In Situ Immobilization of Alkaline Protease during Inverse Suspension Polymerization of Polyacrylamide and Poly(acrylamide-*co*-methacrylic acid) Hydrogel Beads

S. KIATKAMJORNWONG,[1] N. SIWARUNGSON,[2] A. NGANBUNSRI[3]

[1] Department of Photographic Science and Printing Technology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[2] Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[3] Multidisciplinary Program of Petrochemical and Polymer Science, Chulalongkorn University, Bangkok 10330, Thailand

Received 14 April 1998; accepted 31 January 1999

ABSTRACT: We report the *in situ* immobilization of alkaline protease in highly porous polyacrylamide and poly(acrylamide-*co*-methacrylic acid) hydrogel beads synthesized by inverse suspension polymerization using ammonium persulfate, *N,N,N′,N′*,-tetraethylmethylenediamine as the redox initiator. The influences of reaction variables on the polymerization, conversion, enzymatic activity, water absorption, and LCST properties were studied in detail. Evaluation of the enzymatic activities on various substrates was carried out for potential applications in the detergent industry. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 73: 2273–2291, 1999

Key words: alkaline protease; immobilization; polyacrylamide; poly(acrylamide-*co*-methacrylic acid); hydrogel beads

INTRODUCTION

Interest in immobilized enzymes and their applications in bioprocessing, analytical systems, and enzyme therapy have been exploited for many years. Immobilized enzymes can often be used more advantageously than the corresponding free enzymes. With immobilized enzymes, the process can be run continuously because of the resulting stabilization of enzymes when subjected to high temperature, pH, and inhibitor concentration.[1] Enzymes can be recovered and reused after reactions in any shape, such as membranes or beads, for fitting to specific reaction processes. Protease is the proteolytic enzyme that can be found in plants, animals, and microorganisms, such as fungi and bacteria. The protease used in this study was produced from a microorganism. Microbial proteases have been used in industrial applications, such as in detergents, tanning, photography, textile industries, and so on.[2] For the present research, we aim to stabilize the alkaline protease enzyme on polyacrylamide and poly(acrylamide-*co*-methacrylic acid) hydrogel beads to make the immobilized enzyme. These polymers have been widely applied in various fields of technology, such as flocculating, gelling, film-forming, and hydraulic-friction-reducing agents. The main synthetic method of acrylamide based polymers is by radical polymerization either in solution, inverse emulsion, or suspension.[3] Acrylamide is widely used as the matrix polymer for immobilized enzymes. Several authors have reported the properties of immobilized enzymes on polyacrylamide and supports, which were synthesized by differ-

Correspondence to: S. Kiatkamjornwong.

Contract grant sponsor: Research Division and also the Graduate School of Chulalongkorn University.

Journal of Applied Polymer Science, Vol. 73, 2273–2291 (1999)

 CCC 0021-8995/99/112273-19

Table I Standard Polymerization Recipes of AM and AM/MAA Polymerization

Parameter	Level				
	1	2	3	4	5
Acrylamide (AM) (m*M*)	3.14	4.57	6.28	9.14	—
N,N′-methylenebisacrylamide (N-MBA) (m*M*)	15	30	60	90	120
Pluronic PE 8100 surfactant (m*M*)	5.3	10.6	15.9	21.2	—
Ammonium persulfate (APS) (m*M*)	3.13	6.56	9.39	12.52	—
Alkaline protease ($\times 10^{-8}$) (m*M*)	5	10	30	50	100
N,N,N′,N′-tetraethylmethylene diamine (TEMED) (m*M*)	47.75	95.50	143.25	191.05	—
Stirring rate (rpm)	100	200	300	400	—
Polymerization temperature (°C)	0	10	20	30	40
Polymerization time (h)	1	2	3	4	—
AM-to-MAA[a] ratio (w/w %)	100 : 0	97.5 : 2.5	95 : 5	90 : 10	—

Paraffin wax used was 200 cm^3, and the quantity of the carbonate-bicarbonate buffer fixed at pH of 10.5 was 200 cm^3.
[a] MAA is methacrylic acid.

ent methods.[4–6] The immobilization of alkaline protease by inverse suspension polymerization through a redox initiation has not been published yet. Papain was immobilized on many polymer supports with spacer arms of varying natures and lengths. As the length of the spacer was increased, there was a marked increase in the extent of enzyme immobilization and activity of immobilization on nature of the support from which hydrophilic supports are more efficient in bringing about immobilization reactions, and they have more affinity towards substrates as indicated by their increased activities.[7] Another immobilization of papain was done on porous poly(γ-methyl *l*-glutamate) beads. The relative activity and stability of the immobilized papain was investigated.[8] Hayashi and Ikeda[9] found that the initial enzymatic activity of the covalently immobilized papain remained approximately unchanged with storage time. The thermal stability of the immobilized papain was also higher than that of the free papain. Chitosan beads were used as a support for protease immobilization. The activity of the immobilized protease was found to be still high toward small ester substrate, for example, *N*-benzyl-*l*-arginin ethyl ether (BAEE), but rather low toward casein, a high-molecular-weight substrate.

In this work, we synthesized polyacrylamide and poly(acrylamide-*co*-methacrylic acid) beads as the matrix polymers for simultaneously immobilizing alkaline protease enzyme from *Bacillus licheneformis* by inverse suspension polymerization, and we also elaborate on the reaction conditions that provided the optimum enzymatic activity and compared the activities of immobilized enzyme and free enzyme for the potential applications.

EXPERIMENTAL

Polymerization and Immobilization of Alkaline Protease

The acrylamide monomer, AR grade (Merck, Germany), and *N,N′*-methylene-bis-acrylamide crosslinker, AR grade (Fluka, Switzerland) were dissolved in 35 cm^3 of carbonate–bicarbonate buffer solution of pH 10.5, and this solution (Merck, Germany) was then mixed with 200-cm^3 paraffin wax (Fluka, Switzerland), which contains Pluronic PE 8100 surfactant (BASF, Thailand). The mixture was purged with nitrogen gas to remove oxygen. After purging, 5 cm^3 of ammonium persulfate (APS, Merck) and 5 cm^3 of alkaline protease P5380, Type VII, (Sigma, USA) with an average molecular weight of 25,000 were added and mixed rapidly. This solution was also purged with the nitrogen gas. After stable droplets of the monomer and crosslinker in the aqueous phase had been formed, *N,N,N′,N′*-tetraethylmethylenediamine (TEMED, from BDH, UK) was then injected into the paraffin phase to initiate the polymerization. The reaction flask was purged with nitrogen gas and stirred vigorously at a specific rate (see Table I) during polymerization. After the polymerization reaction had been completed, the mixture was transferred to a beaker, and an excess amount of the buffer solution

was added to separate the system into two phases. The beads were washed several times with the buffer solution and were then freeze-dried and stored in a refrigerator. The effects of monomer, enzyme, initiator, accelerator, crosslinker, surfactant concentrations, the acrylamide-to-methacrylic acid ratio, stirring rate, polymerization time, and temperature were studied. The dried samples were investigated for the protease enzyme activity, its percentage of immobilization, and the percentage of conversion of the monomer.

The products obtained were characterized for their protease activity. Infrared (IR) and thermal properties, morphology, water absorption, and protein staining were performed to identify the functional groups, appearance, and existence of the enzyme. Table I shows the polymerization recipes of AM and AM–methacrylic acid (MAA) polymerization. The optimum reaction conditions for homopolymerization and copolymerization were chosen for investigations of the effects of pH, temperature, protein digestion, and storage stability on activity. The durability of immobilized beads was also analyzed. Detailed procedures are described in the following sections.

Functional Group Identification

The acrylamide homopolymer and acrylamide–methacrylic-acid-crosslinked copolymer were identified for functional groups by a Fourier transform infrared (FTIR) spectrophotometer (1460X, Perkin–Elmer, USA). Standard FTIR spectra are used for comparison with those of the reaction products.

In Situ Immobilization of Alkaline Protease in Poly(acrylamide-*co*-methacrylic acid) by Inverse Suspension Polymerization

AM and methacrylic acid, MAA (Siam Resin & Chemical Thailand) were copolymerized in the presence of alkaline protease by the optimum conditions of PAM polymerization. AM : MAA in the ratios of 100 : 0, 97.5 : 2.5, 95 : 5 and 90 : 10 were used for the synthesis.

Thermal Analysis

The glass transition temperatures of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) were measured by the differential scanning calorimetric (DSC) technique.

Morphology of Polyacrylamide and Poly(acrylamide-*co*-methacrylic acid) Beads

Polyacrylamide and poly(acrylamide-*co*-methacrylic acid) beads were examined using a scanning electron microscope (JSM-35 CF, Jeol, Japan) for the surface and interior morphologies. The sizes of the beads were also measured by the scale on the micrographs.

Determination of Protease Activity

Protease activity was assayed by the modified method of Richardson and Whaiti.[10] The activities of free protease and immobilized protease were determined by using Hammersten-type casein (BDH, UK) as a substrate. The reaction mixture consisted of 1.9 cm^3 of 0.1*M* carbonate–bicarbonate buffer of pH 10.5, 0.1 cm^3 of free enzyme solution, or 0.05–0.1 g of the immobilized enzyme suspension in buffer which contained 1.0 cm^3 of 0.5% w/v casein solution (BDH, UK). This reaction mixture was incubated in a water bath at 45°C for 20 min, followed by stopping the reaction with 10% w/v of trichloroacetic acid (TCA, Fluka). Unhydrolyzed casein was removed by centrifugation at 3500 rpm for 20 min. The absorbance of the solution or the supernatant of free tyrosine liberated was measured at 280 nm with the ultraviolet (UV) spectrophotometer (DU 650, Beckman, USA) in a 1-cm quartz curvette. The enzyme activity was calculated as the amount of enzyme that liberated 1 μg of tyrosine from casein per minute per gram of enzyme (free or immobilized) under the above condition. The concentrations could be obtained from a calibration curve by measuring the absorbance of tyrosine samples at 280 nm.

The effect of pH on the activity of the immobilized and free protease was studied in several buffer solutions. The pH was adjusted with the following buffering systems: phosphate buffer (pH 7.5 and 8.0, Merck), tris-hydrochloride buffer (pH 8.5–9.0 from Merck), and carbonate–bicarbonate buffer (pH 9.5–11.0). The casein solution (Sigma) was prepared in the same buffering pH that was used for checking the enzymatic activity.

The effect of thermal stability on the enzymatic activity was carried out. The optimum temperatures for the activity of immobilized and free protease were determined by varying incubated temperatures at 25, 37, 45, and 60°C.

The effect of enzymatic activity on protein digestion was studied. The casein, bovine serum albumin (BSA, Sigma), gelatin (Sigma), animal

hair, and blood were used as substrates for checking the enzymatic activity. The ability of protein digestion by both the free and immobilized enzymes on activity were compared.

The effect of storage stability on the enzymatic activity was also examined. The free enzyme and immobilized enzyme were stored at the various temperatures of −20, 4, 25, 37, 45, and 60°C, and the residual enzymatic activity was checked every week for a total of one month.

Determination of Percentage Conversion

The experimental procedure for polymerizations was carried out as mentioned above. The percentage of conversion was defined as the weight of the total amount of polymer obtained from the weight of monomer charged.

Determination of Percentage of Enzymatic Immobilization

The enzymatic activities of free and immobilized enzymes were carried out as mentioned above. The total enzymatic activity of the immobilized enzyme was calculated by multiplying the enzymatic activity of the immobilized enzyme with the total weight of polymer obtained in the reaction. The percentage of enzymatic immobilization was then calculated by dividing the total enzymatic activity of the immobilized enzyme by the total enzymatic activity of the enzyme in the feed, as shown in eq. (1).

$$\% \text{ Enzyme immobilization} = \frac{A_i}{A_f} \times W_t \times 100 \quad (1)$$

where A_i is the activity of the immobilized enzyme in 1 g of the polymer, A_f is the total activity of the enzyme added to the feed, and W_t is the total weight of the polymer formed (g).

Determination of Water Absorption

Water absorption of the polyacrylamide and poly(acrylamide-*co*-methacrylic acid) beads was measured gravimetrically at room temperature. Dry bead samples were weighed (0.1 g) and equilibrated in 100 cm^3 of deionized water for 30 min. The swollen gels were poured in the filtering system and were left for 2 h to render a full swelling before recording the gel weight. The water absorption ratio was defined as the difference in weights of beads before and after being equilibrated in deionized water as grams of water/grams of the dry beads.

The water absorption in salt solutions was also studied. The same experimental procedure as described above was carried out, except that a series of salt solutions of 0.9% w/v sodium chloride [British Drug House (BDH)], potassium chloride, magnesium chloride (BDH), and calcium chloride (BDH) were used instead of the deionized water.

Protein Staining

This method was adapted from an electrophoretic analysis method for protein purification methods.[11] The staining solution was prepared by dissolving 1.0 g of Coomassie blue R-250 (Fluka) in 450 cm^3 of methanol (BDH), 450 cm^3 of distilled water, and 100 cm^3 of glacial acetic acid (Fluka). The destaining solution was prepared by mixing 100 cm^3 of methanol and 100 cm^3 of glacial acetic acid in 800 cm^3 of distilled water.

The free enzyme and enzyme immobilized beads were stained with the staining solution for 5–10 min in a small container, and the supernatant was then discarded. The destaining solution was slowly added into the beads with occasional shaking. The procedure could take up to 24 h but it could also be accelerated by using several changes of the destaining solution. The protein is then visualized as an intense blue color on the beads.

RESULTS AND DISCUSSION

IR Identification

Polyacrylamide and poly(acrylamide-*co*-methacrylic acid) were characterized by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) are compared with the standard spectra.[12] The polyacrylamide peaks were quite similar to those of poly(acrylamide-*co*-methacrylic acid). The poly(acrylamide-*co*-methacrylic acid) showed a peak difference from polyacrylamide spectra at around 1350–1250 cm^{-1} (C—O stretch) and 1500–1600 cm^{-1} (NH-bending band).

Thermal Analysis

Glass transition temperatures (T_g) of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) were measured by DSC. The sample weight used for the characterization was 10–12 mg. In the

present work, T_g of the crosslinked polyacrylamide is 220°C, while that of the uncrosslinked one[13–15] is 165 or 188°C,[14] depending on synthesis and measuring methods. Poly(acrylamide-*co*-methacrylic acid) shows the following two endothermic peaks: T_{g1} and T_{g2} at 228 and 258°C, respectively. Poly(methacrylic acid) gives a reference T_g at 106,[15] 130,[16] 141,[15] or 185°C,[16] depending on measuring techniques. The T_{g2} values of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) are higher than the reference values due to the effect of the crosslinking agent. The glass transition temperature is increased additionally by the formation of salts due to the incorporation of buffer solution.[17] Incorporation of *N,N′*-methylene-bis-acrylamide (MBA) restricts the mobility of polymer segments and, thus, increases T_g of polyacrylamide and poly(acrylamide-*co*-methacrylic acid).[18] An additional endothermic peak at around 80–110°C was also found. Based on several previous works, we confirm that imidization could take place in the polymerization of acrylamide in low nitrogen,[16] at high temperature,[19] and at very extreme pH values (lower than 2.5 or higher than 9),[20,21] which leads to partially insoluble products known as the imidization of polyacrylamide. In this study, we polymerized and immobilized alkaline protease on polyacrylamide and poly(acrylamide-*co*-methacrylic acid) in the carbonate–bicarbonate buffer of pH 10.5. The side reaction of intramolecular imidization can take place. The imidization affects the T_g of polyacrylamide and poly(acrylamide-*co*-methacrylic acid). The T_g values of both polyacrylamide and poly(acrylamide-*co*-methacrylic acid) are shifted to higher values than those in the literature.[13–16]

Effect of the Monomer Concentration on the Enzymatic Activity

The different concentrations of acrylamide monomer of 3.14, 4.57, 6.28, and 9.14 m*M* in 35 cm^3 of carbonate-bicarbonate buffer solution of pH 10.5 were investigated for enzymatic activity, the percentage of immobilization, and the percentage of conversion. The reaction system had a fixed concentration of MBA (120 m*M*), alkaline protease (5.0 mg/5 cm^3, or 1.0×10^{-6} m*M*), ammonium persulfate (APS) (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), a stirring rate of 300 rpm, and a time and temperature of polymerization of 2 h and 30°C, respectively (see Table I). The total enzymatic activity of free enzyme added in the reaction was 12,490 units. The enzymatic

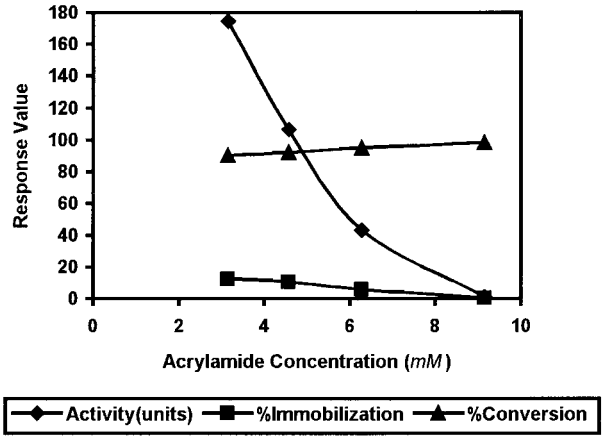

Figure 1 Effect of monomer concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

activity of immobilized enzyme, the percentage of immobilization, and the percentage of conversion on polyacrylamide for different acrylamide concentrations are given in Figure 1. It can be stated that the enzymatic activity and the percentage of immobilization of the immobilized enzyme decreased with increasing acrylamide concentration. The maximum of enzymatic activity of polyacrylamide beads was 175 units, with 12% immobilization and 90% conversion. The bead size increased with increasing acrylamide concentration, due to more diffusion of the monomers to reaction sites. The enzymatic activity decreased with increasing bead size. It could possibly be stated that bead size was affected by enzymatic activity of immobilized enzyme. The percentage of conversion of acrylamide increased with increasing acrylamide concentration. High concentrations of AM lead to more polymer chain networks that possibly increased the chance of crosslinking and decreased the soluble materials. For all the subsequent experiments, the acrylamide concentration used was 3.14 m*M,* based on the highest enzymatic activity.

Effect of the Enzyme Concentration on the Enzymatic Activity

Various amounts of alkaline protease of 0.25, 0.5, 1.5, 2.5, and 1×10^{-6} m*M* were investigated for enzymatic activity. The reaction system had fixed concentrations of AM (3.14 m*M*), MBA (120 m*M*), APS (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), a stirring rate of 300 rpm, and a time and temperature of polymeriza-

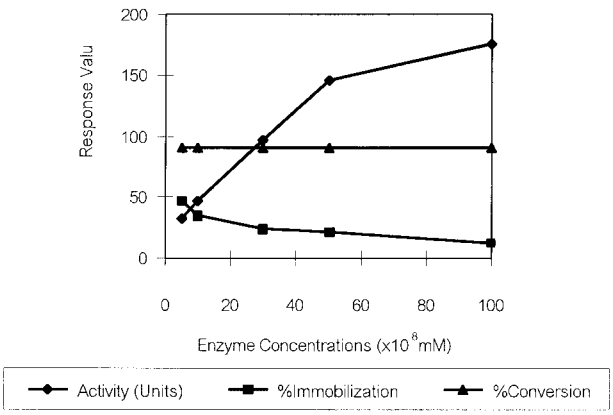

Figure 2 Effect of enzyme concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

tion of 2 h and 30°C, respectively (see Table I). Figure 2 reveals the results of enzymatic activity immobilized on the polyacrylamide for different protease concentrations. The enzymatic activity of immobilized alkaline protease increased with increasing amounts of alkaline protease and with a declining percentage immobilization of alkaline protease. The decreasing percentage immobilization on alkaline protease is almost proportional to the initial alkaline protease concentration from 5×10^{-8} to 5×10^{-7} m*M*. The current percentage of immobilization indicates clearly that limited amounts of enzyme can be immobilized on polyacrylamide beads. The free enzyme is claimed also to contribute to the increasing activity of the enzyme. The amount of enzyme used to further the study of reaction parameters was evaluated. Note that the low enzyme concentrations of 5×10^{-8} m*M* and 1×10^{-7} m*M,* giving the enzymatic activity of 32 and 47 units, are not used in our experiments and in the biotechnology industry owing to their low activity. The concentrations of the enzyme of 5×10^{-7} to 1×10^{-6} m*M,* which gave the enzymatic activity of 145 and 175 units, respectively, seem to be more appropriate for industrial applications. However, for the present experiments, we found that the free enzyme of 80–85% could not be immobilized on the beads, which is indeed wastage. The suitable concentration of enzyme for further investigations of the reaction parameters is essentially 3×10^{-7} m*M,* which gives the enzymatic activity of 97 units with 24% immobilization and 90% conversion. The percentage of conversion of polyacrylamide was not altered by changing the enzyme concentration because the percentage of conversion of acrylamide was constant at 90%.

Effect of the Polymerization Stirring Rate on the Enzymatic Activity

The stirring rates of 100, 200, 300, and 400 rpm were investigated for the enzymatic activity. The reaction was for fixed concentrations of AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (120 m*M*), APS (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), and for a time and temperature of polymerization of 2 h and 30°C, respectively (see Table I). Figure 3 shows the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized enzyme on polyacrylamide. The optimum of enzymatic activity was 97 units with 24% immobilization and 90% conversion. The percentage of conversion was not affected by the stirring rate. The polymerization stirring rate affected the bead sizes. The bead size decreased with increasing stirring rate, as shown in Figure 4. At the stirring rate of 400 rpm, a substantial amount of tiny beads were produced. The enzymatic activity increased with increasing stirring rate from 100 to 300 rpm and decreased slightly while increasing the stirring rate to 400 rpm. The slow stirring rate (100 rpm) could not migrate the monomers to the growing radicals. Once the initiation took place, the monomers propagated at the reactive sites. This reaction is thus a diffusion controlled kinetic reaction. The higher stirring rate (400 rpm) moved both the monomers and radicals freely to enable collisions among them to occur. Therefore, finer-sized particles were obtained. The finely sized particles could also aggregate to form a gel for the enzyme immobilization and also affected the enzymatic activity.

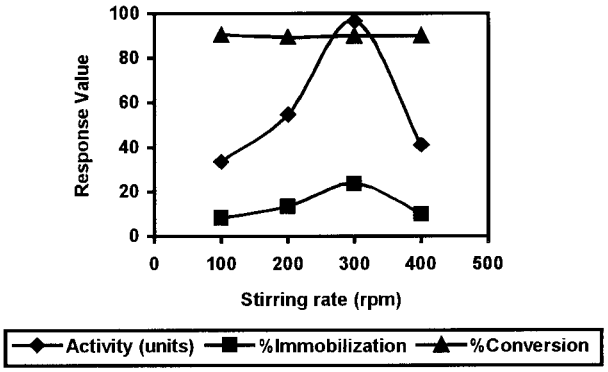

Figure 3 Effect of polymerization stirring rate on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

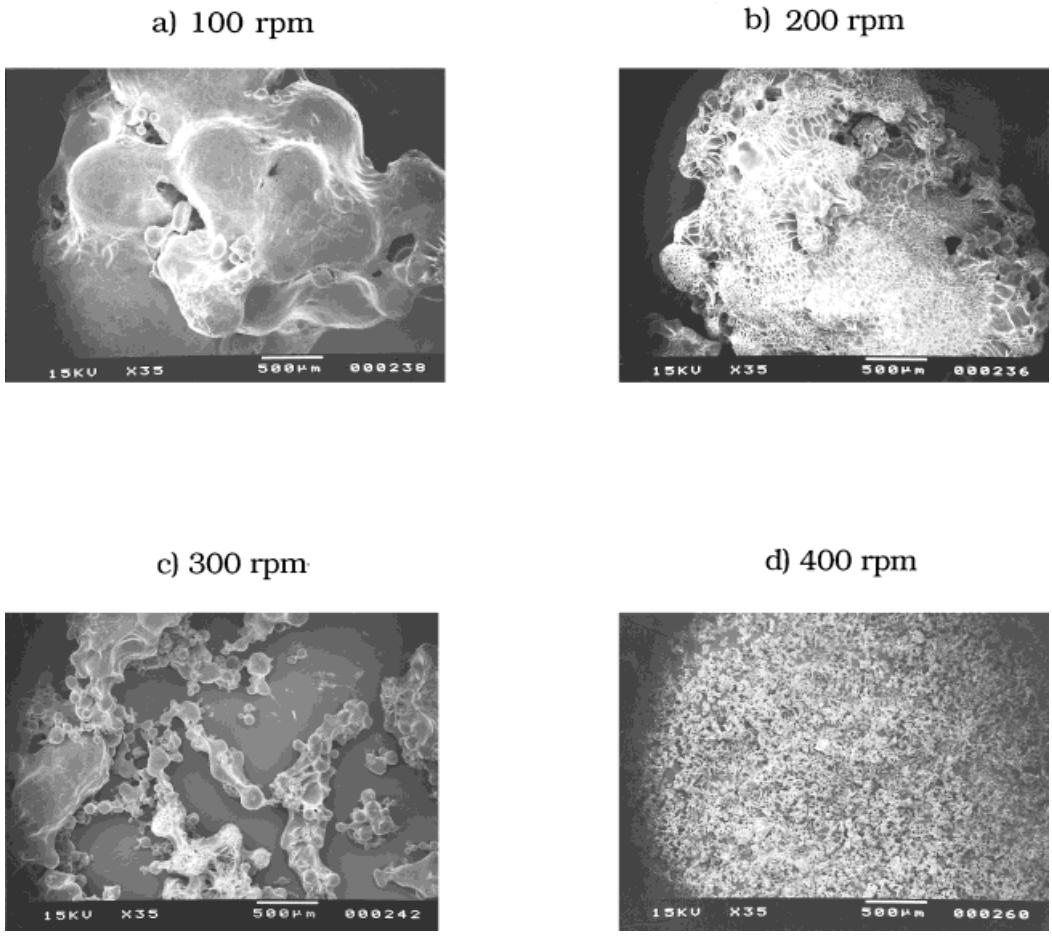

Figure 4 Effect of stirring rate on the beads morphology: (a) 100, (b) 200, (c) 300, and (d) 400 rpm.

Effect of the Polymerization Time on the Enzymatic Activity

The variation of polymerization time was investigated at 1, 2, 3, and 4 h. The reactions were carried out with fixed AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (120 m*M*), APS (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), a stirring rate of 300 rpm and a polymerization temperature of 30°C (see Table I). The results can be seen in Figure 5. The desirable polymerization time that gave the optimum enzymatic activity of immobilized enzyme onto polyacrylamide beads was 2 h with 97 units of enzymatic activity. Polymerization time affected the enzymatic activity, and the percentage of conversion decreased slightly (about 90% throughout the reaction time of 4 h). The 90% conversion from a 2 h polymerization time provided the highest enzymatic activity and percentage immobilization. The bead sizes of different polymerization times ranged from 80–100 μm, as shown in Figure 6. The beads polymerized for 1 and 2 h showed more surface porosity than those obtained for the longer reaction times. Smaller beads with

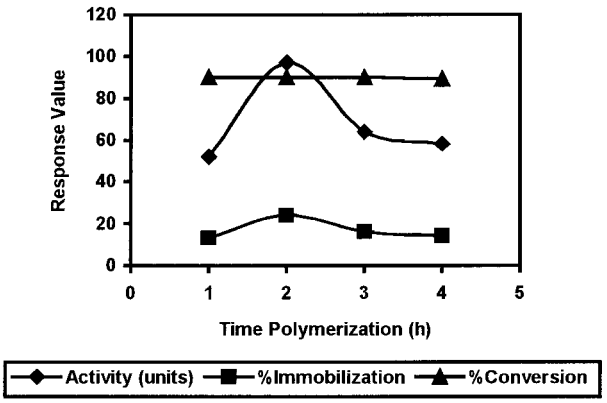

Figure 5 Effect of polymerization time on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

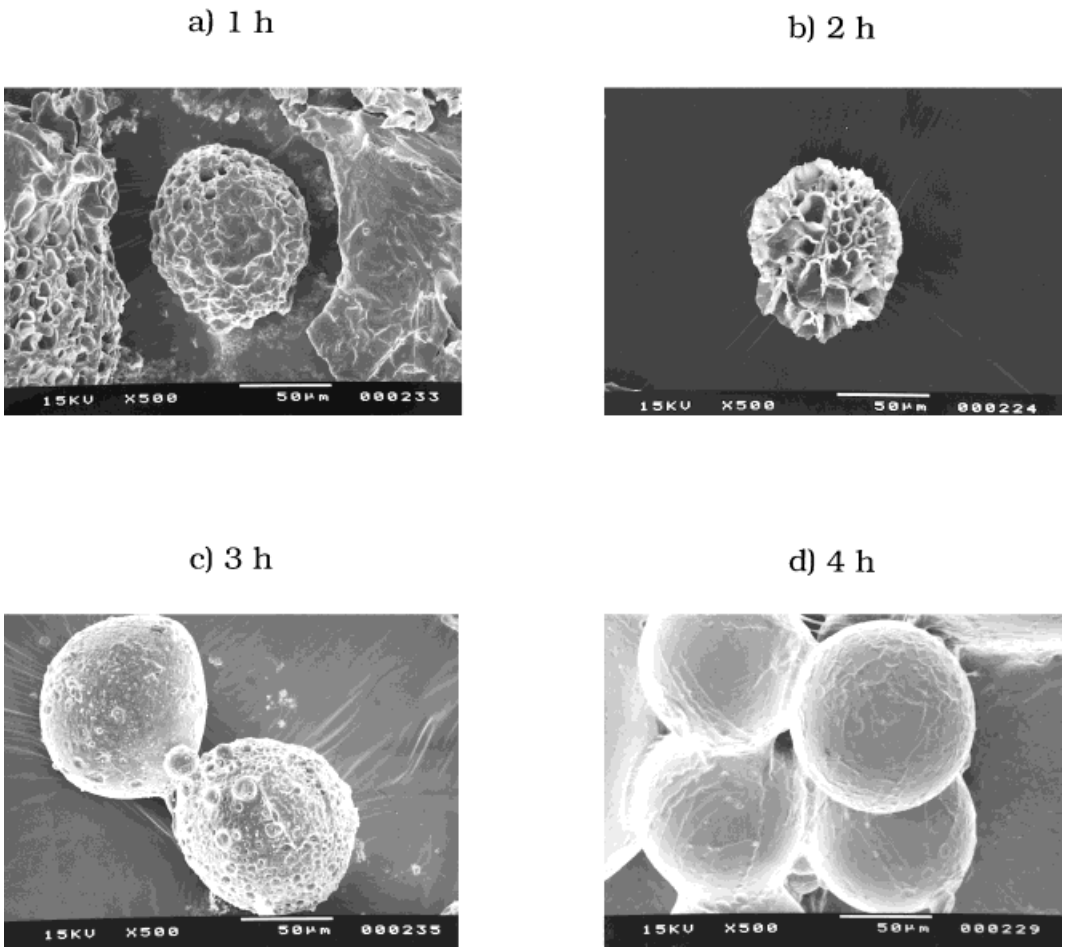

Figure 6 SEM of polyacrylamide beads produced by the polymerization times of (a) 1, (b) 2, (c) 3, and (d) 4 h.

more small pores obstructed the ability of the substrate to penetrate into the pores to react with the entrapped enzyme and to release the soluble reaction products to the outside of the bead. Therefore, 2 h of polymerization time was then chosen for further studies of the reaction parameters.

Effect of the Polymerization Temperature on the Enzymatic Activity

Effect of polymerization temperature was studied at 0, 10, 20, 30, and 40°C. The reaction conditions had fixed concentrations of AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (120 m*M*), APS (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), a stirring rate of 300 rpm, and a polymerization time of 2 h. Figure 7 shows the enzymatic activity, the percentage of immobilization, and the percentage of conversion of polyacrylamide beads at different polymerization temperatures. The optimum temperature was found at 30°C for the enzymatic activity of 97 units with 24% immobilization and 90% conversion. The percentage conversion was almost con-

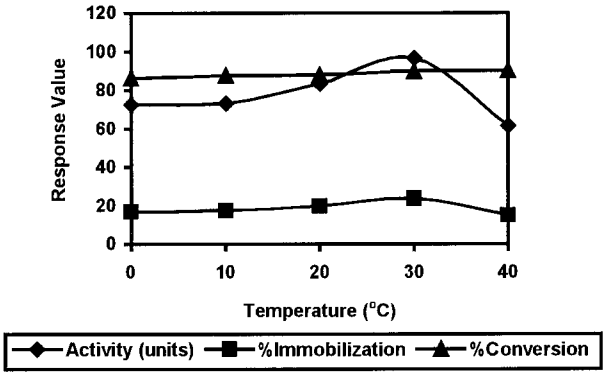

Figure 7 Effect of polymerization temperature on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

stant with an increasing reaction temperature. At the lowest temperature (0°C), the initiation and propagation rates were relatively slow, leading to a somewhat lower conversion. The percentage immobilization of the enzyme was highest at 30°C and decreased when the temperatures were further increased. For the lower reaction temperatures, the enzyme molecules moved slowly to the beads due to their molecular bulkiness. The enzyme was consequently less active than that at 30°C. At the temperatures higher than 40°C, the enzyme loses its enzymatic activity through a protein denaturation. The increase in bead sizes with decreasing polymerization temperatures (shown in Fig. 8) may be due to coagulation, which may possibly be caused by the longer kinetic chain length of polymer growing chains. The beads gelled together (polymerized) below 30°C, and the gelled beads were cloudy before freeze drying, but the beads polymerized at 30°C or higher temperatures are clear and transparent. At lower temperatures, the overall activation energy was not sufficient to diffuse the initiator, crosslinker, monomer, and enzyme to enable the reaction to occur. Once an initiation at one active site could occur, the active components in the close vicinity diffused slowly to each other to form many pieces of tiny beads, which fused into one giant bead. At 40°C polymerization temperature, the active ingredient could diffuse much better, which allowed a higher frequency of collisions upon encounters. However, the bead surface was rather more porous than for low temperature polymerizations (0, 10, and 20°C), and the bead sizes were also relatively smaller. It is already well known that the enzymatic activity was affected by the bead size and its morphology. On a porous bead, there is a higher ability of the substrate to penetrate into the porous polyacrylamide surface to react with the immobilized enzyme to give a high value of enzymatic activity. From the results, the suitable polymerization temperature of 30°C was then chosen for further studies of the reaction parameters.

Effect of the Crosslinker Concentration on the Enzymatic Activity

We used *N,N′*-methylene-bis-acrylamide (MBA from Fluka) as a crosslinker. Figure 9 shows the effects of the crosslinker on enzymatic activity, the percentage of immobilization, and the percentage of conversion. The different concentrations of MBA were tested with the fixed concentrations of AM (3.14 m*M*), alkaline protease (3 $\times$ 10^{-7} m*M*), APS (6.56 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*), a stirring rate of 300 rpm, a polymerization time 2 h, and a polymerization temperature of 30°C. The enzymatic activity of the immobilized enzyme was decreased with increasing MBA concentration. The results indicate that the enzymatic activity was dependent on the rigidity of the polyacrylamide chains due to crosslinking (since the polyacrylamide beads containing 120 m*M* of MBA only gave an enzymatic activity of 97 units with 24% immobilization, whereas the beads having 15 m*M* of MBA produced an enzymatic activity of 150 units with 34% immobilization). Percentage conversion was relatively constant when increasing the MBA concentration. We anticipate that the higher crosslinking network takes up the available molecular spaces within the pores. The incoming enzyme molecules cannot fit in those smaller molecular spaces, and this slightly reduces the immobilization of the enzyme. Based on the results in Figure 9, the concentration of 25 m*M* MBA is the upper limit of the crosslinking reaction. Above this concentration, there was almost a constant percentage immobilization. The enzymatic activity shows the same trend.

Effect of the Initiator Concentration on the Enzymatic Activity

Ammonium persulfate (APS) was used as an initiator. Figure 10 shows the effect of initiator concentrations of 3.13, 6.56, 9.39, and 12.52 m*M* on the enzymatic activity, the percentage of immobilization, and the percentage of conversion. The reaction was carried out by fixing the concentrations of AM (3.14 m*M*), alkaline protease (3 $\times$ 10^{-7} m*M*), MBA (15 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (5.6 m*M*), and with a stirring rate 300 rpm, a polymerization time of 2 h, and a polymerization temperature of 30°C. As the APS concentration increased, the enzymatic activity and the percentage of immobilization increased and reached a maximum at the APS concentration of 6.56 m*M*. When the APS concentration was higher than 6.56 m*M,* the enzymatic activity and percentage immobilization decreased. Higher APS concentrations lead to an increase in the number of radicals, thereby giving an increase in the polymerization rate. The short kinetic chain lengths of the network increased the hydrophilic nature due to the presence of many units of acrylamide functionality on the chain. The ability of the immobilized enzyme to anchor on the highly hydrophilic support decreased with

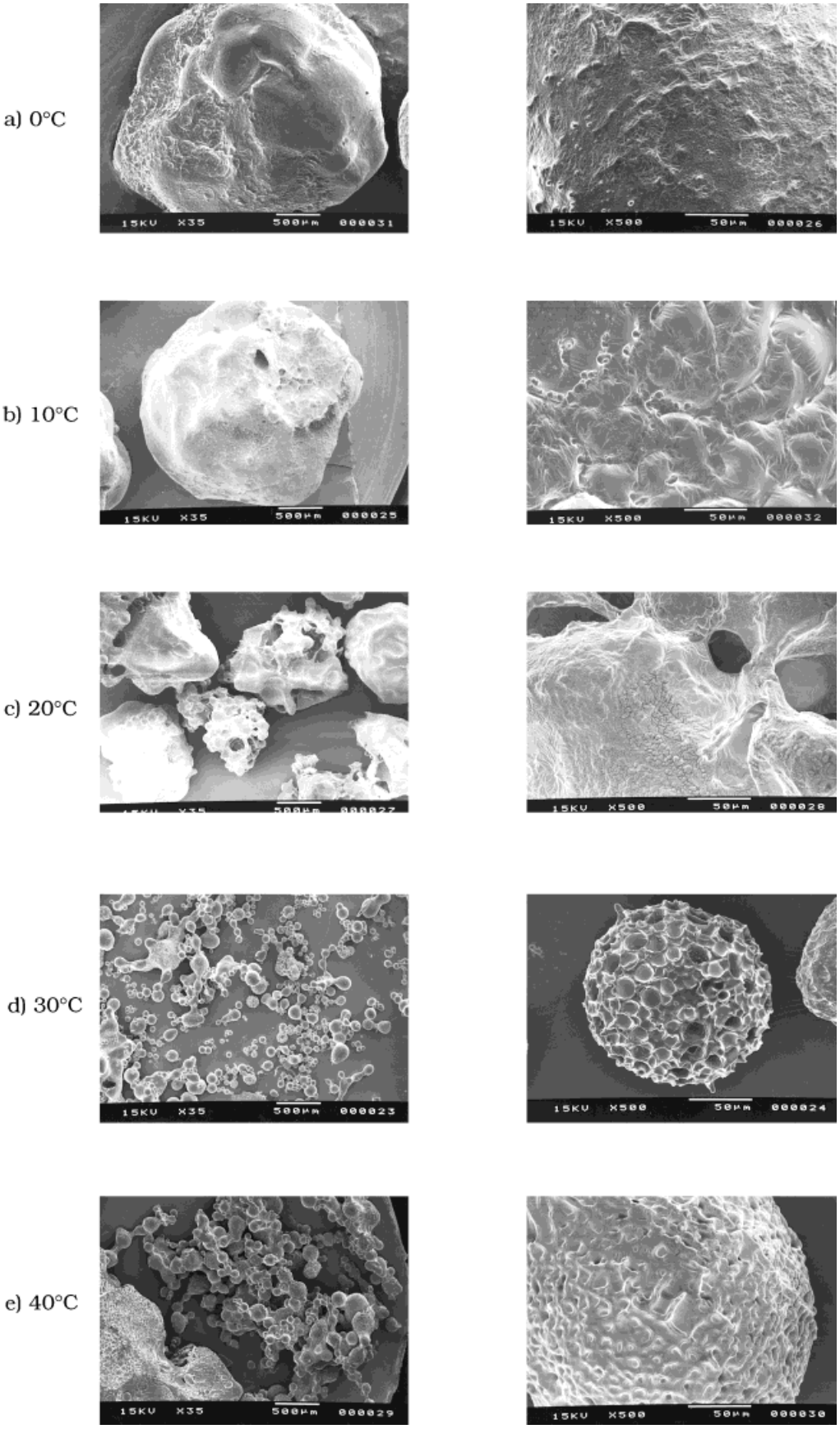

Figure 8 Effect of polymerization temperature on bead sizes at different times of (a) 0, (b) 10, (c) 20, (d) 30, and (e) 40°C.

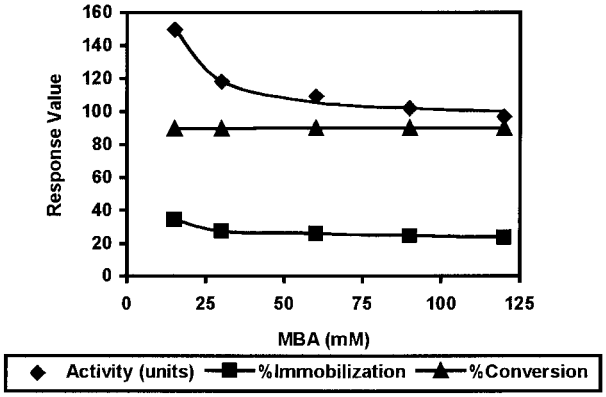

Figure 9 Effect of crosslinker concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide beads.

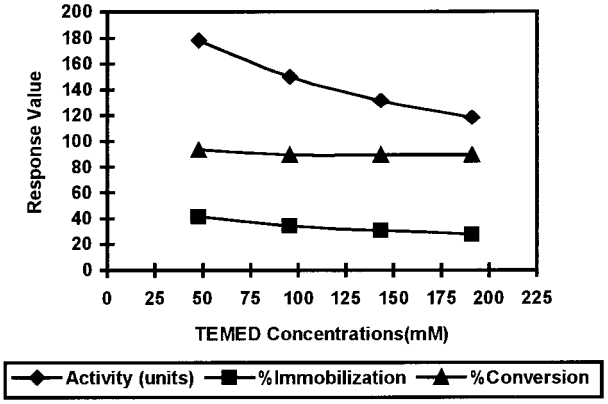

Figure 11 Effect of accelerator concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

increasing APS concentration because of the many short chains of polyacrylamide, the presence of the sulfate group, and the electrostatic repulsion between the sulfate and acrylamide functional groups. The enzymatic activity decreased when the APS concentration was greater than 6.56 m*M*. Changing the concentration of APS did not affect the percentage of conversion. The conversion was nearly constant with any increase in the APS concentration.

Effect of the Accelerator Concentration on the Enzymatic Activity

We used *N,N,N′,N′*-tetraethylmethylenediamine (TEMED) with ammonium persulfate as a redox accelerator. The different TEMED concentrations were 47.75, 95.50, 143.25, and 191.05 m*M*. The reaction was carried out by fixing the concentration of AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (15 m*M*), APS (6.56 m*M*), and Pluronic PE 8100 (10.6 m*M*) and the stirring rate (300 rpm), time (2 h) and temperature (30°C). Figure 11 depicts the enzymatic activity, the percentage of immobilization, and the percentage of conversion. As the TEMED concentration increased, the enzymatic activity and percentage immobilization decreased. The optimum enzymatic activity observed was 178 units for a TEMED concentration of 47.75 m*M*. Conversion and immobilization decreased slightly with increasing TEMED concentration. Increasing the TEMED concentration increased the amount of radicals for initiation, giving an increase in the polymerization rate. At this point, a greater amount of polyacrylamide chains with low molecular weights could dissolve, which limited the amount of insoluble PAMs.

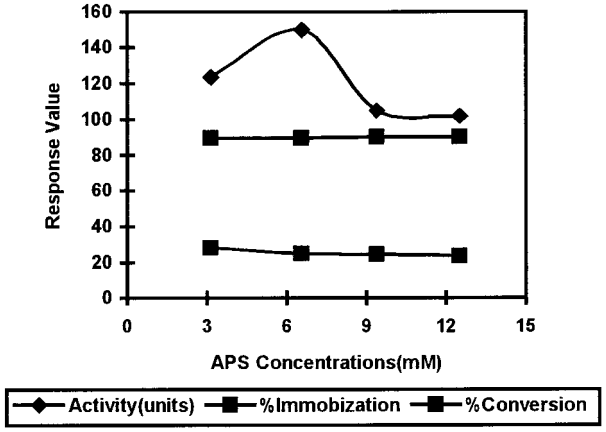

Figure 10 Effect of initiator concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

Effect of the Surfactant Concentration on the Enzymatic Activity

Pluronic PE 8100 was used as surfactant. Various Pluronic PE 8100 concentrations were investigated for enzymatic activity, the percentage of immobilization, and the percentage of conversion. The reaction was carried out by fixing the concentrations of AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (15 m*M*), and TEMED (47.75 m*M*), as well as the stirring rate (300 rpm), time (2 h), and temperature (30°C) (see Table I). The optimal enzymatic activity, the percentage of immobilization, and the percentage of conversion

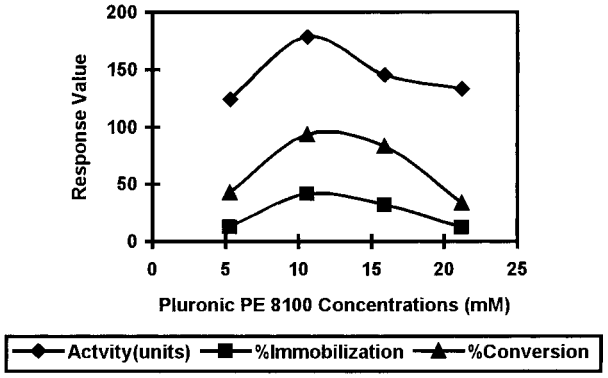

Figure 12 Effect of surfactant concentration on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

were found to be 178 units, 42% immobilization, and 93% conversion, respectively, as shown in Figure 12. The different concentrations of surfactant affected the immobilization and the conversion of monomers. The bead sizes were also affected by the surfactant concentration. At low surfactant concentration, there was not enough surfactant to stabilize monomer droplets. At high surfactant concentration, the surfactant can form many small monomer droplets. Beads having both high and low molecular weights were obtained. The beads with the low molecular weights could not precipitate out to give the stable beads, so the percentage conversion decreased with increasing surfactant concentration. Only at 10.6 m*M* of Pluronic PE 8100 did the surfactant concentration permit the formation of stable monomer droplets for polymerization, which, consequently, gave a high conversion of 93% along with good properties of enzymatic activity and the percentage of immobilization of the enzyme.

Effect of the Acrylamide-to-Methacrylic Acid Ratio on the Enzymatic Activity

The ratios of acrylamide to methacrylic acid of 100 : 0, 97.5 : 2.5, 95 : 5, and 90 : 10% w/w were investigated by fixing the concentration of AM (3.14 m*M*), alkaline protease (3×10^{-7} m*M*), MBA (15 m*M*), TEMED (95.50 m*M*), and Pluronic PE 8100 (10.6 m*M*) and the stirring rate (300 rpm), polymerization time (2 h), and temperature (30°C). The results are indicated in Figure 13. The reactivity of poly(AM-*co*-MAA) in the carbonate–bicarbonate buffer ($NaCO_3$–$NaHCO_3$) with a pH of 10.5 and polymerization temperature of 30°C has not been reported elsewhere. Regarding the reactivity of poly(AM-*co*-MAA) in a solution containing a sodium salt and at the temperature of 30°C,[15] the r_1 and r_2 of acrylamide and methacrylic acid were 0.42 and 0.59, respectively. When $r_1r_2 = 0.25$ ($0 < r_1r_2 < 1$), that is $r_1 < 1$ and $r_2 < 1$, the behavior of the comonomer lies between extremes of ideal copolymerization ($r_1r_2 = 1$) and alternating ($r_1r_2 = 0$) copolymerization. As r_1r_2 decreases from unity toward zero, there is an increasing tendency toward alternation.[22] The enzymatic activity and the percentage of immobilization decreased with increasing methacrylic acid monomer concentration due to the effect of ionic side chains. The COO^- side chains of the MAA affect the conformation of the alkaline protease because it becomes sterically hindering. Polyacrylamide beads gave the optimum activity of 150 units, whereas the poly(acrylamide-*co*-methacrylic acid) provided the optimum activity of 144 units at 97.5 : 2.5% w/w of acrylamide/MAA comonomers. The MAA comonomer was used for enhancing the hydrophilicity of the polyacrylamide support. The water absorption of poly(acrylamide-*co*-methacrylic acid) increased with increasing MAA concentration. The gel strength thereby decreased, so the ability to function as the support for immobilizing decreased substantially. The increasing methacrylic acid concentration unfortunately decreased the polymerization conversion slightly. The conversion of polyacrylamide was higher than that of poly(acrylamide-*co*-methacrylic acid) at the same reaction conditions. The advantage to include the MAA moiety is to increase mass transport in terms of water absorption in relation to the solution temperature.

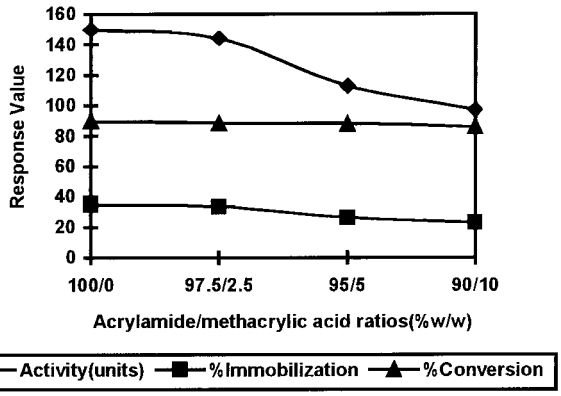

Figure 13 Effect of the acrylamide-to-methacrylic acid ratio on the enzymatic activity, the percentage of immobilization, and the percentage of conversion of immobilized alkaline protease on polyacrylamide.

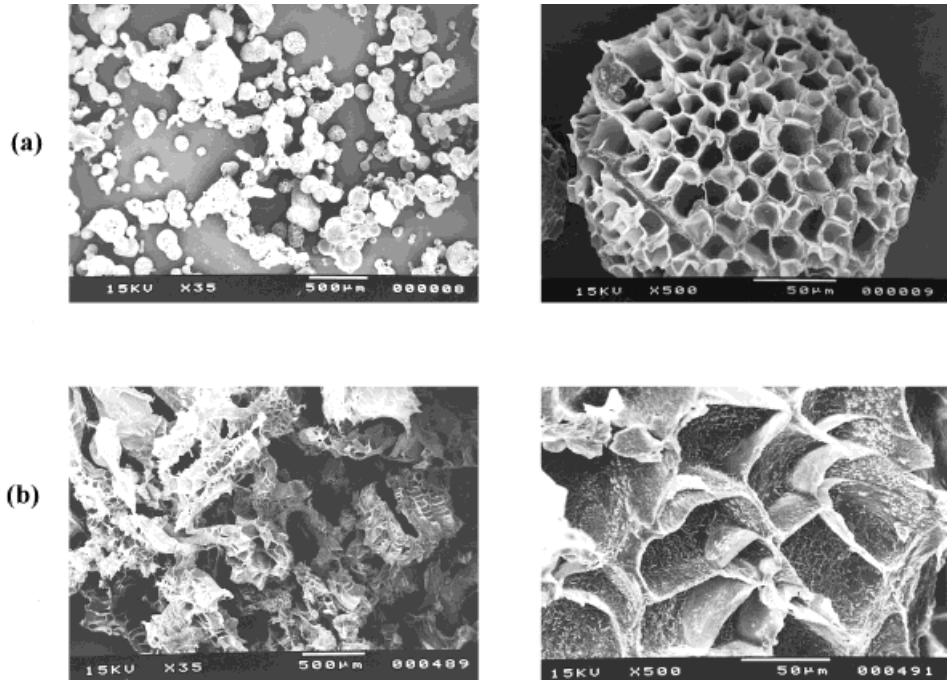

Figure 14 SEMs of polyacrylamide and poly(acrylamide-*co*-methacrylic acid): (a) polyacrylamide and (b) 90 : 10% w/w of poly(acrylamide-*co*-methacrylic acid): left, ×35 magnification; right, ×500 magnification.

The beads examined with a scanning electron microscope, as shown in Figure 14. The electron micrographs of the beads revealed a porous structure with netting on the surface. Changing the reaction condition of polymerization affected the surface, sizes and formation of beads. The major concern is the content of acrylamide and MAA in the reaction. The presence of MAA leads to a larger pore size with netting on the beads.

Water Absorption

The absorptions in deionized water and saline solutions of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) were investigated. Table II shows the effects on water absorption in deionized water, 0.9% NaCl, 0.9% KCl, 0.9% $MgCl_2$, and 0.9% $CaCl_2$ of polyacrylamide and poly(acrylamide-*co*-methacrylic acid) hydrogel beads. Water absorption was determined for acrylamide and methacrylic acid monomer ratios of 100 : 0, 97.5 : 2.5, 95 : 5, 90 : 10% w/w. The water absorption increased with increasing the MAA concentration. The MAA concentration increased the hydrophilicity through hydrogen bonding and charge repulsion. The water absorption was found to be of 37, 67, 86, and 99 $g \cdot g^{-1}$ for 100 : 0, 97.5 : 2.5,

Table II Absorption in Deionized Water and Saline Solutions

	Absorption Ratios				
AM/MAA Ratios (% w/w^{-1})	Deionized Water	0.9% NaCl	0.9% KCl	0.9% $CaCl_2$	0.9% $MgCl_2$
100 : 0	37 ± 9	37 ± 3	36 ± 1	39 ± 3	35 ± 2
97.5 : 2.5	67 ± 4	38 ± 3	37 ± 4	39 ± 3	37 ± 2
95 : 5	86 ± 6	38 ± 2	38 ± 1	39 ± 2	37 ± 3
90 : 10	99 ± 6	37 ± 2	37 ± 1	33 ± 4	31 ± 2
90 : 10[a]	84 ± 1	30 ± 2	29 ± 2	29 ± 1	22 ± 3

[a] The polymeric beads without immobilization of the enzyme.

95 : 5, and 90 : 10% w/w, respectively. Water absorptions of the beads composed of AM–MAA (90 : 10) without immobilization of alkaline protease were lower both in distilled water and 0.9% saline solutions. The enzyme immobilization affected water absorption of the beads of PAM/PMAA, which was possibly caused by the physicochemical interaction. The immobilized enzyme on the polymer chains restricts the carboxylate ions to bind with the cations of the saline solution. The free PAM or PAM/PMAA chains had more freedom to interact with the cations of the saline solution, therefore, the polymer membrane can absorb less water. The absorption abilities of polyacrylamide in deionized water and in saline solutions were not very different. It is believed that the basicity of the amide group contributed to the constant equilibrium osmotic pressure for both deionized distilled water and saline solutions at the isotonic point (0.9%). The water absorption of poly(acrylamide-*co*-methacrylic acid) in deionized water was higher than for 0.9% saline solutions. In saline solutions, the effects of different AM : MAA ratios were not significant. The ionic strength and oxidation states of the cations do not affect the water absorption. This property is of great importance, as it implies that the membrane properties of various poly(AM-*co*-MAA)s were almost identical to permit a constant equilibrium osmotic pressure. The membrane properties can plausibly be controlled partly by the number-average molecular weight of the crosslinks, that is, the crosslink density of each copolymer is constant. An additional attribute could be the buffering effect. The water content in a hydrogel has been known to be a critical factor in determining overall transport rate[23] since the permeation of solute across the membrane occurs mostly by diffusion. The porous structure of hydrogel and their connections between pores, porosity, and tortuousity determine the overall effective diffusivity.

Effect of Temperature on Water Absorption

Water absorption of the beads in deionized, distilled water depends slightly on temperature, as shown in Table III. Increasing the temperature from 25 to 45°C does not affect the water absorption of polyacrylamide, but the water absorption of poly(acrylamide-*co*-methacrylic acid) hydrogel beads does decrease. We can observe changes of clarity and volume of the polymer beads at different temperatures. The clarity of the hydrogel beads increased with increasing MAA concentration. The results indicate that poly(acrylamide-*co*-methacrylic acid) hydrogel exhibits a lower critical solution temperature (LCST) at around 30–35°C in distilled water because it swells below 35°C (higher water absorption) and shrinks above 35°C (lower water absorption). The decrease of pore volume consequently reduced the amount of free water in the hydrogel at higher temperatures. In this case, the aqueous/PAM and aqueous/P(AM-MAA) undergo a temperature-induced phase transition at approximately 30–35°C. According to Park and Hoffman,[23] enzymatic activity by thermal cycling of the gel below its LCST can be enhanced. They explained the enhancement as due to the increased mass transport (reduced mass transport resistance) of the substrate in and out, as the shrinking and reswelling of the gel beads act to "pump the gel pore fluid out and draw in". Any product inhibition would also be reduced by this system. When the gel beads reswell, the product reequilibrates into the pore from the environment. When the temperature once again rises, there is a rapid and efficient removal of the product as the gel beads shrink. This reduces the influences of product inhibition within the enzyme, which is a common problem in many immobilized systems.

Table III Effect of Temperature on Water Absorption

Temperature (°C)	Acrylamide-to-Methacrylic Acid Ratio (w/w %)			
	100 : 0	97.5 : 2.5	95 : 5	90 : 10
25	37 ± 9	62 ± 4	88 ± 6	99 ± 6
30	34 ± 2	59 ± 6	86 ± 9	98 ± 3
35	34 ± 1	54 ± 2	77 ± 1	90 ± 1
40	30 ± 3	47 ± 2	61 ± 2	81 ± 4
45	35 ± 8	49 ± 5	64 ± 2	88 ± 5

Kawaguchi[24] synthesized poly(acrylamide-*co*-methacrylic acid) in uniform-sized microspheres that can swell and shrink at a transition temperature of 32°C. The particle size swelled to a diameter about 3 μm at low temperatures and shrank to 0.5 μm at temperatures higher than about 32°C. More support can be obtained from the work of Gutoaska et al.,[25] who prepared a thermal sensitive hydrogel of *N*-isopropyl acrylamide, acrylic acid, and butyl methacrylate. The incorporation of hydrophobic and hydrophilic comonomers strongly influences the swelling/shrinking behavior of thermal sensitive hydrogels. The hy-

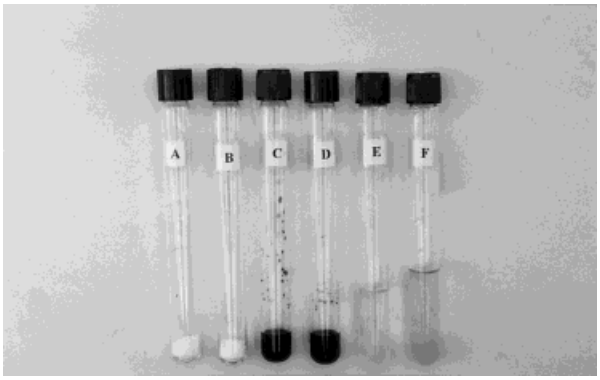

Figure 15 The protein staining of polyacrylamide beads in the presence and absence of immobilized protease enzyme (see text for details).

drophobic comonomer decreased the gel collapse temperature, whereas the hydrophilic comonomer increased the gel collapse temperature. For our case, we did observe the change in the vicinity of 20% of the size of the bead. We cannot indicate precisely the exact diameter since our beads are not perfectly spherical. Therefore, it is logical to state that the present poly(acrylamide-*co*-methacrylic acid) is a thermally reversible hydrogel with an LCST. More hydrophilic interaction of methacrylic acid units increases the LCST when increasing the methacrylic acid concentration.

The inclusion of MMA units improves the mass transfer of the materials in the beads, and the exhibition of LCST helps diffuse the fluid in and out of the gel pore. This will increase the overall transport rate of the whole process. This advantage can outweigh the disadvantage of the lower enzymatic activity.

Protein Staining

After binding the immobilized enzyme on polyacrylamide beads, the intense blue color was observed. Figure 15 shows the results of protein staining with and without immobilized enzyme on polyacrylamide beads. Tubes (A), (C), and (E) contain the enzyme-free polyacrylamide beads, and tubes (B), (D), and (F) contain the immobilized enzyme on polyacrylamide beads. After binding the enzyme, Tube (F) shows the blue color of the immobilized enzyme, whereas Tube (E), with enzyme-free beads, shows a clear solution. This result indicates that the enzyme is possibly entrapped in the polyacrylamide beads. The crosslinked polyacrylamide has a constrained structure, which is tight enough to hold the enzyme and prevents the enzyme from diffusing out into the surrounding medium. The intensity of blue color of protein staining was used to quantify the amount of enzyme immobilized in the polymeric beads for a primary screening before determining the enzymatic activity by using a casein substrate. Figure 16 shows the binding of immobilized enzyme in the polyacrylamide and poly(acrylamide-*co*-methacrylic acid) beads. The beads containing acrylamide : methacrylic acid of 100 : 0, 97.5 : 2.5, 95 : 5, and 90 : 10% w/w, as shown in tubes (A)–(D), were prepared; and the enzymatic activities of the immobilized enzyme were found to be 150, 144, 113, and 97 units, respectively. The activities imply that a polyacrylamide bead is a better enzymatic immobilizer. The enzyme-free polyacrylamide beads as a control shown in tube (E) (colorless) help indicate that the intensity of blue color increased with increasing enzyme content in the polymeric beads.

Effect of Protein Digestion

Immobilized alkaline protease in polyacrylamide with 0 or 2.5% w/w of poly(acrylamide-*co*-methacrylic acid) was used as a model for investigating the effect of protein digestion. Different substrates of casein Hammerstein, Bovine Serum Albumin (BSA), blood solution, gelatin, and animal hairs were investigated (Table IV). The concentrations of casein, BSA, and gelation were 0.5% w/v in a carbonate–bicarbonate buffer solution of pH 10.5. The blood solution was diluted 20 times with the carbonate–bicarbonate buffer solution of pH 10.5. The animal hair was weighed to 0.020 g and suspended in 1 cm^3 of the carbonate–bicarbonate buffer of pH 10.5. Alkaline protease

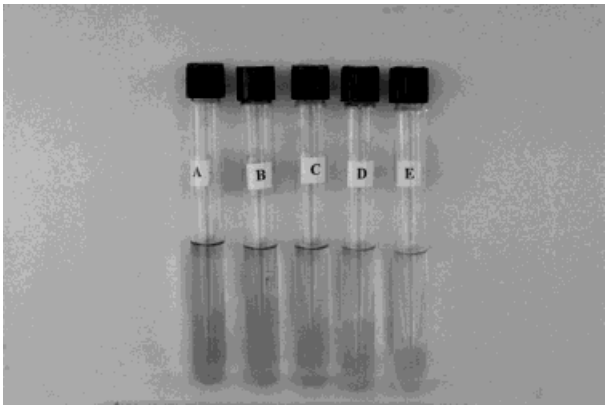

Figure 16 The binding of immobilized enzyme in the polyacrylamide and poly(acrylamide-*co*-methacrylic acid) beads (see text for details).

Table IV Effect of Free and Immobilized Enzymes on Protein Digestion

	% Relative Activity		
Substrates	Free Enzyme	Immobilized Enzyme on Polyacrylamide	Immobilized Enzyme on Poly(AM-*co*-MAA)
Casein	97.4 ± 1.8	99.2 ± 0.6	96.0 ± 0.9
BSA	55.6 ± 1.2	26.8 ± 2.1	22.9 ± 1.3
Gelatin	—	—	—
Blood	105.4 ± 1.6	26.7 ± 1.9	25.5 ± 2.3
Animal hair	17.4 ± 0.6	—	—

has performed excellently in detergents for many years. This enzyme is suitable for use in the detergent industry for removal of the protein from body secretions and skin particles and food, such as milk, egg, meat, and fish, and plant materials, such as grass.[2] In the washing process, this enzyme can be used under alkaline conditions and in the presence of heat or a surfactant. Accordingly, we selected casein, BSA, and blood as the proteinaceous dirt adsorbed in clothes for evaluation of immobilized enzyme activities. The immobilized enzyme showed a higher stability than the free alkaline form. The results of enzyme digestion on different proteins are shown in Table IV as percentage relative activity (% RA), in which the enzymatic activities of free and immobilized enzymes of 1.2×10^6 and 150 units are set 100% RA, respectively. The free enzyme shows a greater ability to digest different substrates than the immobilized enzyme does because the immobilized enzyme is entrapped in a polymeric support for prevention of thermal denaturation. The proteolytic velocity of the immobilized enzyme was therefore slower than for the free enzyme. The free and immobilized enzymes could digest blood, casein, and BSA. In the protein-staining test, we anticipated that the enzyme was entrapped in the pores of polyacrylamide gel and no leakage under washing could occur. The substrates with a low molecular size can diffuse easily and penetrate into the pores of the polymer support to react with the enzyme and to release the product to outside the beads. Casein, having a lower average molecular weight (approximately 23,600)[26] than that of BSA (approximately 66,000)[27] and blood, shows a higher enzymatic activity than the latter. Our result shows the similar direction with those of Hayashi and Ikeda.[9] The protease immobilization on chitosan beads gave rather low activity to high-molecular-weight substrate like casein compared to BAEE. In addition, protease is used for unhairing and batting in the tanning industry. In this study, the gelatin and animal hairs are represented as protein sources found on the hide. The results show that the gelatin could not be digested by both the free and immobilized enzyme because gelatin formed a gel solution that is too viscous to react with the enzyme. In the suspension of animal hair, the free enzyme can diffuse to this substrate, but the immobilized enzymes cannot, and the enzymatic activity was therefore not detected. The suspended animal hair solution as a substrate lowers the activity of the immobilized enzymes because the diffusion limit of the animal hair substrate to the active site of the enzyme due to steric hindrance caused by the enzyme immobilization is the main attribute, leading to a reduced activity of the immobilized enzyme in the polymer supports. We suggest that the immobilized enzyme may be suitable for the detergent industry but not for the tanning industry.

Effect of Washing on the Leakage of Enzyme Molecules

Figure 17 presents the enzymatic activity of immobilized enzymes, using casein as substrate at

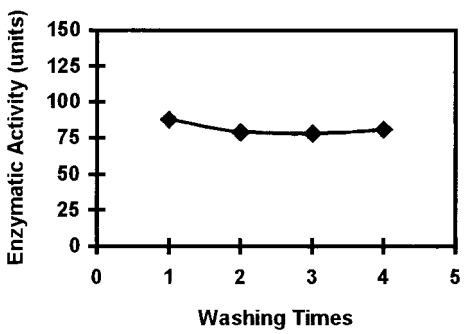

Figure 17 Amount of the leakage enzyme molecules on the beads under washing.

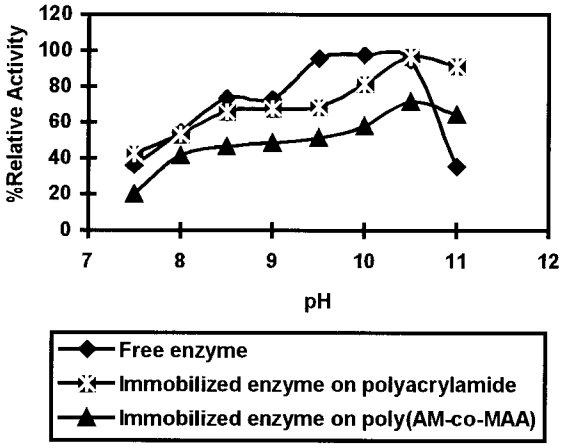

Figure 18 Effect of pH on the enzymatic activity.

pH 10.5 and at 45°C. The dried beads with a known enzymatic activity were washed for one to four times by the carbonate–bicarbonate buffer solution of pH 10.5. The washed beads were dried, and the enzymatic activity was determined. A single washing time gives a higher enzymatic activity than the washing of two to four times. In addition, freeze-drying may cause a greater effect on enzymatic activity.[28] The enzymatic activity did not significantly decrease after washing the wet beads with the excess buffer solution for three times. After each washing, the enzymatic activity was determined. It was found that the amount of the immobilized enzyme was not retained after four washings. This suggests that the enzyme be entrapped in the polymer support, and no leakage of the immobilized enzyme can occur after buffer washings.

Effect of the pH on the Enzymatic Activity

The pH effects on the enzymatic activity of the free and immobilized enzymes for casein hydrolysis at 45°C in various buffer solutions of pH 7.5 to 11.0 are shown in Figure 18. The enzymatic activity was determined by the amount of L-tyrosine released. The results were reported as the percentage of relative activity that fixes the enzymatic activity of free and immobilized enzymes of 6.3×10^6 and 172 units, respectively, as 100% RA. The optimum pH was about 10.0 for the free enzyme.

The optimum pH of immobilized enzymes in polyacrylamide and poly(acrylamide-*co*-methacrylic acid) was shifted to pH 10.5. The pH effect of the immobilized enzyme in the alkaline range was stronger than for the free enzyme.

The temperature dependence of enzymatic activity of free and immobilized enzymes was determined in 0.1*M* carbonate–bicarbonate buffer solution of pH 10.5 in the temperature range of 25 to 70°C, using casein as the substrate. Figure 19 shows that optimum temperature for the free and immobilized enzymes to function well is at 45°C. At low temperatures, the enzyme is not active. At high temperatures, the enzyme loses its activity. The results are presented as the percentage of relative activity that fixes the enzymatic activities of free and immobilized enzymes of 4.4×10^6 and 172 units, respectively, as 100% RA. From the temperature plot, the immobilized enzyme is more thermally stable than is the free enzyme in the range of higher temperatures. The polymer supports dissipate the heat that prevent the immobilized enzyme from denaturation, especially at high temperatures.

Effect of Storage Stability on the Enzymatic Activity

The storage stability and the enzymatic activity of immobilized alkaline protease were compared with those of free alkaline protease at various temperatures from −20 to 60°C. Table V illustrates the residual enzymatic activity as the percentage of relative activity that fixes the enzymatic activities of free enzymes and immobilized enzymes on polyacrylamide and poly(acrylamide-*co*-methacrylic acid) of 1.5×10^4, 145, and 124 units as 100% RA, respectively. The enzymatic activity was checked every week for 1 month. Likewise, the immobilized enzyme is more stable than the free enzyme at the higher temperatures. The immobilized enzyme on polyacrylamide is

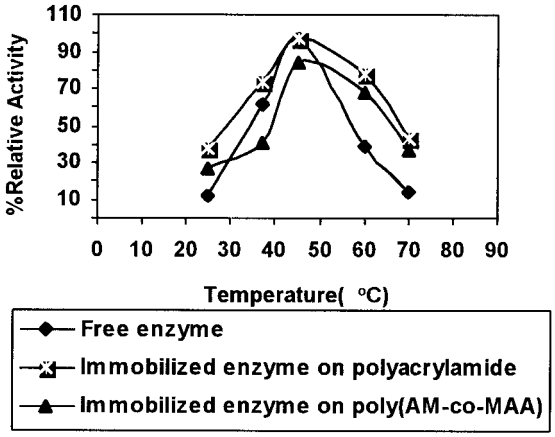

Figure 19 Effect of thermal stability on the enzymatic activity.

Table V Effect of Storage Stability on the Enzymatic Activity

Temperature (°C)	Enzyme	Percentage Relative Activity				
		0 Day	7 Days	14 Days	21 Days	30 Days
−20	Free	100.0 ± 1.8	101.3 ± 1.1	99.2 ± 2.8	102.0 ± 0.8	100.8 ± 2.2
−20	On PAM	100.0 ± 1.7	99.6 ± 0.4	102.3 ± 0.3	99.9 ± 1.9	101.4 ± 0.7
−20	On Poly(AM-*co*-MAA)	100.0 ± 2.3	100.6 ± 1.8	100.1 ± 1.5	100.3 ± 1.2	100.4 ± 0.4
4	Free	100.0 ± 1.8	100.1 ± 0.5	100.0 ± 0.3	100.4 ± 1.5	100.0 ± 2.1
4	On PAM	100.0 ± 1.7	100.1 ± 0.7	100.0 ± 1.7	100.1 ± 0.9	100.0 ± 0.3
4	On Poly(AM-*co*-MAA)	100.0 ± 2.3	100.2 ± 1.4	100.6 ± 1.5	99.9 ± 2.2	100.1 ± 0.5
25	Free	100.0 ± 1.8	98.3 ± 1.5	94.4 ± 0.5	90.9 ± 1.5	70.7 ± 0.7
25	On PAM	100.0 ± 1.7	101.5 ± 2.1	92.1 ± 0.8	85.6 ± 0.8	79.5 ± 0.9
25	On Poly(AM-*co*-MAA)	100.0 ± 2.3	100.2 ± 0.7	97.3 ± 0.7	86.4 ± 0.4	74.8 ± 1.2
37	Free	100.0 ± 1.8	97.0 ± 1.3	82.2 ± 0.8	69.1 ± 1.3	59.2 ± 0.8
37	On PAM	100.0 ± 1.7	97.3 ± 0.4	87.8 ± 1.7	78.8 ± 0.9	76.2 ± 2.4
37	On Poly(AM-*co*-MAA)	100.0 ± 2.3	96.1 ± 0.9	89.7 ± 2.3	76.8 ± 1.6	70.1 ± 0.5
45	Free	100.0 ± 1.8	78.8 ± 1.5	65.2 ± 2.2	59.4 ± 1.8	55.6 ± 0.7
45	On PAM	100.0 ± 1.7	90.9 ± 1.5	85.4 ± 2.6	77.3 ± 1.1	71.9 ± 1.1
45	On Poly(AM-*co*-MAA)	100.0 ± 2.3	89.8 ± 1.6	86.6 ± 3.2	73.9 ± 0.5	63.3 ± 0.3
60	Free	100.0 ± 1.8	60.5 ± 1.3	58.8 ± 0.9	52.9 ± 2.1	49.3 ± 0.7
60	On PAM	100.0 ± 1.7	87.5 ± 0.4	77.0 ± 0.4	73.1 ± 1.7	63.3 ± 0.8
60	On Poly(AM-*co*-MAA)	100.0 ± 2.3	84.1 ± 0.8	75.4 ± 1.5	62.8 ± 0.7	58.0 ± 0.6

Free means the free enzyme.
PAM means the immobilized enzyme on polyacrylamide.
Poly(AM-*co*-MAA) means the immobilized enzyme on poly(acrylamide-*co*-methacrylic acid).

more stable than that on poly(acrylamide-*co*-methacrylic acid). The free and immobilized enzymes can be kept at a temperature range of −20 to 4°C for 1 month without losing any enzymatic activity. The free enzyme loses enzymatic activity to 50% RA after being kept at 60°C for 1 month. The higher stability of the immobilized enzyme can be attributed to the prevention of auto-digestion and thermal denaturation due to fixation of the enzyme molecules onto the polymer supports.

CONCLUSION

Alkaline protease can be entrapped on polyacrylamide and poly(acrylamide-*co*-methacrylic acid) hydrogel beads by inverse suspension polymerization under careful controls of reaction parameters. The extent of enzymatic activities of immobilized alkaline protease on polyacrylamide hydrogel and poly(acrylamide-*co*-methacrylic acid) depended significantly on the concentrations of acrylamide, MBA, and TEMED, whereas those of the poly(acrylamide-*co*-methacrylic acid) decreased with increasing MAA concentration. The enzymatic activity of the free-alkaline protease is higher than the immobilized one. The relative activities of immobilized enzyme, in general, are relatively less than the free enzyme for those large molecular size substrates, for example, BSA (66,000) compared with casein (23,600). Regardless of the amount of enzyme immobilized, the immobilized enzyme has a much better performance and thermal stability. Additionally, the immobilization of enzyme on PAM or poly(acrylamide-*co*-methacrylic acid) beads has another advantage of hydrogels as a carrier. The enzymatic activity of polyacrylamide was higher than that of poly(acrylamide-*co*-methacrylic acid). The water absorption of polyacrylamide was lower than that for poly(acrylamide-*co*-methacrylic acid)s due to the inclusion of more hydrophilic comonomers. The water absorption of the latter increased with increasing methacrylic acid concentration. Protein digestion and staining of the immobilized alkaline protease revealed that the enzyme should be entrapped in the polymeric hydrogels, as no leakage of enzymatic activity after some washings could be detected. The immobilized enzyme possessed a higher storage stability than the free enzyme. These hydrogels exhibit an LCST at 30–35°C, which is very important for reversible de-mixing in aqueous solution. The results indicate their applications in the biotechnology industry.

The authors acknowledge the financial support from the Research Division of Chulalongkorn University as the pilot project and the Graduate School of the same university for the partial financial support to one of authors (A.N.). The discussions and suggestions of Prof. Shinzo Omi are also highly appreciated. We appreciate the Department of Photographic Science and Printing Technology, Faculty of Science of Chulalongkorn University for the provision of research facilities for the entire research period.

REFERENCES

1. Kokufuta, E. Prog Polym Sci 1992, 17, 647.
2. Aunstrup, K. in Economic Microbiology, Vol. 114; Rose, A. H., Ed.; Academic Press: London, 1980; pp. 49–113.
3. Kurenkov, V. F.; Myagchenkov, V. A. Polym Plast Technol Eng 1991, 30, 367.
4. Kotorman, M.; Siman, L. M.; Szajani, B.; Boross, L. Biotechnol Appl Biochem 1986, 8, 53.
5. Kawamura, Y.; Nakanishi, K.; Matsuno, R.; Kamikubo, T. Biotechnol Bioeng 1981, 23, 1219.
6. Mosbach, K.; Nelson, K. U.S. Pat. 4,647,536, 1987.
7. Iayakumari, V. G.; Rajasekharan Pillai, V. N. J Appl Polym Sci 1991, 42, 583–590.
8. Hayashi, T.; Hirayama, C.; Iwatsuki, M. J Appl Polym Sci 1992, 44, 143–150.
9. Hayashi, T.; Ikeda, Y. J Appl Polym Sci 1991, 42, 85–92.
10. Richardson, B. C.; Whaiti, I. E. NZ J Dairy Sci Technol 1978, 13, 172.
11. Dunn, M. J. in Protein Purification Methods: a Practical Approach; Harris, E. L. V.; Angel, S., Eds.; IRL Press: New York, 1990; pp. 18–37.
12. Hummel, D. O. Atlas of Polymer and Plastics Analysis, Vol. 1, 3rd ed.; VCH Verlagsgesellschaft: Weinheim, Germany, 1991; pp. 683–684.
13. Harrison, L. A.; Flint, K. P. Industrial Biological Testing; Hopton, J. W.; Hill, E. C., Eds.; Blackwell Scientific Publications: Oxford, UK, 1987; pp. 181–195.
14. Klein, J.; Heitzamnn, R. Makromol Chem 1978, 179, 1895.
15. Brandrup, J.; Immergut, E. H., Eds. Polymer Handbook, 2nd ed.; John Wiley & Sons: New York, 1975; pp. (III) 111–112, (III) 146–147.
16. Nemec, J. W.; Bauer, Jr., W. Encyclopedia of Polymer Science and Engineering, Vol. 1, 2nd ed.; John Wiley & Sons: New York, 1985; pp. 211–234.
17. Thomas, D.; Hill, F. Water-Soluble Synthesis Polymers: Properties and Behavior, Vol. 1; Molyneux, P., Ed.; CRC Press: New York, 1985; pp. 75–117.
18. MacWilliams, D. C., Ed. Functional Monomers; Their Preparation, Polymerization, and Application, Vol. 1; Dekker: New York, 1973; pp. 2–197.
19. Graillat, C.; Pichot, C.; Guyot, A.; El Aasser, M. S. J Polym Sci, Polym Chem Ed 1986, 24, 427.
20. Bikales, N. M.; Kolodny, E. R.; Bikales, N. G. Kirk-Otmer Encyclopedia of Chemical Technology, Vol. 1, 2nd ed.; Mark, H. F.; Gaylord, Eds.; John Wiley: New York, 1963; pp. 274–284.
21. Nuyken, O.; Lattermann, G. Handbook of Polymer Synthesis: Part A; Krichekdorf, H. R., Ed.; Marcel Dekker: New York, 1992; pp. 223–336.
22. Odian, G. Principles of Polymerization, 3rd ed.; John Wiley & Sons: New York, 1991; pp. 452–531.
23. Park, T. G.; Hoffman, A. S. Biotechnol Bioeng 1990, 35, 152.
24. Kawaguchi, H. New Technol Jpn 1995, 22, 40.
25. Gutoaska, A.; Bae, Y. H.; Feijen, J.; Kim, S. W. J Controlled Release 1992, 22, 95.
26. Windholz, M.; Budacari, S.; Stroumtsos, L. Y.; Fertig, M. N. The Merck Index: An Encyclopedias of Chemicals and Drugs, 9th ed.; Merck & Co.: Rahway, NJ, 1976; pp. 240.
27. Biochemical Organic Compounds for Research and Diagnostic Reagents; Sigma Chemical Company: USA 1995; p. 64.
28. Harris, E. L. V.; Angal, S., Eds., Protein Purification Methods: A Practical Approach; IRL Press, Oxford, UK, 1990; pp. 148–149.