Preparation and Characterization of Superparamagnetic Chitosan Microspheres: Application as a Support for the Immobilization of Tyrosinase

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Received 11 August 2004; accepted 6 February 2005
DOI 10.1002/app.22086
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Superparamagnetic chitosan microspheres were prepared by a water-in-oil suspension-crosslinking technique. To this end, magnetite particles were dispersed in a chitosan solution in acetic acid. The dispersion was added to toluene containing Span 20 as a surfactant with stirring. Chitosan solution droplets were hardened with glutaraldehyde. The magnetic chitosan microspheres obtained were characterized with scanning electron microscopy, differential thermal analysis, and vibrational magnetometry. The microspheres had a wide size distribution, ranging from 43 $\pm$ 25 to 255 $\pm$ 55 μm, that depended on the reaction conditions. The mean particle size decreased with an increase in the concentration of Span 20 or the amount of glutaraldehyde and with the addition of NaCl. However, a major size reduction was achieved by an increase in the stirring rate. Tyrosinase was immobilized on the microspheres. The immobilized enzyme retained 70% of its activity, as determined by the capacity to degrade phenolic compounds. The immobilized tyrosinase resulted in greater stability than the free enzyme. In addition, the enzyme maintained 65% of its phenol oxidation activity after 10 cycles of reuse. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 651–657, 2005

Key words: chitosan; microspheres; magnetite; tyrosinase

INTRODUCTION

Superparamagnetic polymer microspheres have been employed in medicine and pharmacy for the specific site delivery of drugs, immunoassays, hemoperfusion, the counting of cell populations, and other uses.[1–5] The superparamagnetic character implies that they respond to a magnet, making sampling and collection easier and faster, but their magnetization disappears once the magnetic field is removed. With magnetic microspheres, animal and plant cells[6] can be separated. Magnetic particles were used recently for selecting and collecting protoplasts of potatoes somatic hybrids.[7] They have also been used for collecting and concentrating *Chlamydia trachomatis* from urine for its further analysis.[8] These polymer particles have also been proposed as useful supports for the immobilization of enzymes because after the reaction they can be easily separated from the products with a magnet and collected for reuse.[9,10]

Chitosan is a biocompatible, biodegradable, nontoxic, and mucoadhesive polymer, and this makes it attractive for applications in medicine and pharmacy.[11–13] These interesting characteristics have led to the development of numerous applications of chitosan and its derivatives in biomedicine, such as surgical sutures,[14] biodegradable sponges and bandages,[15–17] matrices in microspheres, microcapsules, membranes, and compressed tablets for the delivery of drugs,[18–20] and orthopedic[21–23] and dentistry materials.[24,25] There are numerous scientific reports and patents on the preparation of chitosan microspheres and microcapsules, including some reviews on the subject.[26,27]

The preparation of magnetic chitosan particles has been reported previously.[28,29] High-magnetic-property chitosan particles have been recently tested successfully as carriers of aprotinin for trypsin affinity purification.[28] The use of immobilized enzymes has the advantage that it usually increases their storage and thermal stability. It avoids the contamination of the treated solution by enzymes because the immobilized enzymes can be separated from the solution.[30] The separation process is facilitated when the enzyme is attached to a magnetic support.

In this article, we report on the preparation of superparamagnetic chitosan microspheres. The particles were then used as supports for the immobilization of tyrosinase. Tyrosinase is a copper-containing enzyme that catalyzes the hydroxylation of monophenols with

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Contract grant sponsor: Third World Academy of Sciences.
Contract grant sponsor: Ministry of High Education of Cuba.

TABLE I
Experimental Conditions for the Preparation of Microspheres and the Mean Particle Size of Dried Microspheres

Sample	Span 20 (g)	Stirring rate (rpm)	Magnetite (g)	CHI solution (g)	GLU solution (mL)	NaCl (g)	Size (μm)
1	1.15	800	0.16	1	0.2	—	229 ± 54
2	2.30	800	0.16	1	0.2	—	71 ± 43
3	1.15	1200	0.16	1	0.2	—	43 ± 25
4	1.15	1600	0.16	1	0.2	—	36 ± 27
5	1.15	800	0.32	1	0.2	—	255 ± 55
6	1.15	800	0.32	1	0.6	—	182 ± 58
7	1.15	800	0.16	1	0.2	0.02	181 ± 44
8	1.15	800	0.16	2	0.2	—	233 ± 52

molecular oxygen to form *o*-hydroquinones. Like other oxidative enzymes from bacteria and fungi, it can play an important role in wastewater treatment.[31] The phenol oxidation activity of the tyrosinase immobilized on the superparamagnetic chitosan microspheres was corroborated.

EXPERIMENTAL

Chitosan (deacetylation degree (DD) = 89.3%, viscosity-average molecular weight = 3.7×10^4) was obtained from lobster shells by a procedure already described.[32] It was purified as follows. Chitosan was dissolved in a 2% acetic acid aqueous solution until a homogeneous 1% chitosan solution was attained. The solution was neutralized to pH 8.0 with a 10% NaOH solution. The precipitated chitosan gel obtained was washed several times with deionized distilled water and vacuum-dried in a desiccator.

Glutaraldehyde (GLU) was purified by successive distillations in a high vacuum line until the sample was free of α/β-unsaturated oligomers, as checked by UV spectroscopy. Tyrosinase (EC 1.14.18.1) was obtained from Sigma Chemical Co. (Steinheim, Germany) and had a specific activity of 3500 units/mg. $FeCl_3 \cdot 6H_2O$ (pure) for analysis was purchased from Riedel-de Haen (Seeize, Germany), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ pure was obtained from BDH (Dorset, England), and 32% ammonia solutions were from Merk (Darmstadt, Germany). All solutions were prepared with redistilled water.

Preparation of superparamagnetic magnetite

Superparamagnetic magnetite was synthesized and characterized in our laboratory as already described.[33] The synthesis procedure was as follows. A 11.1% $FeCl_3 \cdot 6H_2O$ solution (7.7040 g in 63.7 mL of H_2O) was added to a 11.1% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ solution (9.293 g in 83.6 mL of H_2O). The mixture was homogenized with magnetic stirring. The solution was cooled to 20°C and stirred at 7 000 rpm. Three hundred milliliters of 10% aqueous ammonia was added quickly to the solution of Fe^{2+} and Fe^{3+} ions, and a dark precipitate of magnetite was formed. The precipitated magnetite (Fe_3O_4) was separated from the solution by decantation, washed thoroughly with redistilled water, and stored. The superparamagnetic character of magnetite was assessed by vibrational magnetometry.

Preparation of the superparamagnetic chitosan particles

Magnetic chitosan microspheres were obtained by the dispersion of magnetite particles in a 2 wt % chitosan solution in 1 wt % acetic acid. The dispersion was added to 70 mL of toluene containing Span 20 as a surfactant. A water-in-oil dispersion was obtained by vigorous mechanical stirring during 15 min. A GLU solution (25 wt %) was added, and stirring was reduced to 300 rpm for 2 h. The microspheres were washed with water and ethanol and dried. The experimental conditions are listed in Table I.

Characterization of the superparamagnetic chitosan particles

The size of the microspheres was determined with a Nikon Eclipse E-400 optical microscope (Tokyo, Japan). The values reported are the averages of 120–140 particles. The morphology of particles was investigated with a Phillips XL30 ESEN environmental scanning electron microscope (Eindhoven, the Netherlands).

The magnetite content of the particles was determined by differential thermal analysis (DTA) with a PerkinElmer TGA7 thermogravimetric analyzer (Norwalk, CT) coupled to a TAC/PC controller. Analyses were performed with 8–10-mg samples in a platinum pan under a nitrogen atmosphere. The nominal gas flow was 5 mL/min, and the heating velocity was 10°C/min.

The magnetic properties of the chitosan microspheres were studied with a VSM Oxford 3001 vibrating sample magnetometer (Oxford, England) with a maximum magnetic field of 1.6 T and a sensibility of 10^{-5} emu. The measurements were performed at room

temperature with a 2-Oe path for the field scanning at the origin of the initial magnetization curve.

Measurement of the tyrosinase activity

The tyrosinase activity was determined from the change in the optical density at 280 nm (A_{280}) of a reaction mixture containing L-tyrosine. The optical density was determined with a Jasco 920 GBC ultraviolet–visible spectrophotometer (Cork, Ireland) with a 1-cm-wide quartz cell. One unit is defined as an increase in A_{280} per minute at pH 6.5 at 25°C in a reaction mixture (3 mL) containing L-tyrosine.

Immobilization of tyrosinase

Tyrosinase (20 mg) was dissolved in 20 mL of a 0.1*M* phosphate buffer solution (pH 7.0). The magnetic chitosan microspheres (500 mg) were added to the tyrosinase solution and stirred at 4°C for 15 h. The immobilized magnetic chitosan microspheres were washed with a buffer solution until the enzyme was not detected in the supernatant.

Enxymatic oxidation of phenol

All reactions were carried out at 25°C in 7 mL of a phosphate buffer (pH 7.0) containing tyrosinase and phenol (0.5 m*M*). The reaction solutions were prepared by a previously reported method (10) and incubated under aerobic conditions with stirring. After a prescribed time, a sample was withdrawn from the solution and assayed for the phenol concentration. The concentration was determined by spectrophotometry at 265 nm.

The adsorption of quinones by chitosan was confirmed with the following experiment. Chitosan (50 mg) was added to 7 mL of the solution resulting from the enzymatic oxidation of phenol. Aliquots of the supernatant were taken at different times, and their absorbance was determined at 380 nm.

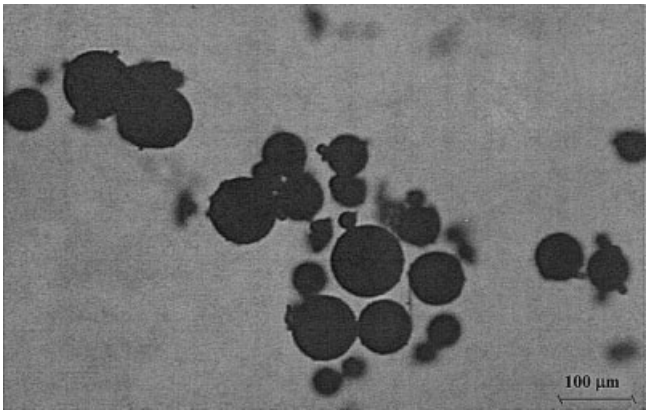

Figure 1 Optical micrograph of superparamagnetic chitosan microspheres (sample 2).

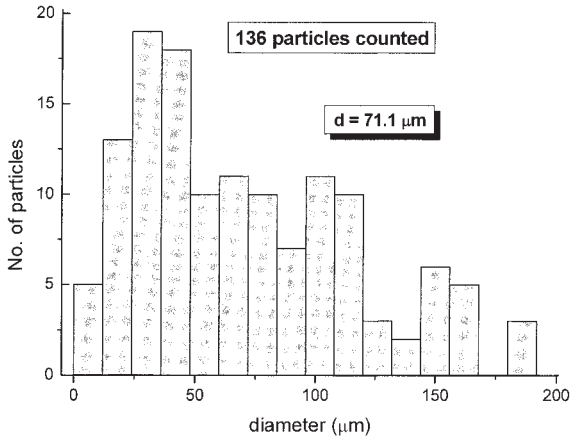

Figure 2 Particle size distribution of superparamagnetic chitosan microspheres, determined from optical micrographs of sample 2.

RESULTS AND DISCUSSION

Characterization of the chitosan magnetic particles

The chitosan magnetic particles had a spherical shape and a broad size distribution, as revealed by the optical micrograph in Figure 1. Figure 2 shows the size distribution diagram for sample 2. The mean particle size varied considerably with the experimental conditions. Table I shows that the mean particle size decreased with an increasing concentration of Span 20 or GLU and with the addition of NaCl. However, a major size reduction was achieved by an increase in the stirring rate. The increase in the chitosan solution increased the size of the microspheres. As clearly shown in the electron micrograph of Figure 3, the magnetic microspheres had a rough surface with embedded magnetite particles within the structure. These surface irregularities are often found when polymer particles loaded with insoluble materials are prepared by the suspension-crosslinking technique.

The chitosan magnetic microspheres had very hydrophilic structures. They swelled rapidly, and equilibrium was achieved in about 15 min. The water uptakes (g of water/g of dry microspheres) at equilibrium swelling were 2.8 at pH 2, 1.7 at pH 5, and 0.8 at pH 7. The increase in the swelling capacity with decreasing pH was due to the protonation of the free amino groups of chitosan.

Determination of the magnetite content of the microspheres

The magnetite content of the microspheres was determined by DTA in a nitrogen atmosphere. This tech-

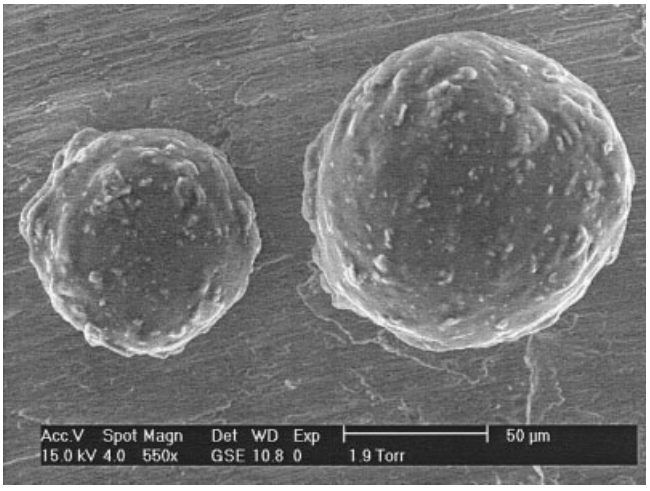

Figure 3 Environmental scanning electron microscopy image of chitosan superparamagnetic microspheres.

nique has been previously used for establishing the magnetite content of magnetic polymer particles.[34] The thermograms of chitosan, magnetite, and samples 2, 4, and 5 are shown in Figure 4. Once it was dried, there was no weight loss in magnetite up to 900°C. On the other side, chitosan experienced an initial loss of water between 60 and 170°C, followed by a 53% weight loss in the interval of 240–400°C, with the maximum decomposition rate at 329°C, due to the decomposition of its acetylated and deacetylated units. All three samples shown in Figure 4 exhibit a similar decomposition pattern, with the main degradation step lying between 200 and 400°C.

Assuming that the ratio between the weight of the residue at 900°C and the weight loss in the interval of 200–400°C for pure chitosan is the same for chitosan in the microspheres, it is easy to show that the content of magnetite in the microspheres can be calculated as follows:

$$\frac{R_q}{\Delta W_q} \times \Delta W_m + M = R \tag{1}$$

where R_q is the residue (wt %) at 900°C of starting chitosan; ΔW_q and ΔW_m are the weight-loss percentages in the interval of 200–400°C for starting chitosan and the magnetic microspheres, respectively; M is the weight percentage of magnetite in the microspheres; and R is the residue at 900°C (in wt %) for the magnetic microspheres.

The magnetite concentrations calculated with eq. (1) for samples 2, 4, and 5 were 4.5, 4.3, and 7.2%, respec-

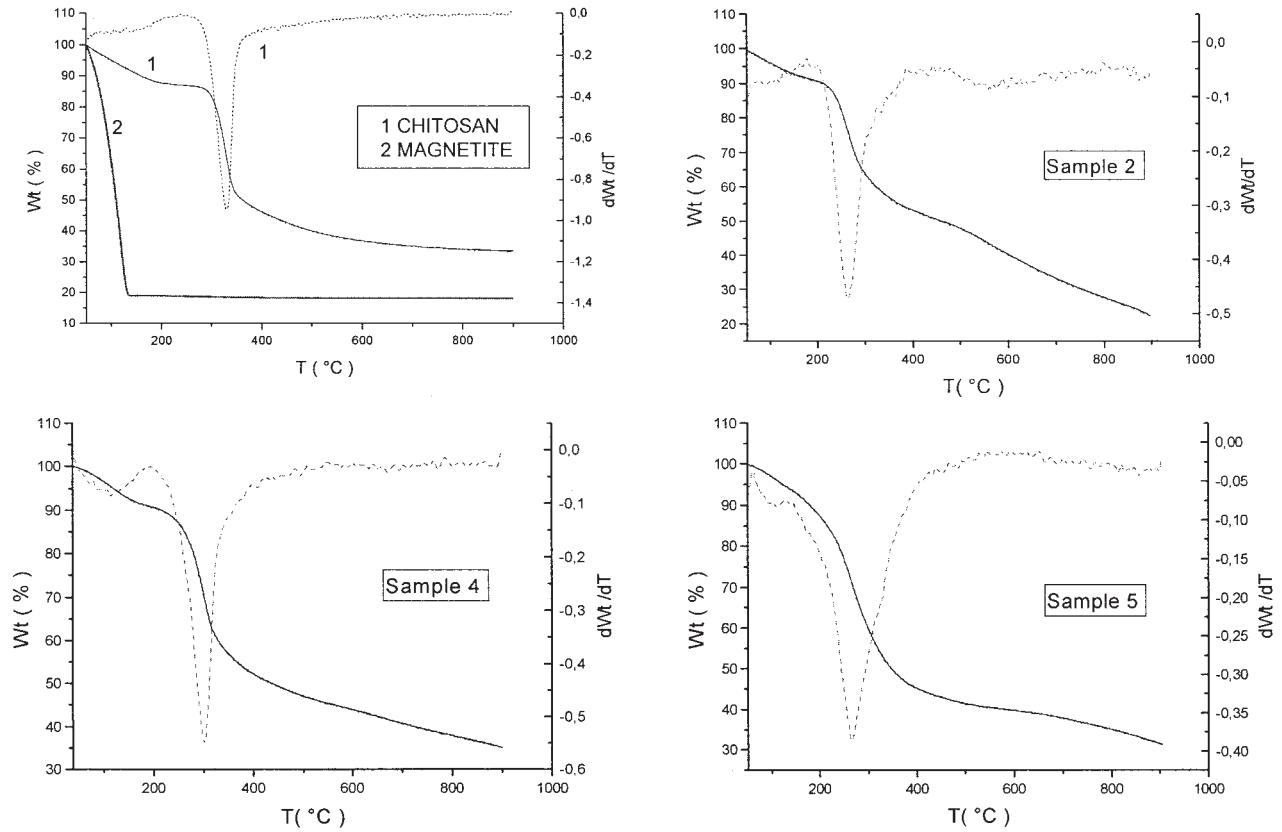

Figure 4 (—) Thermogravimetric and (- - -) differential thermogravimetric curves of chitosan, magnetite, and chitosan superparamagnetic microspheres.

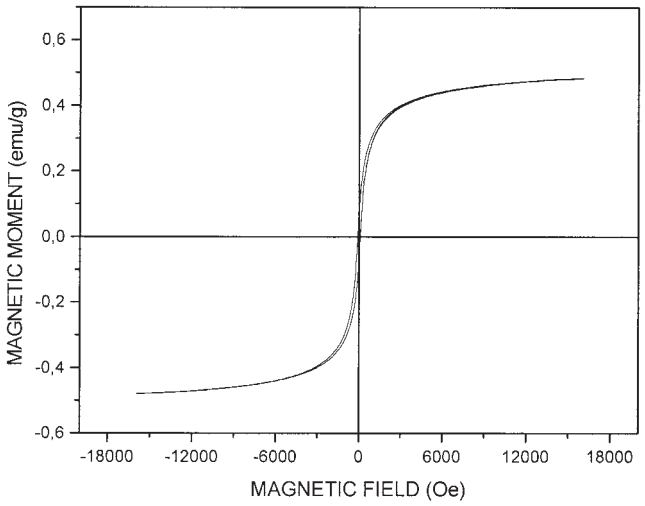

Figure 5 Magnetic hysteresis loop of chitosan superparamagnetic microspheres.

tively. The higher magnetite concentration in sample 5 was due to the fact that in this experiment the load of magnetite was twice those of samples 2 and 4. However, the encapsulation efficiency was lower in sample 5.

Magnetic behavior of the chitosan magnetic particles

The superparamagnetic character of the particles is evident from the magnetic hysteresis loop of sample 2, represented in Figure 5. The remanent magnetization intensity, that is, the sample magnetization when the applied magnetic field is zero, was only 0.05 emu/g. This magnetization could be decreased to zero by the application of a reverse field known as the coercive field or the coercitivity. Ideally, in a superparamagnetic material, this coercitivity should be equal to zero. In our microspheres, we obtained a coercitivity of 101 Oe, similar to that of pure magnetite with good enough superparamagnetic behavior. This result indicates that the magnetite particles entrapped inside the microspheres were dispersed in the chitosan matrix, thus preserving their superparamagnetic behavior.

In contrast, the magnetic chitosan particles prepared by An and Su[28] exhibited a broad magnetic loop with a remanent magnetization intensity of 14.5 emu/g and a coercitivity of 2000 Oe. This indicates that the magnetite particles that they used were not superparamagnetic or that they were highly aggregated inside the polymer particles.

Enzymatic treatment with immobilized tyrosinase

Immobilization imparts an increased storage stability to the enzyme as well as better control of the catalytic process. In addition, immobilization provides operational stability and avoids contamination of the treated solution by enzymes because the immobilized enzymes can be separated from the solution.[10] A variety of polymer supports have been employed to immobilize enzymes. Magnetic polymer particles as a support have the advantage of being easily separated from the solution by the use of magnetic devices.

Several methods have been developed for the immobilization of an enzyme on a polymer surface. The simplest one is based on physical adsorption. However, this method has the inconvenience that the enzyme is easily released to the solution. In this sense, chemical attachment is usually preferred. It can be achieved with crosslinking agents such as GLU or epichlorohydrin. In this work, we made use of the active aldehyde groups present in the magnetic chitosan microspheres for the immobilization of tyrosinase. Aldehyde groups reacted with the free amino groups of the tyrosinase, bringing about the attachment of the enzyme. About 80% of the enzyme was immobilized on the superparamagnetic chitosan particles, and the retained activity was around 70%. Figure 6 shows the degradation of phenol by immobilized tyrosinase with time. It was almost the same as that by soluble tyrosinase.

The stability in time of immobilized tyrosinase at room temperature was compared with that of the free enzyme (Fig. 7). Immobilized tyrosinase was more stable than free enzyme. After 10 days of storage at the ambient temperature, the activity loss of immobilized tyrosinase was only 10%, whereas it reached almost 80% for the free enzyme. These results indicate that the immobilization method of tyrosinase was effective.

The tyrosinase activity was investigated at different temperatures (between 15 and 50°C). Figure 8 shows

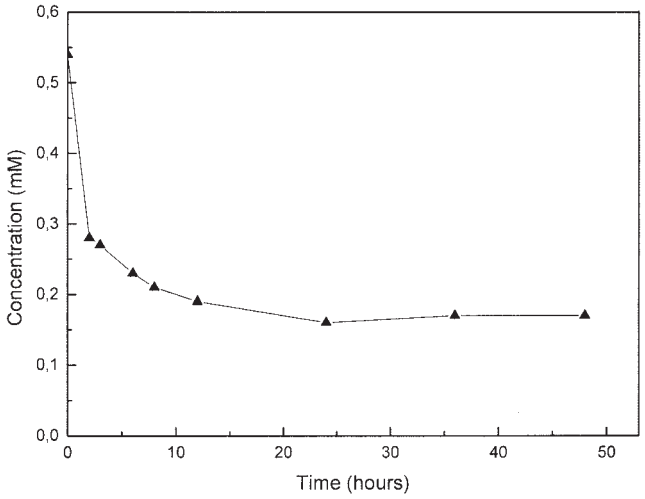

Figure 6 Reduction of the phenol concentration by incubation with tyrosinase immobilized on superparamagnetic chitosan microspheres.

that up to 30°C, the temperature had almost no effect on the activity of immobilized tyrosinase. However, as the temperature was raised from 30°C, there was a noticeable decrease in the enzyme activity.

The ability of tyrosinase immobilized on magnetic chitosan microspheres for repeated use was assessed. To this end, immobilized tyrosinase was immersed into a phenol solution (0.5 m*M*), the reaction was left to proceed for 24 h, and the degradation of phenol was determined. This procedure was carried out repeatedly with the same immobilized enzyme, the phenol solution being replaced by a fresh one of the same initial concentration (0.5 m*M*) after each treatment. Tyrosinase immobilized on the magnetic chitosan microspheres maintained 65% of the phenol oxidation activity even after 10 cycles of reuse.

A comparative experiment was performed with the free enzyme. In this case, it was not possible to isolate the enzyme after each treatment. Instead, more phenol was added to adjust the concentration to the initial value (0.5 m*M*) before each run was started. The initial enzymatic activity was the same as that of the treatments with immobilized tyrosinase (20 units/mL). However, soluble tyrosinase was rapidly inactivated by the reaction with phenol. This inactivation was provoked by the quinones produced in the reaction.

In this connection, another advantage of the immobilization of tyrosinase in chitosan is that quinones produced during phenol degradation are chemisorbed on chitosan as they are formed. It is known that *o*-quinones can undergo a reaction with amino groups of chitosan at more than a single site through the formation of Michael's type and Schiff base adducts.[35] This was confirmed by the solution of the reaction products being put into contact with chitosan. This solution had a brownish color due to the presence of quinones. As a result of the sorption of quinones by chitosan, the absorbance of the solution at 380 nm decreased. At the end of the experiment, the originally dark solution had turned clear.

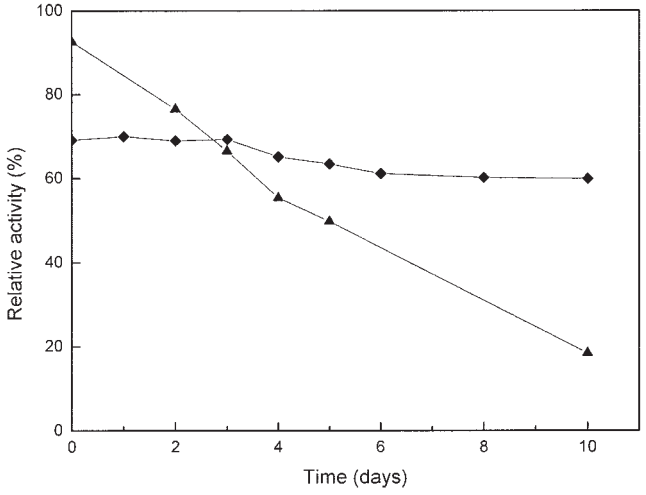

Figure 7 Storage stability of (▲) soluble by free (◆) immobilized tyrosinase at room temperature.

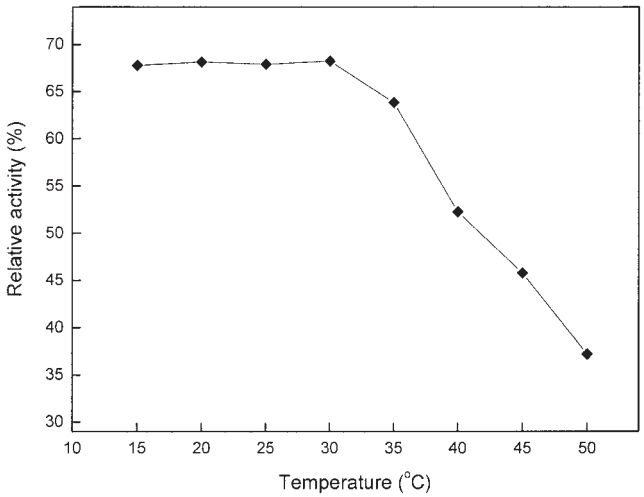

Figure 8 Effect of the temperature on immobilized tyrosinase.

CONCLUSIONS

Superparamagnetic chitosan microspheres were prepared by the encapsulation of superparamagnetic magnetite on chitosan with a suspension-crosslinking technique. The average size of the microspheres depended on preparation parameters, such as the surfactant concentration, stirring rate, chitosan solution volume, and amount of the crosslinking agent. The superparamagnetic chitosan microspheres were suitable for the immobilization of enzymes. Tyrosinase immobilized on these microspheres resulted in more stability than the free enzyme and maintained a high phenol oxidation activity after 10 cycles of reuse.

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