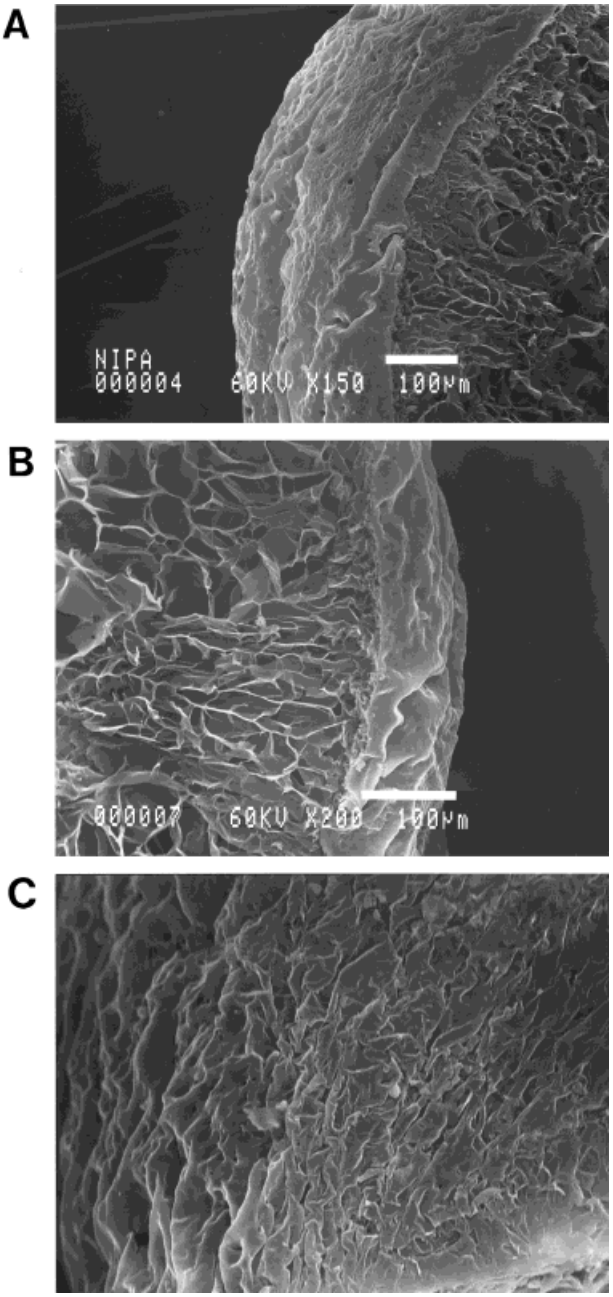

Figure 5 The SEM photographs showing the interior part and the surface of the enzyme gel beads. The bars in the all SEM photographs indicate 100 μm. (A) The surface and the internal structure, (B) the internal structure, (C) the detailed surface morphology.

uid content of gel increased continuously with the increasing ethanol concentration.

The surface morphology and the internal structure of the enzyme-gel beads were exemplified by the electron micrographs given in Figure 5. As seen in the electron micrograph taken with 150× original magnification, the enzyme-gel beads were comprised of a highly macroporous interior and a shell placed around this matrix [Fig. 5(A)]. The macroporous matrix could be observed in more detail in Figure 5(B). Note that these micrographs were taken with the freeze-dried forms of the enzyme-gel beads equilibrated at 15°C. Therefore, the electron micrograph in Figure 5(B) showed the macroporous structure of the swollen enzyme-gel beads. The internal structure of enzyme-gel beads was also very similar to that of crosslinked poly(NIPAM) gel prepared by block polymerization.[26] On the other hand, the macroporosity of the shell was not as high as that of the internal part. However, it was possible to see the presence of large pores on the surface of the enzyme-gel beads [Fig. 5(A)]. Although the crosslinked alginate part was possibly dominant on the surface, the shell may be also considered as an interpenetrating network comprised of Ca-alginate and poly(NIPAM) chains. The surface morphology of the enzyme-gel beads was also exemplified with higher magnification in Figure 5(C). The presence of large pores on the bead surface could be seen more clearly in this electron micrograph.

It should be noted that the change of bead diameter (i.e., the change of particle volume) was reasonably small between 15 and 25°C at which most of the batch runs for the hydrolysis of BTEE were conducted. For this reason, the macroporous structure at 25°C should be reasonably similar to that observed at 15°C.

Free Enzyme

The behavior of free α-chymotrypsin in the hydrolysis of BTEE within the reaction medium including ethanol and water was investigated in our previous article.[27] To test the effect of initial substrate concentration on the hydrolysis rate, BTEE initial concentration was changed between 15 and 400 μM. All runs were performed in a batch reactor at 25°C within 20 mL of reaction volume. The reaction medium at a pH of 7.8 was prepared by mixing 95% borate buffer and 5% ethanol (by volume). For accurate determination of free enzyme kinetic parameters, the enzyme concentration was fixed to a sufficiently low level (i.e., 0.25 μg/mL) by utilizing 5 μg enzyme within a 20 mL reaction volume. The reaction kinetics between BTEE and CT was adequately described by the Michaelis–Menten model.[27] K_m and r_m values by the Lineweaver–Burk plot were found as 63.06 μM BTEE and 11.01 μM BTEE/min., respec-

tively.[27] The maximum activity based on per mg of free enzyme (r'_m) was calculated from the estimated r_m value and found as 44.04 μmol BTEE/mg CT-min to compare the maximum activities obtained with free enzyme and enzyme-gel beads.

In this article, the effect of temperature on the activity of free enzyme was determined by fixing the initial BTEE concentration to 400 μM. In these experiments, temperature of the reaction medium was changed between 15 and 50°C and the other conditions were the same with the those used in the experiments relating to the effect of initial BTEE concentration. The maximum value of BTEE hydrolysis rate was 42.11 μM BTEE/min at 40°C with the free enzyme.

Enzyme-Gel Beads

Monodisperse and thermally reversible gel beads carrying α-chymotrypsin could be synthesized by the selected immobilization method. The selection of aqueous medium as the continuous phase makes possible the synthesis of large size and uniform gel beads. The average size of gel beads with this procedure was 3 mm in the production conditions. In addition to the batch system, these large beads can also be used in the packed or fluidized bed reactors without creating a large pressure drop. The isolation of produced beads from the reaction medium is easy due to their size.

A set of preliminary experiments were performed to determine the immobilization conditions leading to the synthesis of enzyme-gel beads having the best activity. It can be stated that the resultant activity of immobilized α-chymotrypsin is mainly controlled by two factors. These are the enzyme leakage to the continuous phase during the formation of gel beads and the extent of enzyme denaturation depending upon the polymerization reaction. In order to determine proper immobilization conditions, the immobilization temperature and the buffer used in the immobilization medium were changed and the initial activities of enzyme-gel beads produced with different immobilization conditions were measured at 25°C with 239.3 μM of initial BTEE concentration in a batch reactor having 20 mL of reaction volume at a pH of 7.8 and including 95% borate buffer and 5% ethanol. In the activity measurements, $\frac{1}{3}$ of the enzyme-gel beads produced by the application of each recipe were used. The tried immobilization conditions are given in Table II.

In recipes 1 and 2, the borate buffer was used either in the disperse phase or in the continuous medium. Recipes 3 and 4 were applied by using distilled deionized water both in the disperse phase and in the continuous medium. In the design of the first two recipes, it was aimed to prevent or to reduce the possible denaturation of enzyme during immobilization, by using borate buffer in the gelation medium, or by performing the gelation at a lower temperature. As seen in Table II, the highest enzymatic activity was obtained with the enzyme-gel beads produced by recipe 4. This result may be explained by the formation rate of gel beads within the aqueous dispersion medium. We observed that slower bead formation rates (i.e., longer gelation times in the droplet phase) relative to recipe 4 were obtained when the enzyme immobilization was carried out at +4°C or the borate buffer was used instead of distilled water in the polymerization medium. It may be postulated that the enzyme leakage from the disperse phase to the continuous medium increases by the increasing gelation time, since most of the enzyme leakage from the disperse phase along the immobilization period takes place before the gelation. On the other hand, the use of borate buffer both in the disperse and continuous phases caused incomplete particle formation at +4°C and some defects in the spherical form, and the monodispersity of gel beads at 20°C. To find the reason for the slower bead formation rate in the presence of borate buffer, we examined crosslinked poly(NIPAM) gel formation by the block polymerization method. But we could not observe any significant difference between the gelation times and the gel properties obtained by using borate buffer or distilled water at 20°C. Then, we examined Ca-alginate gel formation both in the aqueous medium and in the borate buffer. We prepared two different solutions by using 1 mL of distilled water or borate buffer. The alginate concentration were fixed to 8.3 mg/mL in both solutions. NIPAM, MBAM, and TEMED were also included in the prepared solutions and their concentrations were fixed to the corresponding values of the droplet phase in Table I. To observe Ca-alginate gel formation in the solution prepared by distilled water, we added 0.25 mL of $CaCl_2$ solution (30 mg/mL) prepared by distilled water into this medium. On the other hand, 0.25 mL $CaCl_2$ solution (30 mg/mL) prepared by borate buffer was added into the borate buffer medium including Na-alginate. Although a monoblock Ca-alginate gel having higher mechanical strength could be achieved in the medium prepared by distilled

Table II Experimental Design for Determination of Proper Immobilization Conditions

Recipe	Medium Type	Temperature (°C)	Initial Activity (μM BTEE/min)[a]
1	Borate buffer (pH: 7.8)	+4	No stable particle formation
2	Borate buffer (pH: 7.8)	20	0.678
3	Distilled deionized water	+4	0.572
4	Distilled deionized water	20	1.728

Disperse phase: NIPAM: 480 mg, MBAM/NIPAM: 0.042, Na-alginat: 8.3 mg/mL, TEMED: 83 μL/mL, α-chymotrypsin: 360 μg, buffer or distilled deionized water: 3.6 mL.
Continuous phase: KPS: 10 mg/mL, $CaCl_2$: 30 mg/mL, buffer or distilled deionized water: 40 mL.
Polymerization conditions: 4 h, 250 rpm.
[a] The initial activities were determined with 239.3 μM BTEE initial concentration at 25°C and at pH 7.8.

water, the medium including borate buffer provided a disintegrated Ca-alginate gel having reasonably poor mechanical strength. The slower bead formation rate observed in borate buffer may be explained by using this result. In the preparation of enzyme-gel beads in borate buffer, the leakage of ingredients from the droplet phase increased due to the poor mechanical properties of Ca-alginate gel mold around the enzyme-pregel droplets in borate buffer medium. This case led to a decrease in the concentration of thermoresponsive monomer (NIPAM) and crosslinker (MBAM) within the droplets, which in turn decreased the observed bead formation rate and caused some defects in the spherical form of the final enzyme-gel beads. The rapid gelation obtained with recipe 4 (i.e., 1–2 min) also reduced the enzyme leakage from the disperse phase which in turn provided higher enzymatic activity with the resultant gel beads. The exact spherical beads with high monodispersity were also obtained with recipe 4.

In order to determine the enzyme leakage from the enzyme-gel beads to the aqueous continuous medium during the immobilization period, the amount of enzyme in the continuous phase was determined after preparation of the enzyme-gel beads. This determination was carried out for CT-NIPAM1 and CT-NIPAM2 (Table I) which were the most widely used enzyme-gel beads in the systematic experiments relating to the effect of process conditions on the behavior of the enzyme-gel system. The amount of enzyme passed to the continuous medium (CT_c), the percent of activity in the continuous medium (A_c), and the percent of activity passed to the first shrinking medium (A_S), are given in Table III.

As seen in Table III, the amount of enzyme passed to the continuous medium increased by the increasing enzyme loading into the disperse phase, but the percent of enzymatic activity in the continuous medium remained roughly constant. The effective diffusion coefficient of enzyme within the disperse phase gradually decreases with the increasing gelation time. The decrease in the effective diffusion coefficient of enzyme also involves a proportional decrease in the diffusion flux according to Fick's first law. This factor significantly reduces the enzyme leakage from the disperse phase and allows the immobilization of enzyme with a satisfactory yield. The results in Table III indicated that although an aqueous bead-forming medium was used in the immobilization, it was possible to load $\sim$ 75–80% of enzyme into the gel beads by adjusting the immobilization conditions. Of course, this conclusion was valid when no significant activity loss occurred in the enzyme passed to the continuous medium. This factor was checked by preparing a continuous medium including dissolved enzyme in the absence of disperse phase, and no significant change was observed in the enzymatic activity at the end of 4 h relative to the initial one at 20°C. On the other hand, the enzyme leakage to the first shrinking medium was reasonably lower relative to that in the continuous medium during polymerization. After the first shrinking of enzyme-gel beads, no significant activity was recorded in the aqueous phase in the repeated shrinking operations.

In order to investigate the variation of activity of enzyme-gel beads with the initial substrate concentration, the synthetic substrate (i.e., BTEE) concentration was changed between 159.5 and 877.4 μM in a reaction medium including 95% borate buffer and 5% ethanol (by volume). All of the enzymatic activity experiments were performed in batch mode at 25°C within 20 mL of reaction medium having a pH of 7.8. The effect of initial BTEE concentration was studied with two types of enzyme-gel beads prepared with 100 and

Table III The Enzyme Leakage in the Immobilization Period and After the First Shrinking of Enzyme-gel Beads

Enzyme-gel Bead	Loaded Enzyme (μg)	CT_c (μg)	A_c (%)[a]	A_S (%)[a]
CT-NIPAM1	360	92	25.6	1.28
CT-NIPAM2	1800	364	20.2	0.91

[a] The activities were given based on the initial enzymatic activity loaded into the disperse phase.

500 μg/mL CT loadings (i.e., CT-NIPAM1 and CT-NIPAM2). One-third (based on weight after washing) of the enzyme-gel beads produced in one batch was utilized in the each activity experiment. Therefore, the amount of enzyme-gel beads (i.e., $\sim$ 100 beads, calculated total volume based on average diameter was 1.4 mL) were kept constant in these experiments. In the selection of the amount of enzyme-gel beads, it was aimed to achieve BTEE hydrolysis rates having approximately the same order of magnitude with the those observed by free enzyme experiments. The tried amounts of CT-NIPAM1 and CT-NIPAM2 included 90 and 480 μg of immobilized CT, respectively. The maximum value of substrate concentration was determined by the solubility of BTEE in the selected medium. The precipitation of BTEE was observed with 1200 μM of BTEE concentration. In order to prevent the precipitation, the maximum value of initial substrate concentration was fixed to $\sim$ 900 μM. The variation of BTEE hydrolysis rate with the initial BTEE concentration is given in Figure 6. As seen here, the BTEE hydrolysis rate increased with the increasing BTEE concentration for both of the enzyme-gel beads. At constant BTEE initial concentration, higher BTEE hydrolysis rates were achieved with the enzyme-gel beads having higher enzyme content.

The Lineweaver–Burk plots of enzyme-gel beads with two different enzyme loadings and the apparent values of kinetic parameters based on the Michaelis–Menten model are given in Figure 7 and Table IV, respectively. Higher apparent K_m values with α-chymotrypsin-containing gel beads in a well-stirred batch reactor indicated that the internal mass transfer limitations were strongly effective on the hydrolysis rate of BTEE. Note that K_m values of the enzyme-gel beads were also significantly higher than the solubility of the substrate in the selected medium, since the precipitation of BTEE occurred at 1200 μM. Therefore, K_m values can only give an idea about the effect of internal mass-transfer resistance in the enzyme-gel beads. However, it was impossible to verify experimentally the estimated values of maximum hydrolysis rates of the enzyme-gel beads, since the corresponding initial BTEE concentrations were significantly higher than the BTEE concentration at which the substrate precipitation was observed in the studied medium. However, the estimated values were roughly proportional to the

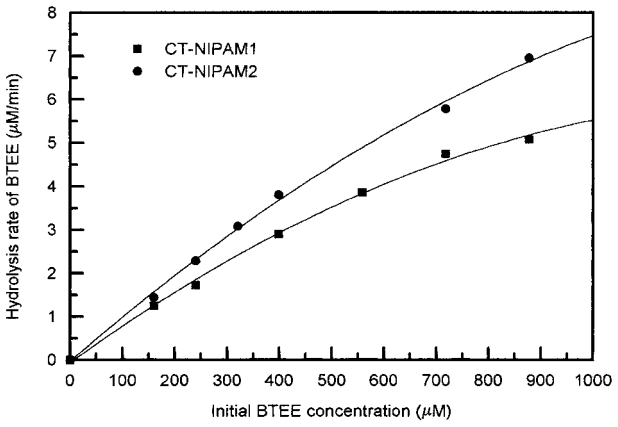

Figure 6 The variation of BTEE hydrolysis rate with the initial BTEE concentration in the presence of enzyme-gel beads prepared with different enzyme loadings.

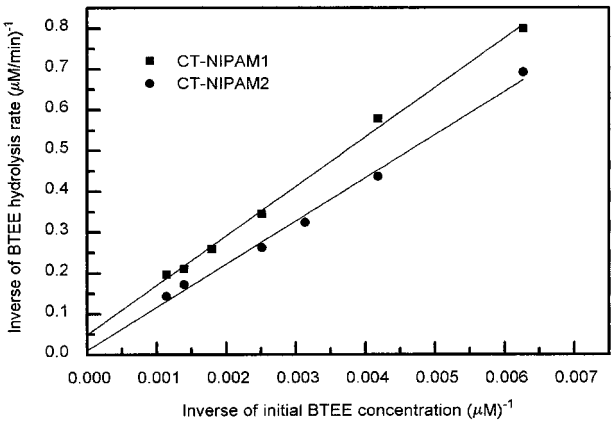

Figure 7 The Lineweaver–Burk plots of enzyme-gel beads prepared with different enzyme loadings.

Table IV The Kinetic Constants of Enzyme-Gel Beads

Carrier Type	CT (μg)	r_m (mM BTEE/min)	r'_m (mmol BTEE/mg CT-min.)	K_m (mM)
Free CT	5[a]	0.011	0.0440	0.063
CT-NIPAM1	90[b]	0.020	0.0045	2.547
CT-NIPAM2	480[b]	0.093	0.0039	9.787

[a] The amount of free CT used in a 20 mL of batch volume.
[b] The immobilized amount of CT included within the gel matrix having a volume of 1.4 mL and used in 20 mL batch volume for testing of the immobilized system activity.

enzyme content of the gel beads since CT-NIPAM2, having 5.3 times higher enzyme content relative to CT-NIPAM1, exhibited 4.7 times higher r_m value. The maximum hydrolysis rate values were also given as r'_m having the unit of mmol BTEE/mg CT-min to compare all maximum reaction rates on the same base (Table IV). As seen here, r'_m values of enzyme gel-beads were $\sim$ 10 times lower relative to that of free enzyme and approximately equal to each other. The lower r'_m values with the enzyme-gel beads relative to free enzyme again indicated the significant effect of internal mass transfer resistance on the hydrolysis rate of BTEE.

It should be stated that the solubility of BTEE within the reaction medium is closely related to the ethanol concentration and increases with the increasing ethanol content of the reaction medium. On the other hand, the activity of free α-chymotrypsin was a function of water concentration in the alcohol–water mixtures as it was reported in the literature.[28,29] Therefore, one of the important parameters controlling the BTEE hydrolysis rate is the ethanol concentration in the aqueous reaction medium in the presence of free enzyme.

It is possible to increase the solubility of substrate by increasing the ethanol content of the reaction medium. Theoretically, it is possible to use higher BTEE concentrations to achieve higher BTEE hydrolysis rates with the enzyme-gel beads. Therefore, 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 beads with the initial BTEE concentration was investigated in these reaction media. One-third of the enzyme-gel beads prepared with the enzyme loadings of 100 and 500 μg enzyme/mL disperse phase (i.e., CT-NIPAM1 and CT-NIPAM2) were utilized in these runs performed at 25°C in 20 mL of batch volume. The variation of BTEE consumption rate with the initial BTEE concentration within the reaction media having different ethanol concentrations is given in Figure 8. As seen here, the BTEE hydrolysis rates obtained with the reaction medium having 25% ethanol were lower than those obtained within the medium including 5% of ethanol. This result may be explained as follows: the effect of ethanol concentration on the hydrolysis rate of BTEE in the presence of free α-chymotrypsin was determined in our previous work by changing the ethanol concentration between 5 and 90% (by volume) and the results indicated that the activity of α-chymotrypsin decreased with the increasing ethanol concentration of the reaction medium.[27] Similar results for the effect of ethanol concentration on the activity of free α-chymotrypsin were also reported in the literature and the decrease in the enzymatic activity was explained by the decreasing amount of hydrated enzyme by the increasing ethanol concentration.[29] The response of gel matrix against ethanol were also effective on the behavior of the enzyme-gel system against the ethanol concentration. As seen in Figure 4, the gel

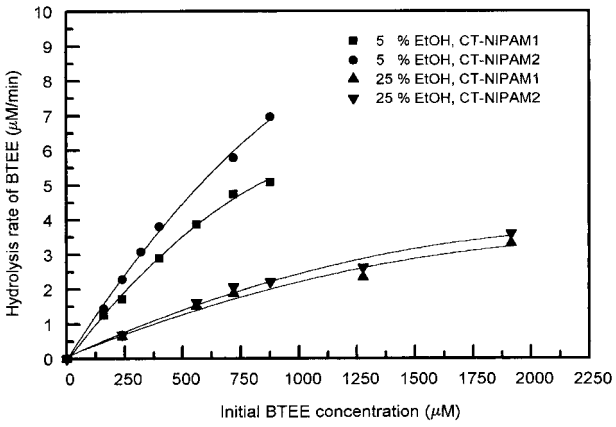

Figure 8 The variation of BTEE hydrolysis rate with the initial BTEE concentration in the presence of enzyme-gel beads within the reaction media having different ethanol concentrations.

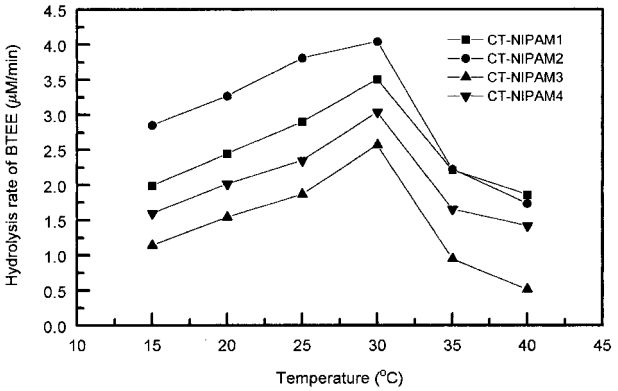

Figure 9 The variation of BTEE hydrolysis rate with the medium temperature in the presence of enzyme-gel beads prepared with different enzyme loadings.

matrix collapsed when the ethanol concentration increased from 5 to 25%, which caused an increase in the internal mass transfer resistance against the BTEE diffusion through the gel matrix. Therefore, the observed BTEE consumption rate decreased in the presence of 25% of ethanol due to these 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 8.

In order to test the effect of temperature on the activity of enzyme-gel beads, the medium temperature was changed between 15 and 40°C. The batch runs were conducted within 20 mL of 95% borate buffer–5% ethanol medium at a pH of 7.8 with an initial BTEE concentration of 400 μM. Four types of enzyme-gel beads prepared with different enzyme loadings and different disperse/continuous phase ratios were tested. In these runs, the volume of enzyme-gel beads were kept constant by including $\frac{1}{3}$ parts of CT-NIPAM1 and CT-NIPAM2 and all of CT-NIPAM3 and CT-NIPAM4 produced in one batch. The detailed preparation conditions of the enzyme-gel beads are also given in Table I. In the preparation of these beads, the disperse phase/continuous phase ratio was changed between 1.2 : 40 and 3.6 : 40. For each disperse phase/continuous phase ratio, two different enzyme loadings were utilized (i.e., 100 and 500 μg/mL disperse phase). The variation of activities of the enzyme-gel beads with the medium temperature is given in Figure 9. For constant enzyme loading (i.e., 100 or 500 μg/mL disperse phase), the enzyme-gel beads prepared with higher disperse phase/continuous phase ratios provided higher BTEE hydrolysis rates at constant temperature. The fraction of enzyme transferred to the continuous medium during the bead formation period possibly decreased by the increasing disperse phase/continuous phase volume ratio, which caused an increase in the final enzyme concentration within the gel beads. Hence, higher enzymatic activities were obtained with the constant amount of gel beads prepared with higher disperse phase/continuous phase ratio. As seen in Figure 9, the maximum activity was observed at 30°C with all enzyme-gel beads. The activity increased with the increasing temperature in the range of 15–30°C and a reasonably sharp decrease was observed between 30 and 40°C. In order to compare this behavior with the free enzyme, Figure 10 was sketched in a way in which the change in the relative activity of free enzyme with the temperature was evaluated together with the relative activity of enzyme-gel beads. The relative activity was defined as the hydrolysis rate of BTEE at any temperature to the maximum hydrolysis rate. As seen in Figure 10, the relative activity of free enzyme sharply increased with the increasing temperature in the range of 15–40°C and the maximum reaction rate was obtained at 40°C. However, the slope of relative activity-temperature curve of the enzyme-gel system was not so high since the relative activity change between 15 and 30°C was smaller in contrast to free enzyme. The temperature in which the maximum activity was observed shifted to 30°C with the enzyme-gel beads. The difference between the response of free enzyme and enzyme-gel beads against the temperature may be explained by the stimulus-responsive character of poly(NIPAM) gel. At the lower temperatures, the mass transfer resistance against the substrate diffusion is lower and the effective diffusion coef-

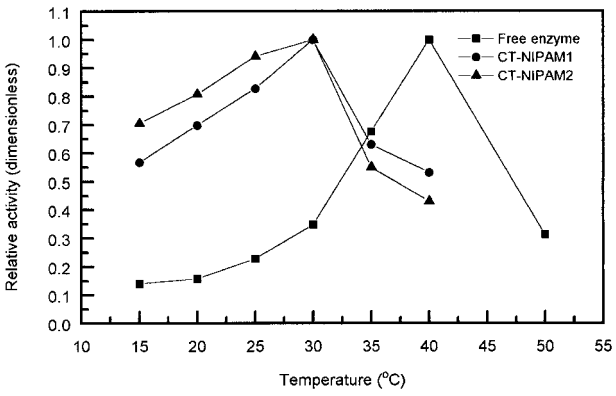

Figure 10 The variation of relative activities of the enzyme-gel beads with the medium temperature.

ficient of substrate is higher due to the presence of larger pores in the swollen form of the thermosensitive gel. The effective diffusion coefficient of substrate decreases by the increasing temperature since the pores become smaller due to the shrinking process. The shrinking of gel volume also causes a decrease in the surface area of the enzyme-gel beads. All these changes involve a decrease in the diffusion rate of substrate since the diffusion rate is directly proportional to the effective diffusion coefficient and the surface area, according to Fick's law.[30] On the other hand, the activity of free enzyme increased with the increasing temperature between 15 and 40°C. However, the increase in the enzyme activity by the increasing temperature is partly compensated by the increasing mass transfer resistance and decreasing surface area (both affects on the substrate diffusion rate according to Fick's law) in the enzyme-gel system and this balance determines the apparent BTEE hydrolysis rate with the enzyme-gel beads. Therefore, the relative activity of enzyme-gel system did not increase dramatically, as observed with the free enzyme in the temperature range of 15–40°C. The increase in the relative activity of enzyme-gel beads completed at 30°C and the maximum point for the hydrolysis rate was obtained at this temperature. Note that the LCST of poly(NIPAM) gel was ~ 33–34°C, hence the gel matrix was completely in the shrunken state at the temperatures higher than LCST.[3,4,8,11] So, the mass transfer resistance within the gel increased and the gel volume (i.e., external surface area) significantly decreased after the LCST value, which caused a sharp decrease in the apparent hydrolysis rate at the temperatures higher than 30°C.

Stability of Enzyme-Gel Beads

CT immobilized poly(NIPAM) gel beads prepared with two different enzyme loadings (i.e., 100 and 500 μg CT/mL disperse phase) were utilized to determine the storage and reaction stability. The enzyme-gel beads were tested for one month by performing one batch run per 24 h. Each run was performed with an initial BTEE concentration of 239.3 μM at 25°C for 4 h within 20 mL of reaction volume at a pH of 7.8. After completion of each run, the enzyme-gel beads were collapsed at 35°C for 30 min to remove any residual substrate or product within the gel matrix, then transferred into a fresh buffer solution (pH 7.8) and stored in the refrigerator at +4°C during the period between the runs. The variation of stability with the run number is given in Figure 11. Here, the stability was defined as the ratio of initial BTEE consumption rate in any run to that observed in the first run with the produced enzyme-gel matrix. As seen here, after the second use, no significant decrease in the stability of CT-NIPAM2 gel was observed over the 30 batch runs performed within one month. The lower activity in the second run relative to the first one may be explained by the leakage of enzyme molecules weakly entrapped, especially on the surface of gel beads during the first run. A similar behavior was also observed with CT-NIPAM1 having a lower enzyme content relative to CT-NIPAM2, except slightly decreasing activity values with the run number were obtained after the second run. The total activity decrease between the 1st and 30th runs was ~ 25% with this gel matrix. These results showed that α-chymotrypsin-immobilized poly(NIPAM) gel beads could be effectively used when their loaded enzyme contents were kept at a sufficiently high level.

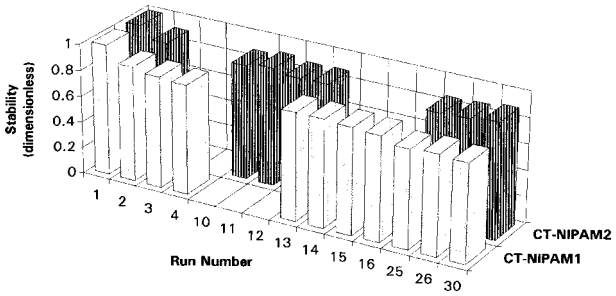

Figure 11 The variation of activity of enzyme-gel beads with the run number.

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