# CHAPTER 4

RESULT AND DISCUSSION

4.1 FT-IR Identification

Polyacrylamide and poly(acrylamide-co-methacrylic acid) were characterized by FT-IR spectroscopy. FT-IR spectra of standard and the sample of polyacrylamide were shown in Figure 4.1 (a,b). The sample spectra was similar with the standard spectra [83]. The IR spectra revealed the primary amide that had, the carbonyl absorption at 1640 cm^{-1} (6.1 μ), the stretch bands of the NH$_2$ at 3450 and 3225 cm^{-1} (2.9 and 3.1 μ) similar in shape to the primary amine. The NH$_2$ deformation band was fused to the C=O stretch absorption at around 1615 cm^{-1} (6.2 μ) and contributes to its broadness. The NH$_2$ wag vibration appeared as a broad sloping band beginning at 770 cm^{-1} (13 μ) with the maximum occurring beyond the 625 cm^{-1} (16 μ) region. FT-IR spectra of standard and sample of poly(acrylamide-co-methacrylic acid) were shown in Figure 4.2 (a, b). The polyacrylamide spectrum was closely similar with poly(acrylamide-co-methacrylic acid) spectra. The C-O streching between 1300-1200 cm^{-1} (8.0 to 8.5 μ) was observed. The C=O streching appeared at around 1670-1640 cm^{-1}. The NH bending band appeared between 1640-1550 cm^{-1}. The oxygen-hydrogen strech appeared between 3335-2500 cm^{-1} (3 and 4 μ). It was overlapped with the NH$_2$ strech band at 3450 and 3225 cm^{-1} (2.9 and 3.1 μ). The CH strech band appeared at around 2940 cm^{-1} (3.4 μ). The C-N strech bands appeared in the vicinity of 1175-1055 cm^{-1} (8.5 to 9.5 μ).
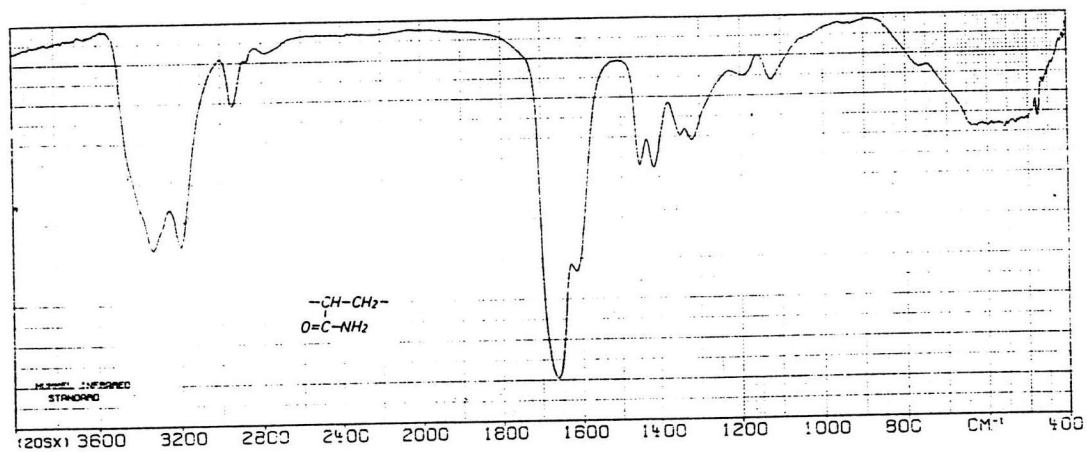
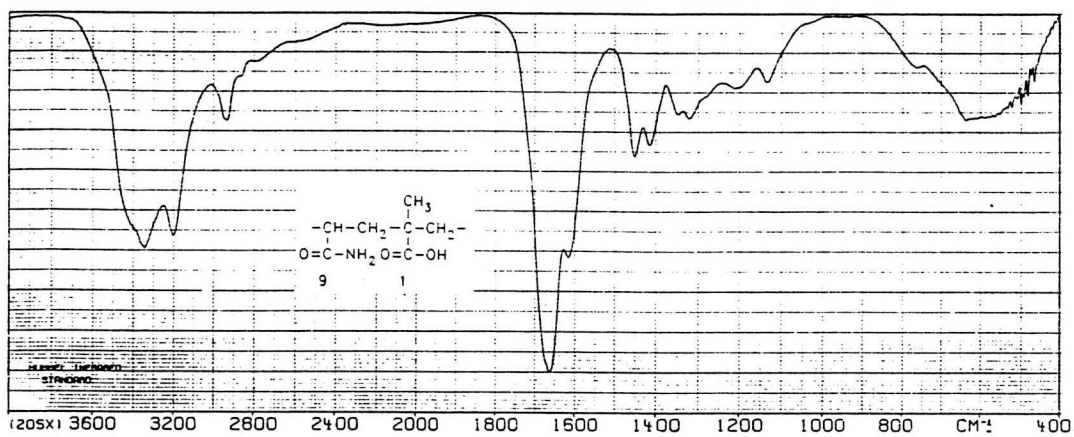(a)
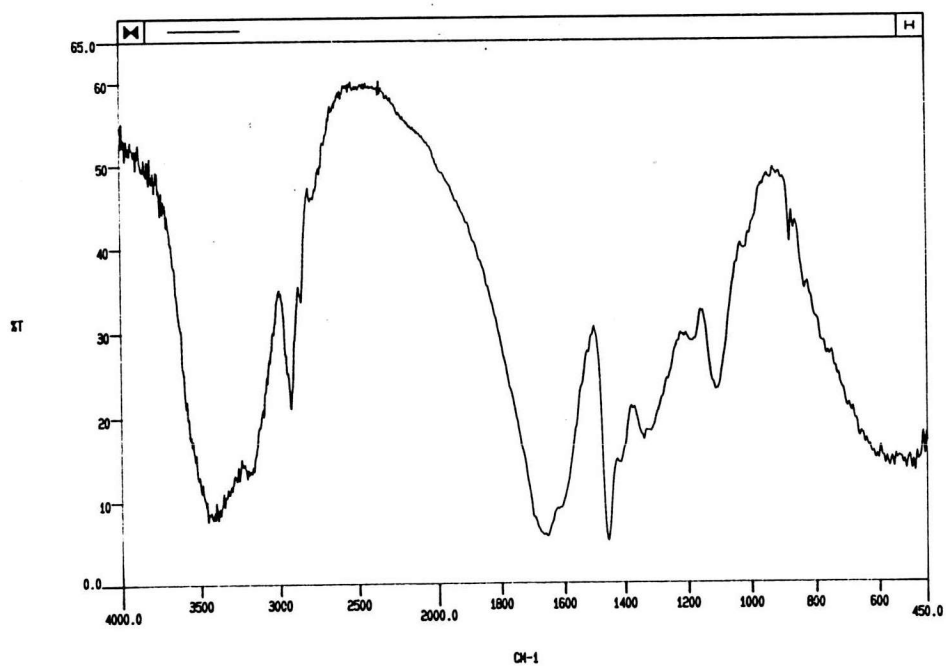
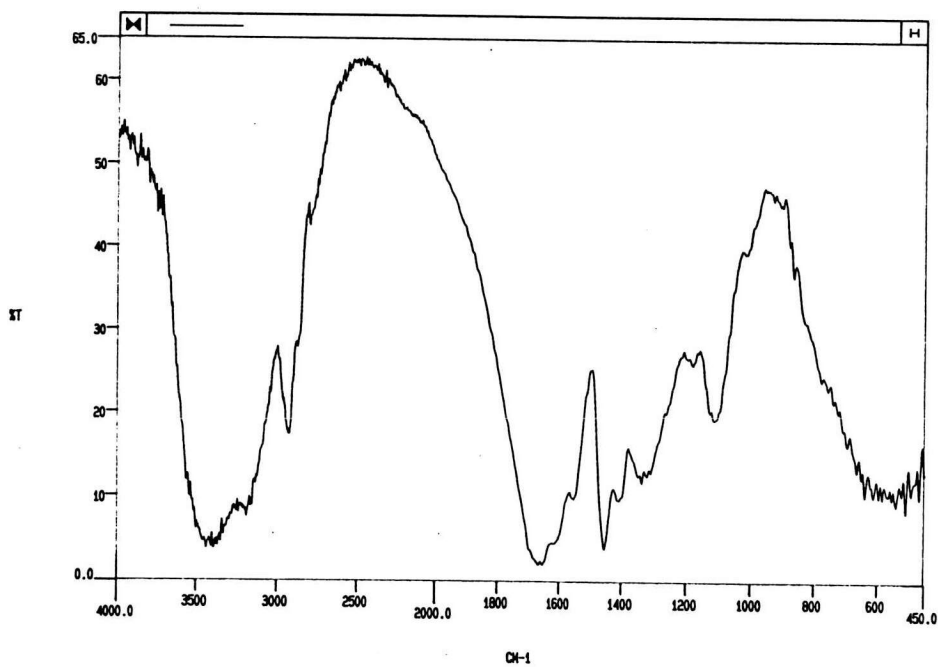

(b)

Figure 4.1 FT-IR polyacrylamide spectra (a) standard spectra (b) sample spectra

(a)

(b)

Figure 4.2 FT-IR poly(acrylamide-co-methacrylic acid) spectra

(a) standard spectra, (b) sample spectra

4.2 Thermal Analysis

Glass transition temperatures (T_g) of polyacrylamide and poly(acrylamide-co-methacrylic acid) were carried out by DSC. Figure 4.3 presents the DSC chromatogram of acrylamide-methacrylic acid copolymer of 100/0, 97.5/2.5, 95/5, 90/10% W/W (Appendix I). The sample weight used for characterization was 10-12 mg. T_g of crosslinked polyacrylamide in the present work was 220°C while that of the uncrosslinked one in the references is 165°C [4, 73, 84], and 188°C [73], depending on synthesis and measuring methods. Poly(acrylamide-co-methacrylic acid)s at 97.5/2.5 and 95/5 give the T_g values at 221, and 228°C, respectively. Since the monomer feed of acrylamide is much higher than that of methacrylic acid, the polymer should contain more feed of acrylamide although the monomer reactivity of methacrylic acid is reletively higher than that of acrylamide. Therefore, single peak for each copolymer could represent mostly the acrylamide feed in the copolymer as the $r_1 r_2 < 1$ (random copolymer) but close to zero (alternating copolymer). Poly (acrylamide-co-methacrylic acid) at 90/10 shows two endothermic peaks; T_{g1} and T_{g2} at 228, and 258°C, respectively. Polymethacrylic acid gives a reference T_g at 106°C [68], or 130°C [68], or 141°C [84],or 185°C [68] depending on measuring techniques. T_gs of polyacrylamide and poly(acrylamide-co-methacrylic acid) are higher than the reference values due to the effect of crosslinking agent. The glass transition temperature is increased additionally by the formation of salts due to the incorporation of buffer solution [85]. Incorporation MBA restricts mobility of polymer segments and thus increases T_g of polyacrylamide and poly(acrylamide-co-
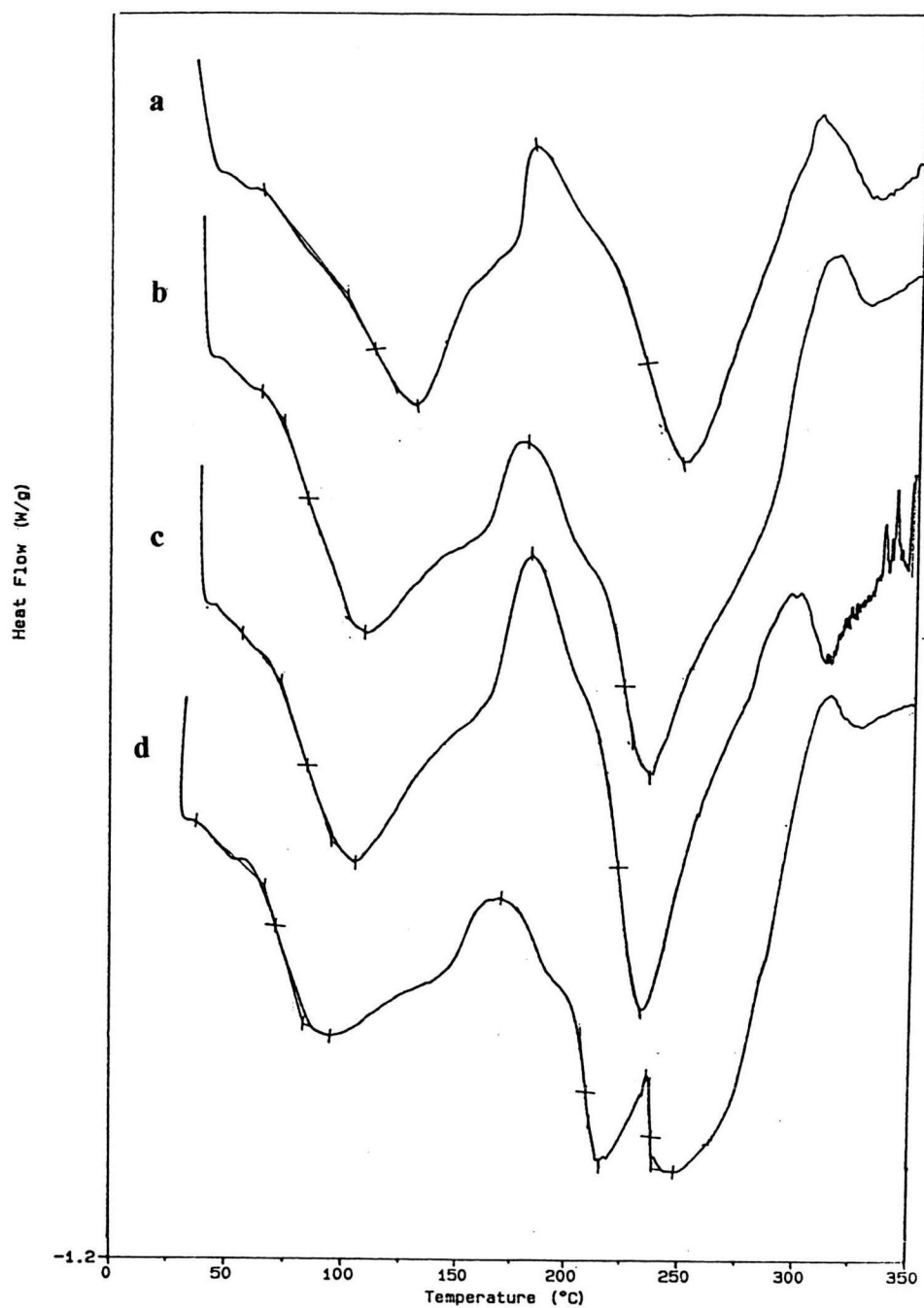
Figure 4.3 DSC thermograms of polyacrylamide and poly(acrylamide-co-methacrylic acid), (a) 100/0, (b) 97.5/2.5, (c) 95/5, (d) 90/10% W/W

methacrylic acid) [72]. Figure 4.3 shows additionally an endothermic peak at around 80-110°C. Based on several previous works, we confirm that imidization should take place in the polymerization of acrylamide in low nitrogen [68], at high temperature [86], at very extreme pH values (lower than 2.5 or higher than 9) [87-88] which leads to partially insoluble products (I) as shown in Figure 4.4. In this study, we polymerized and immobilized alkaline protease on polyacrylamide and poly (acrylamide-co-methacrylic acid) in the carbonate-bicarbonate buffer pH 10.5. The side reaction of intramolecular imidization can take place. The imidization of polyacrylamide can occur at the temperature range of 75-110°C [68]. Imidization affects T_g of polyacrylamide and poly(acrylamide co-methacrylic acid). T_g values of both polyacrylamide and poly(acrylamide-co-methacrylic acid) are shifted to higher values than those values in the literature.
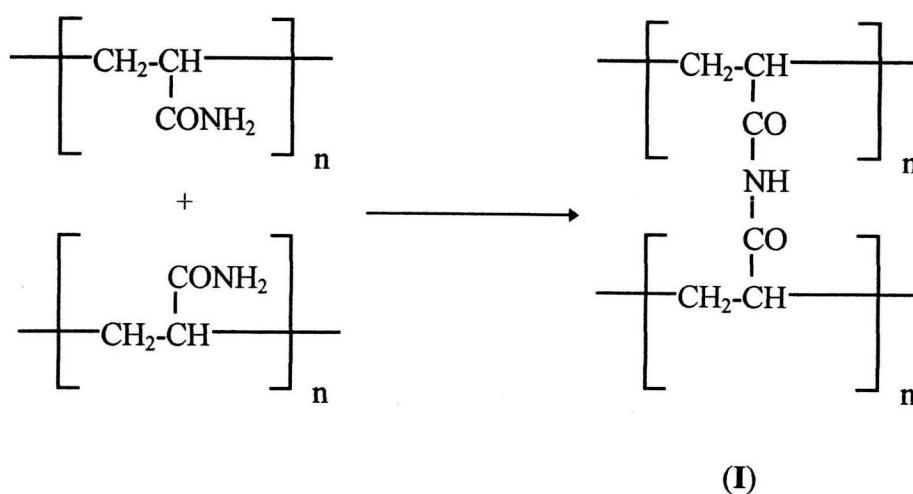

(I)

Figure 4.4 Imidization of polyacrylamide

4.3 Investigation the Number Washings for Removing the Paraffin wax and Free enzyme After Immobilization

After reaction, the beads was washed with carbonate-bicarbonate buffer pH 10.5 for ridding the paraffin wax and free enzyme. The gel (10 g) was washed with 100 cm^3 of carbonate-bicarbonate buffer pH 10.5 with 1-6 times washing. The washed beads in each condition was dried by freeze drying and determined for the enzymatic activity. Table 4.1 and Figure 4.5 presented the enzymatic activity of immobilized enzyme, using casein as substrate. The enzymatic activity did not significantly decrease after washing the wet beads with the excess buffer solution for six times. So in our experiment, we washed the beads at least 4 times for removing the paraffin wax and free enzyme before freeze drying.

Table 4.1 Enzymatic activity on the washing times

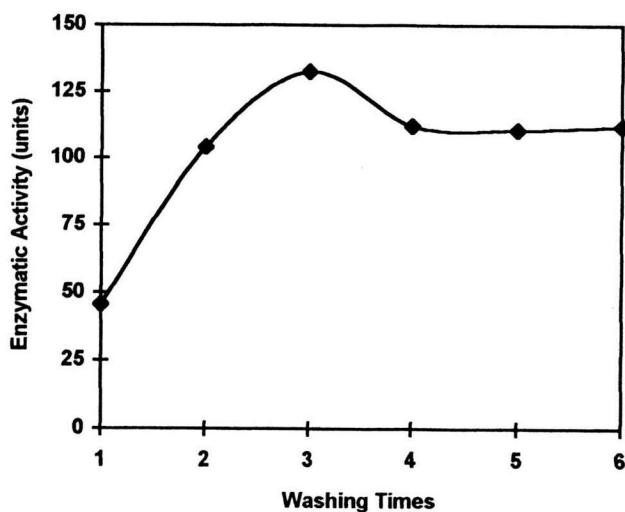| Washing times	Enzymatic Activity (units)[a]
1	46 ± 3
2	104 ± 4
3	133 ± 8
4	112 ± 4
5	111 ± 7
6	112 ± 2

[a] Sample size, n = 3

Figure 4.5 Enzymatic activity on the washing times

4.4 Effect of Monomer Concentrations on the Enzymatic Activity

The different concentrations of acrylamide monomer of 3.14, 4.57, 6.28, and 9.14 mM in 35 cm^3 of carbonate-bicarbonate buffer solution of pH 10.5 were investigated for enzymatic activity, percentage immobilization, and percentage conversion. The reaction system was fixed, the concentration of MBA (120 mM), alkaline protease (5.0 mg/5 cm^3), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, time and temperature of polymerization of 2 h and 30°C, respectively. The total enzymatic activity of free enzyme added in the reaction was found as 12,490 units. The enzymatic activity of immobilized enzyme, percentage immobilization, and percentage conversion on polyacrylamide supports with different acrylamide concentrations were given in Table 4.2 and Figure 4.6. It

Table 4.2 Effect of monomer concentrations on the enzymatic activtiy, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

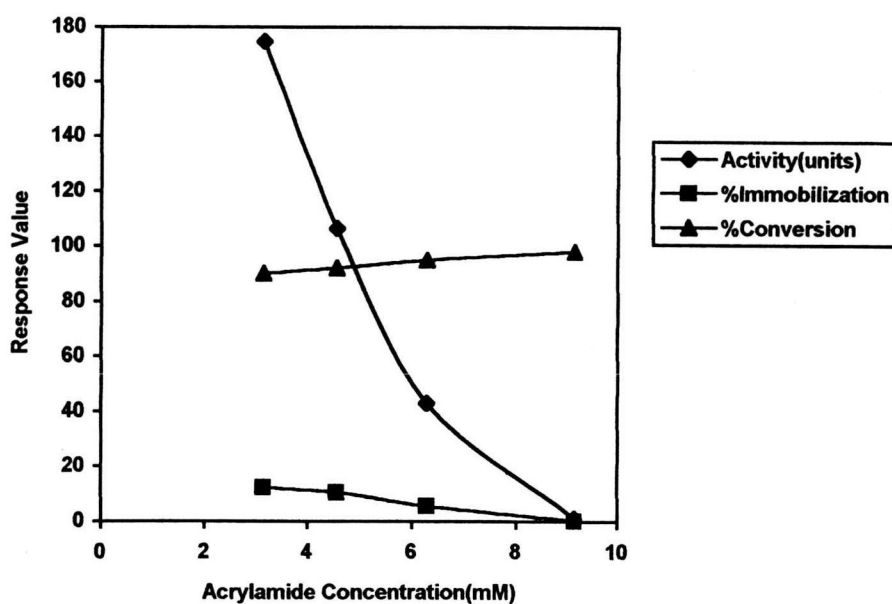| Acrylamide (mM)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
3.14	175 ± 5	12 ± 1	90 ± 2
4.57	106 ± 3	11 ± 1	92 ± 3
6.28	43 ± 5	6 ± 1	95 ± 1
9.14	1 ± 1	0.2 ± 0.3	98 ± 3

Figure 4.6 Effect of monomer concentrations of enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.
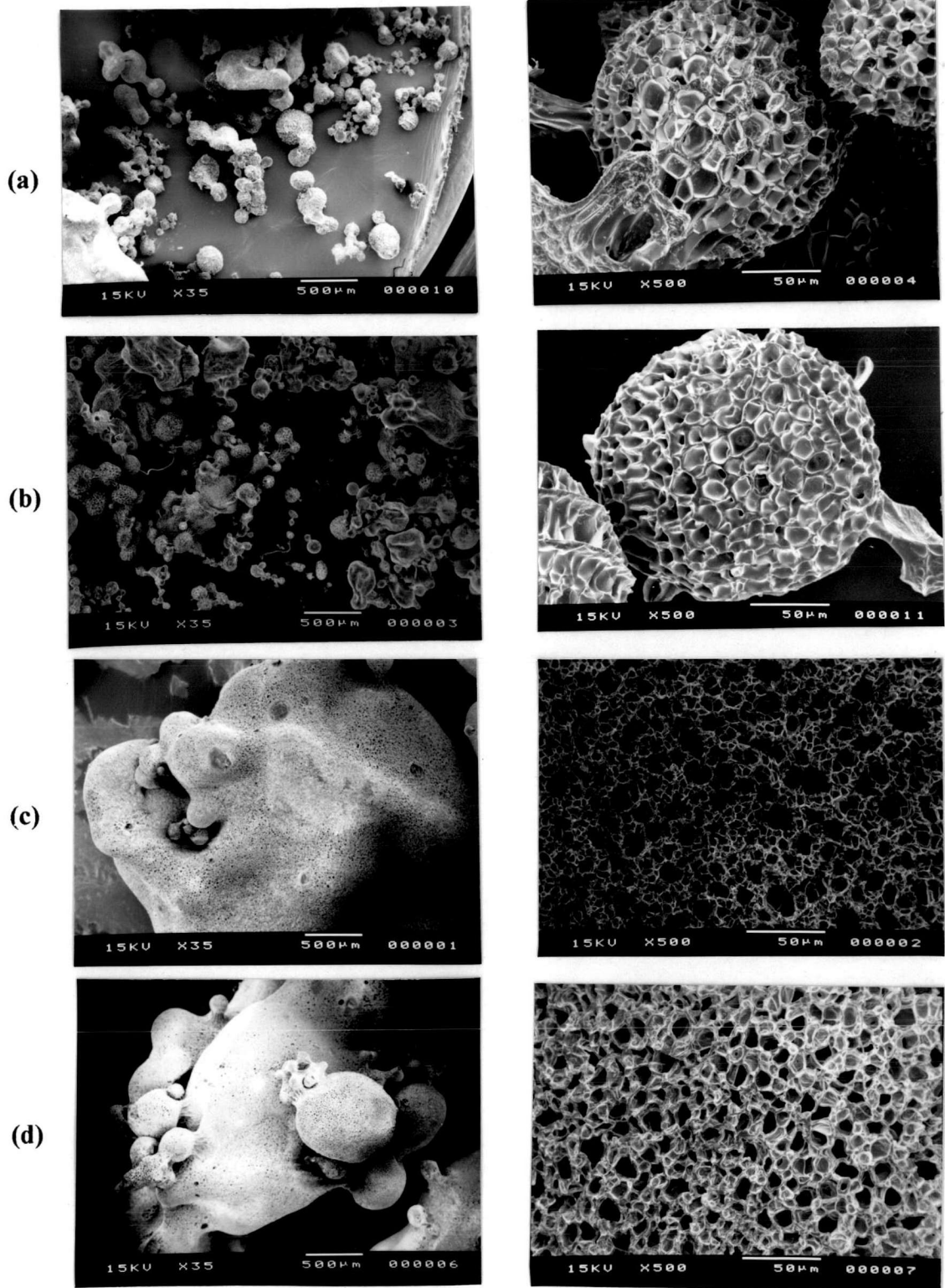
Figure 4.7 SEMs of polyacrylamide at different acrylamide concentrations

(a) 3.14 mM, (b) 4.57 mM, (c) 6.28 mM, (d) 9.14 mM,

Left: x 35 magnification, Right: x 500 magnification.

can be stated that the enzymatic activity and percentage immobilization of immobilized enzyme was decreased with increasing acrylamide concentrations. The maximum of enzymatic activity of polyacrylamide beads was 175 units, with 12% immobilization and 90% conversion. The beads were electron micrographed with scanning electron microscope (SEM) whose morphologies were shown in Figure 4.7. The electron micrographs of the beads revealed a porous structure with netting on the surface. The bead sizes were increased with increasing acrylamide concentrations. The enzymatic activity was decreased with increasing the bead sizes. It can be illustrated that bead size was affected by enzymatic activity of immobilized enzyme. Percentage conversion of acrylamide was increased with increasing acrylamide concentrations. 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 sequent experiments, the acrylamide concentration was kept to 3.14 mM, based on the highest enzymatic activity.

4.5 Effect of Enzyme Concentrations on the Enzymatic Activity

Various amounts of alkaline protease of 0.25, 0.5, 1.5, 2.5, and 5.0 mg/5 cm^3 were investigated for enzymatic activity. The reaction system was fixed the concentrations of AM (3.14 mM), MBA (120 mM), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), at a stirring rate 300 rpm, time and temperature of polymerization of 2 h and 30°C, respectively. Table 4.3 and Figure 4.8 revealed the results of enzymatic acitity immobilized on the polyacrylamide with different concentrations. The enzymatic activity of immobilized alkaline protease increased

Table 4.3 Effect of enzyme concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

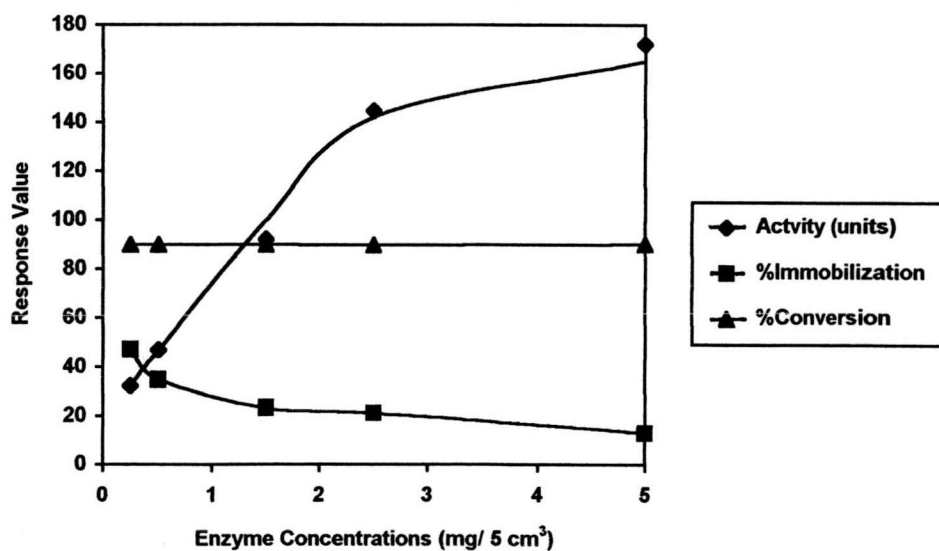| Enzyme Concentration (mg/5 cm^3)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
0.25	32 ± 1	47 ± 2	90 ± 3
0.5	47 ± 1	35 ± 1	90 ± 4
1.5	97 ± 1	24 ± 1	90 ± 2
2.5	145 ± 3	21 ± 1	90 ± 2
5.0	175 ± 5	12 ± 1	90 ± 4

Figure 4.8 Effect of enzyme concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.
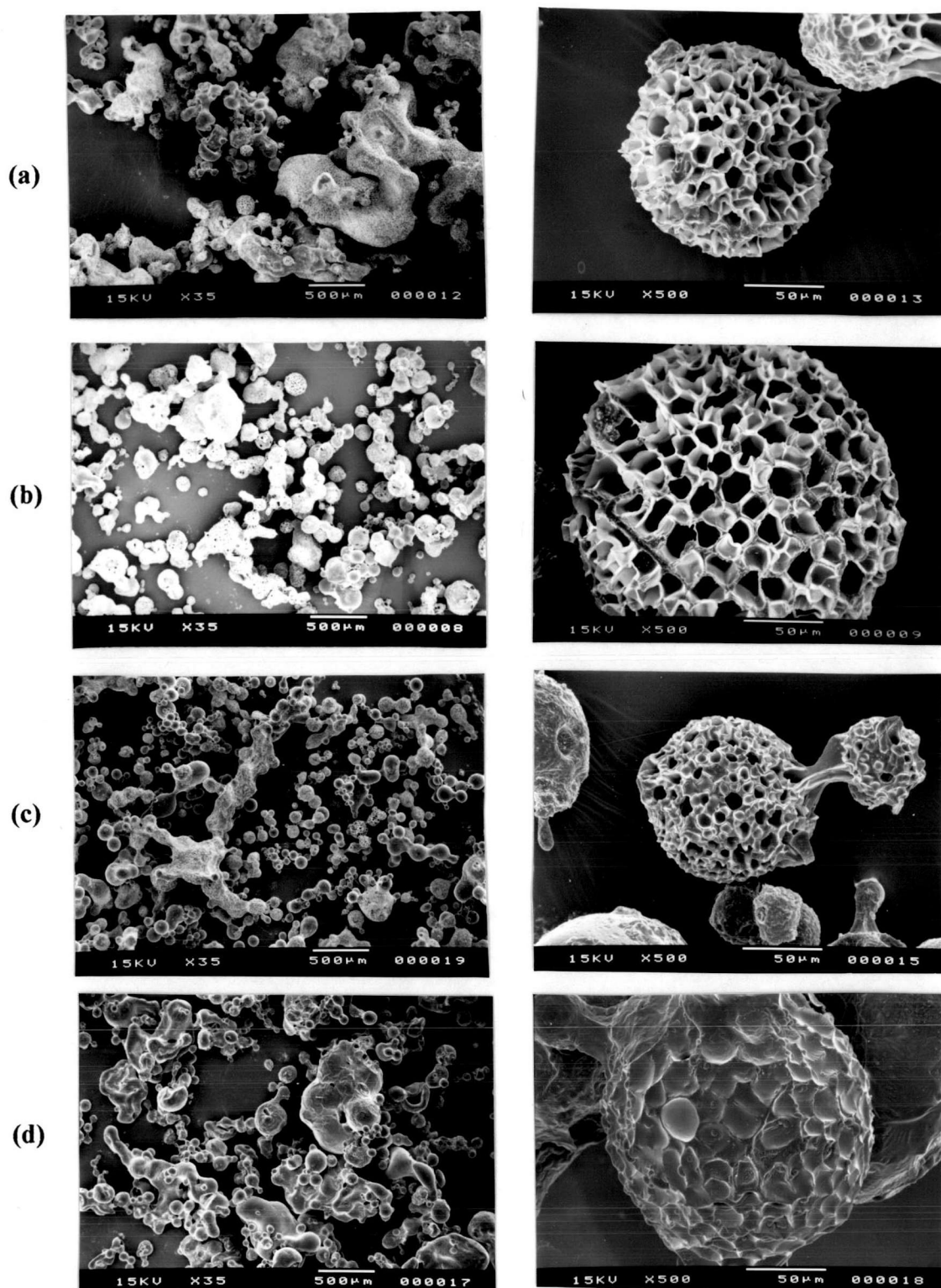
Figure 4.9   SEMs of polyacrylamide at different enzyme concentrations

(a) 0.25, (b) 0.5, (c) 15, (d) 2.5, Left x 35 magnification,

Right x 500 magnification.

with increasing amounts of alkaline protease, and with a declinning percentage immobilization of alkaline protease. The amounts of immobilized alkaline protease is almost proportional to the initial alkaline protease concentration from 0.25 to 2.5 mg/5 cm^3. Percentage of immobilization indicated clearly the limitation of amounts of enzyme that can be immobilized on polyacrylamide beads. The free enzyme is claimed to also contribute to the increasing activities of the enzyme. The amounts of enzyme that used to further study of reaction parameters was evaluated. Amounts of enzyme of 0.25 and 0.5 mg/5 cm^3 giving the enzymatic activity 32 and 47 units are not used in our experiments and the biotechnology industry owing to their low activities. The amounts of the enzyme of 2.5 and 5.0 mg/5 cm^3, which gave the enzymatic activity of 145 and 175 units, respectively, was appropriate, however, for the present experiments, but the free enzyme 80-85% not being immobilized on the beads is a problem of wastage. The suitable concentration of enzyme for further reaction parameters was essentially 1.5 mg/5 cm^3 giving 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 concentrations. The percentage of conversion of acrylamide was constant at 90%. The SEMs of the beads shown in Figure 4.9 disclosed that changing the concentration of enzyme might affect the bead sizes.

4.6 Effect of 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 fixed for the concentrations of AM (3.14 mM), alkaline protease (1.5 mg/5 cm^3), MBA (120 mM), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), time and temperature of polymerization of 2 h and 30°C, respectively. Table 4.4 and Figure 4.10 show the enzymatic activity, percentage immobilization and percentage conversion of immobilized enzyme on polyacrylamide. The optimum of enzymatic activity was 97 units with 24% immobilization and 90% conversion. The percentage conversion was not affected by stirring rate. The polymerization stirring rate affected the bead sizes that can be seen on the SEMs shown in Figure 4.11. The bead size was decreased with

Table 4.4 Effect of polymerization stirring rate on enzymatic activity, percentage immobilization, percentage conversion of immobilized alkaline protease on polyacrylamide.

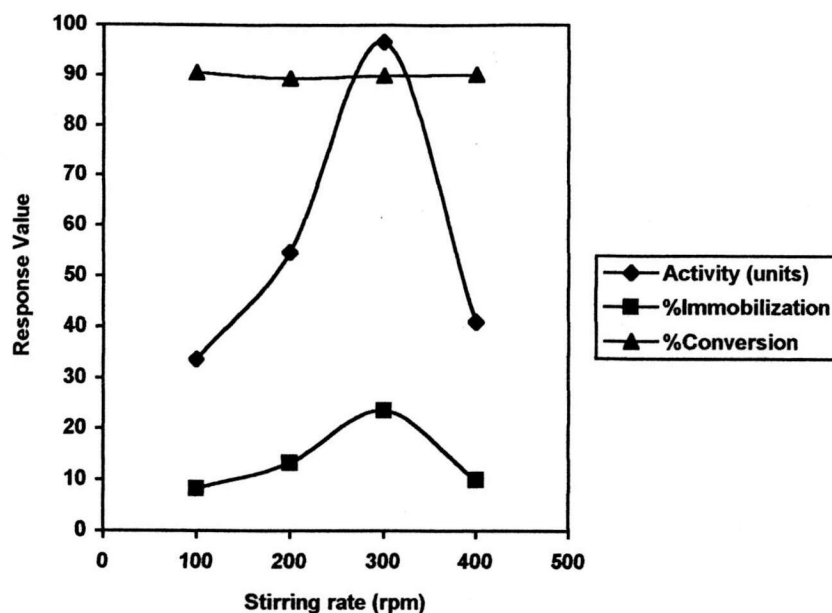| Stirring Rate (rpm)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
100	34 ± 2	8 ± 1	91 ± 1
200	55 ± 1	13 ± 1	89 ± 3
300	97 ± 1	24 ± 1	90 ± 2
400	41 ± 1	10 ± 1	90 ± 3

Figure 4.10 Effect of polymerization stirring rate on the enzymatic activity,

percentage immobilization, and percentage conversion of immobilized

alkaline protease on polyacrylamide.

increasing stirring rate. The enzymatic activity was increased with increasing the

stirring rate from 100 to 300 rpm and was decreased slightly while increasing the

stirring rate to 400 rpm. The slow stirring rate (100 rpm) could not migrate the

monomer to the redicals. Once the initiation took place, the monomers propagate at

the same reactive site. This reaction is thus a diffusion controlled kinetics. The

higher stirring rate (400 rpm) moved both the monomers and radicals freely to enable

collisions among them to occur. Therefore a fines sizes of the particles were

obtained. The fine particle size could also form gel for the enzyme immobilization

and enzymatic activity.

(a)

(b)

(c)

(d)
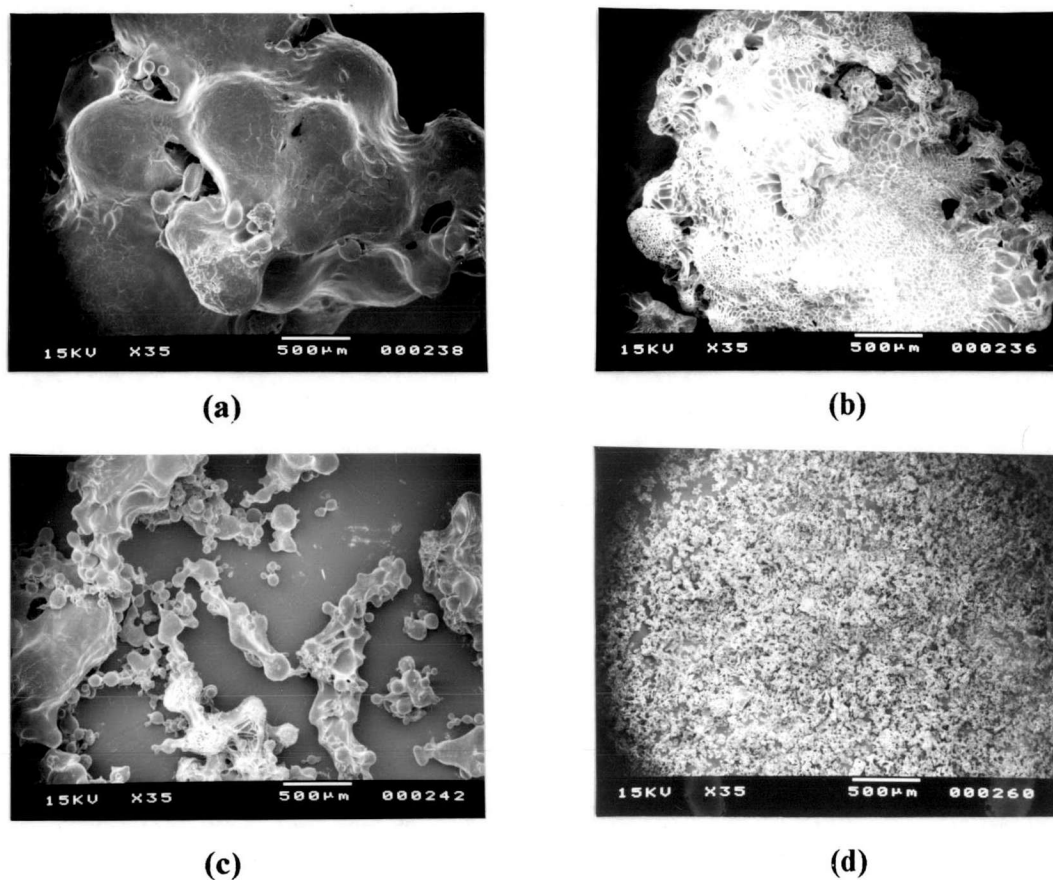
Figure 4.11 SEMs of polyacrylamide at different polymerization stirring rate (a) 100, (b) 200, (c) 300, (d) 400 rpm.

4.7 Effect of Polymerization Time on the Enzymatic Activity and Bead Formation

4.7.1 On the Enzymatic Activity, Percentage Immobilization and Percentage Conversion

The variation of any time for polymerization was investigated at 1, 2, 3, and 4 h. The reaction were carried out by AM (3.14 mM), alkaline protease (1.5 mg/5

cm^3), MBA (120 mM), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, polymerization temperature 30°C. The results can be seen from Table 4.5 and Figure 4.12. The desirable time for polymerization 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 but not affected to percentage of conversion (~90% throughout the reaction time of 4 h). The 90% conversion from 2 h polymerization time provided the highest enzymatic activity and percentage immobilization. The micrographs shown in Figure 4.13 disclose that the bead sizes of different polymerization times ranged from 80-100 μm. The beads polymerized for 1 h and 2 h showed the porosity on the surface higher than the beads from the longer reaction times. More pores on the beads

Table 4.5 Effect of polymerization time on enzymatic activity, percentage immobilization, percentage conversion of immobilized alkaline protease on polyacrylamide

Time (h)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
1	52 ± 1	13 ± 1	90 ± 1
2	97 ± 1	24 ± 1	90 ± 2
3	64 ± 2	16 ± 1	90 ± 3
4	58 ± 1	14 ± 1	89 ± 3
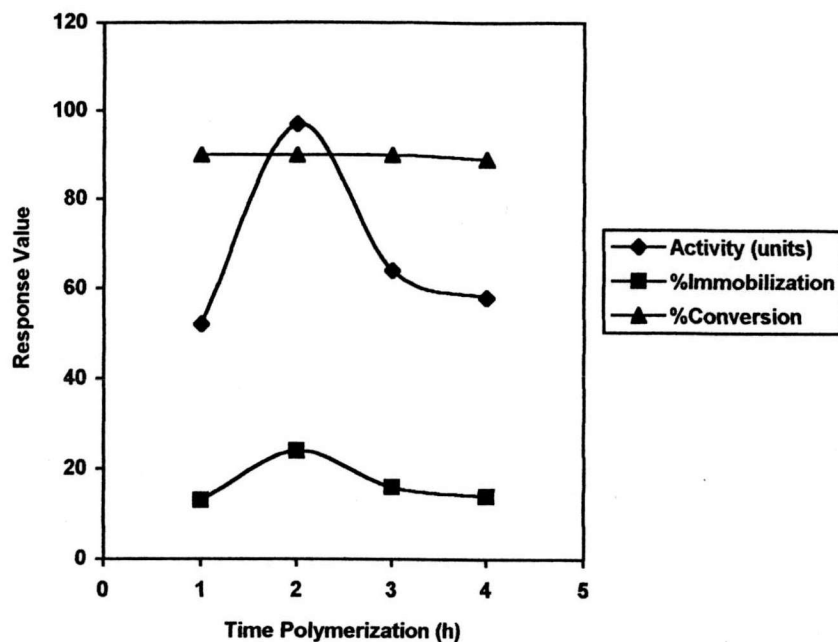
Figure 4.12 Effect of polymerization time on the enzymatic activity, percentage immobilizaton, and percentage conversion of immobilized alkaline protease on polyacrylamide.

affected the ability of the substrate to penetrate to inside of the pores to react with the entrapped enzyme and to release the soluble product to outside of the bead. So the maximum enzymatic activity was indicated by 2 h of the polymerization time that was then chosen for further studies of the reaction parameters.

(a)

(b)

(c)

(d)
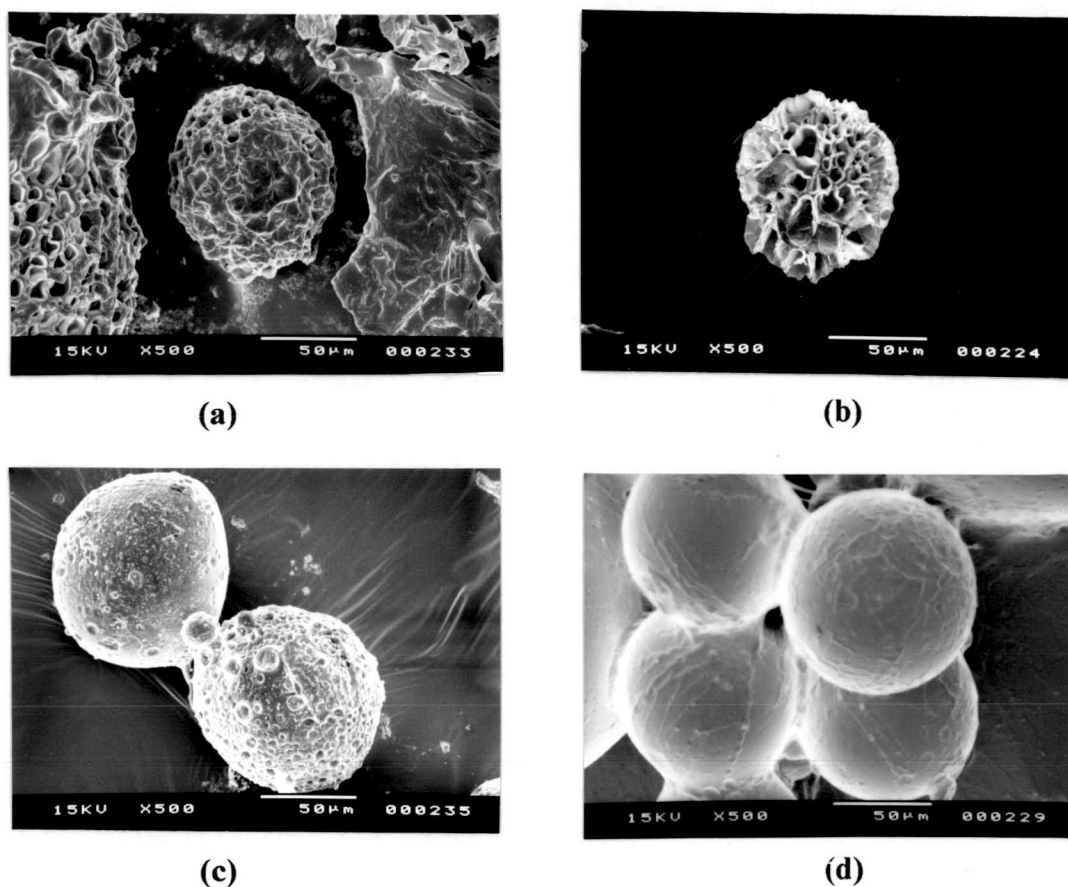
Figure 4.13 SEMs of polyacrylamide at different of polymerization time

(a) 1 h, (b) 2 h, (c) 3 h, (d) 4 h

4.7.2 On the Percentage Conversion and Bead Formation as a Function of Time

The measured residual monomer concentrations were used to calculate the conversion as the function of polymerization time. Conversions were inferred from measurements of the residual monomer concentrations by the High Performance Liquid Chromatographic technique. The calibration curve of acrylamide monomer, HPLC chromatogram of standard acrylamide monomer and residual monomer that

were discharged several times were given in Appendix II. The calibration slope of the standard acrylamide monomer was 9.6305×10^6. The quantity for reactor volume, monomer charged, and injection volume were 246 cm^3, 8.0206 g, and 5×10^{-3} cm^3, respectively. The residual monomer and conversion were calculated by eqs. 2.3 and 2.4. More than 90% conversion occured after a polymerization had taken place for 10 min. The limit of conversion was observed at 96% conversion as shown in Table 4.6 and Figure 4.14.

Table 4.6 Effect of conversion-time on acrylamide polymerization

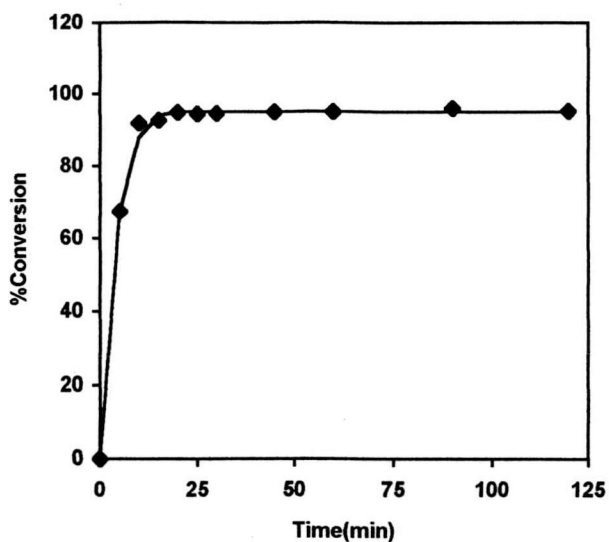| Time (min)	Peak Area	Percentage Conversion
5	454804	67
10	112149	92
15	102516	93
20	71580	95
25	76824	95
30	74990	95
45	69396	95
60	69042	95
90	53864	96
120	65660	96

Figure 4.14 Conversion as a function time of acrylamide polymerization

Hunkeler, D. [77] determined the polymerization rate of acrylamide at high monomer concentration. Acrylamide was polymerized in inverse microsuspension using potassium persulfate as the initiator. Experiments were performed between 40 and 60°C. The limiting conversions have been observed. The limit conversion at 0.2 mM of potassium persulfate, at 6.41-3.35 mM of acrylamide concentration was observed between 92.0-99.7%.

This type of polymerization, we used, can be confirmed that the time of 2 h for polymerization enables a complete conversion to occur and the associated reactions of either grafting or crosslinking are possible to entrapped the enzyme onto the polymer supports. The enzymatic activity and average molecular weights of the polymeric supports are optimized within the 2 h polymerization.

Figure 4.15 SEMs of bead formations in each interval time. (a) 5 min, (b) 10 min, (c) 15 min, (d) 30 min, (e) 45 min, (f) 60 min, (g) 90 min, (h) 120 min, left x 35 magnification, Right x 500magnification.
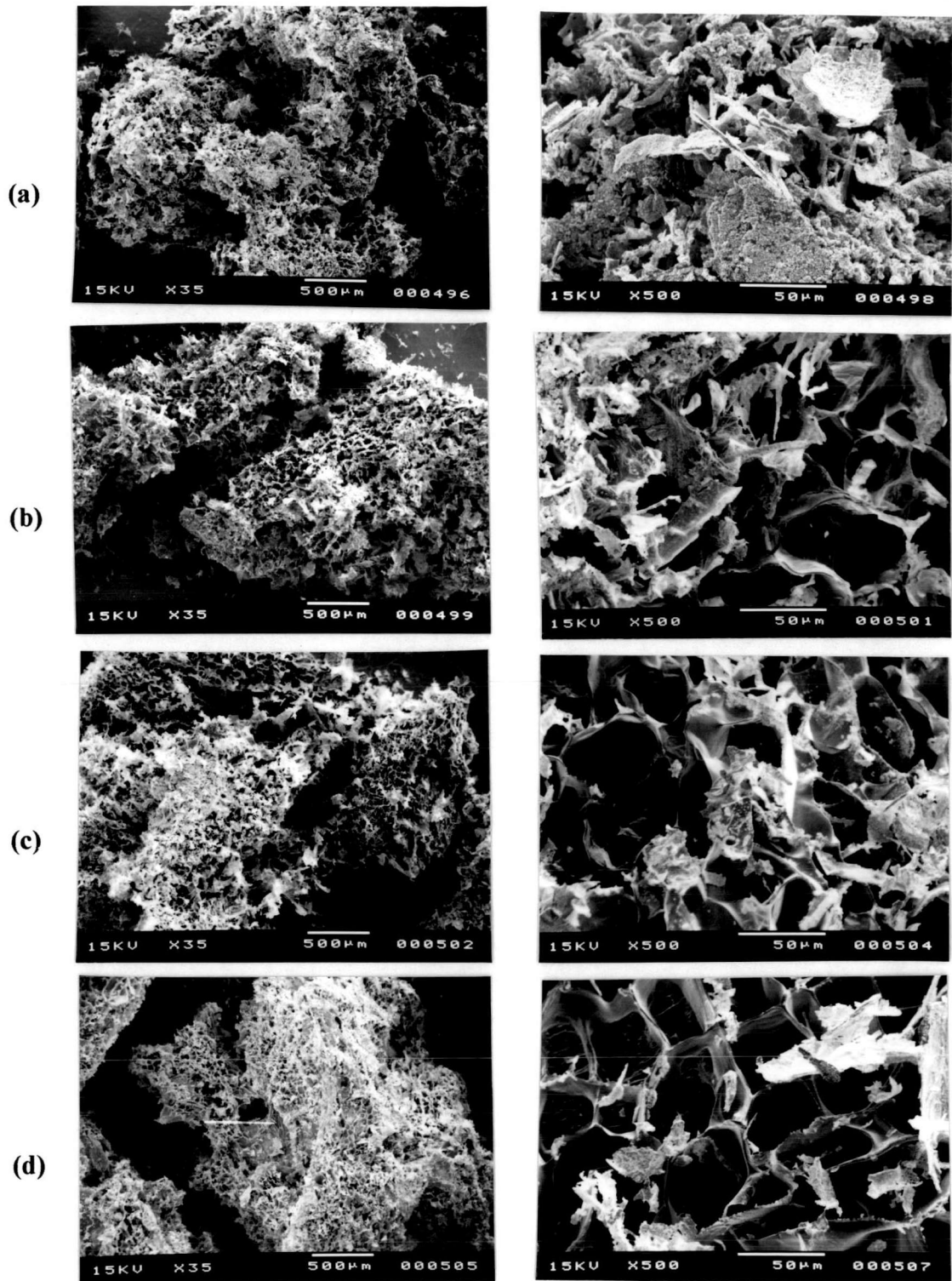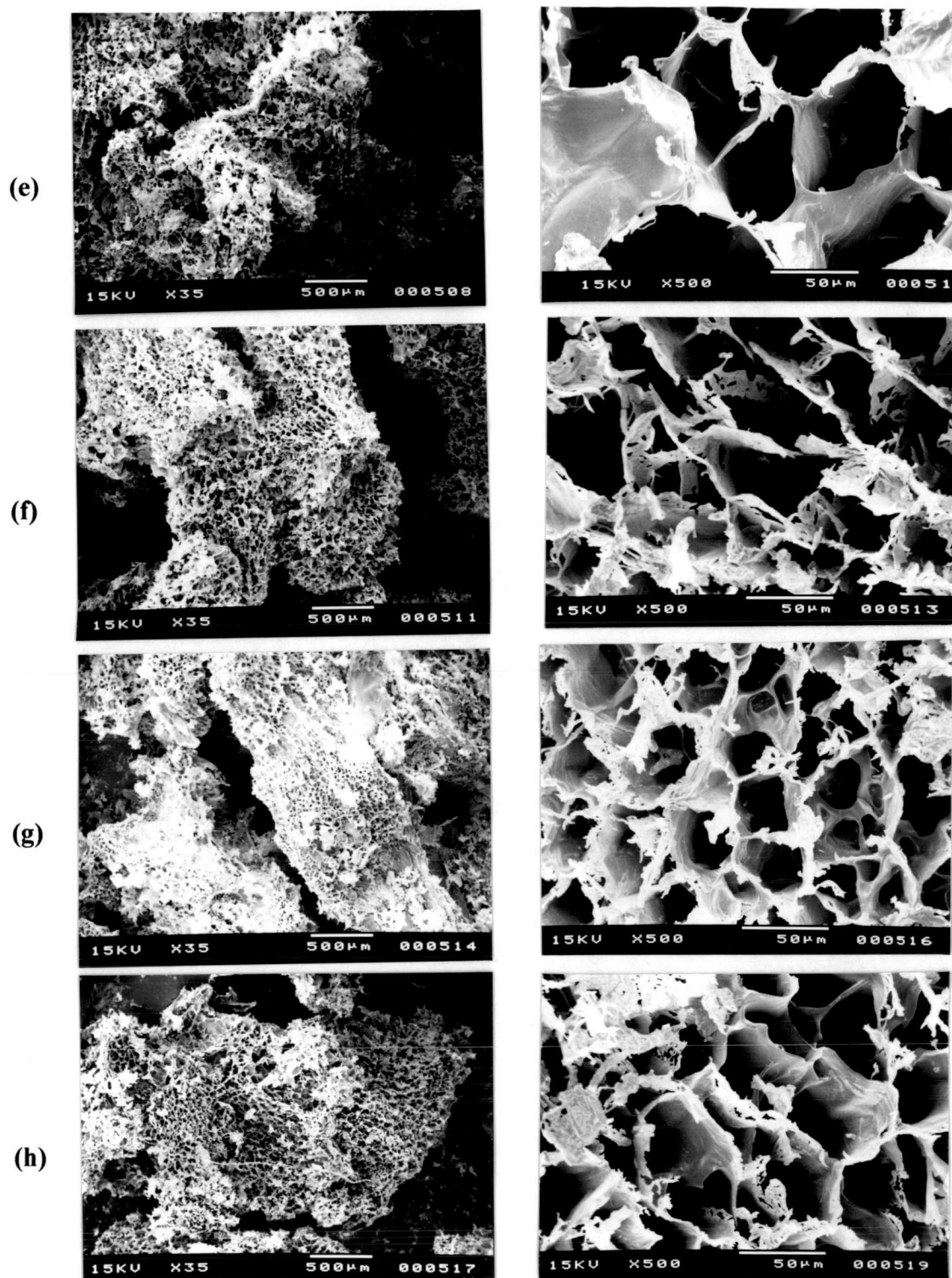
Figure 4.15 (continued)SEMs of bead formations in each interval time. (a) 5 min,

(b) 10 min, (c) 15 min, (d) 30 min, (e) 45 min, (f) 60 min, (g) 90 min,

(h) 120 min, left x 35 magnification, Right x 500 magnification.

The polymer formation at several time was taken by SEM shown in Figure 4.15. Polyacrylamide formed the sheet morphologies and showed the porous on the interior surface. Discharged the residual monomer in the reactor of each interval time affected to the formation of the polymer beads. The beads can not form the spherical form after reaction. This condition is very sensitive. In this study, we found that the polymer could occur when the purged nitrogen gas was pure. The reaction cannot be distributed, due to stoichiometric amount, before the reaction is completed.

4.8 Effect of Polymerization Temperature on the Enzymatic Activity

Effect of polymerization temperature was studied at 0, 10, 20, 30, and 40°C. The reaction condition was fixed for the concentrations of AM (3.14 mM), alkaline protease (1.5 mg/5 cm^3), MBA (120 mM), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, polymerization time of 2 h. Table 4.7 and Figure 4.16 indicate the enzymatic activity, percentage immobilization and percentage conversion of acrylamide at different polymerization temperatures. The optimum temperature was found at 30°C with the enzymatic activity 97 units, 24% immobilization, and 90% conversion. Percentage conversion was increased with increasing the reaction temperature. At low temperature (0°C), the initiation rate was relatively slow leading to a some what lower conversion. Percentage immobilization of enzyme showed a maximum at 30°C and decreased when the temperature were increased. For the lower reactor temperature, the enzyme molecules move slowly to the beads due to its stiffness. The entrapped enzyme was

Table 4.7 Effect of polymerization temperatures on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

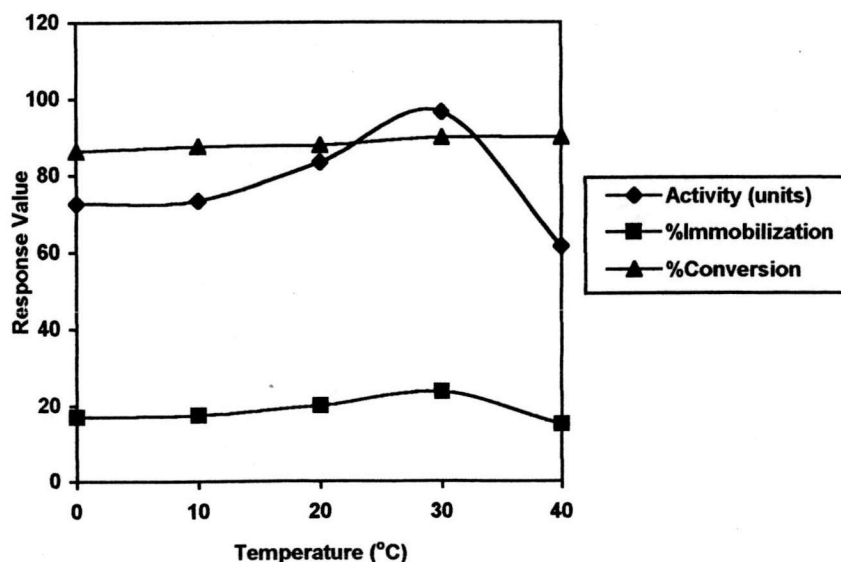| Temperature (°C)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
0	73 ± 2	17 ± 1	86 ± 2
10	73 ± 4	18 ± 1	88 ± 2
20	83 ± 2	20 ± 1	88 ± 3
30	97 ± 1	24 ± 1	90 ± 2
40	62 ± 1	15 ± 1	90 ± 1

Figure 4.16 Effect of polymerization temperatures on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.
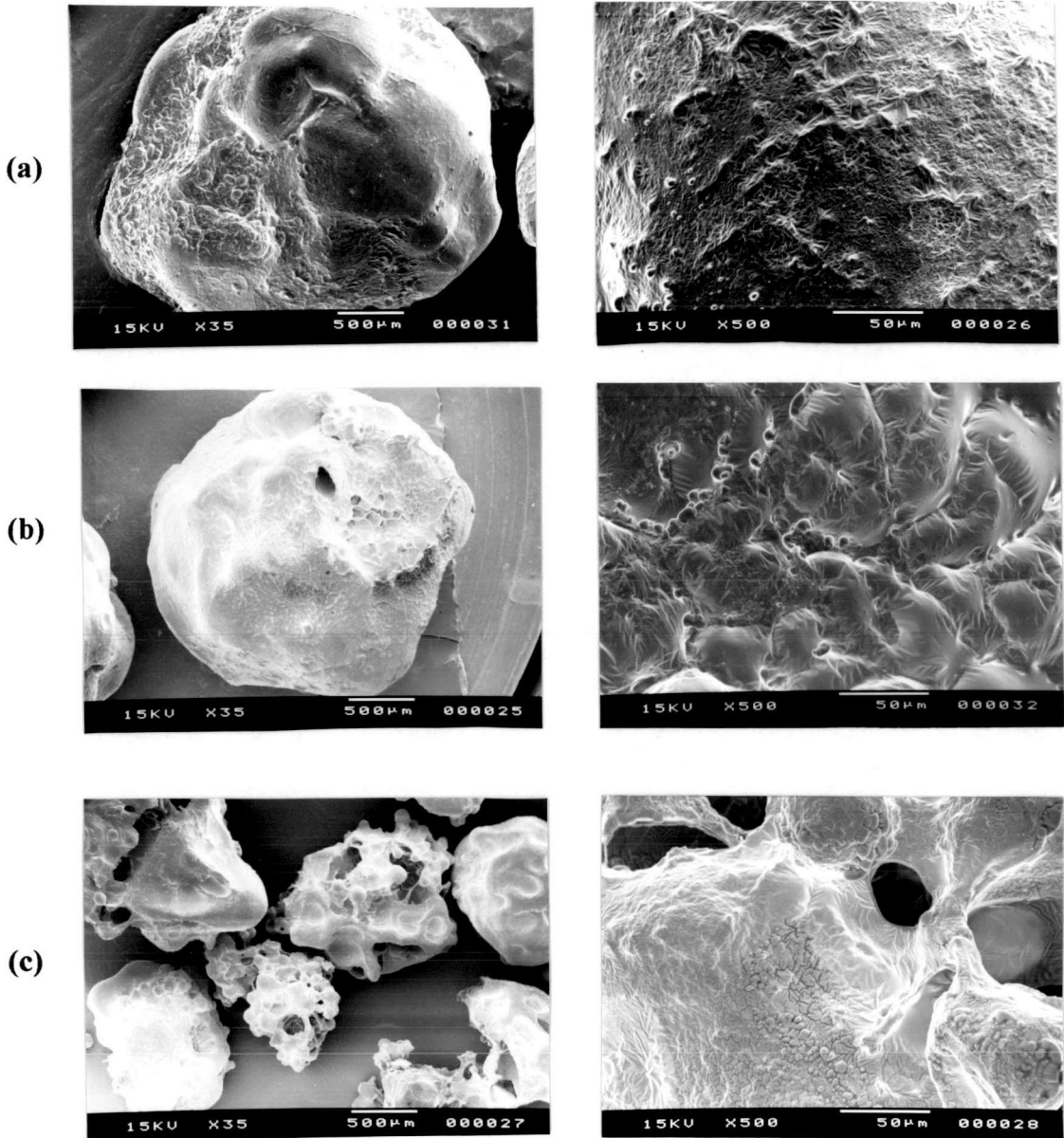
Figure 4.17 SEMs of polyacrylamide at different polymerization temperatures
(a) 0°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, Left x 35 magnification,
Right x 500 magnification.
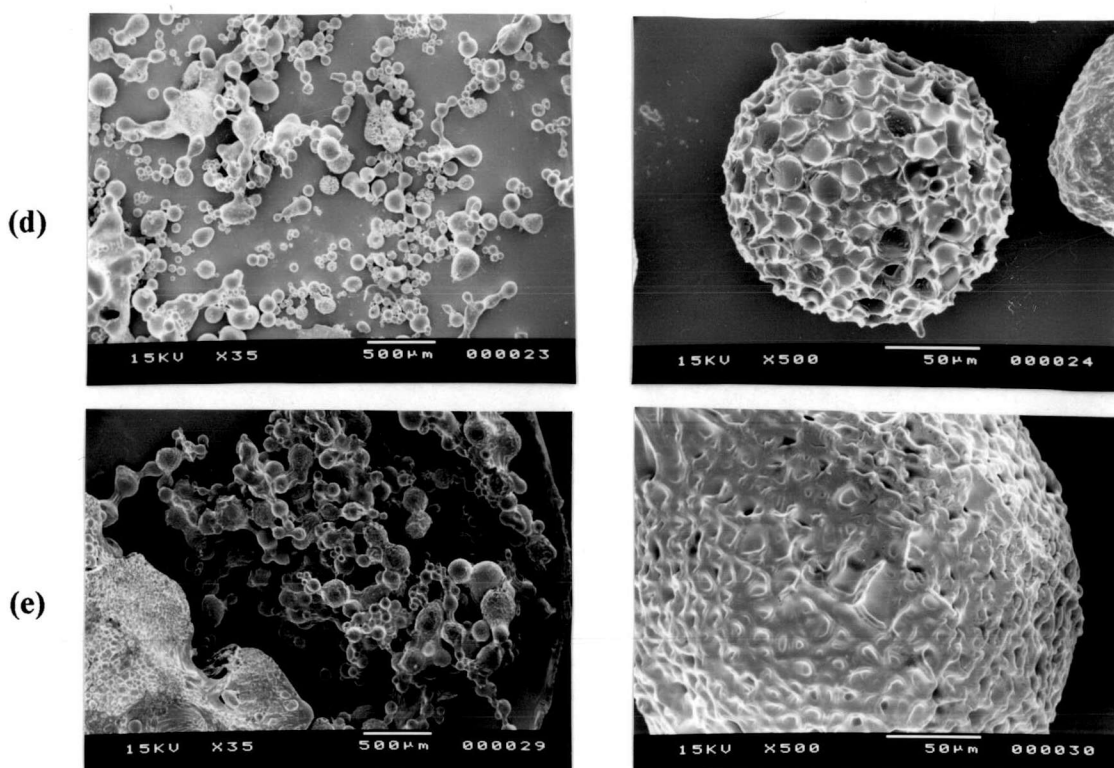
Figure 4.17 (continued)   SEMs of polyacrylamide at different polymerization temperatures (a) 0°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, Left x 35 magnification, Right x 500 magnification.

consequently less than that at 30°C. At the high temperture, higher than 40°C, the enzyme lost its enzymatic activity through a protein denature effect. The morphology of the beads on the SEMs as shown in Figure 4.17 is very interesting. The beads sizes were increased with decreasing the polymerization temperatures. The beads gel together polymerized below 30°C, the gelled beads were cloudy before freeze drying, beads polymerized at 30°C or higher show the clearity beads. At lower temperature,

the overall activation energy was not sufficient to diffuse of the initiator, crosslinker, monomer and enzyme in the reactor. Once an initiation at one active site could occur, the active components in the close vicinity slowly diffused to it to form a large piece of tiny beads fused into one giant bead. The high temperature of 40°C, the active ingredient could diffuse much better that allowed more frequencies of collision when they encountered. However, the beads surface was rather porous than those of low temperature polymerizations (0, 10, and 20°C) and the bead sized were also relatively smaller. More interestly, the enzymatic activity was affected by the bead sizes. On a porous bead, the 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 at 30°C that was chosen for further studies of the reaction parameters.

4.9 Effect of Crosslinker Concentration on the Enzymatic Activity

N,N′-methylene-bis-acrylamide (MBA) was used as a crosslinker. Table 4.8 and Figure 4.18 show the effects of crosslinker on enzymatic activity, percentage immobilization, and percentage conversion. The different concentrations of MBA were experimented with the fixed concentrations of AM (3.14 mM), alkaline protease (1.5 mg/5 cm³), APS (6.56 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, polymerization time 2 h and polymerization temperature 30°C. The enzymatic activity of immobilized enzyme was decreased with increasing the MBA concentrations. The results indicate that the enzymatic activtiy was dependent on the rigidity of the polyacrylamide support due to crosslinking,

since, the polyacrylamide beads containing 120 mM of MBA only gave the enzymatic activity of 97 units, with 24% immobilization whereas, the beads having 15 mM of MBA produced the enzymatic activity of 150 units, with 34% immobilization.

Jayakumari, V. G. and Pillai, V. N. R. [32] immobilized papain on polystyrene-divinylbenzene (DVB) resins and DVB-crosslinked acrylamide resins by a DCC (dicyclehexylcarbodiimide) method. The effects of the crosslink concentrations on enzymatic activity and immobilization yields were carried out. They found that the enzyamatic activity was depent on the rigidity of the matrix. The enzymatic activity and enzyme content of DVB-crosslinked acrylamide resins was higher than those of polystyrene-DVB resins, because of the hydrophilic nature of DVB-crosslinked acrylamide resins. Percentage conversion was relative constant

Table 4.8 Effect of crosslinker concentrations on the enzymatic activity, percentage immobilization, percentage conversion of the immobilized alkaline protease on polyacrylamide.

MBA Concentration (mM)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
15	150 ± 2	34 ± 1	89 ± 2
30	118 ± 4	27 ± 1	89 ± 1
60	109 ± 1	26 ± 1	90 ± 2
90	102 ± 2	24 ± 1	90 ± 3
120	97 ± 1	24 ± 1	90 ± 2
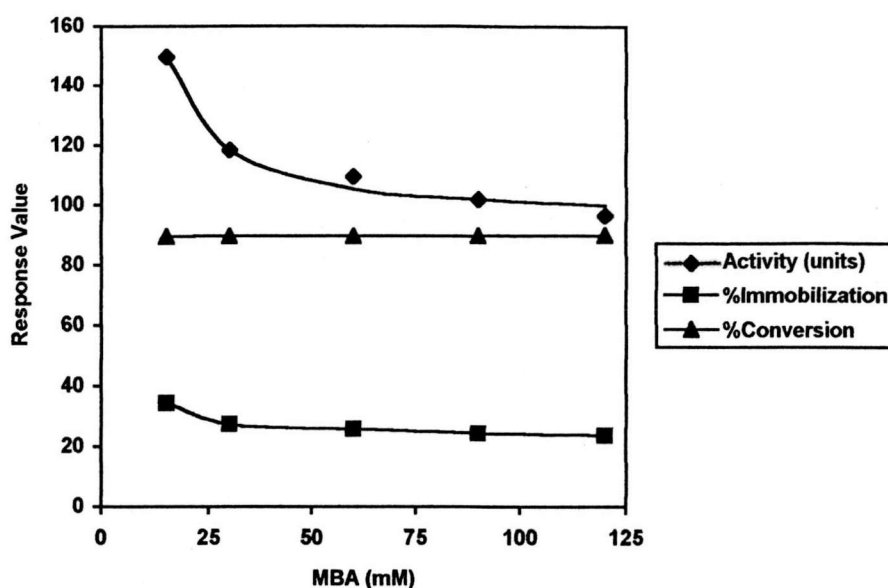
Figure 4.18 Effect of crosslinker concentrations on enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide beads.

with increasing the MBA concentration. The morphologies of the polyacrylamide in the SEMs as shown in Figure 4.19. revealed that the spherical size of the beads was believed to increase with increasing the MBA concentrations owing to its internal crosslinking. The MBA concentration of 15 mM was selected for investigation of other reaction parameters.

Figure 4.19 SEMs of polyacrylamide at different crosslinker concentrations (a) 15, (b) 30, (c) 60, (d) 90, (e) 120 mM, Left x 35 magnification, Right x 500 magnification

(d)

(e)
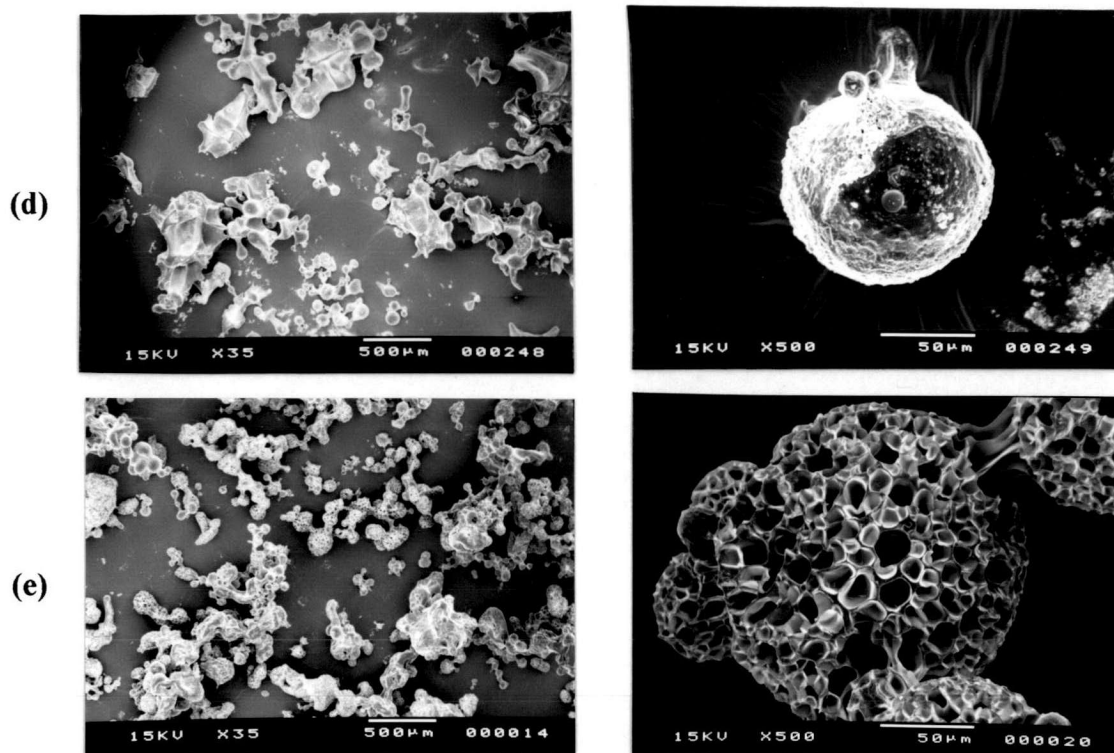
Figure 4.19 (continued)   SEMs of polyacrylamide at different crosslinker concentrations (a) 15, (b) 30, (c) 60, (d) 90, (e) 120 mM, Left x 35 magnification, Right x 500 magnification

4.10 Effect of Initiator Concentrations on the Enzymatic Acitivity

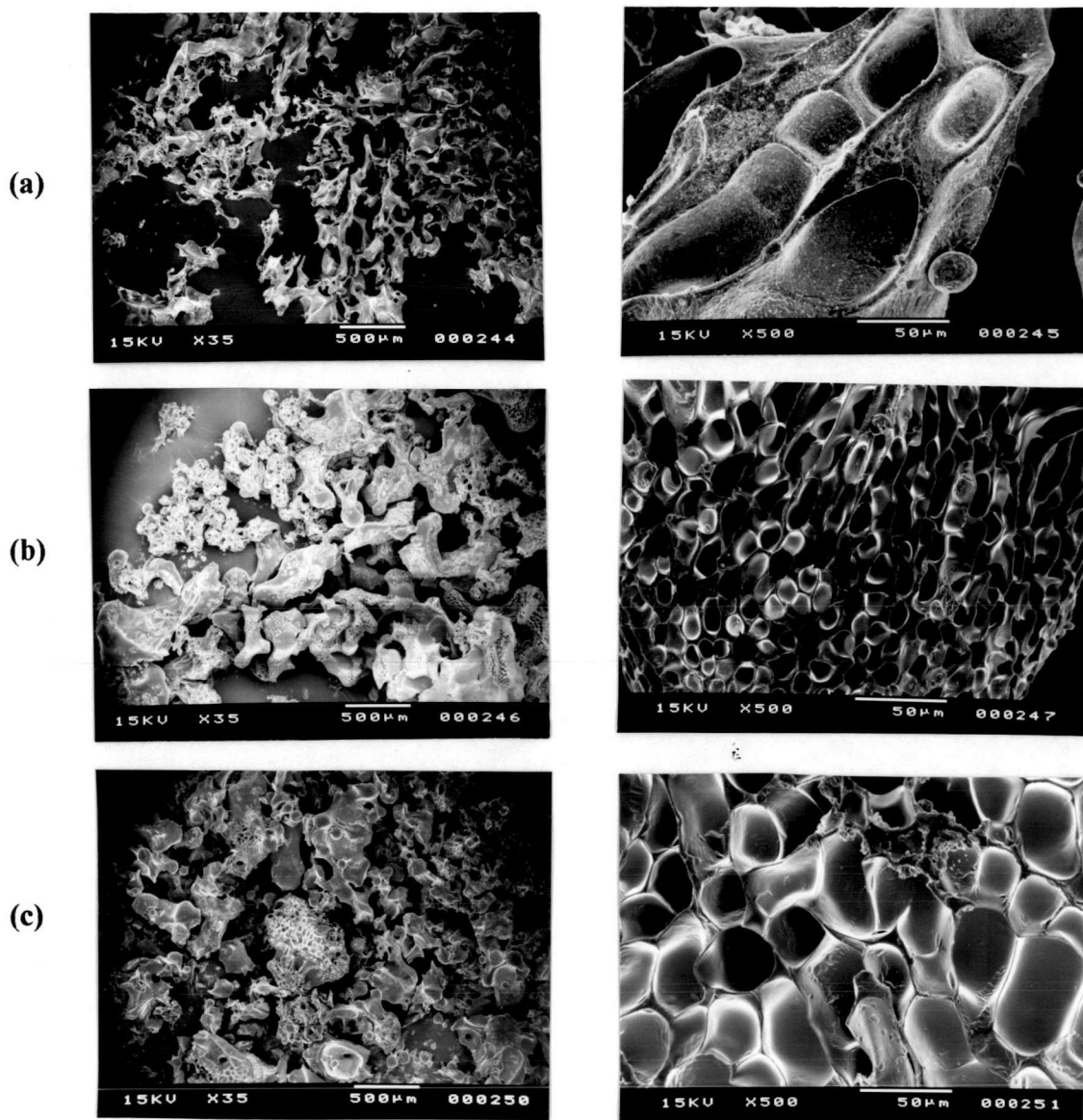Ammonium persulfate (APS) was used as an initiator. Table 4.9 and Figure 4.20 show the effect of initiator concentrations of 3.13, 6.56, 9.39, and 12.52 mM on the enzymatic activity, percentage immobilization and percentage conversion. The reaction was fixed for the concentrations of AM (3.14 mM), alkaline protease (1.5 mg/ 5cm^3), MBA (15 mM), TEMED (95.50 mM), Pluronic PE 8100 (5.6 mM), stirring rate 300 rpm, polymerization time 2 h and polymerization temperature 30°C.

stirring rate 300 rpm, polymerization time 2 h and polymerization temperature 30°C. The APS concentrations increased, the enzymatic activity and %immobilization increased and reach a maximum at APS concentration of 6.56 mM. When APS concentration was higher than 6.56 mM, the enzymatic activtiy and %immobilization were decreased. Higher APS concentrations lead to an increase in the number of radicals, thereby giving an increase in polymerization rate. The short kinetic lengths of the chain network increase the hydrophilic nature due to the presence of many acrylamide functionalities on the chain. The ability of immobilized enzyme on the high hydrophilic support decreased with increasing the APS concentration because of many short chains of polyacrylamide. The enzymatic activity decreased when the APS concentrations were greater 6.56 mM. Changing the concentration of APS did not affect the percentage conversion. The conversion was nearly constant with any

Table 4.9 Effect of intiator concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

APS Concentration (mM)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
3.13	123 ± 4	28 ± 1	89 ± 1
6.56	150 ± 2	34 ± 1	89 ± 2
9.39	105 ± 1	24 ± 1	90 ± 3
12.52	101 ± 1	24 ± 1	90 ± 3

Figure 4.20 Effect of initiator concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide

increase in the APS concentration. Figure 4.21 shows SEMs of polyacrylamide beads at different initiator concentrations. Morphology of polyacrylamide shows the porous sheet formation. Changing the initiator concentrations did not affect the formation of the polymer beads.

Figure 4.21 SEMs of polyacrylamide at different initiator concentrations

(a) 3.13 mM, (b) 6.56 mM, (c) 9.39 mM, (d) 12.52 mM,

Left x 35 magnification, Right x 500 magnification.

4.11 Effect of Accelerator Concentrations on the Enzymatic Activity

N,N,N',N'-tetraethylmethylenediamine (TEMED) was used with ammonium persulfate as a redox initiation. The different TEMED concentrations were carried out at 47.75, 95.50, 143.25, and 191.05 mM. The reaction was fixed for the concentration of AM (3.14 mM), alkaline protease (1.5 mg/5 cm^3), MBA (15 mM), APS (6.56 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, time 2 h and temperature 30°C. Table 4.10 and Figure 4.22 depict the enzymatic activity, percentage immobilization, and percentage conversion. As the TEMED concentrations increased, the enzymatic activity and percentage immobilization were
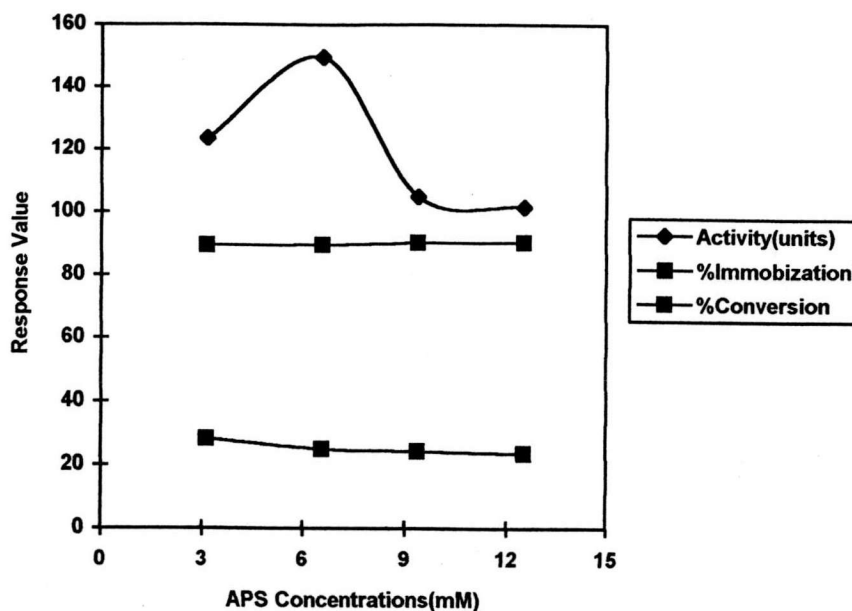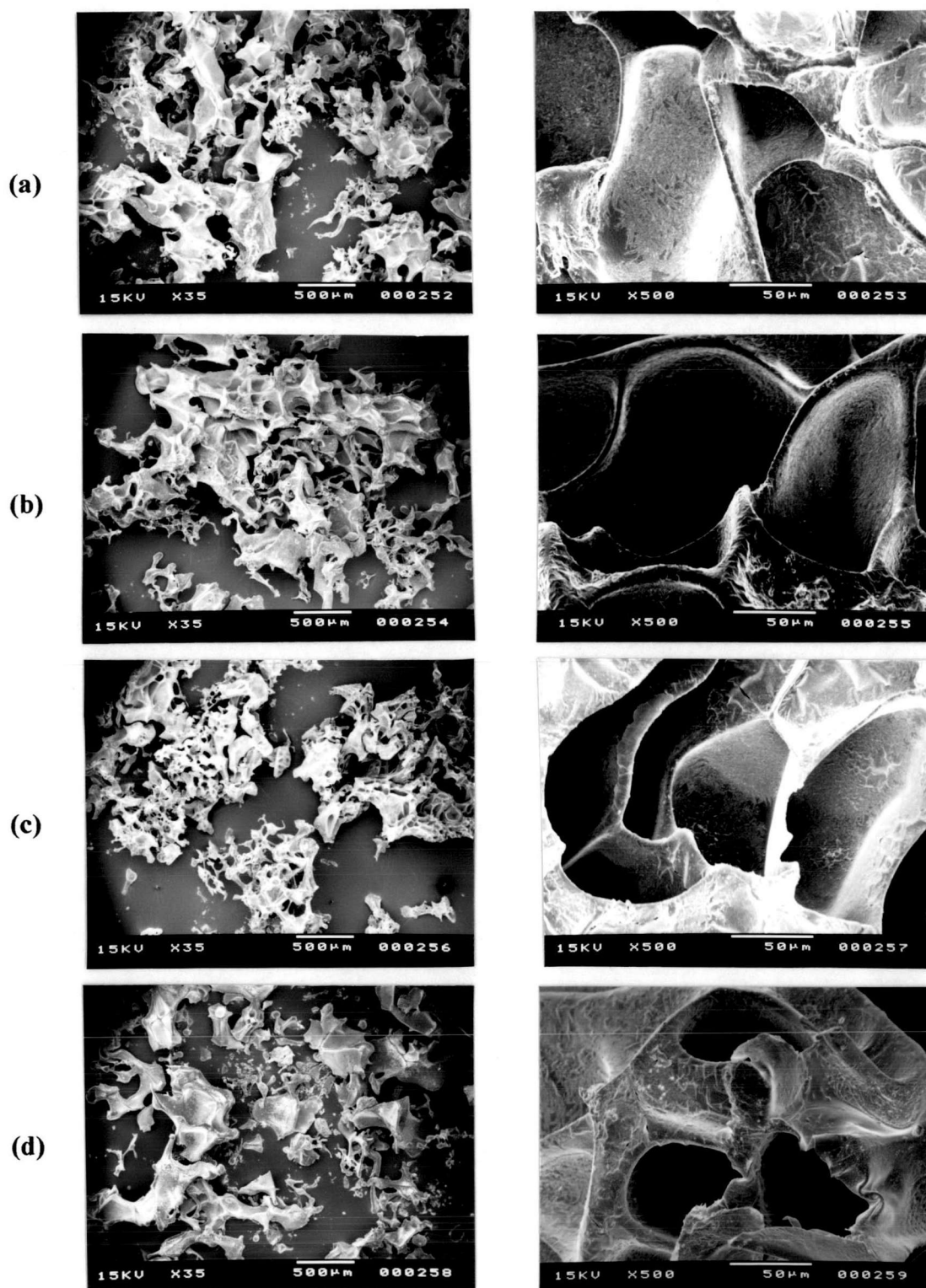
Table 4.10 Effect of accelerator concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

TEMED Concentration (mM)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
47.75	178 ± 2	42 ± 1	93 ± 2
95.50	150 ± 2	34 ± 1	89 ± 2
143.25	131 ± 4	31 ± 1	89 ± 3
191.05	118 ± 1	28 ± 1	89 ± 1
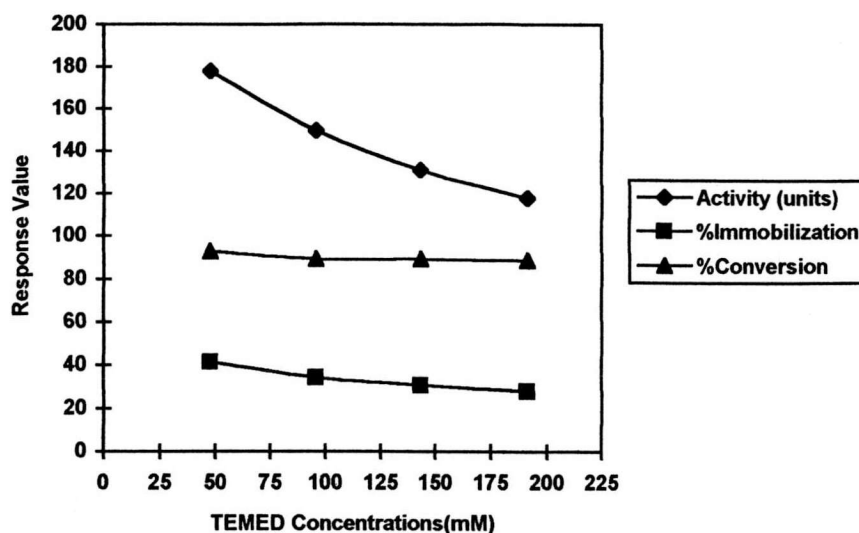
Figure 4.22 Effect of accelerator concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

decreased. The optimum enzymatic activity was observed at 178 units by a TEMED concentration of 47.75 mM. Conversion and immobilization were decreased with increasing the TEMED concentrations. Increasing the TEMED concentration increased the radicals for initiation giving an increase in the polymerization rate. At this point a greater amount of low molecular weights of polyacrylamide could solubilize that limited the amount of insoluble PAM which was regarded as conversion. Figure 4.23 presents SEMs of polyacrylamide beads at different TEMED concentrations. Polyacrylamide formed a porous sheet morphology. Changing TEMED concentrations did not affect the morphology of polyacrylamide bead.

Figure 4.23 SEMs of polyacrylamide at different of TEMED concentrations

(a) 47.75 mM, (b) 95.50 mM, (c) 143.25 mM, (d) 191.05 mM,

Left x 35 magnification, Right x 500 magnification.

4.12 Effects of Surfactant Concentration on the Enzymatic Activity
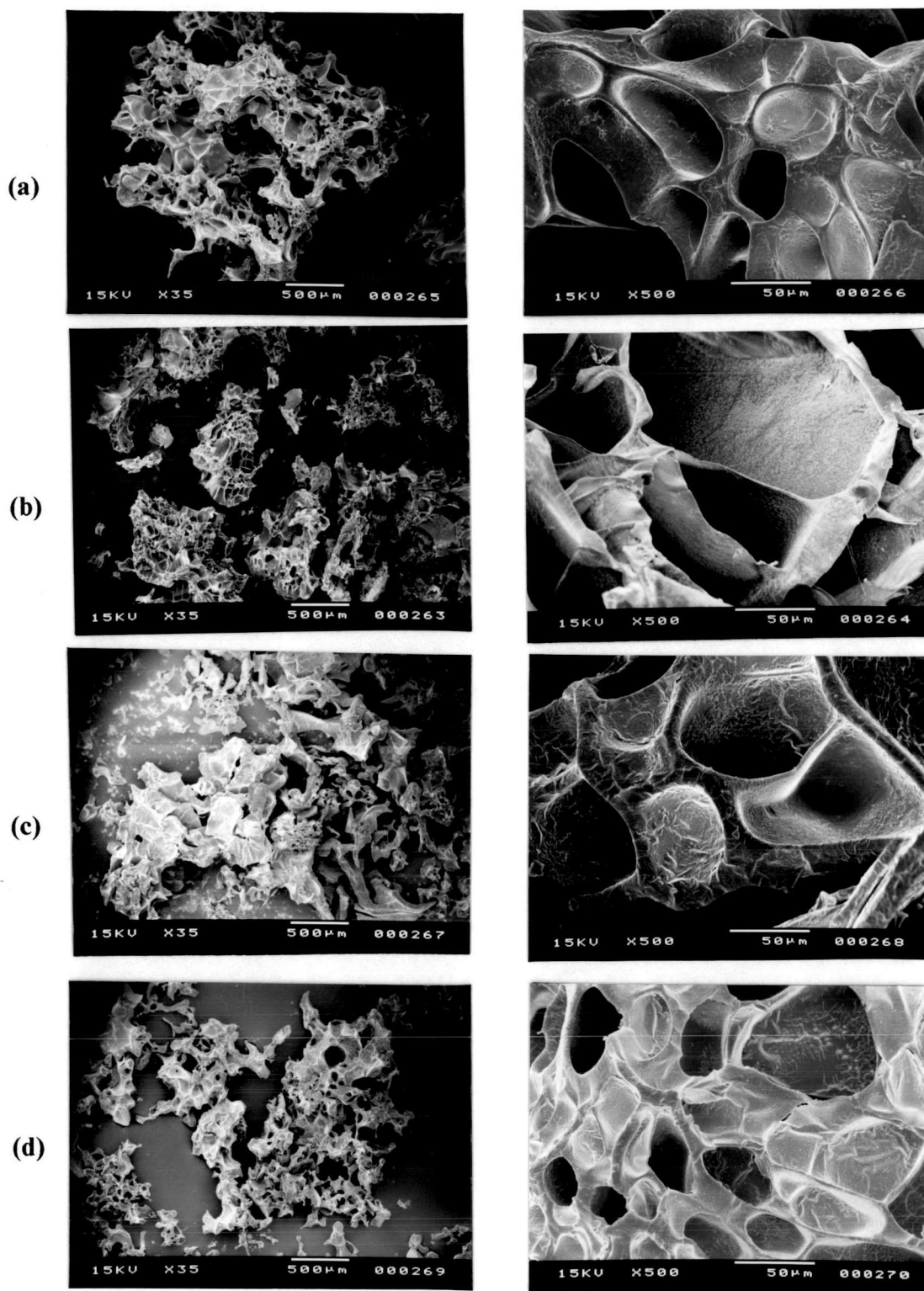
Pluronic PE 8100 was used as surfactant. Various pluronic PE 8100 concentrations were investigated for enzymatic activity, percentage immobilization and percentage conversion. The reaction was fixed the concentrations of AM (3.14 mM), alkaline protease (1.5 mg/5 cm^3), MBA (15 mM), TEMED (47.75 mM), stirring rate 300 rpm, time 2 h and temperature 30°C. The optimum enzymatic activity, percentage immobilization, and percentage conversion were found to be of 178 units, 42% immobilization and 93% conversion, respectively as shown in Table 4.11 and Figure 4.24. The different concentrations of surfactant affected the immobilization, and conversion of monomer. The bead sizes were also affected with surfactant concentrations. At the low surfactant concentration, the monomer droplet did not have enough surfactant to stabilize its droplets. At the high surfactant concentrations, the surfactant can form many small monomer droplets. The beads having both high

Table 4.11 Effect of surfactant concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

Pluronic PE 8100 Concentration (mM)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
5.3	124 ± 1	13 ± 1	43 ± 4
10.6	178 ± 2	42 ± 1	93 ± 2
15.9	145 ± 2	32 ± 1	83 ± 5
21.2	133 ± 1	12 ± 1	33 ± 1

Figure 4.24 Effect of surfactant concentrations on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

and low molecular weights were obtained. The beads with the low molecular weights were not formed and could not precipitate out to give the stable beads. So the percentage conversion was decreased with increasing the surfactant concentraions. Only at 10.6 mM of Pluronic PE 8100, the surfactant concentration permitted the formation of stable monomer droplets for polymerization, which consequently gave a high conversion of 93% along with good properties of enzymatic activity and percentage immobilization of the enzyme. Figure 4.25 shows SEM micrographs of polyacrylamide at different Pluronic PE 8100 concentrations. polyacrylamide formed the porous sheet. Changing surfactant concentrations did not chage morphologies of the polyacrylamide beads.

Figure 4.25 SEMs of polyacrylamide at different surfactant concentrations

(a) 5.3 mM, (b) 10.6 mM, (c) 15.9 mM, (d) 21.2 mM,

Left x 35 magnification, Right x 150 magnification

4.13 Effect of Acrylamide/Methacrylic Acid Ratios 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. The reaction was fixed for the concentration of AM (3.14 mM), alkaline protease (1.5 mg/5 cm^3), MBA (15 mM), TEMED (95.50 mM), Pluronic PE 8100 (10.6 mM), stirring rate 300 rpm, polymerization time 2 h and temperature 30°C. The results were indicated in Table 4.12 and Figure 4.26. The reactivity of poly(AM-co-MAA) in carbonate-bicarbonate buffer (NaCO$_3$-NaHCO$_3$) pH 10.5 and polymerization temperature 30°C has not reported. Consideration reactivity of poly(AM-co-MAA) in sodium salt and temperature 30°C instead [84]. The r$_1$ and r$_2$ of acrylamide and methacrylic acid were 0.42 and 0.59, respectively. When r$_1$r$_2$ = 0.25 (0<r$_1$r$_2$<1). That is r$_1$< 1 and r$_2$ < 1. The behavior of comonomer lies between extremes of ideal (r$_1$r$_2$ = 1) and alternating (r$_1$r$_2$ = 0) copolymerization.
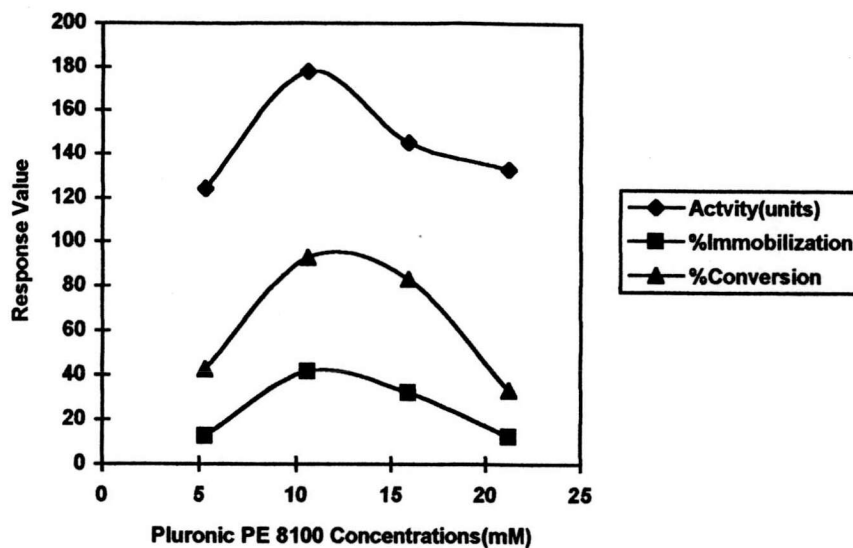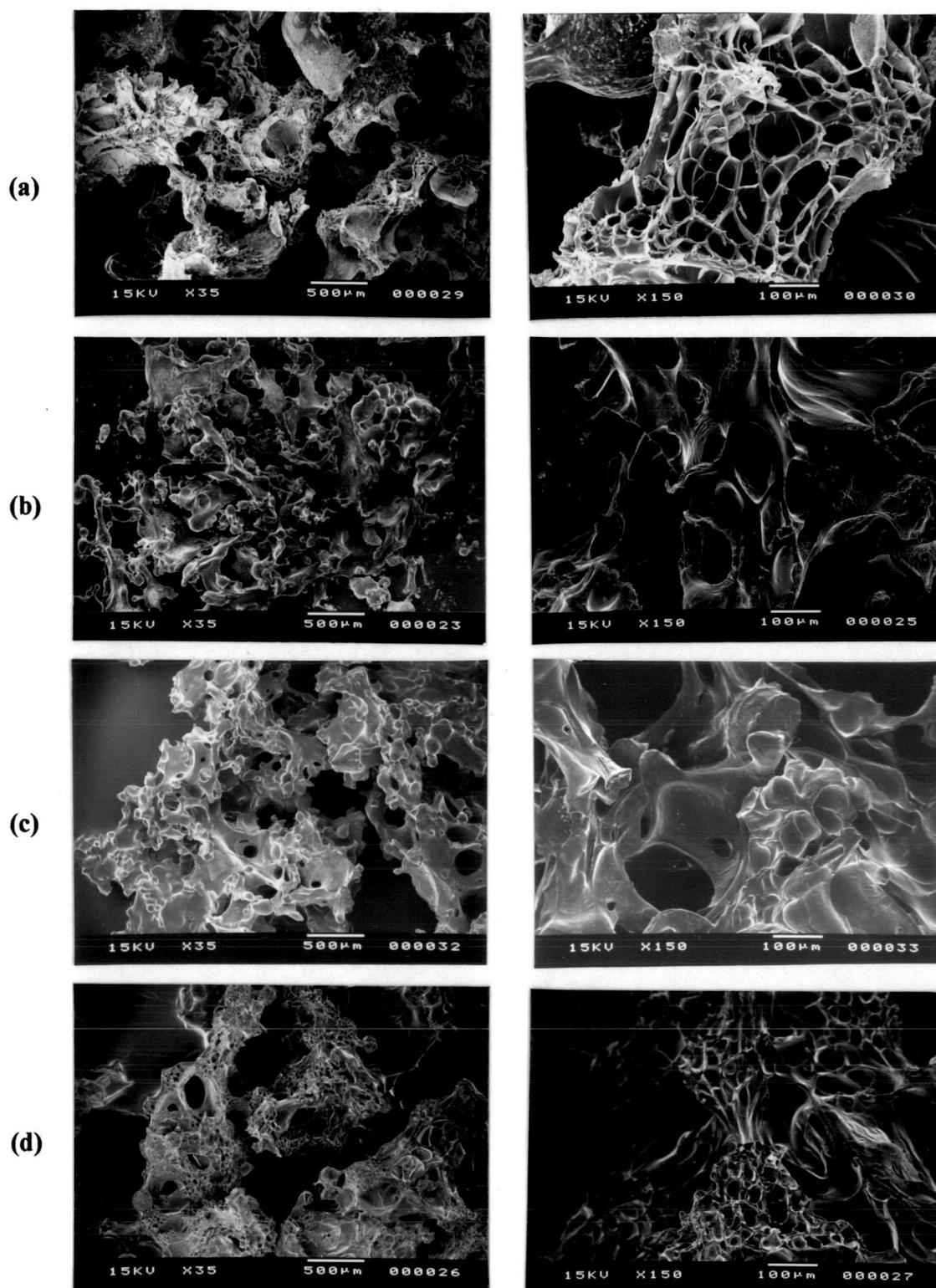
Table 4.12 Effect of acrylamide/methacrylic acid ratios on the enzymatic activity, percentage immobilization, and percentage conversion of immobilized alkaline protease on polyacrylamide.

AM/MAA Ratios (%W/W)	Enzymatic Activity (units)	Percentage Immobilization	Percentage Conversion
100/0	150 ± 2	34 ± 1	89 ± 2
97.5/2.5	144 ± 1	34 ± 1	88 ± 3
95/5	113 ± 2	26 ± 1	88 ± 5
90/10	97 ± 1	23 ± 1	86 ± 3
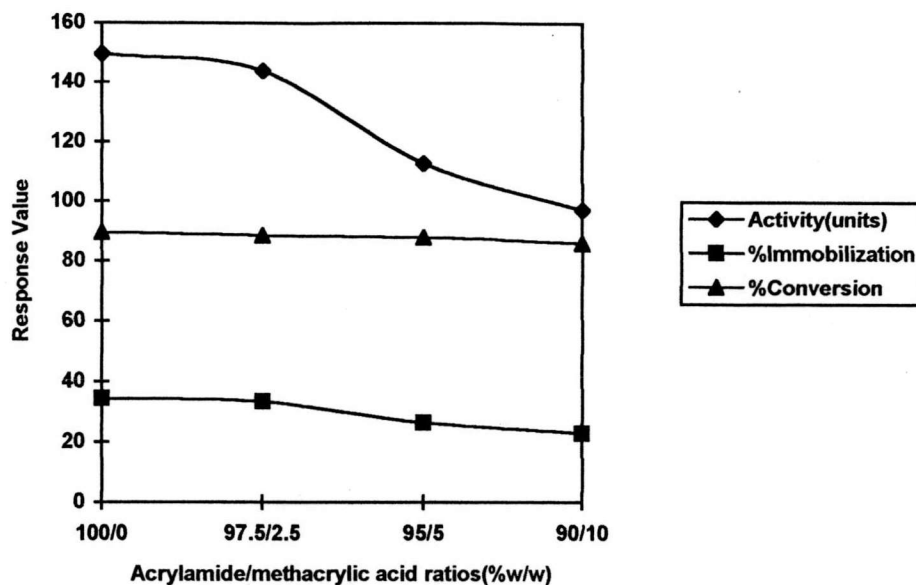
Figure 4.26 Effect of acrylamide/methacrylic acid ratios on the enzymatic activity, percentage immobilization, percentage conversion of immobilized alkaline protease on polyacrylamide

As r_1r_2 decreases from unity toward zero, there is an increasing tendency toward alternation [89]. The enzymatic activity and percentage immobilization was decreased with increasing the methacrylic acid monomer concentrations. Polyacrylamide beads gave the optimum activity of 150 units where as the poly (acrylamide-co-methacrylic acid) provided the optimum activity of 144 units at 97.5/2.5% W/W acrylamide/methacrylic acid comonomer. Methacrylic acid comonomer was used for enhancing the hydrophilic polyacrylamide support. The water absorption of poly(acrylamide-co-methacrylic acid) increased with increasing the methacrylic acid concentration. The gel strength was thereby decreased, so the

Figure 4.27   SEMs of polyacrylamide and poly(acrylamide-co-methacrylic acid),

(a) 100/0, (b) 97.5/2.5, (c) 95/5, (d) 90/10% W/W,

Left x 35 magnification, Right x 500 magnification

ability to function as the support for immobilizing rather decreased. The increasing methacrylic acid concentrations unfortunately decreased 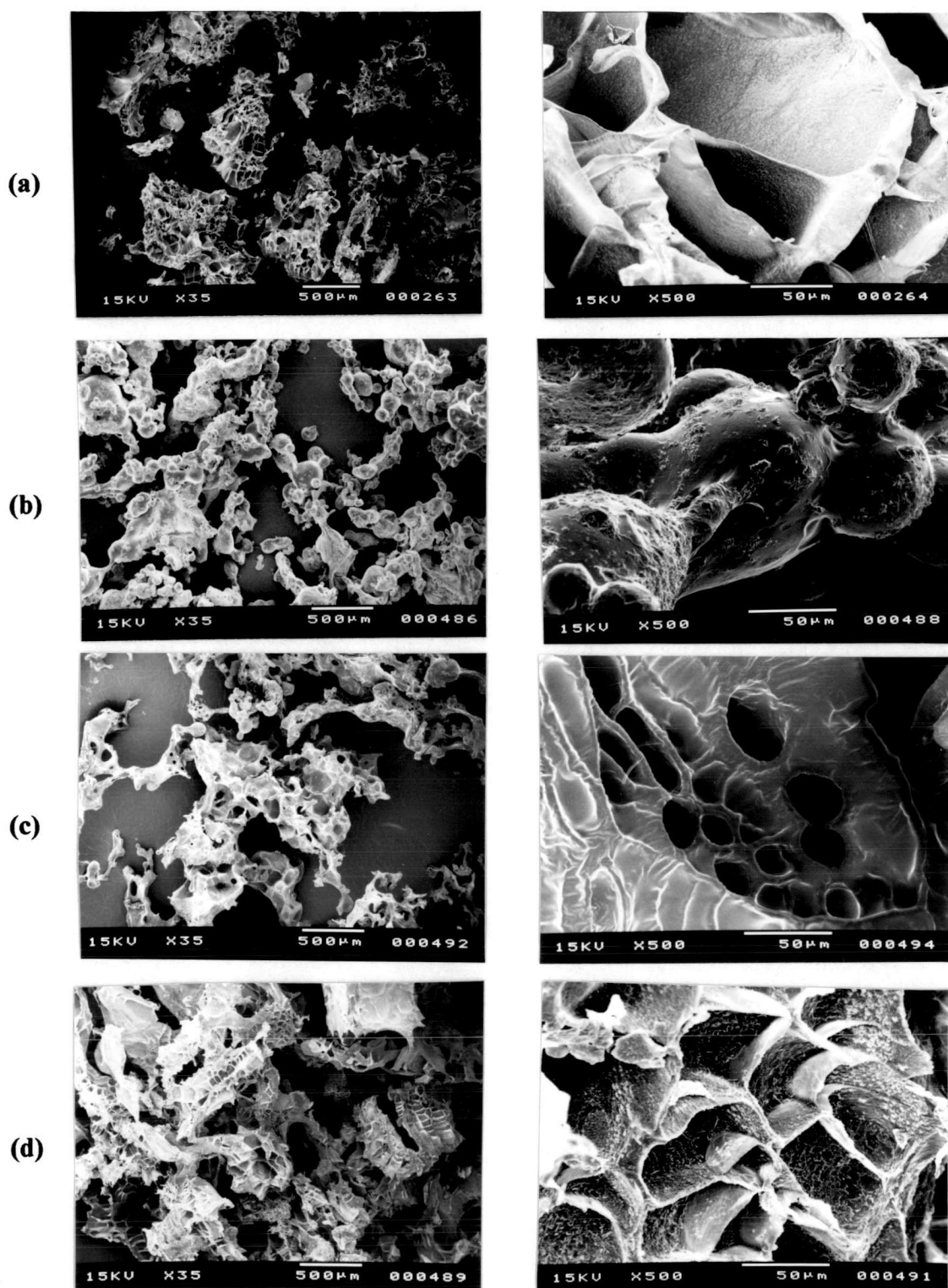the polymerization conversion. The conversion of polyacrylamide was higher than that of poly (acrylamide-co-methacrylic acid) at the same reaction condition. SEMs of polyacrylamide and poly(acrylamide-co-methacrylic acid) are showed in Figure 4.27. Changing acrylamide/methacrylic acid ratios did not affect morphologies of the polymer beads.

4.14 Water Absorption

4.14.1 In Deionized Water and Saline solutions

The absorption in deionized water and saline solutions of polyacrylamide and poly(acrylamide-co-methacrylic acid) were investigated. Table 4.13 and Figure 4.28 show the effects of 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). Copolymer absorption was determined as a function ratio of acrylamide and methacrylic acid monomer of 100/0, 97.5/2.5, 95/5, 90/10% W/W. The water absorption increased with increasing the methacrylic acid concentrations. Methacrylic acid concentration increased the hydrophilicity through the hydrogen bonding and charge repulsion. The water absorption was found to be of

Table 4.13 Absorption in deioned water and saline solutions

AM/MAA ratios (% W/W)	Absorption Ratios				
	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**	84 ± 1	30 ± 2	29 ± 2	22 ± 1	22 ± 3

* = with the enzyme, ** = with out the enzyme
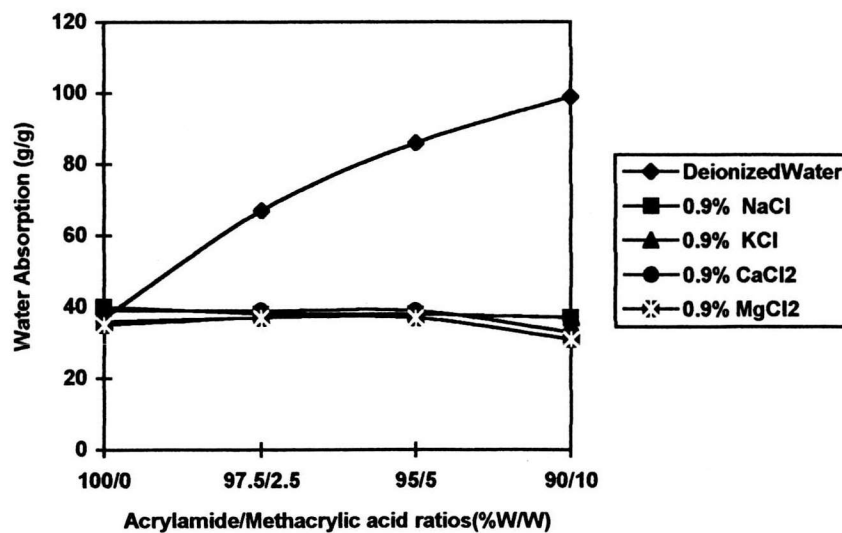

Figure 4.28 The absorption of polyacrylamide and poly(acrylamide-co-methacrylic acid) in deionized water and saline solutions.

37, 67, 86, and 99 g/g, for 100/0, 97.5/2.5, 95/5, 90/10, respectively. The absorption abilities of polyacrylamide in deionized water and in saline solutions were not different. It is anticipated that the basicity of amide group contributed to the constant equilibrium osmotic pressure for both deionized distilled water and saline solutions at isotonic point (0.9%). The water absorption of poly(acrylamide-co-methacrylic acid) in deionized water was higher than those of 0.9% saline solutions. In saline solutions, the effects of different AM/MAA ratios were not significant. Ionic strength and oxidation states of the cation ions do not affect the water absorption. This property is of very importance. It implied 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 be plausibly controlled by the number and average molecular weights of the crosslinks, i.e. the crosslink density of each copolymer is constant.

In addition, the water absorption of with and without enzyme of 90/10% W/W poly(acrylamide-co-methacrylic acid) were compared. The results show that the amounts of enzyme affect at some extent on the water absorption. This may plausibly suggest that the enzyme could be entrapped in the polymer support via some physico-chemical interaction. Such an interaction affect the equillibrium osmotic pressure for water absorption.

4.14.2 Effect of Temperature on Water Absorption

The result of water absorption at various temperatures of polyacrylamide and poly(acrylamide-co-methacrylic acid) were shown in Table 4.14 and Figure 4.29.

Table 4.14 Effect of temperature on water absorption

Temperature (°C)	Acrylamide/Methacrylic Acid Ratios (% W/W)			
	100/0	97.5/2.5	95/5	90/10
25	30 ± 2	60 ± 2	81 ± 6	96 ± 7
30	31 ± 2	59 ± 6	86 ± 9	98 ± 3
35	34 ± 1	54 ± 2	77 ± 1	90 ± 1
40	30 ± 3	47 ± 2	61 ± 2	91 ± 4
45	35 ± 8	49 ± 5	64 ± 2	88 ± 5
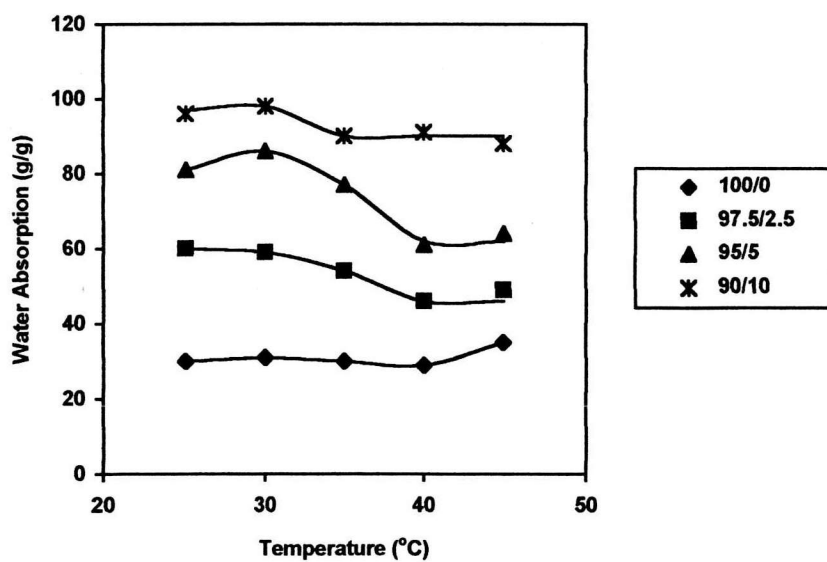
Figure 4.29 Effect of temperature on water absorption

Water absorption of acrylamide and methacrylic acid ratios of 100/0, 97.5/2.5, 95/5, 90/10 was carried out. Increasing the temperature did not affect the water absorption of polyacrylamide but the water absorption of poly(acrylamide-co-methacrylic acid) decreased at around 30 to 35°C.

Kawaguchi, H. produced poly(acrylamide-co-methacrylic acid) hydrogel in alcohol solution. These microspheres swelled at low temperatures and shrank at temperatures higher than 32°C. The homopolymers of acrylamide and methacrylic acid alone do not display the changing pattern, but the change occurs when the two monomers are copolymerized. This is conceived to be due to hydrophobic interaction among methacrylic acid units, and a further of interaction hydrogen bonding formed between the molecular chains with amide groups [80].

The clarity of polymeric beads at different temperatures was observed. The clarity of the beads increased with decreasing temperatures. The polyacrylamide beads are less clear than that of poly(acrylamide-co-methacrylic acid). The clarity of copolymer beads increased with increasing the methacrylic acid concentrations. From the results, poly(acrylamide-co-methacrylic acid) is a thermally reversible hydrogel. Poly(acrylamide-co-methacrylic acid) hydrogel exhibits a lower critical solution temperature (LCST) at around 30-35°C in distilled water because the hydrogel swells below 35°C and shrinks above 35°C. The present work is analogous to the work of Kawaguchi, H. [80] who synthesized poly(acrylamide-co-methacrylic acid) uniform-sized microspheres by which the microsheres swells and shrank at a transition temperature of 32°C. The particles 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

recent works done elsewhere also support our finding such as Gutowska, A. et al.[90], synthesized thermosensitive hydrogels of N-isopropyl acrylamide, N-isopropyl acrylamide/acrylic acid, N-isopropyl acrylamide/butyl methacrylate. The incorporation of hydrophobic and hydrophilic comonomers strongly influences the swelling/shrinking behavior of thermosensitive hydrogels. The hydrophobic comonomer decreased the gel collapse temperature, whereas the hydrophilic comonomer increased the gell collapse temperature. As suggested earlier that the poly(acrylamide-co-methacrylic acid) copolymer is the thermally reversible hydrogel, which shows the LCST. This may be plausibly due to a hydrophobic interaction among methacrylic acid units. The LCST of poly(acrylamide-co-methacrylic acid) increased with increasing the methacrylic acid concentration.

4.15 Effect of Protein Digestion

Immobilized alkaline protease on polyacrylamide and 97.5/2.5% W/W poly (acrylamide-co-methacrylic acid) was used as a model for investigaing the effect of protein digestion. The different substrates of casein Hammerstein, Bovine Serum Albumin (BSA), blood solution, gelatin, and aminal hair were investigated. The concentrations of casein, BSA, and gelation were 0.5% W/V in a carbonate-bicarbonate buffer solution of pH 10.5. Blood solution was diluted to 20 times the with carbonate-bicarbonate buffer solution of pH 10.5. The aminal hair was weighed to 0.020 g and suspended in 1 cm^3 of the carbonate-bicarbonate buffer of pH 10.5. Alkaline protease 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, food such as milk egg, meat, and fish, and plant materials such as grass [2]. In the washing process, this enzyme can be used in the alkaline conditions, heat and in the presence of surfactant. In this study, we selected the casein, BSA and blood as the proteinaceous dirt adsorbed in clothes, for testing this immobilized alkaline protease. It showed a higher stability than the free alkaline protease at higher temperature. The results of enzyme digestion on different proteins are shown in Table 4.15 as percentage relative activity (%RA) which fixing the enzymatic activity of free and immobilized enzyme of 1.2×10^6 and 150 units as 100% RA, respectively. The free enzyme shows the higher ability to digest different substrates than does the immobilized enzyme. Because the immobilized enzyme is entrapped in a polymeric support for preventing from the thermal denaturation. The proteolytic velocity of immobilized enzyme was therefore slower than the free

Table 4.15 Effect of free and immobilized enzyme on protein digestion

Substrates	% Relative Activity		
	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	-	-

The free and immobilized enzymes could digest blood, casein, and BSA. In Section 4.17 we suggested that the enzyme was entrapped in the pore of polyacrylamide gel and no leakage under washing could occur. The substrates with the low molecular size can penetrate into the pores of the polymer support to react with enzyme and to release the product to outside the beads. Casein having an average molecualar weights (approx.23,600) [91] less than BSA (approx. 66,000) [92] and blood show enzymatic activity higher than BSA and blood. In addition, protease is used for unhairing and batting in the tanning industry. In this study, the gelatin and aminal hair are represented as protein sources found on the hide. The results show that the gelatin could not be digested by the free and immobilized enzymes. Because gelatin formed gel solution that cannot pass the pores of the polymer supports to react with the enzyme. In the suspension of aminal hair, the free enzyme can diffuse to this substrate, but the immobilized enzymes cannot, the enzymatic activity was therefore not detected. The suspended animal hair solution as a substrate lower the activities of the immobilized enzymes because the diffussion limit of the animal hair substrate is the main attribute to the reduced activities of the immobilized enzyme on the polymer supports. We suggest that the immobilized enzyme be suitable for the detergent industry not suitable for the tanning industry.

4.16 Protein Staining

After binding the immobilized enzyme on polyacrylamide beads, the intense blue colour was observed. Figure 4.30 shows the results of protein staining with and without immobilized enzyme on polyacrylamide beads. Tubes A, C, and E are the free enzyme polyacrylamide beads and tubes B, D, and F are the immobilized enzyme on polyacrylamide beads. After binding the enzyme, Tube F shows the blue colour of the immobilized enzyme whereas Tube E, the beads was free from the enzyme shows the clarity solution. This result indicates that the enzyme is entrapped on the polyacrylamide beads. The crosslinked polyacrylamide has a the constraining structure, and tight enough to entrap the enzyme and prevent the enzyme from diffusing into the surrounding medium. The intense and pale blue colours of protein staining were used to qualified the amounts of enzyme that immobilized on the polymer beads for the primary screening before determining the enzymatic activity by using a casein substrate. Figure 4.31 shows the binding of immobilized enzyme on the polyacrylamide and poly(acrylamide-co-methacrylic acid) beads. The ratios of acrylamide/methacrylic acid of 100/0, 97.5/25., 95/5, 90/10% W/W as shown in the tubes A-D were carried out. The enzymatic activities of the immobilized enzyme were 150, 144, 113, 97 units, respectively. The enzyme free polyacrylamide bead as a control tube as shown in tube E was also tested. The results indicate that the blue colour was increased with increasing the enzyme content in the polymer beads. Tube E was colourless in the absence of enzyme. The result concludes that the enzyme forms a strong interaction with the molecules of supports that often hinder the free movement of enzyme molecules, resulting in decreased enzyme activity.
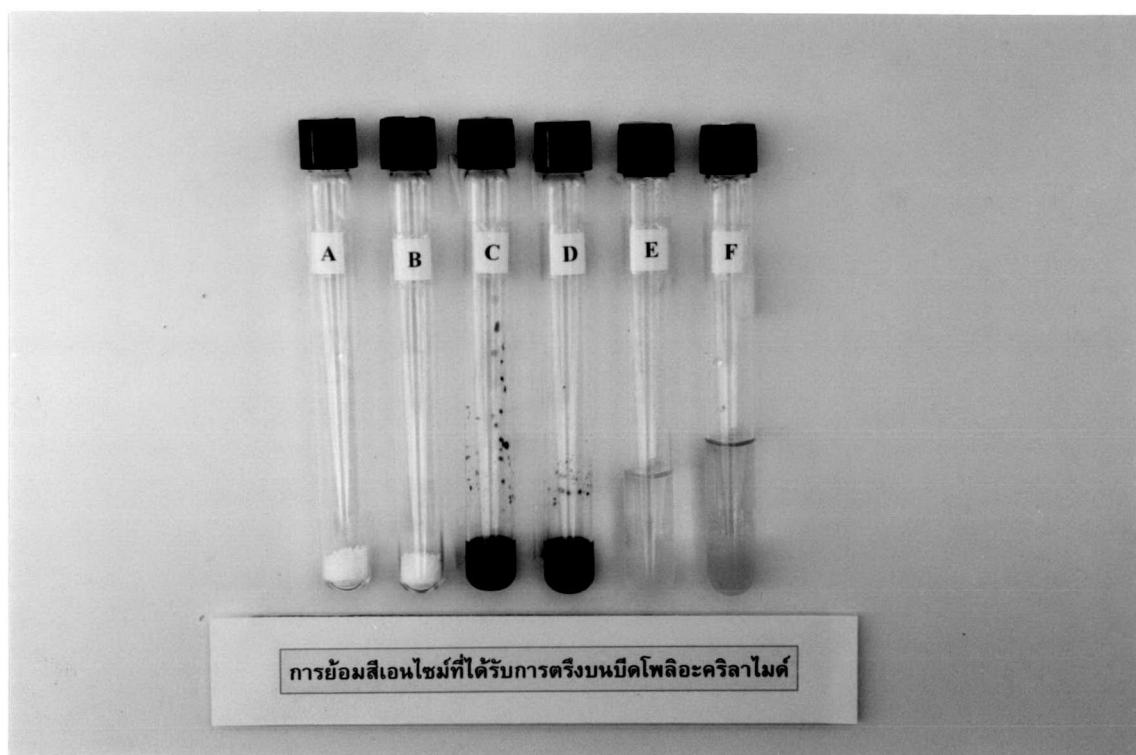
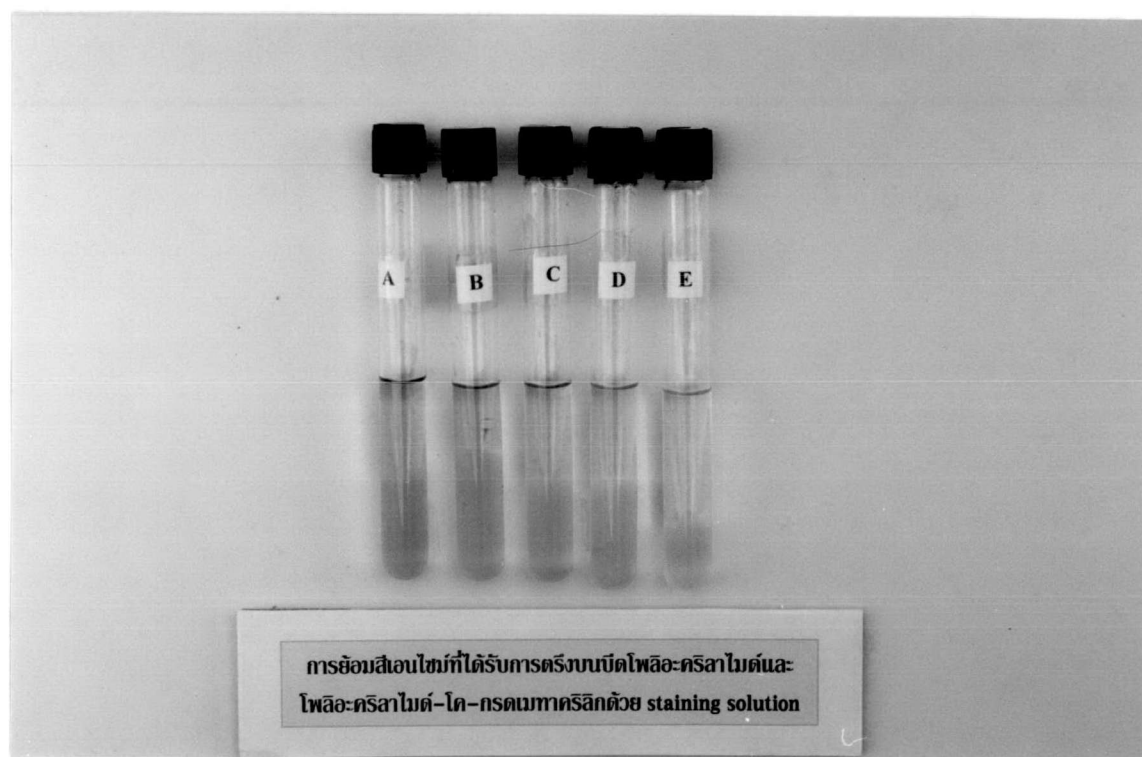**Figure 4.30** The protein staining of polyacrylamide

Figure 4.31 The protein staining of polyacrylamide and

poly(acrylamide-co-methacrylic acid)

4.17 Determination of Amount of the Leakage Enzyme Molecules on the Beads Under Washing

Table 4.16 and Figure 4.32 present the enzymatic activity of immobilized enzyme, using casein as substrate at pH 10.5 and 45°C. The dried beads that known the enzymatic activity were washed again with 1-3 times of carbonate-bicarbonate buffer solution of pH 10.5. The washed beads were dried and determined the enzymatic activity. One washing time show higher enzymatic activity than 2-4 washing times. In addition, freeze drying may cause greater effect in enzymatic activity [93]. 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 that of was determined. It was found that the amount of the immobilized enzyme retained after 4 washing. This suggests that the enzyme be entrapped in the polymer support and no leakage of the immobilized enzyme can occur as a result of the a numerous repeatedly buffer washings. As stated in Section 2.1.1.4, the enzyme can be immobilized by crosslinking it on an inert support. PAM or poly(AM-co-MAA) can be considered as an inert membrane where the physically

Table 4.16 Amount of the leakage enzyme molecules on the beads under washing

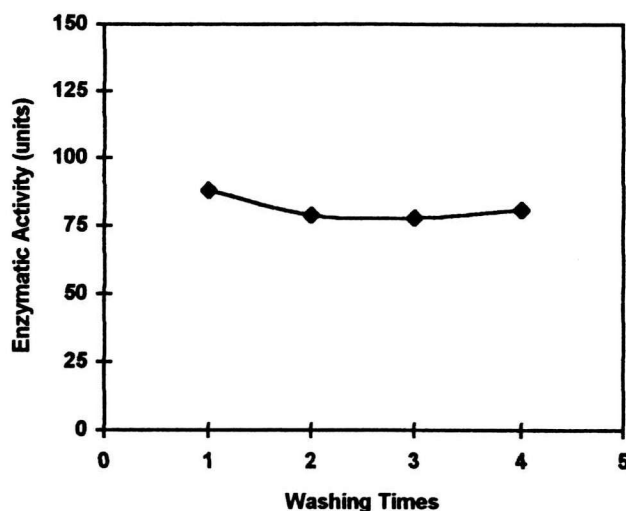| Washing times	Enzymatic Activity (units)
1	88 ± 2
2	79 ± 3
3	78 ± 1
4	81 ± 3

Figure 4.32 Amount of the leakage enzyme molecules on the beads under washing

absorbed enzyme is crosslinked on the membrane surface. Another suggestion is that

the type of functional groups on the enzyme can covalently bond with the support

material either by grafting or crosslinking [2]. The protein functional groups that can

be used for the covalent binding of enzymes to polymeric supports include (1) amino

groups, (2) carboxy groups, (3) phenol rings of tyrosine, (4) sulfohydryl groups of

cysteine, (5) hydroxyl group of serine, threonine, and tyrosine, (6) imidazole groups

of histidine and (7) indole groups of tryptophan. Because of the tight binding, the

enzyme does not leak or detach from PAM or poly(AM-co-MAA) during use. The

immobilized enzymes can easily come into contact with substrates because the

enzymes are localized on the supports. An increase in heat is often observed as

described in Section 4.19 because of the strong interaction between the enzyme

molecules and supports.

4.18 Effect of pH on Enzymatic Activity

The pH effects on the enzymatic activity of the free and immobilized enzyme

for casein hydrolysis at 45°C in various buffer solutions of pH 7.5 to 11.0 are shown

in Table 4.17 and Figure 4.33. The enzymatic activity was determined by the amounts

of tyrosine released. The calibration curve of tyrosine in various buffer solutions are

shown in Appendix III. The results were reported as the percentage relative activity

(%RA) that fixes the enzymatic activity of free and immobilized enzymes of 6.3 x 10^6

and 172 units as 100% RA. The optimum pH was at about 10.0 for the free enzyme.

Table 4.17 Effect of pH on enzymatic activity

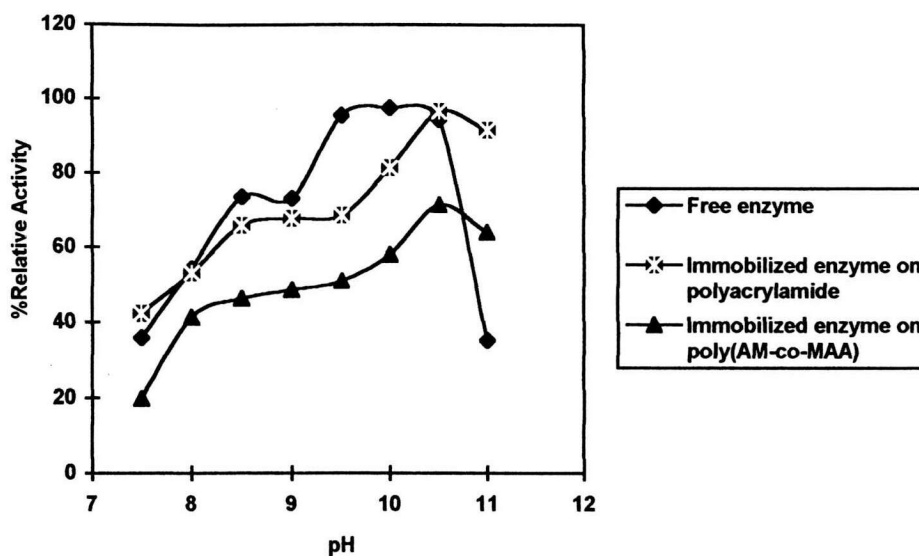| pH	% Relative Activity		
	Free enzyme	Immobilized enzyme on PAM	Immobilized enzyme on poly(AM-co-MAA)
7.5	35.9 ± 2.9	42.3 ± 3.1	19.7 ± 1.2
8.0	54.2 ± 0.9	53.2 ± 0.4	41.4 ± 1.1
8.5	73.5 ± 3.4	65.9 ± 0.8	46.3 ± 2.4
9.0	73.1 ± 1.0	67.7 ± 0.4	48.6 ± 1.2
9.5	95.6 ± 1.2	68.6 ± 1.3	51.1 ± 2.1
10.0	97.4 ± 2.8	81.3 ± 1.4	58.0 ± 0.3
10.5	94.3 ± 2.5	96.6 ± 1.1	71.4 ± 3.3
11.0	35.2 ± 3.0	91.5 ± 1.3	64.0 ± 1.2

Figure 4.33 Effect of pH on enzymatic activity

Aunstrup, K. [42] reported that the pH optimum of alkaline protease in a hydrolysis of casein was at about 10. Over 80% of the enzymatic activity was maintained in the pH range of 8-11. Inactivation of the enzyme was very rapaid at pH values below 4 or above 11.5.

The optimum pHs of immobilized enzyme on polyacrylamide and poly (acrylamide-co-methacrylic acid) were shifted to pH 10.5. The pH profile of immobilized enzyme was more pronouncly displayed the pH dependence in the alkaline range than the free enzyme.

4.19 Effect of Thermal Stability on Enzymatic Activity

The temperature dependence of enzymatic activity of free and immobilized enzymes were determined in 0.1 M of carbonate-bicarbonate buffer solution of pH

10.5 in the temperature range of 25 to 70°C, using casein as the substrate. Table 4.18 and Figure 4.34 show that optimum temperature for the free and immobilized enzyme to function well is at 45°C. At low temperatures, the enzyme are not active. At high temperature the enzymes lose their activity. The results are presented as percentage relative activity (%RA) that fixes the enzymatic activities of free and immobilized enzyme of 4.4×10^6 and 172 units as 100% RA. From the temperature profile, the immobilized enzyme is more thermally stable than is the free enzyme in the range of higher temperatures. The polymer supports dissipate the heat especially at high temperatures that prevent the immobilized enzyme from denaturation.

Table 4.18 Effect of thermal stability on the enzymatic activity

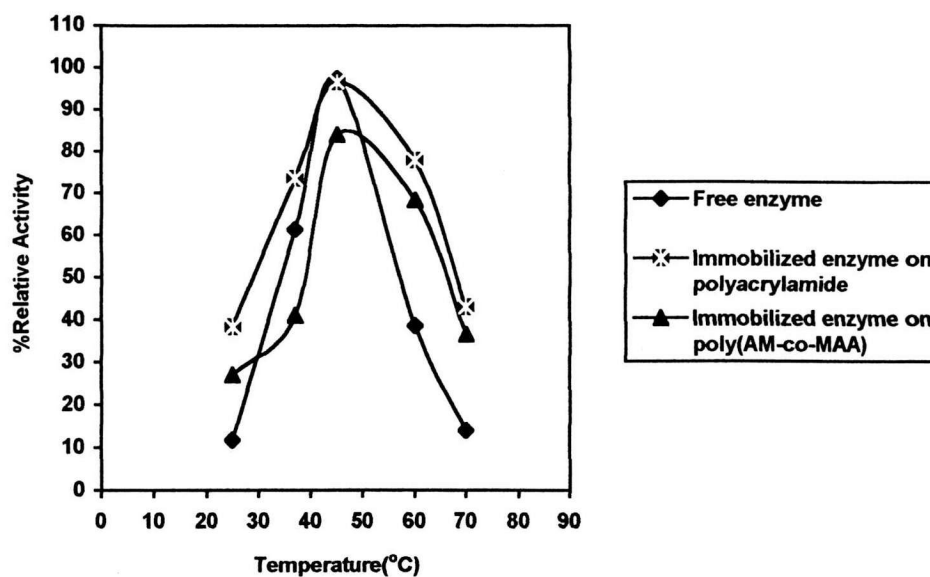| Temperature (°C)	% Relative Activity		
	Free Enzyme	Immobilized enzyme on PAM	Immobilized enzyme on poly(AM-co-MAA)
25	11.7 ± 0.6	38.3 ± 2.5	27.0 ± 0.9
37	61.2 ± 3.3	73.4 ± 0.6	41.0 ± 0.8
45	97.4 ± 1.3	96.6 ± 1.5	84.0 ± 0.1
60	38.6 ± 2.6	77.8 ± 0.6	68.3 ± 2.1
70	14.0 ± 1.8	43.0 ± 1.5	36.5 ± 0.4

Figure 4.34 Effect of thermal stability on the enzymatic activity

4.20 Effect of Storage Stability on the Enzymatic Activity

The storage stability on the enzymatic activity of immobilized alkaline protease was compared with the free alkaline protease at various temperatures from -20°C to 60°C. Table 4.19 illustrates the residual enzymatic activity as percentage relative activtiy (%RA) that fixes the enzymatic activities of free and immobilized enzyme on polyacrylamide (polyAM) 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 one month. The immobilized enzyme was more stable than the free enzyme at the higher temperature. The immobilized enzyme on polyacrylamide is more stable than is the immobilized enzyme on poly(acrylamide-co-methacrylic acid). The free and immobilized enzyme can be kept at a temperature

Table 4.18 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
	PAM	100.0 ± 1.7	99.6 ± 0.4	102.3 ± 0.3	99.9 ± 1.9	101.4 ± 0.7
	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
	PAM	100.0 ± 1.7	100.1 ± 0.7	100.0 ± 1.7	100.1 ± 0.9	100.0 ± 0.3
	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
	PAM	100.0 ± 1.7	101.5 ± 2.1	92.1 ± 0.8	85.6 ± 0.8	79.5 ± 0.9
	Poly(AM-co-MAA)	100.0 ± 2.3	100.2 ± 0.7	97.3 ± 0.7	86.4 ± 0.4	74.8 ± 1.2

Table 4.18 (continued) Effect of storage stability on the enzymatic activity.

Temperature (°C)	Enzyme	Percentage Relative Activity					
		0 day	7 days	14 days	21 days	30 days
37	Free	100.0 ± 1.8	97.0 ± 1.3	82.2 ± 0.8	69.1 ± 1.3	59.2 ± 0.8
	PAM	100.0 ± 1.7	97.3 ± 0.4	87.8 ± 1.7	78.8 ± 0.9	76.2 ± 2.4
	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
	PAM	100.0 ± 1.7	90.9 ± 1.5	85.4 ± 2.6	77.3 ± 1.1	71.9 ± 1.1
	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
	PAM	100.0 ± 1.7	87.5 ± 0.4	77.0 ± 0.4	73.1 ± 1.7	63.3 ± 0.8
	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 = Free enzyme, PAM = Immobilized enzyme on polyacrylamide,

Poly(AM-co-MAA) = Immobilized enzyme on poly(acrylamide-co-methacrylic acid)

of range -20°C to 4°C for one month without losing the enzymatic activity. The free enzyme loses the enzymatic activity to 50% RA after being kept at temperature of 60°C for one month. The higher stability of the immobilized enzyme can be attributed to the prevention of autodigestion and thermal denaturation as a result of the immobilization of the enzyme molecules onto the polymer supports.