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Graft copolymers with immobilized peroxidase for organic synthesis

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Abstract

The graft copolymer poly(propylene)-graft-poly(acrylic acid) (PP-G-AA) was prepared by radiation-induced graft copolymerization of acrylic acid onto polypropylene spheres and characterized by thermal analysis and scanning electron microscopy (SEM). Maximum percentage of grafting (70%) was obtained at a total dose of 12 kGy using 30% (w/w) of acrylic acid. The Michaelis constant, K_M, and the maximum reaction velocity, V_{Max}, were determined for the free horseradish peroxidase and for the immobilized horseradish peroxidase. The enzyme affinity for the substrate (K_M/V_{max}) remains quite good after immobilization. The sulfoxidation reaction of a ketosulfide was investigated with the immobilized peroxidase. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

The application of biocatalysis to organic synthesis is at present a well-defined area of research, to which several books and reviews have already been devoted (Drauz and Waldmann, 1995; Faber, 1997; Steckhan, 1994; Wong et al., 1990). Biocatalysts of synthetic utility to organic chemists for the preparation of chiral products can be constituted by whole cells of animals, plants, microorganisms or enzymes (Morihara et al., 1992; Nakamura et al., 1991; Santaniello et al., 1992).

The native enzymes can be modified by well-established immobilization techniques in order to recover the biocatalyst and improve its stability toward the usual conditions in which they are used by organic chemists (de Queiroz et al., 1996; Iwuoha et al., 1997; Sheffield et al., 1995). Enzyme immobilization is excellent due its high storage stability and better control of the catalytic process. In addition, immobilization allows for operational stability and reduced contamination of the solution treated by enzymes, because the immobilized enzymes can be easily separated from the solution. Sulfoxidations catalysed by enzymes have widely been employed in organic syntheses (Holland, 1988; Colonna et al., 1992; Lee et al., 1996; Van Deurzen et al., 1997). The sulfoxides obtained have attractive pharmacological or biological activities. They may be used for controlling the tereoselectivity of a large number of reaction types: alkylation of carbanions, Michael additions, aldolisation reactions,

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cyclo-additions, Pummerer rearrangements and so on (Carreño, 1995).

Applications involving the use of immobilized horseradish peroxidase for the removal of chlorophenols or chlorolignins from wastewater (Dezotti et al., 1995; Tatsumi et al., 1996) have been documented.

In addition enzyme electrodes (Tatsuma et al., 1994; Zhang et al., 1996) in a variety of insoluble supports (Batra et al., 1997) have been prepared and evaluated. The results obtained encouraged us to study the catalytic properties of the immobilized peroxidase and to explore the synthetic utility of the copolymers in the sulfoxidation of ketosulfides.

2. Experimental

2.1. Materials

The horseradish peroxidase (EC 1.11.1.7) (HRP) was purchased from Sigma and had a specific activity of 1000 units/mg. The polypropylene spheres (PP), 3 mm diameter, were washed with water followed by methanol, filtered and dried to a constant weight. Acrylic acid (AA) was obtained from Aldrich Chemical Co. and was used as received. All the other reagents were commercially available products of analytical grade.

2.2. Grafting procedure

A simultaneous radiation grafting method was used as a technique for the preparation of poly(propylene-g-acrylic acid) (PP-G-AA) spheres. Dry PP spheres (10.0 g) were added in water/AA mixture of known (30% w/w) bulk monomer concentration. The PP spheres, monomer and solvent were put in a glass ampoule. The ampoule was connected to a vacuum system (0.013 Pa) and it was evacuated by a freeze–thaw cycle which was repeated five times. After evacuation, the ampoule was irradiated by using gamma-rays from a ^{60}Co source at a dose rate of 0.37 kGy/h, and a total dose of 12 kGy to give 70% (w/w) of graft yield. The grafted spheres were washed throughly with hot distilled water and soaked in distilled water for 48 h to extract the residual monomer, and the homopolymer, poly(acrylic acid), occluded in the spheres.

The PP spheres were then dried in a desiccator under vacuum at room temperature until a constant weight was reached. The degree of grafting was determined gravimetrically.

2.3. Surface characterization

Electron microscopy was used to study the morphology of the grafted and ungrafted polypropylene. Thus, PP and PP-G-AA spheres were sputter-coated with gold and observed by scanning electron microscopy, (SEM), (JEOL JXA-6400 electron probe microanalyzer).

2.4. Thermal analysis

The thermal properties of the grafted (PP-G-AA) and ungrafted (PP) spheres were investigated using a Mettler thermal gravimetric analyser (TGA 50). The TGA analyses were carried out under a nitrogen atmosphere (20 mL/min) at a heating rate of 10°C/min.

2.5. Peroxidase immobilization

Horseradish peroxidase was immobilized onto PP-G-AA by covalent coupling. In order to attach the enzyme to the copolymer surface covalently, the support had to be activated using the Coulet method (Coulet et al., 1974). A variety of procedures has been reported for activating graft copolymers for enzyme immobilization (da Silva et al., 1990; Chen et al., 1993). The Coulet's method for binding the enzyme is particularly mild since the enzymes never come into contact with the chemical reagents, and, thus, denaturing processes are avoided. The copolymer spheres (10 g) were actived by acyl azide formation. After removal of the reagents by repeated washing, the coupling of the enzyme was performed by immersion of the activated spheres in the HRP solution (0.4 mg/mL, 50 mL). The components were shaken for 24 h at 277 K, after which the solids were filtered and washed. The protein content was determined using the Bradford assay procedure (Bradford, 1976), with bovine albumin (Sigma, fraction V powder) as the standard protein. The coupling process is shown in Fig. 1 (step IV).

2.6. Determination of horseradish peroxidase activity

Assays of peroxidase activity for both the free HRP and the immobilized HRP were performed by measuring the initial rate of oxidation of *o*-dianisidine by H_2O_2, using the two substrates in saturating concentrations (Ugarova et al., 1979). The concentrations of HRP were determined spectrophotometrically at 403 nm and a molar extinction coefficient of 102 000 $M^{-1}cm^{-1}$ was used (Ohlson and Paul, 1976). The spectrophotometric measurements were undertaken at a wavelength of 460 nm using a Hitachi V 2000 spectrophotometer.

2.7. Sulfoxidation reaction

The ketosulfide used in this work, 1-methylthioacetone (Fig. 2a), was prepared according to the reaction of thiolate anions with α-halogeno carbonyl compounds (Prelog et al., 1944). The obtained ketosulfide

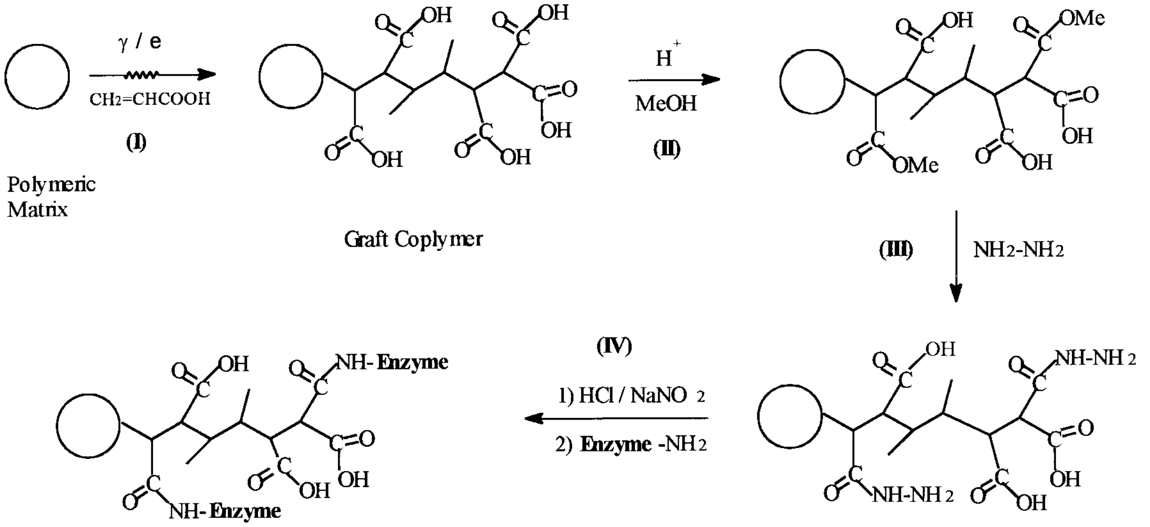

Fig. 1. Schematic diagram of the HRP immobilization onto PP spheres functionalized with radiation-induced graft copolymerization of acrylic acid.

(1.0 mmol) and immobilized HRP (5.0 g) were magnetically stirred in 20.0 mL of 0.05 mol.dm^{-3} phosphate buffer, pH 6.0 at 25°C for 5 min and H_2O_2 (1.5 mmol) in 2.0 mL of buffer, pH 6.0, was added in 18 equal aliquots at 3 min intervals. The spheres were filtered off and the reaction quenched with sodium sulfite, extracted with diethyl ether (4 x 100 mL), dichloromethane (4 x 100 mL) and dried with magnesium sulfate.

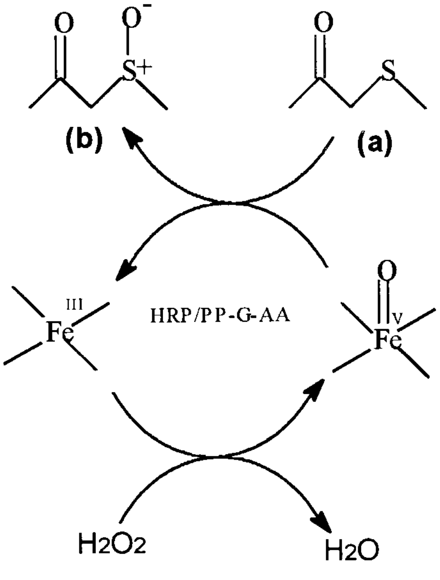

Fig. 2. Oxidation of ketosulfide by immobilized HRP. (a) 1-methylthioacetone (ketosulfide) and (b) 1-(methylsulfinyl)acetone (ketosulfoxide).

The catalytic activity of HRP, in the oxygenation of sulfides in the presence of H_2O_2, is low. Thus, an enzyme:substrate ratio of 1:500 was used to maximize the contribution of the catalysed reaction (Colonna et al., 1992). The obtained product was characterized by ^{1}H-NMR by using a Bruker AC-200 (200 MHz) spectrometer and Me_4Si as an internal standard. The ^{1}H-NMR data for obtained ketosulfoxide, 1-(methylsulfinyl)acetone (Fig. 2b), in this work are 2.37(s, 3H), 2.70(s, 3H), 3.70(d, 1H, $J = 13.7$ Hz), 3.86(d,1H, $J = 13.7$ Hz).

The enantiomeric excess of ketosulfoxide, 1-(methylsulfinyl)acetone (Fig. 2b), were analysed by a polarimetric method (Jasco model DIP 370). The racemic sulfoxide, 1-(methylsulfinyl)acetone, was prepared by periodate oxidation of the sulfide (Leonard and Johnson, 1962).

3. Results and discussion

The morphology of the samples was investigated by SEM (Fig. 3). The increased roughness with the grafting process may be due to the hydrophilic copolymer poly(acrylic acid) chains onto the sphere surface.

The thermal stability of the polypropylene (PP) and poly(acrylic acid)-graft-polypropylene (PP-G-AA) was evaluated by thermal analysis. Fig. 4 shows the TG and DTG curves recorded for the original PP sample and for the graft copolymer PP-G-AA. It is observed from the TG and DTG curves of PP sphere (Fig. 4a) that the weight loss begins at 633 K, beyond which rapid weight loss occurs continuously up to 733 K

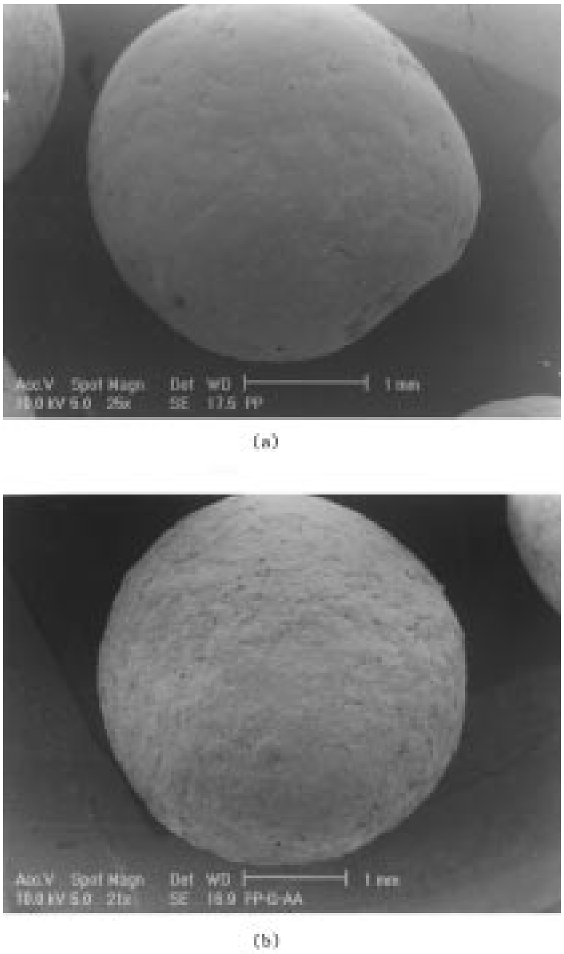

Fig. 3. Scanning electron micrographs of (a) ungrafted PP and (b) PP-G-AA (G: 70%). G(%): grafting degree.

leaving 0% residue. This may be due to the oxidative degradation of PP sphere. The TG and DTG curves of the PP-G-AA are shown in Fig. 4b. The initial decomposition of the grafted film begins at 523 K and continues slowly up to 733 K, with a total weight loss of 100%. The degradation region, weight loss of 10% between 493 and 573 K, is due to the dehydration of the pendant carboxylic groups of the grafted AA chains leading to the formation of the corresponding anhydride (Misra et al., 1995). Thus, the thermal analysis of the grafted and ungrafted PP provided additional evidence for the grafting of acrylic acid onto PP spheres.

Covalent coupling of HRP directly onto the grafted PP spheres was carried out by reaction of the diazonium salts, generated in the graft copolymers, with the amino groups in the enzyme, as shown in Fig. 1 (step IV). The results obtained are shown in Fig. 5. It can be seen that coupling of HRP did occur onto the grafted PP spheres. The data in Fig. 5 show that the surface amount of the bound active HRP rose with increasing reaction time.

A similar effect has been reported for the immobilization of lipase and papain onto acrylic acid graft copolymers (Hayashi and Ikada, 1990; Emi et al., 1990).

The quantity of the immobilized enzyme on the polymeric surface which presented catalytic activity was 360 U.mg^{-1} or 60% of the total activity (relative to the *o*-dianisidine method). This result may be explained in terms of structure deformation, i.e., the structure of the enzyme molecule, immobilized by covalent fixation, may undergo strong deformation, especially in the lower surface concentration region, being apt to decrease the activity with decreasing surface concentration (Chen et al., 1993). The ketosulfide was oxidized with hydrogen peroxide in the presence of immobilized HRP, as shown in Fig. 2, affording the ketosulfoxide in a yield of 66%. The chemical oxidation of the ketosulfide in the presence of hydrogen peroxide without the enzyme afforded only 2% of the ketosulfoxide. During the immobilization procedure by covalent attachment, some loss of activity due to conformational changes of the enzymes is always observed. The residual activities do not exceed 60–80% of the activity of the native enzyme and values of around 50% are normal (Faber, 1997).

The properties of the biocatalyst after immobilization, such as its selectivity of operation may be significantly altered (Christen and Crout, 1987). In our results, the free enzyme and the HRP immobilized onto PP-G-AA induced no chirality on the studied ketosulfoxide according to the results emanating from polarimetric measurements. Probably deformation at the receptor site was not enough to affect the stereoselectivity of the immobilized enzyme.

The ketosulfide was recovered in 15 and 20% yields of the final reactional mixture. This mixture arose as a consequence of the Pummerer rearrangement of the ketosulfoxide, catalysed by acid sites at the spheres acquired during the step I (Fig. 1) of the immobilization procedure.

It is known that ketosulfoxides can react with acid aqueous solution affording a mixture of products such as (-ketothioesters, α-keto ortho, trithio esters and (-ketoaldehydes (Becker et al., 1963).

The Michaelis constant (K_M) and the maximum velocity (V_{max}) of the immobilized HRP on PP-G-AA was determined and compared with the same parameters for the free enzyme. Table 1 shows that the enzyme affinity for the substrate (K_M/V_{max}) remains consistent after immobilization. The same molar quantity of the free enzyme and of immobilized enzyme was used for comparisons between the chemical yield and the enzyme activity.

The relationship between the enzyme activity and

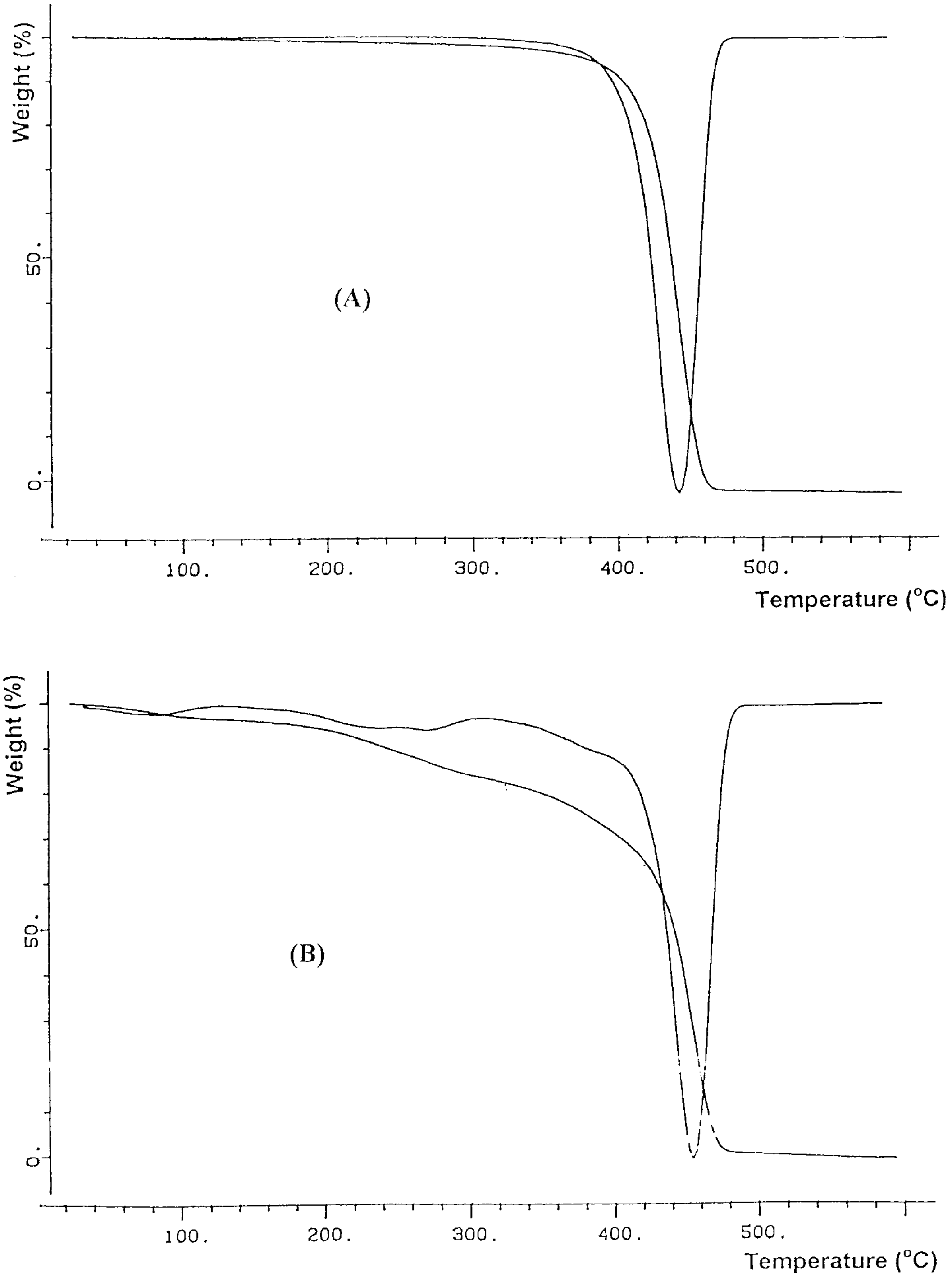

Fig. 4. Thermogravimetric analysis of (A) PP and (B) PP-G-AA, G: 70%. G(%): grafting degree.

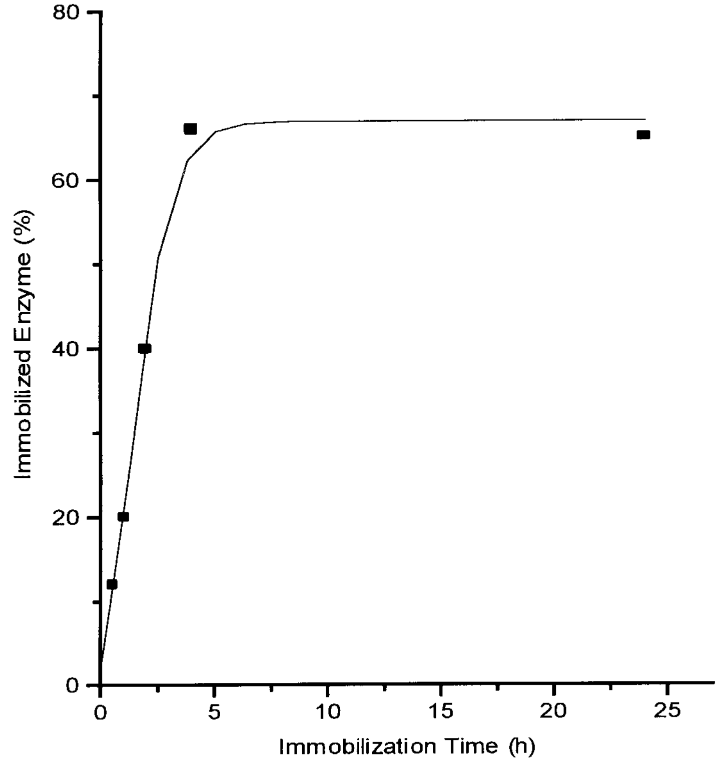

Fig. 5. Effect of reaction time on the immobilization of HRP.

the reaction yield was very close. Thus, the HRP immobilized onto PP-G-AA by covalent coupling, retained an enzyme activity of 65%.

The influence of temperature on the yields arising from the HRP catalysed oxidation of ketosulfide are reported in Table 2. It is clear that the catalytic activity of the immobilized HRP rapidly decreases above 298 K, contrary to the behaviour of the free enzyme. These changes in the temperature dependence of the catalytic activity may be due to a reduction in mobility

Table 1
Michaelis–Menten constant (K_M) and maximum velocity (V_{max}) of the immobilized HRP as compared with the free enzyme

Kinetic parameter	K_M (mol.dm^{-3})	V_{max} (mmol.min^{-1}.mg^{-1})	K_M/V_{max}
Free HRP	3.3	2.04	1.6
Immobilized HRP	2.45	2.47	1.0

Table 2
Influence of temperature on horseradish peroxidase catalysed oxidation of ketosulfide to ketosulfoxide[a]

Temperature (K)	Yield (%) (immobilized HRP)	Yield (%) (free HRP)
277	7.5	10.0
298	65.0	100.0
313	45.0	100.0

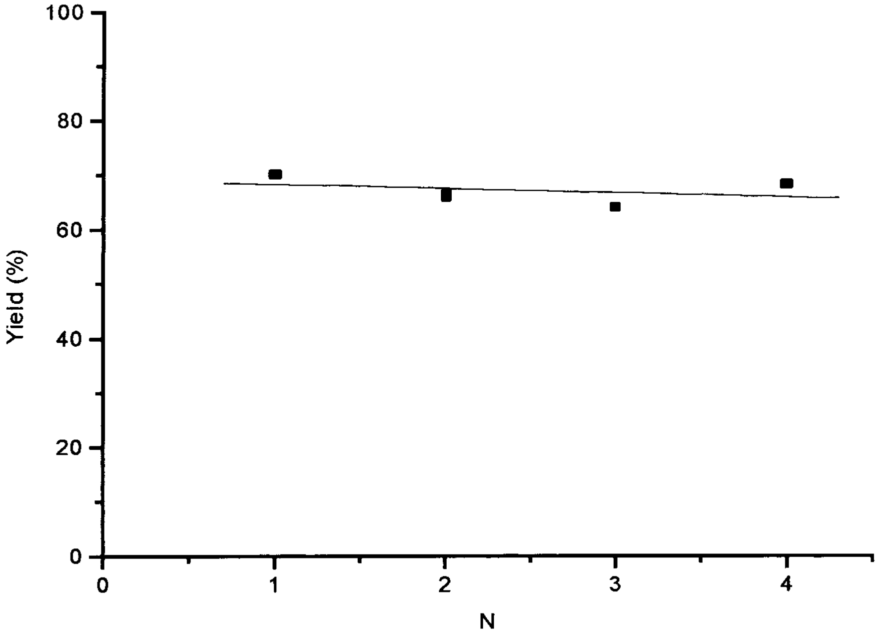

Fig. 6. Effect of repeated use (N) on the yield (%) of the sulfoxidation reactions at room temperature (25°C).

or changes in the physicochemical properties of the enzyme arising from immobilization procedure (Ulbrich et al., 1986; Ugarova et al., 1979). The thermal activation energy values ($\Delta H*$) calculated by the application of the conventional Arrhenius method (Atkins, 1987), for the free enzyme and for the immobilized enzyme were 20.8 and 16.4 kJ/mol, respectively.

The decrease of the $\Delta H*$ for immobilized peroxidase shows alteration of the tertiary structure of the insoluble enzyme (Owusu et al., 1992).

Reusability of the immobilized HRP was also investigated. The activity of the immobilized HRP after repeated use in sulfoxidation reactions is shown in Fig. 6. After being used four times, the yield of the sulfoxidation reactions is 65% for reaction times that were relatively short (1 h) at room temperature (25°C). This result indicates that the immobilized horseradish peroxidase is stable for repeated use without severe loss of activity.

4. Conclusions

The synthesized enzyme carrier, poly(acrylic acid)-graft-poly(propylene) appears to be a promising horseradish peroxidase carrier. The enzyme affinity for the substrate (K_M/V_{max}) remains after HRP immobilization. The results obtained in sulfoxidations of ketosulfides indicate that the immobilized horseradish peroxidase is relatively stable for repeated use without severe loss of activity and that the immobilized enzyme could be used for other proposes in which HRP, in a free form, is used.

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