Immobilization of Lipase on a Polymeric Microsphere with an Epoxy Group Prepared by Radiation-Induced Polymerization

Seong-Ho Choi,[1] Kwang-Pill Lee,[1] Hee-Dong Kang[2]

[1]*Department of Chemistry Graduate School, Kyungpook National University, Daegu 702-701, South Korea*
[2]*Department of Physics, Kyungpook National University, Daegu 702-701, South Korea*

Received 1 March 2002; accepted 23 May 2002
Published online 18 February 2003 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/app.11737

ABSTRACT: A polymeric microsphere (PM) with an epoxy group was prepared by the radiation-induced polymerization of glycidyl methacrylate and diethylene glycol dimethacrylate in reaction conditions with variations in solvents, irradiation dose, and monomer composition. The epoxy group of the PM was analyzed by solid-state ^{13}C-NMR, Fourier transform infrared spectroscopy (FTIR), Fourier transform Raman spectroscopy, and elemental analysis (EA) after amination. In EA after amination, the epoxy group content was in the range 0.20–0.50 mmol/g. The lipase was immobilized to the epoxy group of the PM in experimental conditions with variations in the pH and the epoxy group content. The activity of the lipase-immobilized PM was in the range 148–342 unit/mg min. The activity of lipase-immobilized PM increased in accordance with the epoxy group content. The lipase-immobilized PM was also characterized by FTIR and EA. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 1153–1161, 2003

Key words: radiation; biomaterials; irradiation

INTRODUCTION

Immobilization is one of the oldest and most practical techniques in enzyme technology. Immobilized enzymes are widely used in the production of various sugars, amino acids, and pharmaceutical drugs.[1–4] The immobilization of antibiotics or other proteins is used both for affinity chromatography and in clinical settings. Immobilized enzymes are also used as biosensors and catalysts in industrial applications. The use of synthetic polymers as supports for enzyme immobilization provides several advantages, such as inertness to microorganism attack, higher chemical resistance, and the option to use a complex buffer component.

Glycidyl methacrylate (GMA) is one of the monomers that is easily modified into various functional groups. As GMA is polymerized, the epoxy groups of the GMA become useful for the introduction of various functional groups, such as amines,[5,6] alcohols,[7] phosphoric acid,[8] and proteins.[9]

Lipases, or triacylglycerol acyl ester hydrolases (EC 3.1.1.3), are enzymes possessing a capacity to catalyze the cleavage of carboxyl ester bonds in triacylglycerols, diacylglycerols, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils).[10,11]

The radiation-induced polymerization of ethylene glycol dimethacrylate (DEGMA) in organic solvents produces a monodisperse polymeric microsphere (PM).[12] The characteristics and the advantages are that the preparation can be carried out in the absence of a surfactant or a stabilizer. The mixture of only monomer and solvent gives the monodisperse microspheres. The fact that the procedure does not need any stabilizer suggests a different mechanism for the microsphere formation from dispersion and emulsion polymerization.

In a previous work,[13] carboxylic acid groups were introduced onto polyethylene (PE) film by radiation-induced graft copolymerization. Subsequently, the CGTases were immobilized on the PE film with a carboxylic acid group with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The activity of the immobilized CGTase on PE film was in the range 0.40–1.04 unit/cm^2 min. Little has been reported on the immobilization of the enzyme onto synthetic PM with an epoxy group prepared by radiation-induced polymerization.

In this study, PMs with an epoxy group were prepared by the radiation-induced polymerization of GMA/DEGMA. The effects of solvents, radiation dose, and monomer composition were examined. The obtained PMs with an epoxy group were ana-

Correspondence to: K.-P. Lee (kplee@knu.ac.kr).
Contract grant sponsor: 2001 Research Program of the Innovative Technology Center for Radiation Safety.

TABLE I
Effects of Solvent on the Radiation-Induced Polymerization of the GMA/DEGMA Mixture[a]

Sample	Solvent	Polymer yield (%)[b]	Shape of PMs	PM diameter (μm)[c]	SEM photograph in Figure 1
1	H_2O	14.1	Isolated spheres	0.2 ± 0.1	1(a)
2	MeOH	97.2	Isolated spheres	0.6 ± 0.1	1(b)
3	THF	—	Soluble spheres	—	1(c)
4	Toluene	80.7	Isolated spheres	0.4 ± 0.1	1(d)
5	EtOH	~100	Isolated spheres	0.9 ± 0.1	1(e)
6	Acetone	—	Soluble spheres	—	1(f)
7	$CHCl_3$	—	Soluble spheres	—	1(g)
8	DMF	—	Not obtained polymer	—	—

DMF = N, N-dimethylformamide.
[a] Reaction conditions: GMA = 10.0 g, MeOH = 180 mL, DEGMA = 10.0 g, total irradiation dose = 30 kGy.
[b] Filtered by Whatman filter paper No. 2.
[c] Determined by SEM photography

lyzed by solid-state ^{13}C-NMR, Fourier transform infrared spectroscopy (FTIR), Fourier transform (FT) Raman spectroscopy, and elemental analysis (EA) after amination. The lipase was immobilized onto PM with an epoxy group in various pH conditions and epoxy group contents. The lipase-immobilized PM was characterized by FTIR and EA, and then the activity (unit/mg min) was also determined.

EXPERIMENTAL

Reagents

The base lipase (activity = 6288 unit/mL min, content of protein = 147 mg/mL) solution from *Aspergillus oryzae* (lipolase 100L, Novozymes) was used as received. Reagent-grade GMA and diethylene glycol dimethacrylate (DEGMA) were supplied by Junsei (Ja-

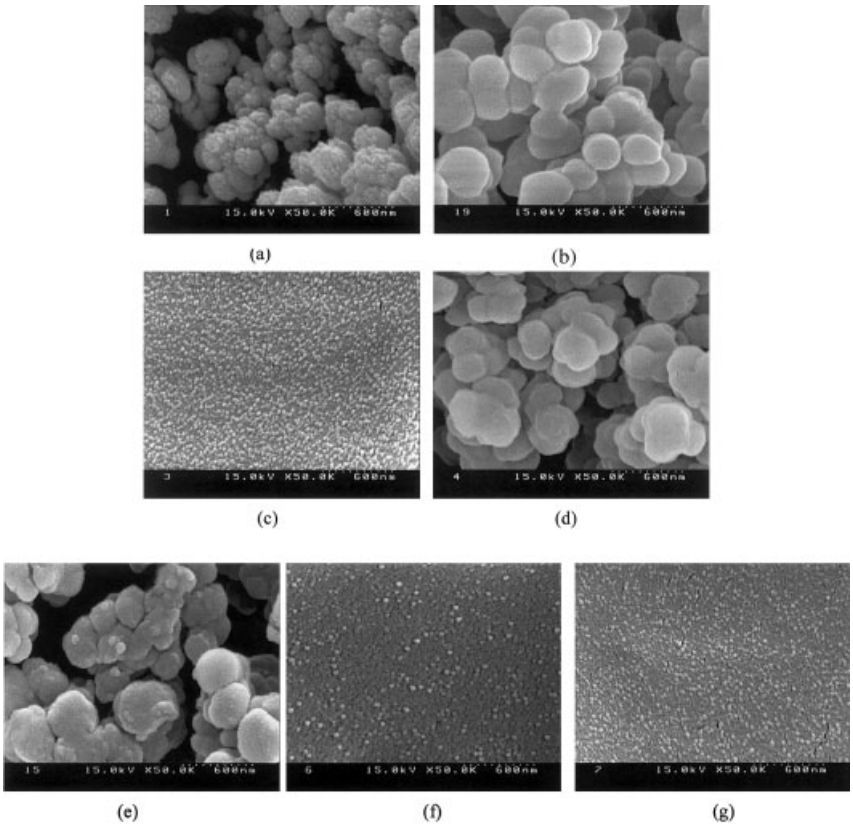


Figure 1 SEM photographs of the PMs obtained by radiation-induced polymerization: samples (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, and (e) 7 from Table I.

TABLE II
Effects of Total Irradiation Dose on the Radiation-Induced Polymerization of the GMA/DEGMA Mixture[a]

Sample	Total irradiation dose (kGy)	Solvent	Shape of PMs	PM diameter (μm)[b]	SEM photograph in Figure 2
9	10	EtOH	Isolated spheres	0.1 ± 0.1	2(a)
10	20	EtOH	Isolated spheres	0.2 ± 0.1	2(b)
11	30	EtOH	Isolated sphere	0.2 ± 0.1	2(c)
12	40	EtOH	Isolated spheres	0.1 ± 0.1	2(d)
13	10	MeOH	Isolated spheres	0.2 ± 0.1	2(e)
14	20	MeOH	Isolated spheres	0.2 ± 0.1	2(f)
15	30	MeOH	Isolated sphere	0.1 ± 0.1	2(g)
16	40	MeOH	Isolated spheres	0.1 ± 0.1	2(h)

[a] Reaction conditions; GMA = 10.0 g, MeOH = 180 mL, DEGMA = 10.0 g, total irradiation dose = 30 kGy, filtered by Whatman filter paper No. 2.
[b] Determined by SEM photography.

pan) and Merck (Korea), respectively. The other chemicals were also reagent grade.

Radiation-induced polymerization

Radiation-induced polymerization was performed in Pyrex tubes. First, the solvent was added, and then, the monomers were added to the reaction tube with a total volume of 50 mL and bubbled by nitrogen gas for 30 min. The monomer mixture solution was irradiated by γ rays from a Co-60 γ-ray irradiator (dose rate = 0.7 $\times$ 10^5 rad/h) under atmospheric pressure and ambient temperatures. The PMs obtained by radiation-induced polymerization were filtered by filter paper (Whatman filter paper No. 2) and dried in a vacuum oven at 60°C for 7 h.

Immobilization of the lipase onto the PM with an epoxy group as a covalent bond

The PMs (100 mg) were immersed in 0.1*M* carbonated buffer solution (1.0 mL, pH = 9.5), then the base lipase solution (1.0 mL) was added to the PMs containing 0.1*M* carbonated buffer solution, and the reaction solution was adjusted to pH = 9.0 with NaOH solution (0.1*M*). The immobilization of lipase on PMs with an epoxy group was performed in a shaking incubator at 37°C for 20 h. The lipase-immobilized PM was rinsed with 0.1*M* carbonated buffer (pH = 8.0) six times and then rinsed in an acetic acid buffer solution (pH = 4.0) two times. Finally, the lipase-immobilized PMs were stored in phosphate buffer (pH = 7.0).

Activity determination of the lipase-immobilized PMs

The triolein (88.5 mg) and gum Arabic (3.0 mg) in phosphate buffer (total volume = 1.0 mL, pH = 7.0) were emulsified by sonication. After the addition of

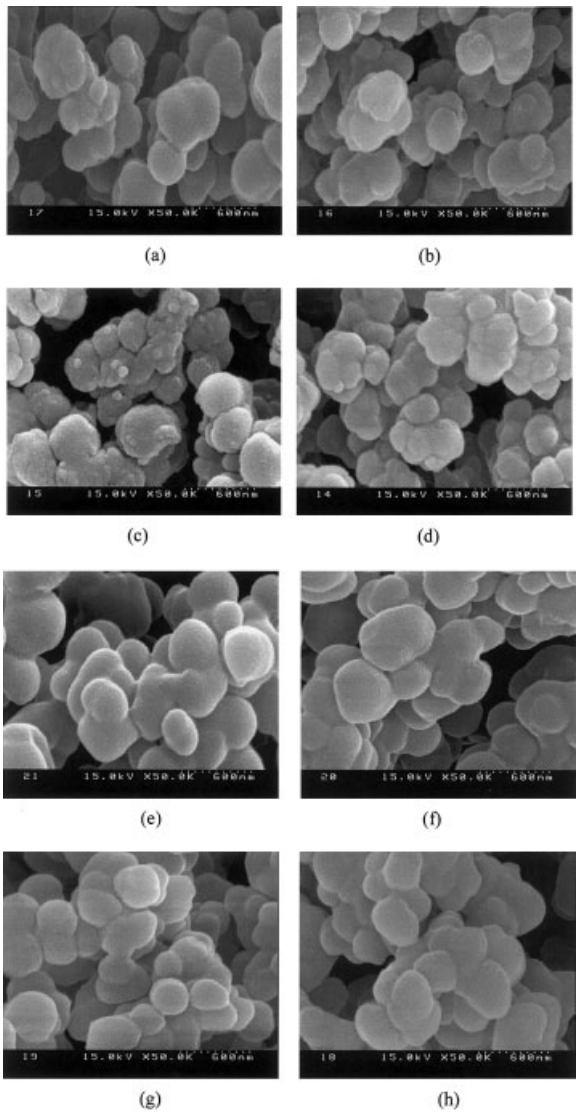


Figure 2 SEM photographs of the PMs obtained by radiation-induced polymerization: samples (a) 9, (b) 10, (c) 11, (d), 12, (e) 13, (f) 14, (g) 15, and (h) 16 from Table II.

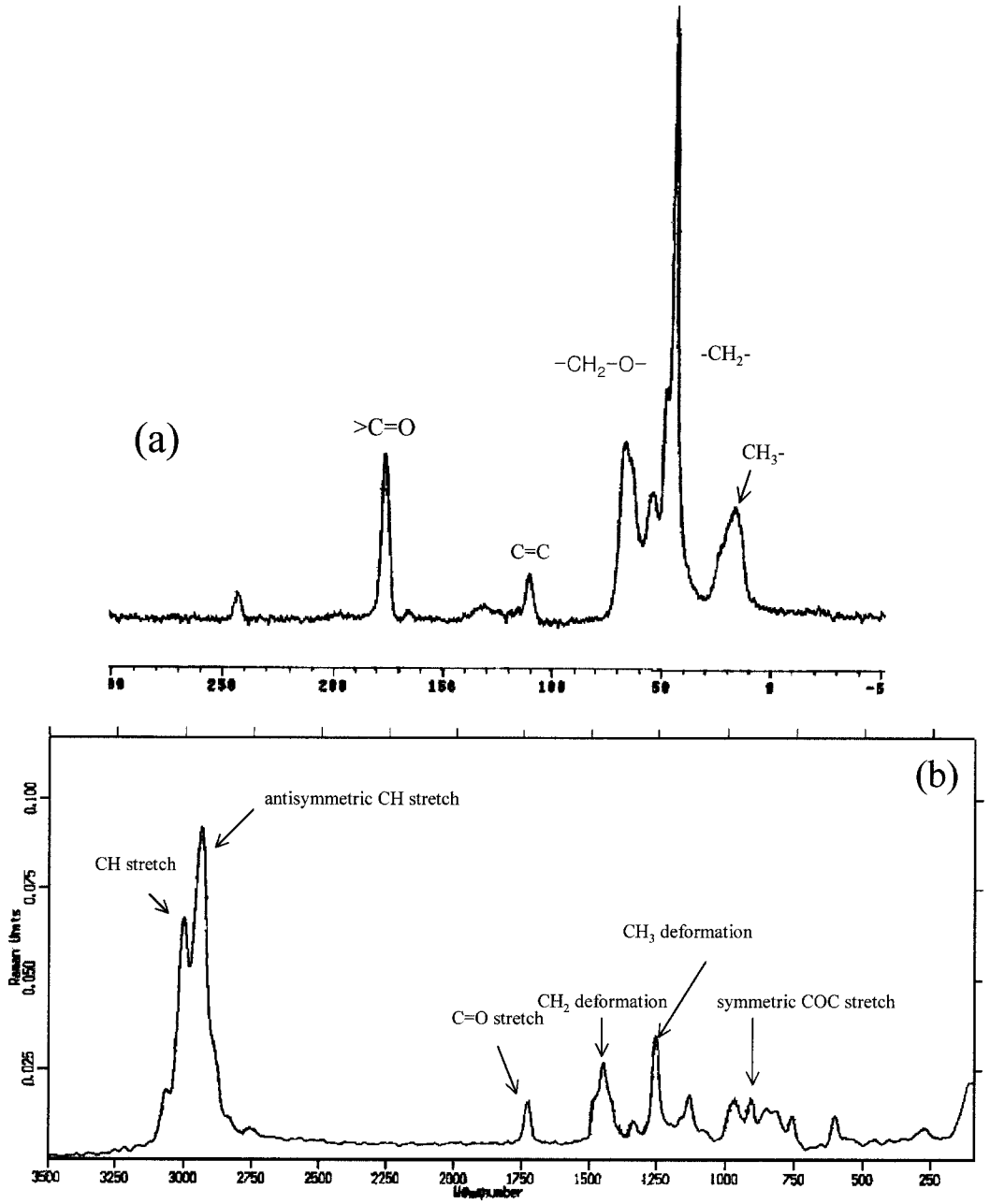


Figure 3 (a) Solid-state ^{13}C-NMR and (b) FT Raman spectra of the pGMA/DGEMA with an epoxy group (sample 11 from Table II).

the lipase-immobilized PMs (10 mg), the solution was reacted in a 200-rpm incubator at 37°C for 30 min. The reaction was stopped by heating at 100°C for 5 min. The reaction solution was separated with isooctane (5.0 mL). The isooctane solution was treated with cupric acetate pyridine solution (1.0 mL) and mixed for 1 min with a vortex. The isooctane was measured with an ultraviolet spectrometer at 715 nm.

One unit of lipase activity was defined as the amount of enzyme needed to liberate 1 μm of oleic acid/min in the conditions described for the assay system.

Characterization

For scanning electron microscopy (SEM), PMs were coated with a gold–palladium alloy before measurement. A sputtered sample was then scanned with a scanning electron microscope (JSM-840A, Jeol, Japan).

TABLE III
Effects of Monomer Composition on the Radiation-Induced Polymerization of GMA/DEGMA[a]

Sample	Monomer composition (mol %)		Polymer yield (%)[b]	EA analysis (%)[c]			PM diameter (μm)[d]	SEM photograph in Figure 4
	GMA	DEGMA		N	C	H		
17	100	0	96.3	5.17	59.42	9.74	0.1 ± 0.1	4(a)
18	80	20	97.2	4.53	59.00	9.30	0.2 ± 0.1	4(b)
19	60	40	96.3	2.94	58.50	8.82	0.2 ± 0.1	4(c)
20	50	50	97.7	1.86	58.78	8.24	0.1 ± 0.1	4(d)
21	40	60	96.3	0.52	57.84	7.69	0.2 ± 0.1	4(e)
22	20	80	97.2	0.21	57.92	7.19	0.2 ± 0.1	4(f)
23	0	100	96.3	0.00	59.40	7.40	0.1 ± 0.1	4(g)

[a] Reaction conditions: EtOH = 180 mL, total irradiation dose = 30 kGy.
[b] Filtered by Whatman filter paper No. 2.
[c] Determined by EA, after amination of GMA onto polymeric microspheres.
[d] Determined by SEM photography.

FT Raman spectra were recorded with a Bruker FT-106 Raman module, equipped with a Ge detector cooled by liquid nitrogen and connected to a Bruker FTIR 66 interferometer. To excite the Raman spectra, a continuous wave diode-pumped Nd:YAG laser with a radiation wavelength of 1064 nm (9398.4 cm^{-1}) was used. In all cases, the laser power was 300 mW, and the spectral resolution was 2 cm^{-1}.

FTIR spectra of the samples in the solid state were obtained with Nujol mulls with an IR spectrophotometer (PerkinElmer model 983).

EA of the microspheres was performed with an EA1110 instrument (Fisons).

RESULTS AND DISCUSSION

Effects of the solvents and irradiation dose

Table I shows the effects of solvent on the radiation-induced polymerization of GMA/DEGMA at 25°C without any stabilizer. In methanol (MeOH) and ethanol (EtOH), PMs with a high yield were ob-

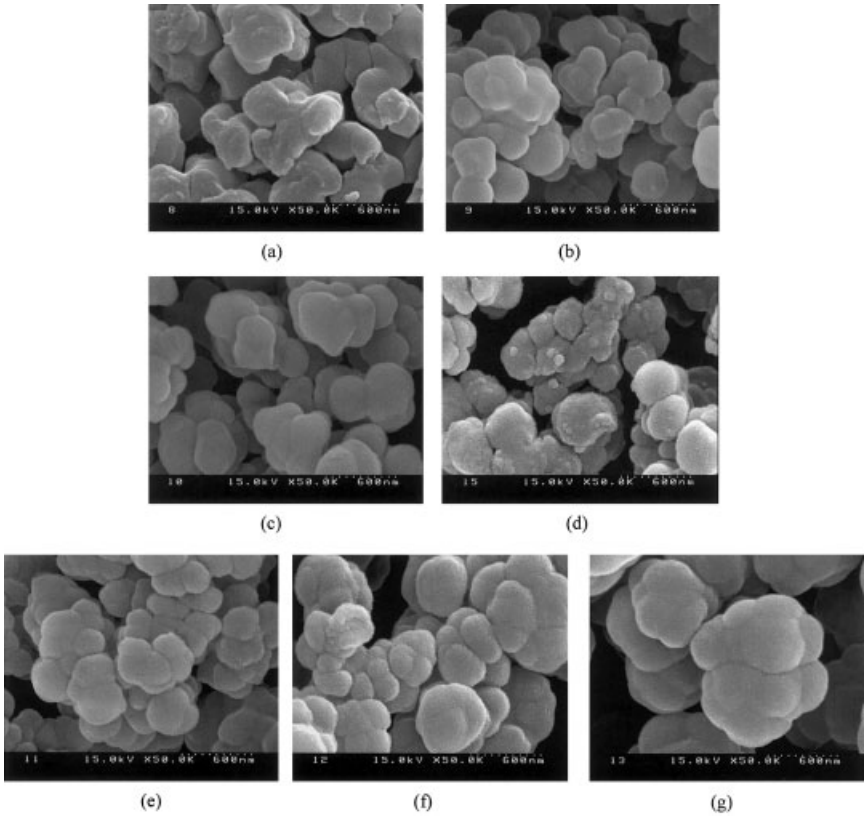


Figure 4 SEM photographs of the PMs obtained by radiation-induced polymerization: samples (a) 17, (b) 18, (c) 19, (d) 20, (e) 21, (f) 22, and (g) 23 from Table III.

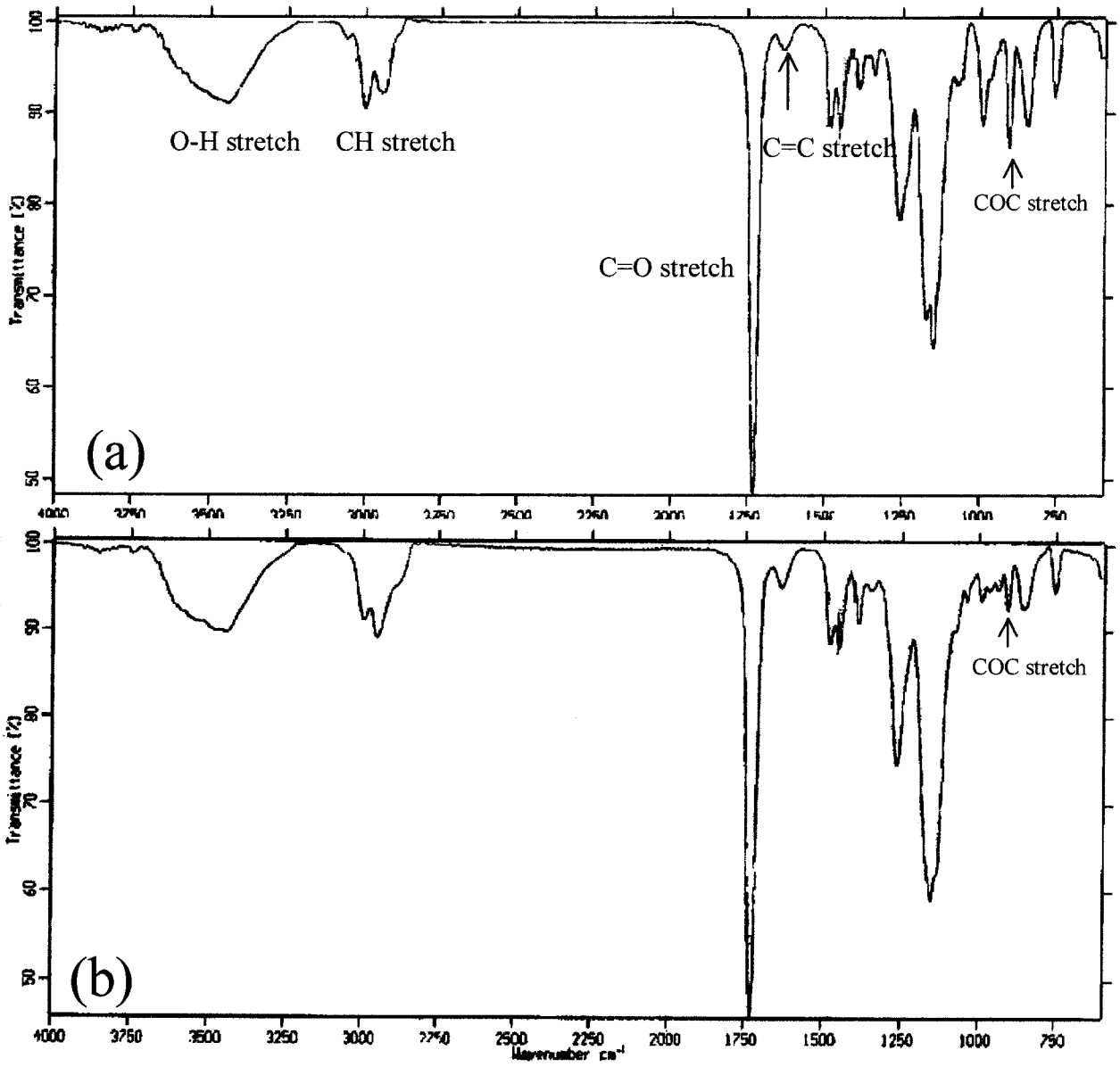


Figure 5 IR spectra of the PMs obtained by radiation-induced polymerization: samples (a) 17, (b) 20, and (c) 23 and (d) TEA-immobilized PM (sample 20).

tained, whereas the polymer was not obtained in DMF. However, soluble polymer was obtained in tetrahydrofuran (THF), acetone, and $CHCl_3$. In H_2O, PMs with a low yield were obtained. PM diameter was in the range 0.1–1.0 μm.

Figure 1 shows the scanning electron micrographs of PMs 1–7 from Table I. The diameter of the PMs in various solvents was in the following order:

$$\text{EtOH} > \text{MeOH} > \text{Toluene} > H_2O$$

The solvents strongly affected the conversion yield and the diameter. Naka and Yamamoto[12] reported the effects of monomer concentration on the radiation-induced polymerization of DEGMA in ethyl acetate. They found that monomer concentration strongly affected the conversion and the shape of the microspheres. However, the effect of solvents and irradiation dose were not described.

Table II shows the effects of the total irradiation dose on the radiation-induced polymerization of GMA/DEGMA at 25°C in EtOH without stabilizer. The size of the PMs was 0.2 ± 0.1 μm. The size of the PMs did not increase with increasing irradiation dose. However, Safranj et al.[14] reported that the size of their microspheres was 0.9 ± 0.2 μm by the radiation-initi-

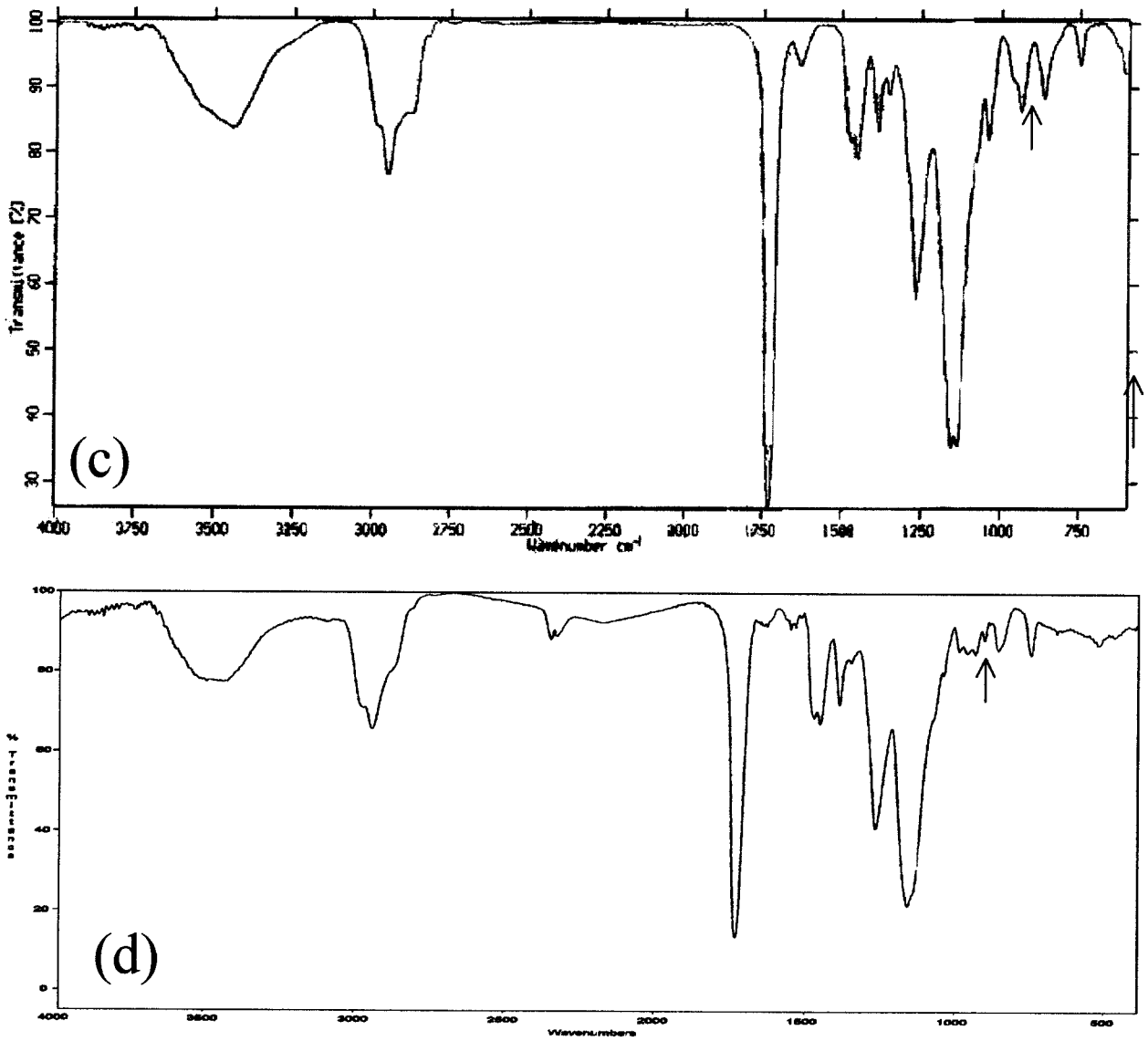


Figure 5 (*Continued from the previous page*)

ated polymerization of DEGMA with an irradiation dose of 5 kGy, whereas after 10 kGy, it increased to 1.1 $\pm$0.3 μm.

TABLE IV
Effects of Epoxy Group Content on Immobilization of the Lipase to PM[a]

Sample	Feed ratio (mol %) GMA	Feed ratio (mol %) DEGMA	Content of epoxy group (mmol/g)	Activity of the lipase-immobilized PM (unit/mg min)
24	100	0	0.50	342
25	80	20	0.44	328
26	50	50	0.19	273

[a] Immobilization conditions: temerature = 37°C, reaction time = 20 h, solvents = 0.1 *M* carbonate buffer (pH = 9.0).

Figure 2 shows the SEM photographs of PMs 9–16 from Table II. The total irradiation dose was not affected by the conversion yield and the size of PM in this study.

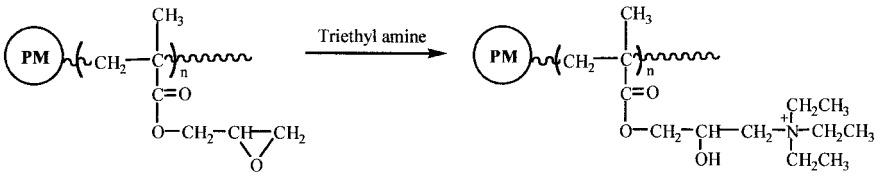
To determine the structure of the PMs with an epoxy group, solid-state ^{13}C-NMR, FT Raman, and FTIR spectroscopy were used. Figure 3 shows the ^{13}C-NMR spectra and FT Raman spectra of PM 11 from Table II. As shown in Figure 2(a), we observed a CH_3— peak at 17 ppm; —CH_2— peaks at 45 and 48 ppm; —CH_2—O— peaks at 54 and 67 ppm; a C═C peak at 110 ppm, which was unreacted; and a carbonyl of the ester peak (C═O) at 176 ppm due to an ester linkage. An unknown peak at 243 ppm was observed. This peak may be considered the triplet

carbon (CC) that is formed in radiation-induced polymerization.

To determine the absorbance of the epoxy group, the PMs were analyzed by FT Raman spectroscopy. As shown in Figure 3(b), predominant peaks at 3004 and 2941 cm^{-1} due to CH stretch and antisymmetric CH stretch were observed. However, peaks were assigned at 1730, 1451, and 1257 cm^{-1} due to C═O stretch, CH_3 deformation, and CH deformation, respectively. A characteristic peak appeared at 910 cm^{-1} due to symmetric COC (epoxy group) stretch. From these results, the epoxy group of the PM was established by FT Raman spectroscopy. The results of the IR spectra of the PMs are shown later in Figure 5.

Effects of GMA/DEGMA composition

Table III shows the effects of monomer composition on the radiation-induced polymerization of GMA/DEGMA in EtOH by irradiation dose of 30 kGy. The conversion yield was above 90%. To determine the content of the epoxy group, the obtained PM was reacted with a triethylamine (TEA) group as follows:



The TEA-immobilized PM was analyzed with an elemental analyzer. N (%) increased with increasing GMA content. The epoxy group content (mmol/g) was in the range 0.20–0.50 mmol/g as determined by EA. As described in a previous article,[15] various amine groups, such as trimethylamine, diethylenetriamine, triethylenetetraamine, ethylenediamine, dimethylamine, and diethyleneamine, were introduced to the GMA-grafted polypropylene film. The amine content was in the range 1.0–1.5 mmol/g. The content (mmol/g) of amine-immobilized polypropylene film was higher than that of amine-immobilized PM.

Figure 4 shows the SEM photographs of PMs 17–23 from Table III. The diameter of the PMs was the range 0.1–0.3 μm.

Figure 5 shows the IR spectra of poly(glycidyl methacrylate) (sample 17 in Table III), poly(glycidyl methacrylate/diethylene glycol dimethacrylate) [p(GMA/DEGMA); 1/1 mol %, sample 20 in Table III], poly(diethylene glycol dimethacrylate) [(pDGEMA), sample 23 in Table III], and TEA-immobilized PM (sample 20 in Table III). As shown in Figure 5(a), a methylene vibration was observed between 2910 and 2940 cm^{-1}. The predominant peak at 1734 cm^{-1} was due to the ester configuration, and a weak peak at 1633 cm^{-1} was due to an acrylic double bond. However, the broad peak between 3380 and 3340 cm^{-1} was due to O—H stretch, and the sharp peak at 910 cm^{-1} was due to an epoxide vibration peak. From these results, it appears that the epoxy group of PM partially decomposed during γ irradiation. The intensity of the epoxide peak decreased with increasing DEGMA content, as shown in Figure 3(b). An epoxide stretching peak in pDEGMA was not observed, as shown in Figure 3(c). However, in the IR spectra of the TEA-introduced PM, an epoxide vibration peak did not appear [see Fig. 5(d)]. From these results, the existence of the epoxy group of the PMs was confirmed.

Immobilization of lipase onto PM

Table IV shows the effects of the epoxy group content on the immobilization of lipase to the PMs at 37°C for 20 h in 0.1*M* carbonate buffer solution (pH = 9.0). The activity of the lipase-immobilized PMs increased with increasing epoxy group content.

Table V shows the activity of the lipase-immobilized PMs with an epoxy group content of 0.19 mmol/g as a function of the pH. The maximum activity of the lipase-immobilized PMs was determined to be at pH = 9.0.

Figure 6 shows the IR spectra of p(GMA/DEGMA), which possessed a ratio of GMA to DEGMA of 1:1 mol %, and the lipase-immobilized PM. As shown in Figure 5(a), the epoxy group was assigned at 910 cm^{-1} of p(GMA/DEGMA). The predominant peak of the carbonyl group appeared at 1730 cm^{-1}, and the broad peak at 3500 cm^{-1} was due to the alcohol group. These

TABLE V
Effects of pH on the Immobilization of Lipase on PM with an Epoxy Group of 0.19 mmol/g[a]

Sample	pH	Activity of the lipase-immobilized PM (unit/mg min)
27	1.0	168
28	8.0	200
29	9.0	273
30	9.5	148

[a] Immobilization temperature = 37°C, reaction time = 20 h.

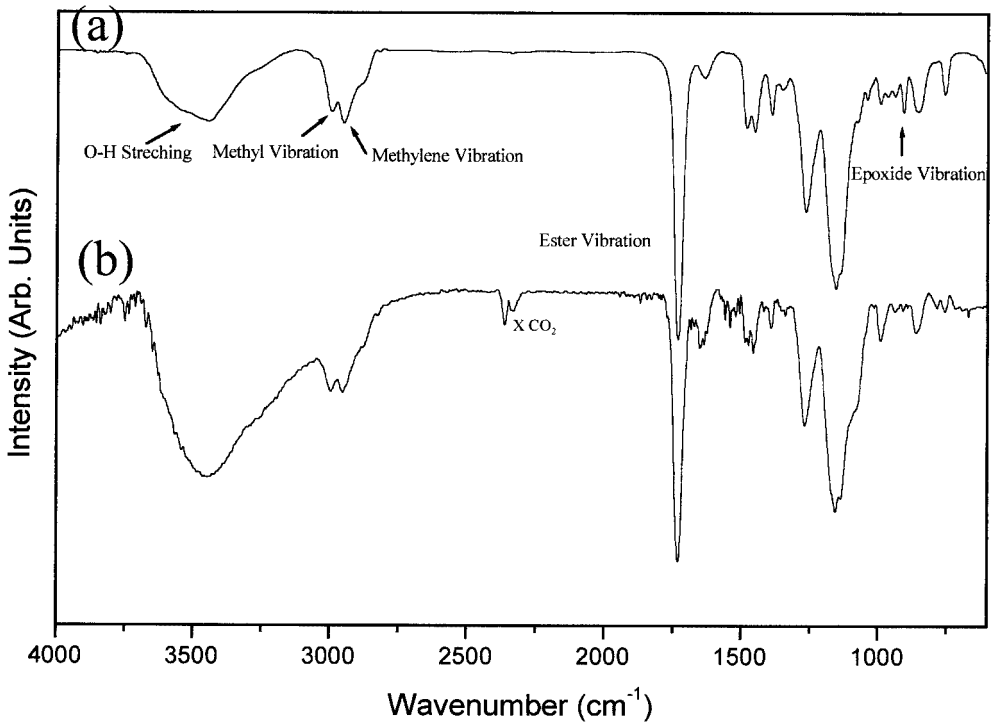


Figure 6 FTIR spectra of (a) the PM with an epoxy group (sample 20 in Table III) and (b) the immobilized PM.

results indicate that the epoxy group of p(GMA/DEGMA) was converted partly to the alcohol group during radiation-induced polymerization. In Figure 6(b), the intensity of the epoxy group peak at 910 cm^{-1} was substantially diminished, and a broad peak around 3500 cm^{-1} due to NH_2 stretch from lipase appeared. These results indicate that the lipase was immobilized to the epoxy group of the PMs. The application of the lipase-immobilized PMs for the chiral HPLC stationary phase is in progress.

CONCLUSIONS

PMs with an epoxy group were prepared by the radiation-induced polymerization of GMA and DEGMA. The immobilization of lipase onto the epoxy group containing PMs was examined. From the results, we conclude that

1. The epoxy group content increased with increasing GMA content in the feed solution.
2. The activity of the lipase-immobilized PMs increased with increasing GMA content.
3. The activity of the lipase-immobilized PMs was in the range 148–342 (unit/mg min).
4. The activity of the lipase-immobilized PMs was highest in 0.1*M* carbonate buffer solution (pH = 9.0) in this study.
5. The lipase-immobilized PMs were successfully characterized by FTIR, ESCA, and EA.

References

1. Chen, C.-S.; Lee, W.-C.; Lin, T.-J. Enzyme Microb Technol 2001, 29, 252.
2. Kawamoto, H.; Oguma, T.; Sekine, H.; Kobayash, M. Enzyme Microb Technol 2001, 28, 515.
3. Yin, B.-D.; Chen, Y.-C.; Lin, S.-C.; Hsu, W.–H. Process Biochem 2000, 35, 915.
4. Devi, S.; Sridhar, P. Process Biochem 2000, 36, 225.
5. Choi, S.-H.; Kim, G.-T.; Nho, Y. C. J Appl Polym Sci 1999, 71, 999.
6. Choi, S.-H.; Lee, K.-P.; Lee, J.-G. Microchem J 2001, 68, 205.
7. Lee, K.-P.; Kang, H.-J.; Joo, D.-L.; Choi, S.-H. Radiat Phys Chem 2001, 60, 473.
8. Park, G.-S.; Chang, J.-H.; Kim, H.-J.; Choi, S.-H.; Nho, Y. C. Anal Sci Technol 1999, 7, 7.
9. Malmsten, M.; Larsson, A. Colloid Surf B 2000, 18, 227.
10. Paiva, A. L.; Balcão, V. M.; Malcata, F. X. Enzyme Microb Technol 2000, 27, 187.
11. Abramič, M.; Leščić, I.; Korica, T.; Vitale, L.; Saenger, W.; Pigac, J. Enzyme Microb Technol 1999, 25, 522.
12. Naka, Y.; Yamamoto, Y. J Appl Polym Sci 1992, 30, 1287.
13. Choi, S.-H.; Kim, M.-S.; Ryoo, J. J.; Lee, K.-P.; Shin, H.-D.; Kim, S.-H.; Lee, Y.-H. J Appl Polym Sci 2002, 85, 2451.
14. Safranj, A.; Kano, S.; Yoshida, M.; Omichi, H.; Katakai, R.; Suzuki, M. Radiat Phys Chem 1995, 46, 203.
15. Choi, S.-H.; Lee, K.-P.; Nho, Y. C. J Appl Polym Sci 2001, 80, 2851.