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Radiolytic Immobilization of Lipase on Poly(glycidyl methacrylate)-grafted Polyethylene Microbeads

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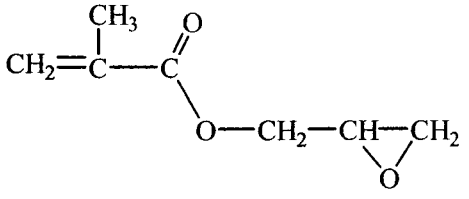
Abstract: Poly(glycidyl methacrylate)-grafted polyethylene microbeads (PGPM) presenting epoxy groups were prepared by radiation-induced graft polymerization of glycidyl methacrylate on the polyethylene microbead. The obtained PGPM was characterized by IR spectroscopic, X-ray photoelectrons spectroscopy (XPS), scanning electron microscope (SEM), and thermal analyses. Furthermore, the abundance of epoxy groups on the PGPM was determined by titration and elemental analysis after amination. The epoxy group content was calculated to be in the range 0.29~0.34 mmol/g when using the titration method, but in the range 0.53~0.59 mmol/g when using elemental analysis (EA) after amination. The lipase was immobilized to the epoxy groups of the PGPM under various experimental conditions, including changes to the pH and the epoxy group content. The activity of the lipase-immobilized PGPM was in the range from 160 to 500 unit/mg·min. The activity of the lipase-immobilized PGPM increased upon increasing the epoxy group content. The lipase-immobilized PGPM was characterized additionally by SEM, electron spectroscopy for chemical analysis (ESCA), and EA.

Keywords: epoxy group, glycidyl methacrylate, immobilization, lipase, lipase activity, poly(glycidyl methacrylate), radiation-induced graft polymerization.

Introduction

Glycidyl methacrylate (GMA), the ester of methacrylic acid and 2,3-epoxy-propanol, bears a reactive epoxide group which reacts with sulfhydryl-,[1,2] amino-,[3,4] carboxy-,[5] or hydroxyl group[6] to form stable covalent bonds with biomolecules without any linker. Such epoxy-modified polymer-surface is stable during long storage periods and is relatively resistant against hydrolysis. Biomolecules, like proteins, are covalently coupled by opening the epoxide bridge in the alkaline media. The chemical structure of glycidyl methacrylate (GMA) was as follows:



Radiation-induced graft polymerization (RIGP) is a beneficial method to introduce functional groups into different polymer materials using specially selected monomers. There have been several reports about RIGP of polar monomers onto polymer film to obtain hydrophilic property for versatile

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applications.[7-9] For example, an ion-exchange membrane can be used in water desalination,[10] as a carrier for immobilization of medical products,[11] as a separator in alkaline batteries,[12,13] and in other application.[14,15]

Enzymes are widely used as biocatalysts in chemical, pharmaceutical and food industrials, and as specific ligands in clinical analysis.[16,17] Since the recovery and the reusability of the free enzyme are limited, immobilization of the enzyme has been proposed. Immobilized enzymes can be used in batch and continuous systems. Such enzymes can also be removed easily from the reaction medium and can provide the facility of the controlled production.

Lipase, or triacylglycerol acyl ester hydrolases (EC 3.1.1.3), are enzymes processing an intrinsic capacity to catalyze cleavage of carboxyl ester bonds in tri-, di-, monoacylglycerols (the major constituents of animals, plant, and microbial fats and oils).[18]

In a previous work,[12] the GMA were grafted onto polyethylene (PE) film, polyethylene hollow fiber membrane,[3,19] polypropylene nonwoven fabric,[13] and other forms,[20] by pre-irradiation grafting technique for removal of heavy metal ions. However, the grafting of the GMA on the PE microbead by radiation-induced graft polymerization technique was not reported, to our knowledge.

In this study, the poly(glycidyl methacrylate) PE microbeads (PGPM) were prepared by radiation-induced graft polymerization of the GMA onto the PE microbead. The obtained PGPM was characterized by IR, XPS, SEM, and thermal analysis. Furthermore, the epoxy group of the PGPM was determined by a chemical modification method. The lipase was immobilized to epoxy group of the PGPM in various experimental conditions such as pH and the epoxy group content. The activity of lipase-immobilized PGPM was also determined.

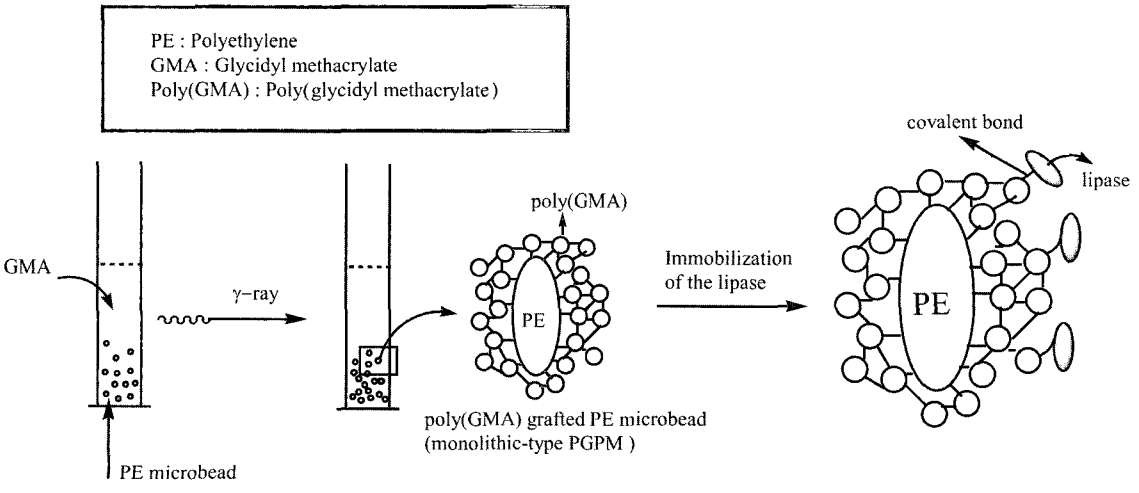
Experimental

Reagents. The base lipase (activity = 6288 Unit/mLmin, content of protein = 147 mg/mL) solution from Aspergillus oryzae (lipolase 100L, Novozymes) were used as received. The polyethylene (PE) microbead prepared by mechanical grinder was used. Reagent-grade glycidyl methacrylate (GMA) were supplied from Junsei and Merck, respectively. The other chemicals were reagent grade.

Radiation-induced Polymerization. Scheme I shows the preparation procedure of PGPM by radiation-induced graft polymerization. The radiation-induced graft polymerization was performed in Pyrex tubes. The GMA (7.0 g, 5.0 wt%) was added in MeOH (150 mL) with the PE microbead (35.0 g) and bubbled by nitrogen gas for 30 min. The reaction mixture was irradiated by γ-ray from Co-60 (Co-60 Gamma-ray Irradiator, the dose rate = 0.7×10^5 rad/hr) under atmospheric pressure and ambient temperatures. The PGPM obtained by radiation-induced polymerization was filtered by filter paper (Whatman Filter Paper No. 2) and removed from the homopolymer using THF. Finally, the PGPM was washed in MeOH and dried in a vacuum oven at 60 °C for 7 hrs.

Immobilization of the Lipase to PGPM with Epoxy Group as Covalent Bond. The base lipase (1.0 mL) was added in 0.1 M carbonated buffer solution (1.0 mL, pH = 9.5) with the PGPM (100 mg). The reaction solution was then adjusted to pH = 9.0 using NaOH solution (0.1 M) before being reacted in a shaking incubator at 37 °C for 20 hrs. The lipase-immobilized PGPM was rinsed using 0.1 M carbonated buffer (pH = 8.0) six times, and then rinsed twice in acetic acid buffer solution (pH = 4.0). Finally, the lipase-immobilized PGPM was stored in phosphate buffer (pH = 7.0).

Activity Determination of the Lipase Immobilized PGPM. The triolein (88.5 mg) and Gum Arabic (3.0 mg) in



Scheme I. Preparation procedure of the PGPM by radiation-induced graft polymerization.

the phosphate buffer (total volume = 1.0 mL, pH = 7.0) was emulsified by sonication, and after the addition of the lipase-immobilized PGPM (10 mg), was reacted in 200 rpm incubator at 37 °C for 30 min. The reaction was stopped by heating 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 by using of Vortex. The isooctane was measured by using of UV spectrometer at 715 nm.

The one unit of lipase activity was defined as the amount of enzyme needed to liberate 1 μm oleic acid per minute at the condition described for assay system.

Characterization. For scanning electron microscopy (SEM), a PGPM was coated with gold-palladium alloy prior to the measurement. Sputtered sample was then scanned by the electron beam in a scanning electron microscope (JSM-840A, JEOL Co., Japan).

FT-IR spectra of the inclusion complex in solid state were obtained using Nujol mulls with an infrared spectrophotometer (Perkin-Elimer Mod. 983).

The X-ray photoelectrons spectra of the samples were obtained using ESCALab 220i (VG Scientific) equipped with a full 180° hemispherical electrostatic analyzer to examine the chemical state of the constituent elements. As a phonon source, Al K_α radiation (1486.6 eV) was used. The half-width at half-maximum of the 4f7/2 line in the XPS spectrum of gold obtained in our XPS spectrometer was smaller than 1.0 eV. The energy scale of the spectrometer was calibrated using the lowest BE component of C 1s peak (285.0 eV). The C 1s spectra were deconvoluted using a Gaussian-Lorentzian model to obtain the best binding energy values.

Table I. Radiation-induced Polymerization of GMA on PE Microbead in MeOH[a]

No.	GMA(wt%)	Content of Epoxy Group[b] (mmol/g)	Content of Epoxy Group[c] (mmol/g)
1	5.0	0.34	0.55
2	10.0	0.31	0.59
3	30.0	0.29	0.53

[a]Reaction condition: PE microbead = 35.0 g, MeOH = 150 mL, total irradiation dose = 30 kGy.
[b]Determined by titration method. [c]Determined by EA analysis after amination.

The Elemental Analysis (EA) of the PGPM using an EA1110 instrument (FISONS) were performed.

Thermogravimetric Analyses (TGA) of the PE microbead and the PGPM were made on a TA instrument of TGA 2950 model (Dupont Co.) with a heating rate of 10 °C min^{-1} in the temperature range of 50-700 °C.

Results and Discussion

Preparation Poly(glycidyl methacrylate)-grafted PE Microbead. Table I shows the results of radiation-induced graft polymerization of the GMA on the PE microbead in MeOH by total irradiation dose of 30 kGy at room temperature as a function of the GMA concentration. The monolithic-type PGPM was obtained in 5 and 10 wt% GMA concentration to the PE microbead, while the bubble-type PGPM in 30 wt% GMA concentration was obtained (see in Figure 2). In order to obtain epoxy group content, the PGPM was reacted with HCl and trimethyl amine (TMA) as

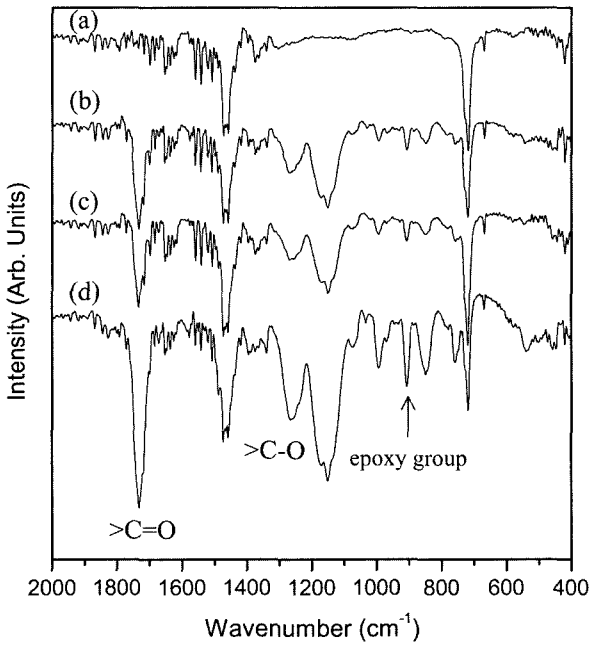
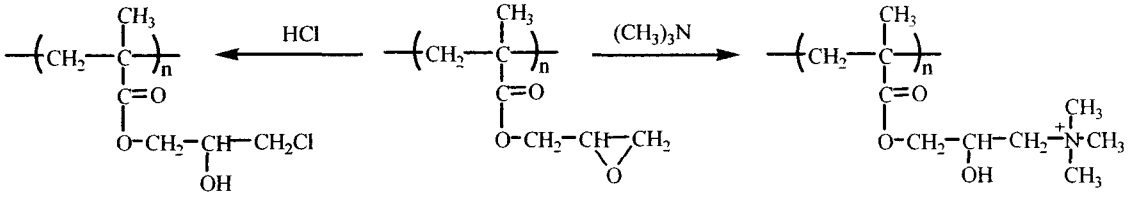


Figure 1. IR spectra of the PGPM obtained by radiation-induced graft polymerization: (a) base PE microbead, (b) No. 1, (c) No. 2, and (d) No. 3 in Table I.



Scheme II. Chemical reaction of epoxy group using HCl and trimethyl amine.

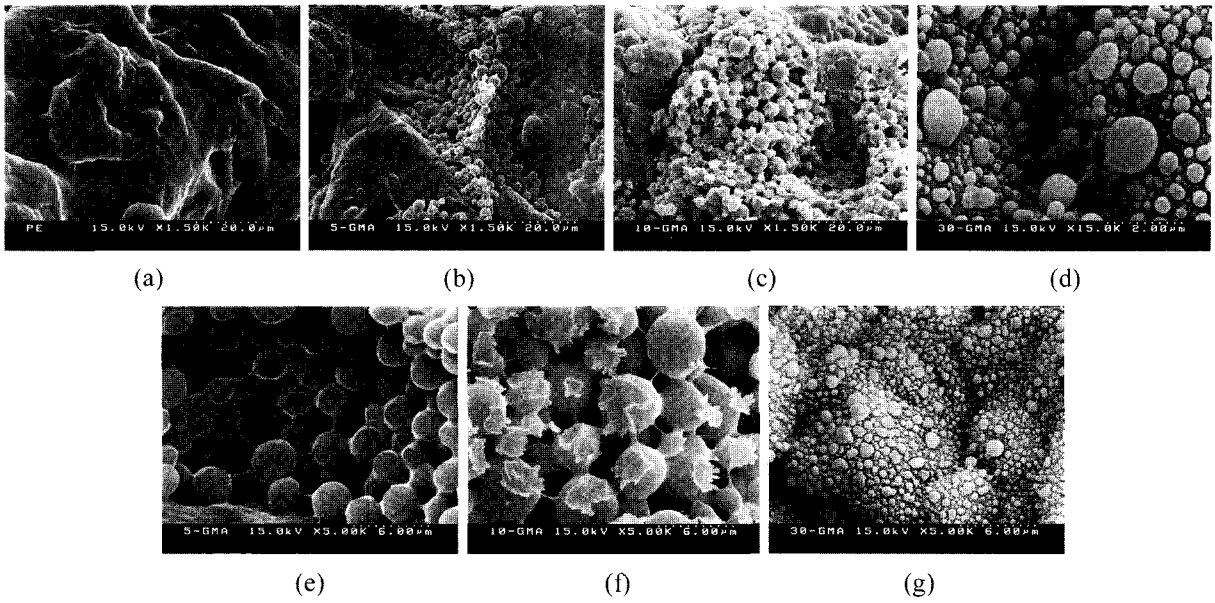


Figure 2. SEM photograph of the PGPM obtained by radiation-induced graft polymerization: (a) base PE microbead, (b) No. 1, (c) No. 2, (d) No. 3, (e) No.1 × 30, (f) No. 2 × 30, and (g) No. 3 × 30 in Table I.

in the following Scheme II.

The epoxy group of the PGPM was in the range of 0.29 ~ 0.34 mmol/g in titration method. On the other hand, the epoxy group content of the PGPM was in the range of 0.53 ~ 0.59 mmol/g by EA analysis after amination. In this study, the amine group content of the PGPM by EA after amination was higher then that of the PGPM by titration method. It may be considered from that the swelling degree of the PGPM in trimethylamine solution was higher than that in HCl solution. Therefore, the trimethylamine deeply penetrated into the poly(GMA) of the PGPM and reacted with the epoxy group. Generally, the HCl-dioxane method,[21] was used to determine the epoxy group content. In a previous paper,[4] the epoxy group was converted to the amine group by chemical reaction and the amine was determined by EA. It was found that the content of the amine group was in the range of 1.0~2.7 mmol/g.

Figure 1 shows the IR spectra of the base PE microbead and the PGPM: (a) base PE microbead, (b) No. 1, (c) No. 2, and (d) No. 3 in Table I. In Figure 1 (b), (c), and (d) show the carbonyl group at 1733 cm^{-1}, the C-O stretch band at 1250 cm^{-1}. The epoxy group peak at 910 cm^{-1} was also determined. However, the base PE microbead did not appeared at the epoxy group peak. These results clearly indicate that the epoxy group was introduced on the PE microbead.

Figure 2 shows the SEM photograph of the base PE microbead and the PGPM: (a) base PE microbead, (b) No. 1, (c) No. 2, (d) No. 3, (e) No. 1× 30, (f) No. 2× 30, and (g) No. 3× 30. The PE microbead structure used in this study was an irregular amorphous bead as shown in Figure 2(a). The diameter of the base PE microbead was in the range of 103~570 μm by SEM analysis. In Figure 2 (b) and (e), the SEM morphology of the PGPM obtained by RIGP was a monolithic-type structure. The diameter of the poly(GMA) on the PE microbead was in the range of 200 ~ 372 μm in Figure 2 (b) and (e). In Figure 2 (c) and (f), the SEM morphology of the PGPM was also determined as a monolithic structure. The size of poly(GMA) on the PE microbead was the range of 370 ~ 495 μm in Figure 2(c) and (f). The size of poly(GMA) on the PE microbead in 10 wt% GMA concentration was higher than that of the poly(GMA) on PE microbead in 5 wt% GMA concentration. On the other hand, in 30 wt% GMA concentration, the SEM morphology of the PGPM was a bubble-type structure.

Table II. Effects of GMA Content on Immobilization of the Lipase to PGPM[a]

No.	GMA (wt%)	Content of Epoxy Group[b] (mmol/g)	Activity (unit/mg min)
4	5.0	0.34	500
5	10.0	0.31	440
6	30.0	0.29	310

[a]Reaction condition: Temperature = 37 °C, Immobilization time = 20 hrs, Solvents = 0.1 M carbonate buffer (pH=9.0).
[b]Determined by titration method.

Figure 3 shows the TGA and DSC curves of the base PE microbead and the PGPM: (a) PE microbead, (b) No. 1, (c) No. 2, and (d) No. 3 in Table I. In Figure 3(a), the weight loss at around 450 °C was due to polyethylene decomposition

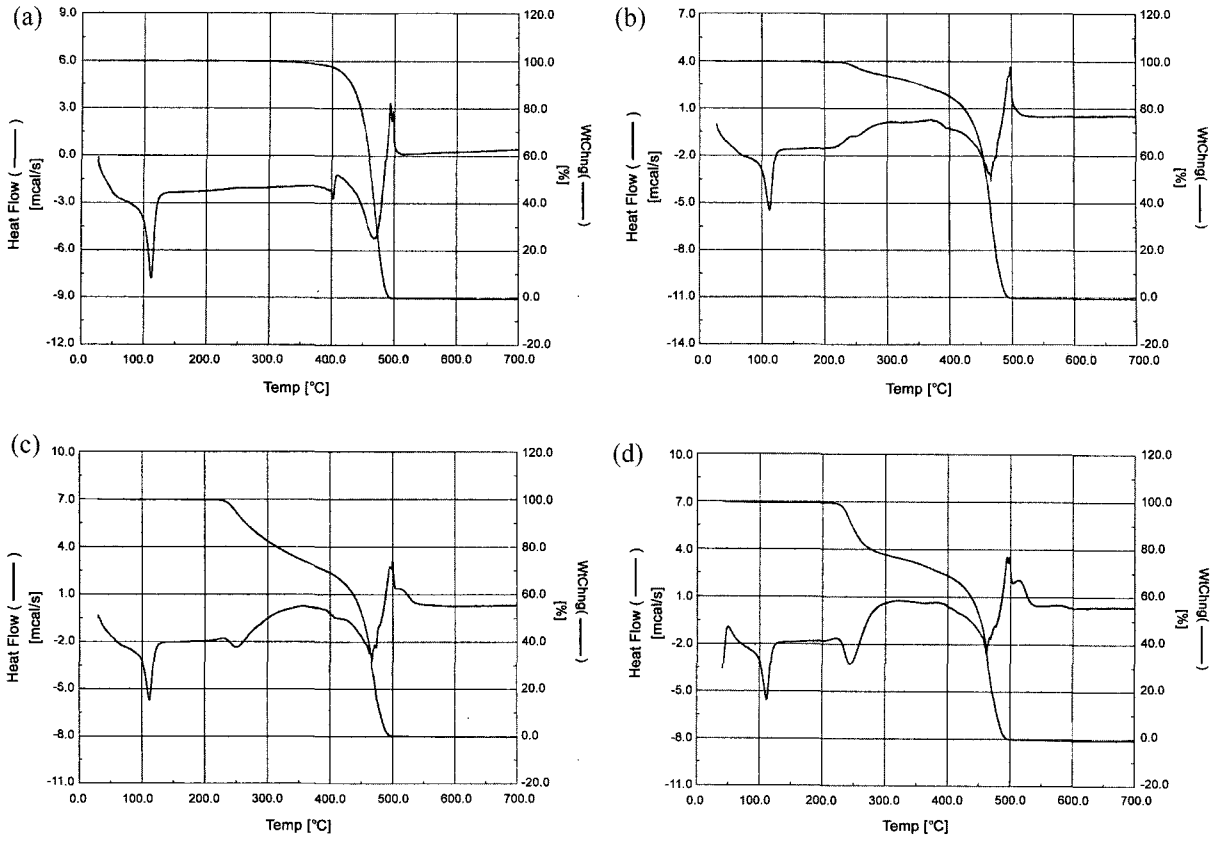


Figure 3. TGA curves of the PGPM obtained by radiation-induced graft polymerization: (a) base PE microbead, (b) No. 1, (c) No. 2, and (d) No. 3 in Table I.

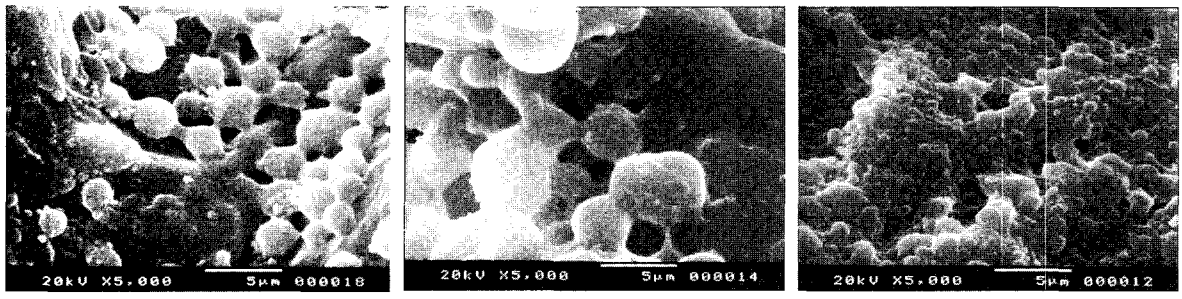


Figure 4. SEM photograph of the PGPM obtained by radiation-induced graft polymerization: (a) No. 4, (b) No. 5, and (c) No. 6 in Table II.

on the TGA curves. It was also determined the endothermic peak at 124 °C was due to melting point of PE on DSC curves. In Figure 3(b), the first weight loss in the range of 220 ~ 300 °C was due to decomposition of ester, the decomposition of the 2,3-epoxypropyl group. The second weight loss from 300 to 420 °C was due to the grafted poly(GMA) decomposition, while the third weight loss from 400 to 480 °C was due to decomposing of the backbone polymer (PE microbead) on the TGA curves. In Figure 3(b), the first endothermic peak at 124 °C was due to the PE melting point, whereas the second endothermic peak at 250 °C was due to the decomposition of 2,3-epoxypropyl group of the PGPM. The patterns of the TGA and DSC curves in Figure 3(c) and (d) were similar to that in Figure 3(b). In the TGA curves, the weight ratios of the grafted poly(GMA)/PE microbead were calculated to be 20/80 wt% (a), 36/64 wt% (b), and 40/60 wt% (c), respectively. From the result in this study, it was found that the content of poly(GMA) of PGPM increased with increasing GMA concentration.

Immobilization of the Lipase onto PGPM. Table II

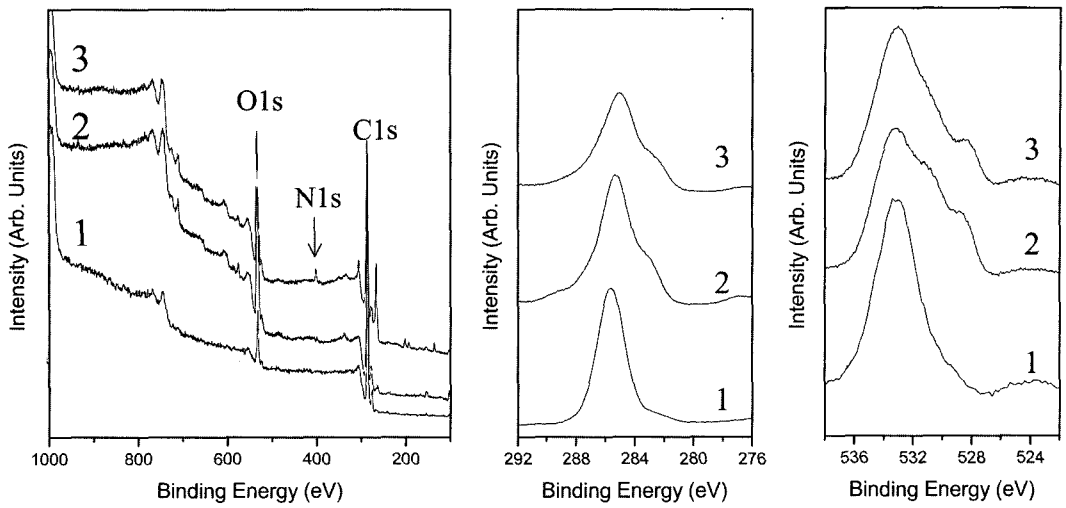


Figure 5. XPS spectra of the base PE microbead (1), PGPM (2) (5 wt%), and lipase immobilized PGPM (3): (a) survey scan spectra, (b) C1s spectra, and (c) O1s spectra.

Table III. Effects of pH on Immobilization of the Lipase to PGPM[a]

No.	GMA (wt%)	Activity (unit/mg min)
7	1.0	160
8	8.0	440
9	9.0	500
10	9.5	460

[a]Immobilization condition: GMA content = 5.0 wt%, Epoxy group content = 0.34 mmol/g, Temperature = 37 °C, Immobilization time = 20 hrs.

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

Figure 4 shows the SEM photograph of the lipase immobilized PGPM. (a) No. 4, (b) No. 5, and (c) No. 6 in Table II. The photograph shows the high immobilization lipase on PGPM with epoxy group. From the results, it can be seen that the lipase was immobilized on the PGPM with epoxy group.

Figure 5 shows the XPS spectra of the PE microbead, the PGPM (5 wt%), and the lipase-immobilized PGPM: XPS survey scan spectra (a), C 1s spectra (b), and O 1s spectra (c) of the base PE microbed (1), PGPM (5 wt%) (2), and lipase-immobilized PE microbead (3). In Figure 5(a), the PE microbead showed two peaks corresponding, to C 1s (binding energy, 285 eV) and O 1s (binding energy, 532 eV), while the lipase-immobilized PGPM showed one additional peak corresponding to N 1s (binding energy, 400 eV). The chemical compositions of the PE microbead, the PGPM, and the lipase-immobilized PGPM, were calculated from the XPS survey scan spectra. The atomic percent (%) of the PE microbead were 81.9% of C and 18.1% of O, whereas the atomic percent (%) of the PGPM were 42.2% of C and 57.8% of O. The oxygen content (%) of the PGPM had increased when compared to the original PE microbead. On the other hand, the atomic percents (%) of the lipase-immobilized PGPM were 31.8% of C, 66.2% of O and 2.0% of N. In EA analysis, the atomic percents (%) of the lipase-immobilized PGPM were determined to be 42.3% of C, 50.0% of O and 1.6% of N. From these results, the lipase was successfully immobilized onto the surface of the GMA-grafted PE microbead. In Figure 5(b), the core level binding energy of the PE microbead is calculated to be 285 eV. As the PE microbead was grafted with GMA, an additional peak was observed at 282-284 eV because of the poly(GMA). The original PE microbead had no peak of poly(GMA) peak. In Figure 5(c), the core level binding energy of the PE microbead is calculated to be 533 eV. In the GMA-grafted PE microbead, the additional peaks were observed to be in the range of 528~539 eV due to O of the poly(GMA).

Table III shows the effects of the pH on immobilization of the lipase to the PGPM at 37 °C for 20 hrs. The maximum activity of the lipase-immobilized PGPM was determined to be pH = 9.0. The immobilization bonding of protein to epoxy group was described earlier.[22] The application of the lipase-immobilized as catalyst and chiral HPLC stationary phase is in progress.

Conclusions

The poly(glycidyl methacrylate) grafted polyethylene

microbead (PGPM) with epoxy group were prepared by radiation-induced graft polymerization of glycidyl methacrylate (GMA) on the polyethylene (PE) microbead. The immobilization of the lipase onto the PGPM was examined. The conclusions of these results are as follows:

(1) The epoxy group content of the PGPM prepared by RIGP was in the range of 0.29 ~ 0.34 mmol/g by titration method, whereas in the range of 0.53 ~ 0.59 mmol/g by elemental analysis after amination.

(2) The activity of the lipase-immobilized PGPM was in the range of 310 ~ 500 (unit/mgmin).

(3) The lipase-immobilized PGPM was also characterized by SEM, ESCA and EA.

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