Synthesis of Magnetic Polymer Microspheres and Application for Immobilization of Proteinase of *Balillus sublitis*

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SYNOPSIS

Core–shell composite magnetic polymer microspheres, containing a magnetic core and a polymer shell, were synthesized by dispersion copolymerization of styrene (St) and 2-hydroxyethyl methacrylate (HEMA) in the presence of magnetic oxide (Fe_3O_4) powder. The Fe_3O_4 powder was ultrasonically dispersed in poly(ethylene glycol) (PEG) and the affinity between the obtained superfine powder and the monomer and initator was improved. It shows that the dispersion medium and stabilizer system have a great effect on the diameter and dispersion parameter of microspheres. In the condition of controlling polymerization, the magnetic polymer microspheres containing surface —OH groups, having 50–500 μm diameter and with better magnetic induction, were synthesized. The proteinase of *Balillus sublitis* was immobilized on magnetic polymer microspheres with an average diameter of 50–60 μm by covalent coupling. The magnetic immobilized proteinase shows an enzyme activity of 1000 U/g, the enzyme yields are usually 20–30 mg/g of carriers, and the activity retention is about 40%. The stability of the immobilized enzyme was obviously improved.

INTRODUCTION

The composite magnetic polymer microspheres usually contain two parts: One is the magnetic core which is always an inorganic magnetic material, such as Fe_3O_4, $BaFeO_4$, etc.; the other part is the polymer shell around the magnetic core. In a magnetic field, the microspheres can move directedly and be separated from the medium, and the polymer shell, always containing some active groups, can covalently bind organic molecules, biomolecules, cells, and so on, so it is widely used in biochemical and biomedical fields, such as immunoanalysis, target drug, enzyme immobilization, and down-stream separation processes.[1-3] There are two ways to prepare these magnetic polymer microspheres: One is by dispersing the magnetic materials, e.g., Fe_3O_4, in gels or macromolecules and then emulsifying them with a surfactant to obtain microspheres;[4] the other way, which is more widely used, is by employing the magnetic materials as the initiative centers to initiate the polymerization of the monomer into the polymer shell.[5,6] In our research, the magnetic polymer microspheres were prepared by dispersion copolymerization of styrene (St) and 2-hydroxyethyl methacrylate (HEMA) into the polymer shell and the magnetic core is Fe_3O_4 powder modified by ultrasonic treatment using poly(ethylene glycol) (PEG).

The proteinase of *Balillus sublitis* [EC 3.4.4.16] is widely used in industrial fields, such as in leather-tanning, drug-producing, and food-making, but its stability is poor and it easily loses its activity.[7] The proteinase is bound by support material containing aldehyde groups[8] and magnetic fluid[9] and its stability is improved. In our research, the free proteinase was immobilized on the magnetic polymer microsphere carriers containing hydroxyl groups, activated by *p*-benzoquinone. As carriers, the magnetic polymer microspheres have several advantages; e.g., the separation of such materials from nonmagnetic materials is easy by use of a magnetic field, which is otherwise difficult or impossible to perform. An-

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other advantage is their use in a magnetically stabilized fluid bed, thereby presenting further options in a continuous reaction system. To compare the properties of free enzyme with that of immobilized enzyme, the optimum temperatures were changed from 50°C to 50–60°C, the optimum pH values from 7.5 to 6.5, and the K_m values from 0.054% to 0.088% casein solution. The thermal stability, pH stability, and storage stability of the immobilized enzyme were obviously improved.

EXPERIMENTAL

Materials

Fe_3O_4 (ϕ: 0.3–0.4 μm) was dried before use. Styrene (St) and 2-hydroxyethyl methacrylate (HEMA) were purified by vacuum-distillation under a N_2 atmosphere before use. Poly(ethylene glycol) (PEG, $\bar{M}$ = 4000), *p*-benzoquinone, ethanol, and potassium peroxosulfate (KPS) were of analytic grade and used without further purification. The proteinase of *B. sublitis* was purified with anion-exchange resin and its activity was about 300,000 U/g; the phosphate-buffered solution (PBS) of the proteinase was used in the experiments. Other reagents were commercially available. Water was doubly distilled before use.

Preparation of Magnetic Polymer Microspheres

A series of microspheres were synthesized by dispersion copolymerization. Fe_3O_4 powder with an average particle size of 0.3–0.4 μm was ultrasonically dispersed in a PEG–water solution. The mixture was carried into a 250 mL round-bottomed four-necked flask with an incubating dispersion medium of H_2O/EtOH; St and HEMA were copolymerized using KPS as the initiator. The reaction mixture was stirred at 400 rpm for 10 h, while the reaction temperature was kept at 65°C and N_2 gas continuously passed through the flask. The reactor was moved from the bath and washing steps were repeated with distilled water and ethanol. The optimum recipe for preparation of microspheres with an average diameter of 52.5 μm is given in Table I.

The average diameter of the microspheres was estimated by optical microscope observations and the dispersion parameter ($\hat{\delta}/\bar{x}$) was calculated from

$$\hat{\delta} = [\sum_{i=1}^{n} (Xi - \bar{X})^2/n - 1]^{1/2}$$

where $\hat{\delta}$ is mean-square deriation; Xi, the diameter of a microsphere, and $\bar{X}$, the average diameter of the microspheres.

Table I Optimum Recipe for Microspheres of 52.5 μm Diameter

Materials	Weight (g)	Materials	Volume (mL)
Fe_3O_4	0.10	St	22.0
50% PEG soln	24.0	HEMA	1.8
H_2O	44.0	DVB	0.4
EtOH	26.0		
2% KPS soln	20.0		

Immobilization of Proteinase of *Balillus sublitis*

The activation of microspheres with an average particle size of 50–60 μm was carried out in 10.0 mL 0.1*M* PBS (pH 7.5, containing 20% ethanol) and the amount of microspheres was 1.0 g. The mixture was allowed to shake continuously at room temperature for 2–3 h. Then, the microspheres were separated from the mixture by a magnetic field of 4200 Gauss and washed with 20% ethanol, 1 mol/L NaCl solution, and water sequently until the liquid washed out was colorless.

The activated microspheres were treated with 10.0 mL phosphate-buffered enzyme solution (pH 6.5) and enzyme activity was 3871.7 U/mL. The mixture was stirred at 0–10°C for 40 h. The effect of pH on immobilization was tested by using different pH values of PBS. The microspheres were separated from the mixture in a magnetic field of 4200 Gauss and the total amount of enzyme washed out was also determined spectrophotometrically. The microspheres were washed with water until no protein existed in the water washed out.

Analytical

The amount of enzyme attached to the microspheres was determined by the material balance according to the amount of enzyme in the supernatant before and after the immobilization, taking the enzyme loss during the posttreatment after immobilization into account:

$$\text{Specific activity retention (\%)} = \frac{C}{A - B}$$

where A is the the total enzyme activity before immobilization; B, the supernatant enzyme activity

after immobilization; and C, the activity of immobilized enzyme.

The determination of the activity of free and bound enzyme was performed by the proteinase-catalyzed reaction of 2% casein solution according to Refs. 10 and 11. The reaction temperature and pH value were 40°C and 6.5, respectively. The effects of temperature and pH were tested by varying the temperature and pH value.

The amounts of protein of the free and bound enzyme were determined by the folin method according to Ref. 10 and the standard sample was the bovine serum containing 75.0 μg/mL protein. The coupling efficiency of the bound enzyme was calculated from

$$\text{Coupling efficiency (\%)} = \frac{A - B}{A}$$

where A is the total protein content of free enzyme before immobilization, and B, the protein content of suppernatant enzyme after immobilization.

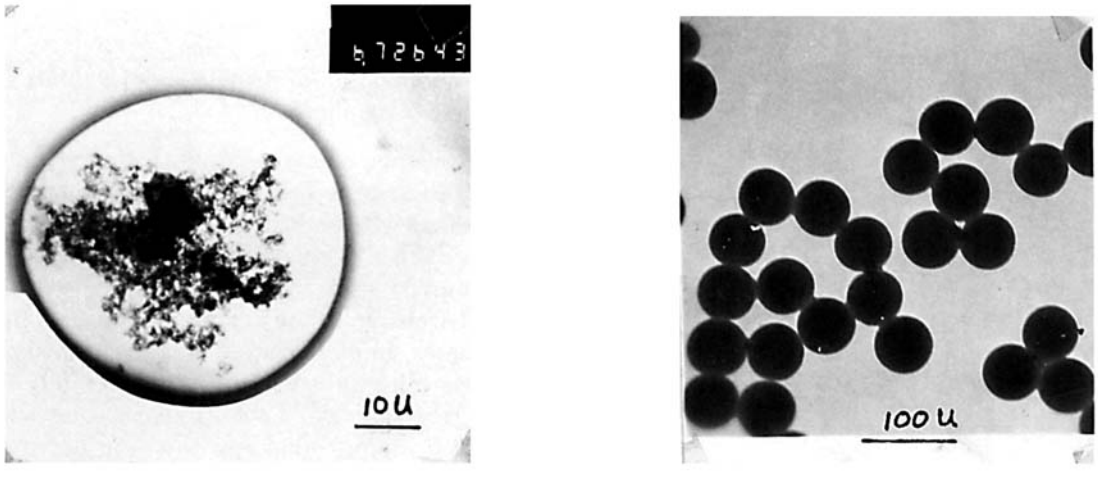
RESULTS AND DISCUSSION

Characterization of Magnetic Polymer Microspheres

The Fe_3O_4 powder, which had poor affinity to an oil-soluble monomer,[12] such as St and HEMA, was dispersed in PEG solution by ultrasonication so that PEG chains were absorbed on the surface of Fe_3O_4 powder. The KPS initiator decomposed at a certain temperature to form radicals abstracting the hydrogen atoms of the PEG molecules on the surface of the Fe_3O_4 powder,[13] and the Fe_3O_4 powder became the initiating center. The increasing polymer chain surrounded the Fe_3O_4 powder and, finally, core/shell composite polymer microspheres were formed. Figure 1(a) is a transmission electron microscope (TEM) photograph of a microsphere (the sample was prepared by ultrathin section), which shows that Fe_3O_4 lies at the core of the microsphere. Figure 1(b) is a macrophotograph of the microspheres, which indicates that the microspheres have excellent monodispersities.

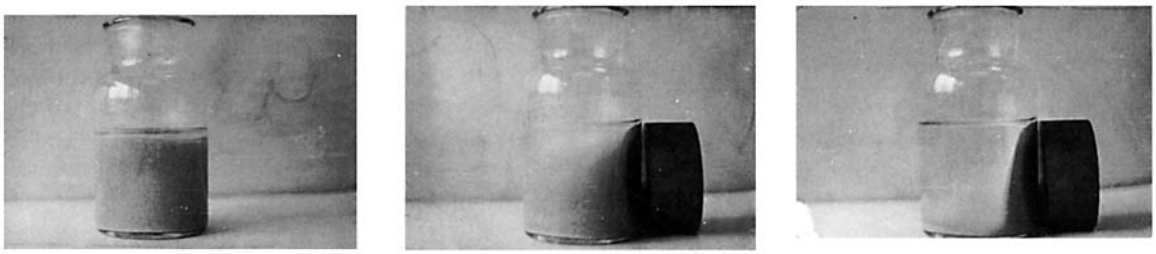
The microspheres containing Fe_3O_4 at the core can move directly in a magnetic field. Figure 2 (graphed by a general camera) shows different states of magnetic polymer microspheres dispersed in a water medium, having (a) an excellent dispersion state without a magnetic field, (b) moving quickly, and (c) being separated from the water medium in a magnetic field of 4200 Gauss.

HEMA can be copolymerized with St to introduce active hydroxyl groups on the microspheres' surface and to make the microspheres hydrophilic. The infrared spectrum of the magnetic polymer microspheres indicated that HEMA was introduced into the polymer shell (1730 cm^{-1}, stretching of $\overset{O}{\overset{\|}{C}}$ of HEMA; 3440 cm^{-1}, stretching of —OH of HEMA) and Fe_3O_4 was introduced into the magnetic core (550 cm^{-1}).



(a) (b)

Figure 1 The (a) TEM photograph and (b) macrophotograph of magnetic polymer microspheres.



(a) (b) (c)

Figure 2 The states of magnetic polymer microspheres dispersed in a water medium: (a) without a magnetic field; (b) in a magnetic field (4200 Gauss) for 15 s; (c) in a magnetic field (4200 Gauss) for 60 s.

Preparation of Magnetic Polymer Microspheres

The affinity between organic and inorganic compounds is usually not good and the affinity between an inorganic superfine powder and an organic compound is much worse;[12] so, it is difficult to form a polymer shell surrounding the Fe_3O_4 powder if incubating undispersed Fe_3O_4 powder into a polymerization system. In our research, the Fe_3O_4 powder was dispersed in a PEG-4,000 water solution by ultrasonication and PEG polymer chains were absorbed on the surface of Fe_3O_4, which not only stabilized the Fe_3O_4 powder into a superfine powder but also improved the affinity between the Fe_3O_4 superfine powder and the monomer and initiator. Figure 3 shows that the increase in the concentration of PEG (in the total polymerization system) led up to the decrease in the diameter of the microspheres, indicating that more initiating centers had formed with increase in the amount of PEG.

In our research, we found that the water/ethanol dispersion medium had a great effect on the formation of microspheres. Figure 4 shows that the increase in the amount of ethanol led up to a decrease in the diameter and had little effect on the dispersion parameter of the microspheres. It is interesting that there is a limit to the concentration of ethanol for the formation of microspheres, indicating that with increase in the amount of ethanol the polarity of the dispersion medium became weak and the majority of the monomer was soluble in the dispersion medium, so that the monomer that is soluble in the Fe_3O_4 superfine powder initiating system is too small for the polymer chain to propagate effectively and it finally formed a viscoloid. When the PEG concentration (in the total polymerization system) was 5%, the concentration of ethanol must be lower than 25% [see Fig. 4(a)], and when the PEG concentration is 10%, that of ethanol must be lower than 30% [see Fig. 4(b)].

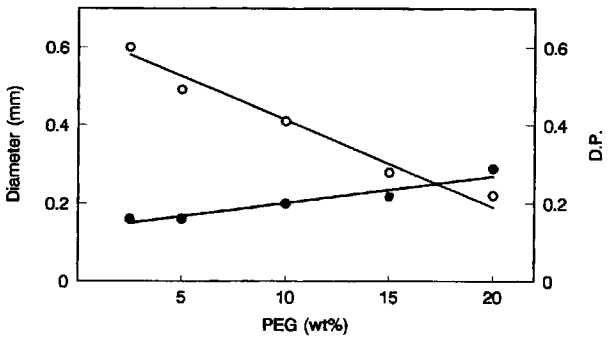


Figure 3 Effect of PEG on (○) diameter and (●) dispersion parameter (D.P.) of microspheres.

Immobilization of Proteinase of *Balillus sublitis* onto Microspheres

The microspheres containing hydroxyl groups on the surface were activated by benzoquinone, which then covalently bound the proteinase of *B. sublitis*. The mechanism of this process was described in the literature.[14]

By changing the amount of the activated microspheres, immobilization was performed under the same conditions by incubating 10.0 mL of the free enzyme solution. Table II shows the results of immobilization, indicating that the decrease in the proportion of supports to enzyme led up to an increase in the bound protein and the specific activity retention varied in the range of 30–40%.

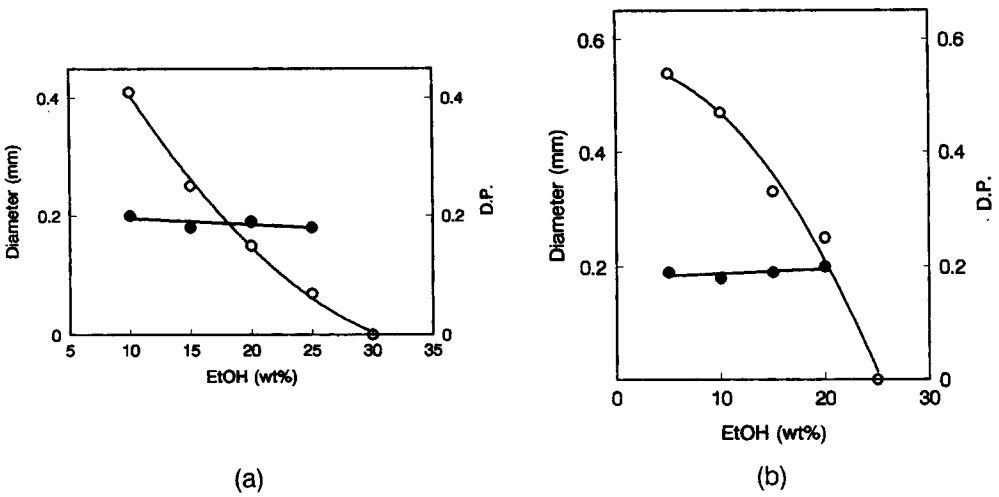


Figure 4 Effect of dispersion medium on (○) the diameter and (●) dispersion parameter (D.P.) of the microspheres: (a) PEG 5%; (b) PEG 10%.

Table II Effect of the Proportion of Supports to Enzyme on Immobilization

		Bound Enzyme Properties		
Activated Microspheres (g)	Enzyme (mL)	Activity (U/g)	Bound Protein (mg/g)	Specific Activity Retention (%)
0.25	10.0	839.4	24.4	42.7
0.5	10.0	839.4	22.0	42.3
1.0	10.0	823.7	21.1	40.9
1.5	10.0	642.5	17.3	33.2
2.5	10.0	642.5	15.4	31.6

Enzyme: 5.45 mg protein/mL, pH 6.5.

Table III Effect of pH on Immobilization

PBS			Bound Enzyme Properties		
Mol/L	pH	Activity of Free Enzyme (U/mL)	Bound Protein (mg/g)	Activity (U/g)	Relative Activity (%)
0.02	5.0	2903.8	24.6	545.0	67.3
0.02	6.0	3291.0	24.0	547.0	67.6
0.02	6.5	3600.7	25.9	809.7	100.0
0.02	7.0	3678.2	24.1	353.8	43.7
0.02	7.5	3871.7	25.9	96.4	11.9
0.02	8.0	2710.2	24.6	70.4	8.7

Activated microspheres: 1.0 g; enzyme (5.45 mg protein/mL): 10.0 mL.

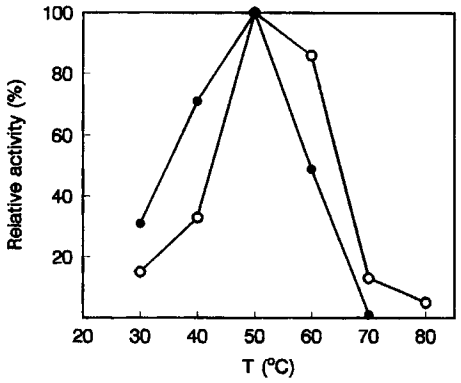


Figure 5 Effect of temperature on activities of (O) bound enzyme and (●) free enzyme.

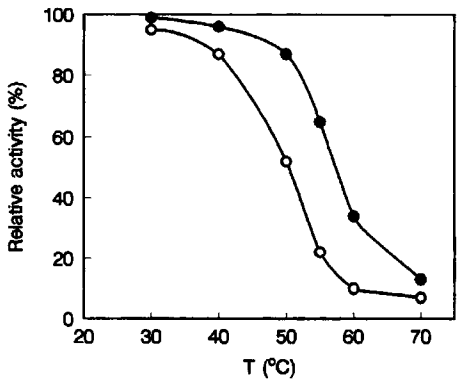


Figure 7 Thermal stability of (●) immobilized enzyme and (O) free enzyme.

The immobilization was carried out in PBS of different pH values; the results are shown in Table III. It shows that the activity of the immobilized enzyme was the highest when the pH value of PBS was 6.5 and that the pH value had little influence on the bound protein of the immobilized enzyme.

Properties of the Immobilized Enzyme

The free enzyme and microsphere-bound enzyme were incubated with a 2% casein solution (pH 6.5) at different temperatures between 30 and 70°C and their activities were determined. Figure 5 shows that the optimum temperature of free enzyme was 50°C and that of bound enzyme was 50–60°C. The activities of free and bound enzymes were determined at 40°C by changing the pH values of casein solution between 5.0 and 8.0, and the results are shown in Figure 6, indicating that the optimum pH value of bound enzyme was 6.5 and that of free enzyme was 7.5.

The free and bound enzymes were stored at 20°C for 30 days; the free enzyme almost lost its activity (its activity retention was only 0.2%), but the activity retention of the bound enzyme was 83.0%. Both of them were stored at 0°C for 30 days, and there was nearly no decrease in the activity of the bound enzyme (its activity retention was 96.4%) and the activity retention of the free enzyme was 62.6%. The free and bound enzymes were kept in a water bath at different temperatures between 30 and 70°C for 1 h, and the activities were determined, respectively. Figure 7 shows that the bound enzyme was stable at 30–50°C, but the free enzyme lost its activity quickly at 50°C, indicating that bound enzyme was more stable than was free enzyme. The structure became tighter and the configuration became more fixed after the enzyme was bound onto the microspheres, and the protein chain of bound enzyme was difficult to stretch and the high-grade structure was

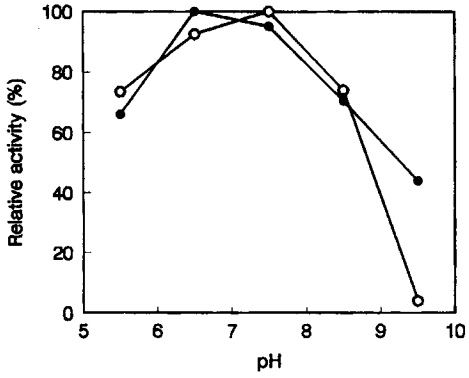


Figure 6 Effect of pH on activities of (●) bound enzyme and (O) free enzyme.

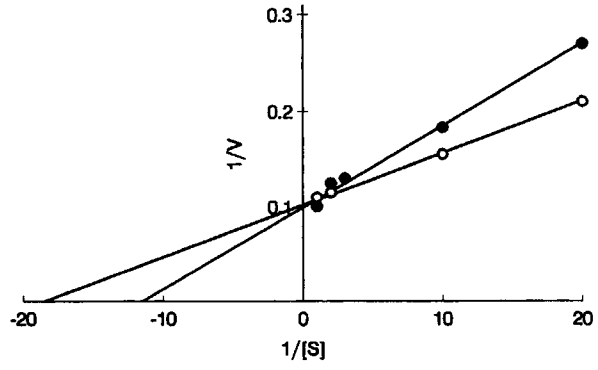


Figure 8 The Lineweaver–Burk plots for (●) immobilized enzyme and (O) free enzyme.

maintained, so the stability of the immobilized enzyme was obviously improved.

The activities of the free and bound enzyme were determined using a casein solution of different concentrations (w/v): 1.0, 0.5, 0.25, 0.1, and 0.05% as the substrate and the Lineweaver–Burk plots are shown in Fig 8. The K_m value of the bound enzyme was 0.088% and that of the free enzyme was 0.054% casein solution calculated from the plots, indicating that the affinity between bound enzyme and substrate was lower than that of free enzyme. The probable reason is that the active centers of bound enzyme were hindered by polymer carriers after immobilization.

CONCLUSIONS

Magnetic polymer microspheres were synthesized. The study of the immobilization of proteinase of *Balillus sublitis* onto magnetic polymer microspheres reveals that the stability of bound enzyme was improved and the magnetic separation of such bound enzyme from substrate was simple to perform.

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