Lactam–Amide Graft Copolymers as Novel Support for Enzyme Immobilization

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ABSTRACT: A graft random copolymer of *N*-vinyl-2-pyrrolidone and *N,N′*-dimethylacrylamide onto polypropylene was synthesized using a simultaneous gamma radiation technique from a ^{60}Co source, so that the hydrogel poly(propylene-*g*-vinylpyrrolidone-*co*-*N,N′*-dimethylacrylamide) [PP-*g*-(VP-*co*-DMAM)], thus produced by grafting, could be used as a support for enzyme immobilization. The grafted spheres showed very good swelling behavior in water due to the incorporation of hydrophilicity in the PP spheres. The influence of pH and temperature on as well as the determination of the kinetic parameters, K_M and V_{max}, for both immobilized and soluble invertase were determined. PP-*g*-(VP-*co*-DMAM) grafting onto the PP spheres caused a significant change in the water content of the support and was more pronounced for the spheres with a high degree of grafting. A porous structure of the polymeric spheres was observed by scanning electron microscopy (SEM). The porous structure contributed to the reaction rate decrease due to diffusional effects, as shown by the larger K_M value observed for immobilized invertase relative to the free enzyme. The enzyme affinity for the substrate (K_M/V_{max}) remains quite good after immobilization. The thermal stability of immobilized invertase was significatively higher than that of the free enzyme and a displacement of 20°C was observed for the immobilized enzyme. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 84: 767–777, 2002; DOI 10.1002/app.10326

Key words: graft copolymers; poly(propylene) (PP); enzymes; functionalization of polymers

INTRODUCTION

The separation and purity of products resulting from biotechnology processes are taking on greater commercial importance. For this reason, the topic of bioactive molecules immobilized on a great variety of supports with various organic or inorganic chemical compositions as well as shape is of increasing current interest.

Various methods of protein immobilization have been studied by an increasing number of authors for many years and have found widespread application in many biotechnology areas such as in clinical analysis,[1,2] therapeutic medicine,[3–8] and the obtention of biomaterials.[9–12] Immobilization of enzymes renders them recyclable, reusable, and easily separable from the products of their catalytic activity. Additional benefits of-

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ten are gained from the immobilization of enzymes or cells include reduced reaction time, side reactions, and increased resiliency to long-term storage, resistance to denaturing agents, and extremes of pH or temperature.[13]

The main problem to solve in the immobilization of biomolecules concerns the support selection. In this sense, a number of criteria such as microbial resistance, thermal stability, chemical inertia, and available functional groups on the surface of the support must be considered in the development of systems for the enzymes.[14,15] Strong interest has been generated in recent years regarding the modification or functionalization of the surface chemistry of polyolefins for the obtention of materials with modulated surface properties.[16]

Polypropylene (PP) is one of the most versatile and cost-effective polyolefins available that enjoys, today, a large range of biotechnological applications.[17–20] Technological PP production evolved into the spheripol process, where a granule of the polymer has a spherical morphology, broad molecular weight distribution, high molecular weight and stereoregularity, and products with both high rigidity and modulus. The uniform spherical morphology of the spheripol PP make it ideal for many applications in bioprocesses that involve the use of an open tubular reactor-type stirred tank or packed bed to convert the substrate into a commercially available product.[21] However, the application of PP in biotechnology fields is limited due to its low surface energy and extremely poor interaction with water.

It is well known that reactive carriers for the immobilization of enzymes should contain hydrophilic components in addition to the reactive groups.[22] This is the requirement necessary when the immobilization and the reaction with the substrate are performed in aqueous solutions.

Increased interest has been observed in the modification of hydrophobic PP to improve the hydrophilic character and functional groups that will be activated for the immobilization of biomolecules. In this sense, radiation-graft copolymerization has attracted the attention of researchers as a process to introduce carriers with active sites on the surface of hydrophobic synthetic polymers due to its ease and wide applicability.[23,24]

The advantages of the radiation-graft method are that almost every synthetic polymer may be used as a support and that a wide variety of vinyl monomers can be grafted onto the polymeric surface to obtain hydrogels: water-swellable three-dimensional polymeric networks possessing both the cohesive properties of solids and the diffusive transport properties of liquids. However, hydrogels show weak mechanical characteristics, particularly in the presence of moisture, and, hence, in considering their bioreactor applications, it is necessary to graft them onto some polymeric support such as polyolefins.

The industrial applications of the graft copolymers, both potential and actual, have expanded to include a wide variety of industries from wastewater treatments[25,26] to biotechnology processes such as the immobilization and purification of proteins and enzymes.[27–30] We have recently been interested in the grafting of hydrogels based on poly(acrylic acid) and their derivatives onto polyolefins for invertase and peroxidase immobilization.[31,32] The obtained materials with immobilized invertase have a potential market attractiveness for their use in bioreactors due to the capacity of the immobilized enzyme to produce an inverted sugar syrup, a valuable commercial product for confectionery and other food processing due to its low crystallization rate and high sweetening power. The enzyme peroxidase (HRP) was immobilized onto PP spheres by diazonium salt. The activity of the immobilized HRP in sulfoxidations of ketosulfides indicate promising results for the applications of the obtained support in organic synthesis of sulfoxides with attractive pharmacological or biological activities. *N*-Vinyl-2-pyrrolidone (VP) copolymers form the basis of a useful group of water-soluble polymers that are relatively insensitive to pH and tonicity, parameters very important in biotechnological applications.

Although numerous studies have been published about invertase immobilization, to our knowledge, no work has been undertaken about invertase immobilization onto VP copolymers to produce inverted sugar syrup from cane sugar. Inverted sugar is used widely in confectionery and other food processing and has various advantages over cane sugar such as the prevention of sugar crystallization, maintenance of softness, and pleasant taste of the finished products and the ability to make highly concentrated liquid sugar because of the higher solubility of fructose.

The aim of this work was to study the properties of invertase after its immobilization onto poly(*N*-vinyl-2-pyrrolidone-*co*-*N*,*N*′-dimethylacrylamide) (VP-*co*-DMAM) grafted onto PP spheres (spheripol). The study was done on copolymer surfaces after activation of the carboxyl groups of the

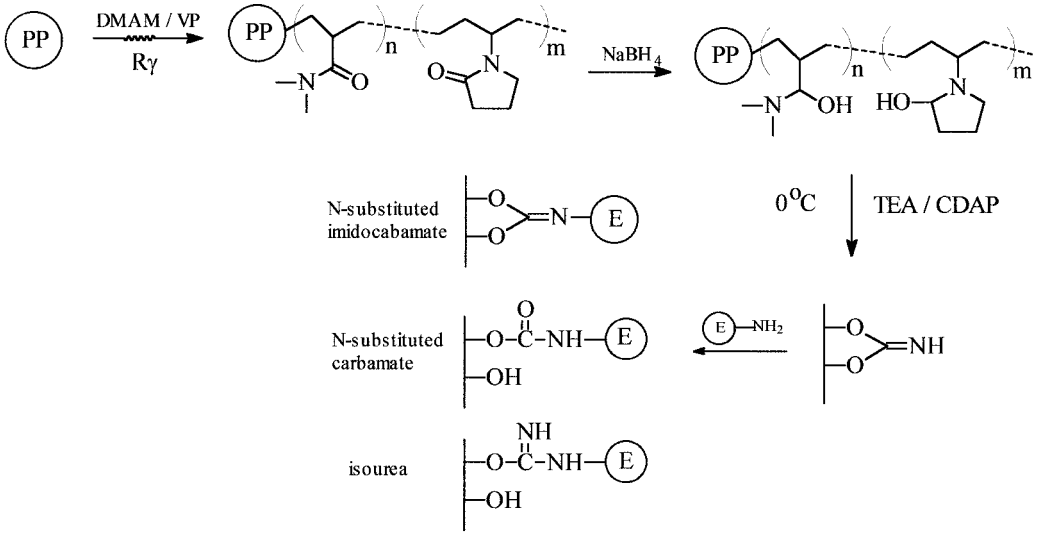


Figure 1 Schematic diagram of the invertase immobilization onto PP spheres functionalized with radiation-graft copolymers of VP-*co*-DMAM.

obtained graft copolymer according the azide method.

EXPERIMENTAL

Materials

Invertase (β-D-fructofuranoside) was purchased from Fluka and had a specific activity of 100 U/mg. PP spheres (spheripol, Poliolefinas Co), 3-mm diameter, were washed with water followed by methanol, filtered off, and dried to a constant weight. *N,N′*-dimethylacrylamide (DMAM) and VP were obtained from the Aldrich Chemical Co and were used as received. All the other reagents were commercially available products of analytical grade.

Grafting Procedure

A simultaneous grafting method was used as a technique for the preparation of [PP-*g*-(VP-*co*-DMAM)] spheres. Dry PP spheres (10.0 g) were added to a benzene/water mixture of a known (30% w/w) bulk monomer concentration (VP/DMAM 1:1 w/w). The PP spheres, monomer, and solvent were put into a glass ampule and then evacuated by a freeze–thaw cycle.

After evacuation, the ampoule was irradiated using gamma rays from a ^{60}Co source at a dose rate of 0.37 kGy/h and a total dose of 12 kGy. The grafted spheres were washed thoroughly with ethanol in a Soxhlet to extract the residual monomer and the homopolymer, poly(VP-*co*-DMAM) occluded in the spheres. The degree of grafting of PP was determined by the percentage increase in weight of the spheres.

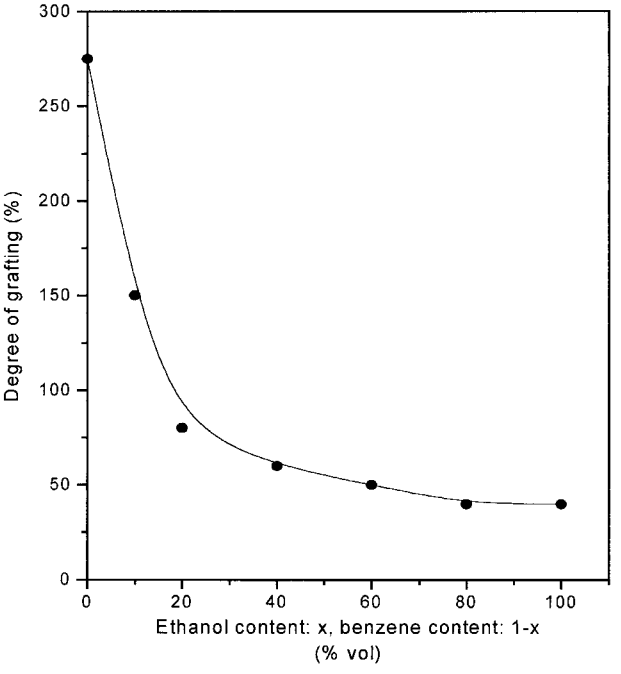


Figure 2 Effects of VP–DMAM (50:50 vol %) concentration on the degree of grafting. Dose rate: 11 Gy/h; total dose: 8 kGy; irradiation temperature: 25°C; VP/DMAM concentration: 25 vol %; solvent: 78 vol %.

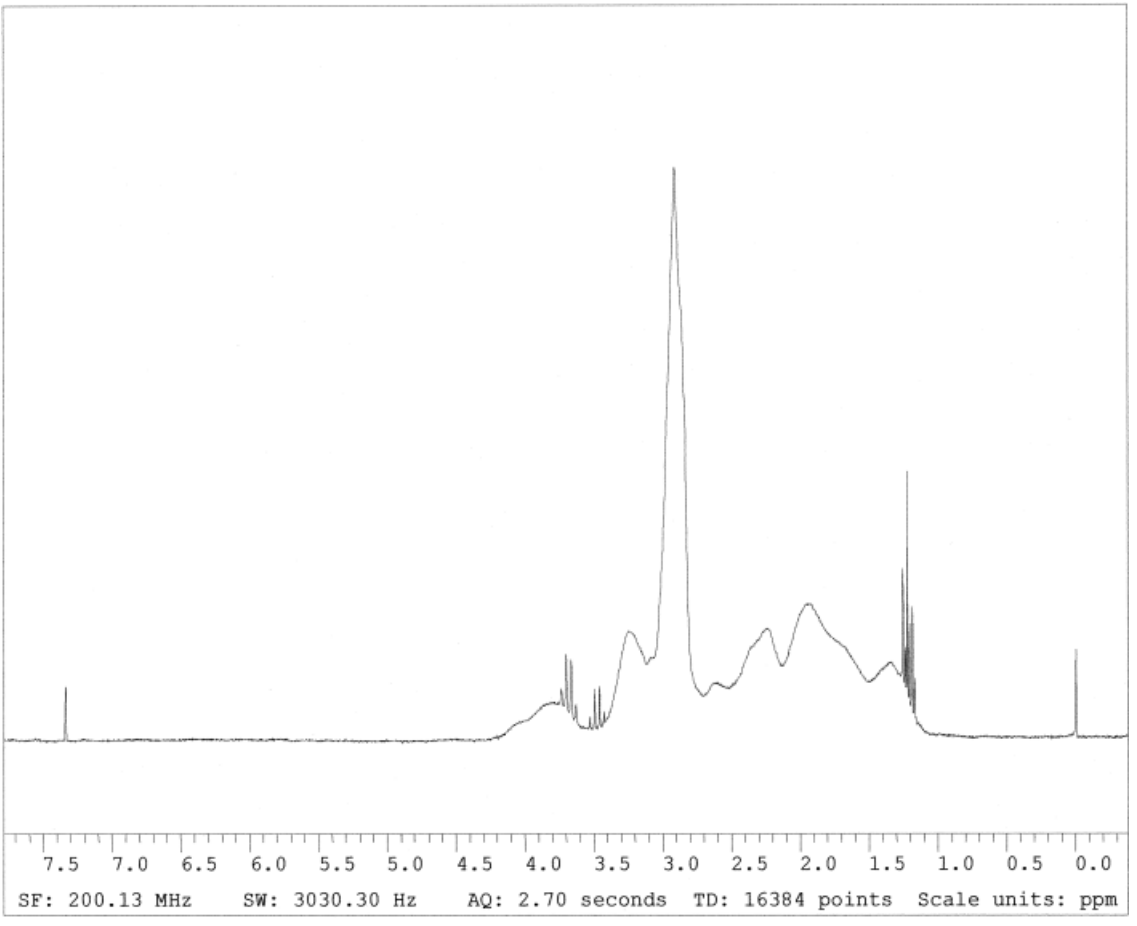


Figure 3 ^{1}H-NMR of the poly(VP-*co*-DMAM) copolymer in $CDCl_3$ at room temperature (298 K).

Polymer Characterization

NMR proton spectroscopy (300 MHz, Bruker AM 300) was used to measure the polymer compositions. ^{1}H-NMR spectroscopy was performed in a solution at 296 K to determine the exact molar compositions of VP and DMAM in the copolymer. Deuterated water (D_2O) was used as the solvent. Small samples (10 mg) of the purified polymer were dissolved in suitable amounts (0.5–1.0 mL) of the solvent and their ^{1}H-NMR spectra were recorded. From the integrated peak area, the molar contributions of the methyl protons [$>N(CH_3)_2$] in DMAM were weighed against the molar contribution of the methylene protons of VP in the copolymer to determine the precise average molar composition of the synthesized polymer. The chemical shifts were referenced from external TMS.

Electron microscopy was used to study the morphology of the grafted and ungrafted PP. Thus, PP and PP-*g*-(VP-*co*-DMAM) spheres were sputter-coated with gold and observed by scanning electron microscopy (SEM Phillips XL 30).

Support Activation and Invertase Immobilization

Activation of the hydroxylated copolymer was accomplished according to a modification of the cyanotransfer methods of Kohn.[33] A single PP-*g*-(VP-*co*-DMAM) sphere was washed with water, acetone–water (35:65), and acetone–water (60:40). The spheres were transferred to a glass bowl containing approximately 50 mL of the continuously stirred acetone–water (60:40) cooled to 0°C with an ice-water bath. Thus, 0.5 mL of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP, Sigma) was added rapidly and the system was allowed to stir for 60 s. A volume of 0.4 mL of triethylamine (TEA, Sigma) was added over a 1–2 min period and stirring was continued for 60 s. Approximately 180 mL of cold 0.05*N* HCl was added and

stirring was stopped. After 15 min, the activated spheres were removed from the reaction mixture and washed with cold water and 50 m*M* cold sodium acetate (pH 5.0). The spheres were transferred to a small glass vial containing approximately 25 mL of the invertase solution (2.0 mg/mL in 50 m*M* sodium acetate buffer, pH 5.0) for the immobilization procedure. The vial was rocked gently on an aliquot mixer at 4°C for up to 24 h. The spheres were removed from the invertase solution and washed with the sodium acetate buffer, pH 5.0. The protein content was determined using the Bradford assay procedure using bovine albumin (Sigma, fraction V powder) as the standard protein.[34] The enzyme-coupling process is shown in Figure 1.

For the assays of invertase activity, the initial rate of the sucrose hydrolysis solution (50 g/L in 0.025 mol/dm^3 sodium acetate buffer, pH 5.0) was carried out for 3–6 h at 25°C under constant stirring. For monitoring the hydrolysis, 1.0-mL samples were taken every 1 h and transferred to a polarimeter (Jasco Model DIP 370) for the measurement of the fructose contents.

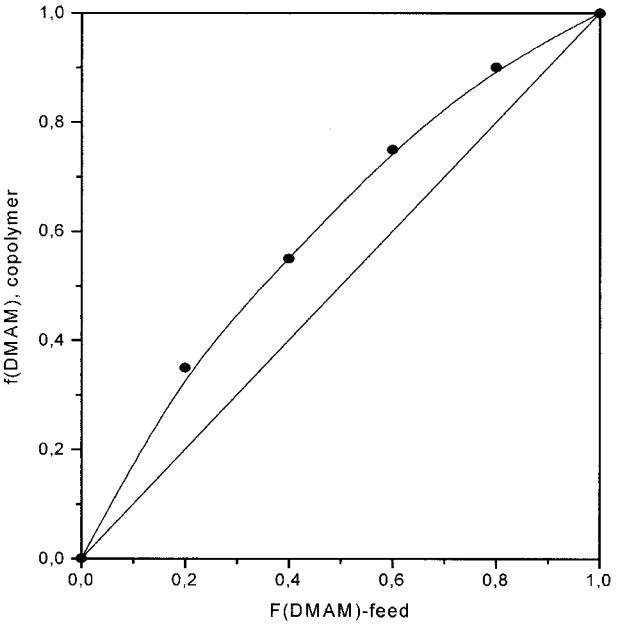


Figure 4 Variation of copolymer composition [*f*(DMAM)] with feed composition [*F*(DMAM)].

RESULTS AND DISCUSSION

In recent years, we have synthesized VP-*co*-DMAM, a nonionic water-soluble copolymer that interacts favorably with proteins without unfolding.[35] Thus, the use of PP-*g*-(VP-*co*-DMAM) in bioreactors must be much gentler and fulfill most of the basic requirements of enzymatic synthesis.

The variation of the grafting yield as a function of the solvent proportion in the grafting solution is illustrated in Figure 2. The increase of the grafting yield with the benzene content of the grafting solution is probably due to a facilitated diffusion of the monomer through the macromolecular VP–DMAM-grafted chains. It is well known that the diffusibility of the monomer diluents into the polymer matrix has a great influence on the grafting process and grafting yield.[36] Thus, the grafting process proceeds by a front mechanism in which the grafted layers initially formed on the polymeric surface can swell in the monomer–diluent mixture, resulting in more progressive diffusion of the monomer into the interior regions of the PP spheres. The selection of a VP/DMAM concentration of 25% was to facilitate the VP–DMAM copolymer extraction with water and to inhibit the crosslinking of the grafted chains that may occur at high concentrations of the VP monomer.[37]

It has been reported that the copolymerization of DMAM and VP gives a copolymer with a random arrangement.[35] This is because DMAM has an electron-donating character while VP has an electron-donating nature, which brings about a very small value of the product of monomer reactivity ratios ($r_{DMAM} \times r_{VP} = 0.015$).

The distribution of protons in the two units is an important means of distinguishing monomers, VP and DMAM, in the copolymer chain. In the VP–DMAM copolymers, the $>N(CH_3)_2$ group protons of DMAm and CH_2 and the CH group protons of VP are distinguishable.[38,39] Hence, in the present study, the distinct peaks of $>N(CH_3)_2$ protons (Fig. 3) are chosen for the estimation of the DMAM composition in the copolymer. The mol fraction of DMAM and VP were calculated by measuring the intensities of methyl protons and the total protons from the spectra of all the copolymer samples.

To ascertain the copolymer kinetic behavior, a plot of the mol fraction of DMAM in the copolymer (f_1) versus that of the feed (F_1) was drawn. The curve shown in Figure 4 indicates that the distribution of monomeric units in the graft copolymer is random, according to the observed values for monomer reactivity ratios.[35]

It is known that PP is a hydrophobic polymer in nature. The grafting of PP with the VP–DMAM copolymer should increase the water uptake of

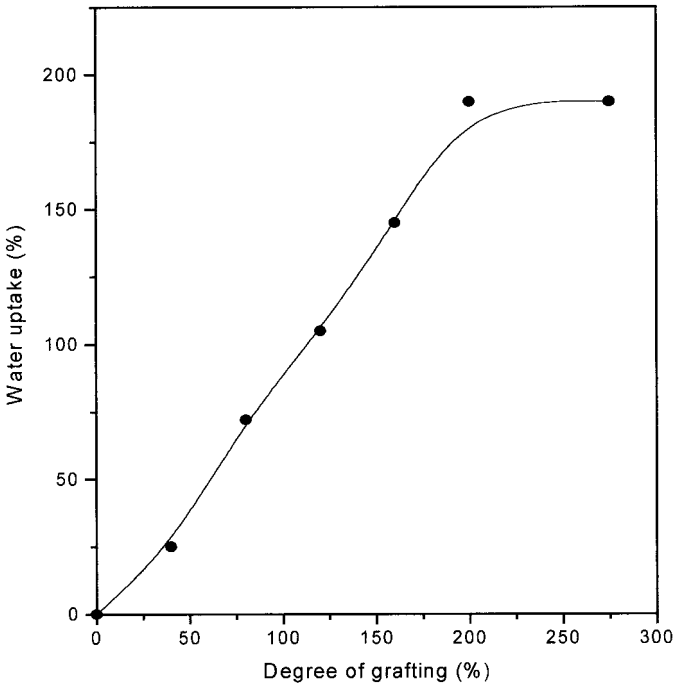


Figure 5 Percent of water uptake versus degree of grafting for PP-*g*-(VP-*co*-DMAM) at room temperature (298 K).

the grafted PP. One can, however, take this measurement as an indication of the concentration of the poly(VP-*co*-DMAM) hydrophilic parts present in the hydrophobic matrix. For instance, a plot of the graft percent against water uptake for PP grafted with poly(VP-*co*-DMAM)

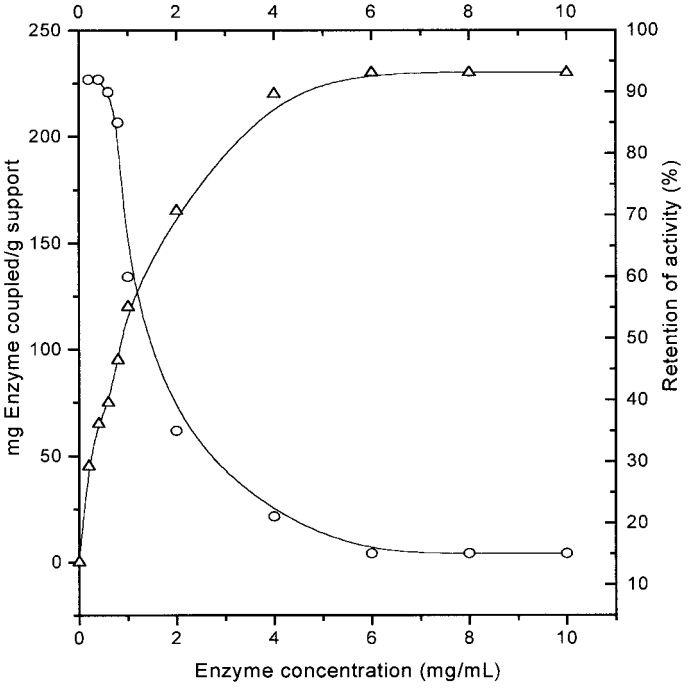


Figure 6 (△) Amount of invertase immobilized on the PP-*g*-(VP-*co*-DMAM) graft copolymer and (○) the retention of activity of the enzyme.

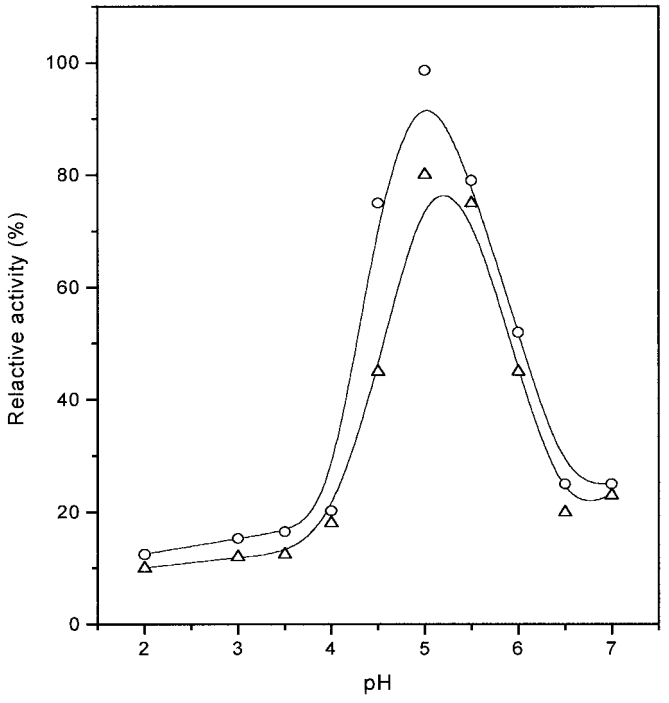


Figure 7 Effect of pH on the activity of (△) free and (○) immobilized invertase in 50 m*M* sodium acetate buffer and 2.0 *M* sucrose concentration.

at 25°C is shown in the Figure 5. It is clear from the figure that the grafted PP swells more than does the ungrafted PP. These swelling characteristics suggest that the grafted PP spheres could perform as a polymeric hydrogel for enzyme immobilization.

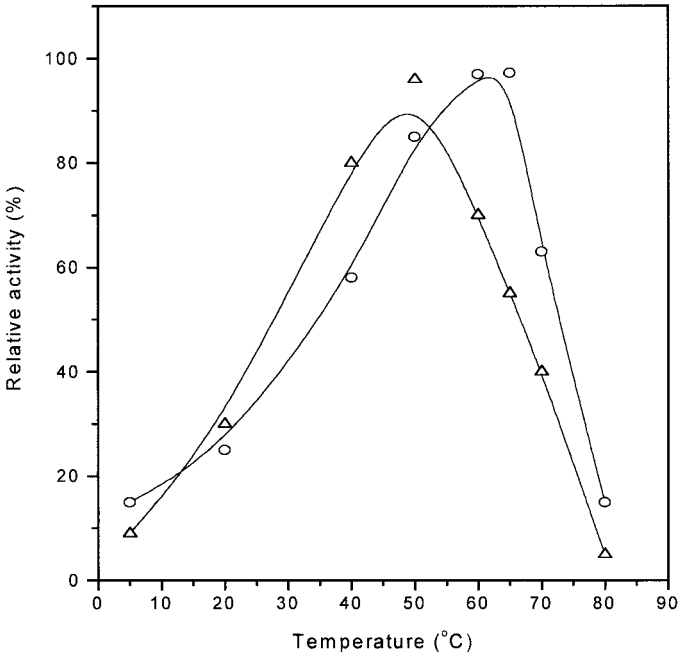


Figure 8 Effect of temperature on the activity of (△) free and (○) immobilized invertase in sodium acetate buffer (50 m*M,* pH 5.0) and 2.0*M* sucrose concentration.

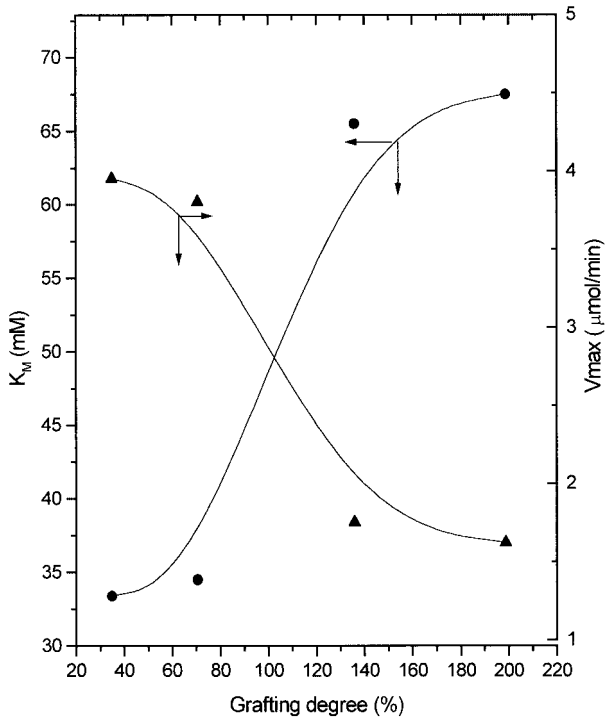


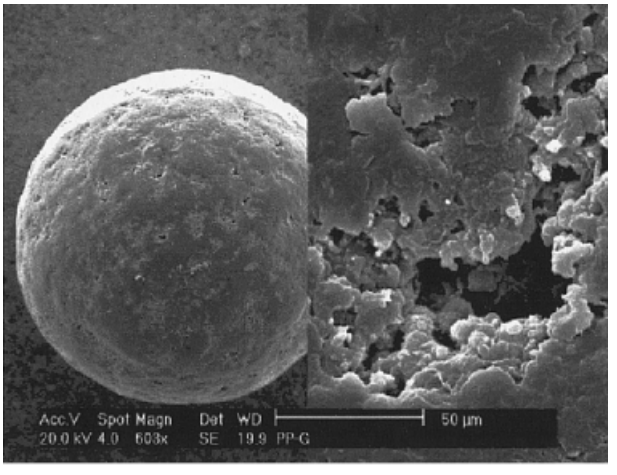
Figure 9 K_M and V_{max} kinetic parameters for immobilized invertase as function of grafting degree. K_M and V_{max} for soluble enzyme are 28.5 mM and 94.7 μmol/min, respectively.

In addition to the composition of the carrier, the amount of enzyme bound also depends on the amount of enzyme available. This dependence was investigated for the case of invertase. Figure 6 shows that the amount of the bound enzyme may reach an upper-limit value corresponding to a saturation of all assessable reactive groups with the enzyme. The yield of the bound enzyme with regard to the available enzyme was most efficient when lower amounts of the enzyme were available. If less invertase was immobilized per gram of the grafted PP spheres, the retained activity of the enzyme was bound. The retained activities were in the range of 80–90%.

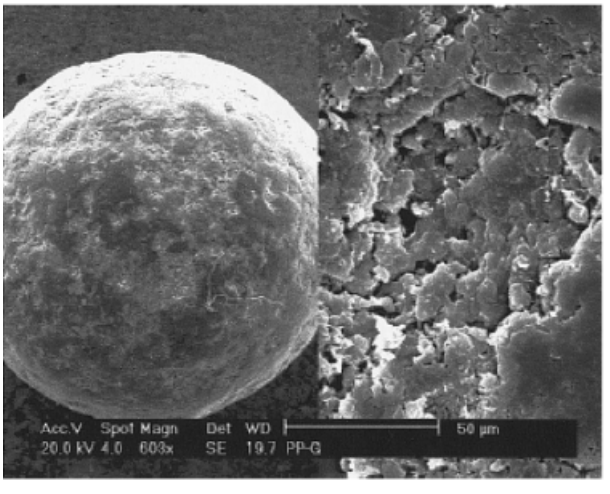
To determine the optimum pH for the enzyme, acetate and phosphate buffers were utilized in the pH range 4.2–7.5. The immobilization process did not affect the optimum pH of the enzyme. For both soluble and immobilized invertase, the optimum pH was 5.0 (Fig. 7). The maintenance of optimum pH for the free and immobilized invertase could be attributed to the fact that the concentration of charged species (hydrogen ions, for instance) in the domain of the immobilized enzyme are similar to that in the bulk solution.[31]

The effect of temperature was determined using a 2.0M sucrose solution at optimum values from 5 to 80°C. As can be seen from Figure 8, the immobilized invertase showed a high thermal stability compared to the free form. The optimum temperature for the free enzyme was 45°C, while the immobilized invertase showed a higher stability against temperature. The optimum temperature for immobilized invertase here was 65°C (Fig. 8).

Lineweaver–Burk plots of $1/V$ versus $1/C_{sucrose}$ of the immobilized invertase were drawn. The V_{max} and Michaelis–Menten constant, K_M, were calculated from the intercept and the slope of the straight line. The K_M and V_{max} for the soluble invertase are 28.5 mM and 94.7 μmol/min, re-



(A)



(B)

Figure 10 SEM micrographs of (A) ungrafted and (B) grafted PP spheres.

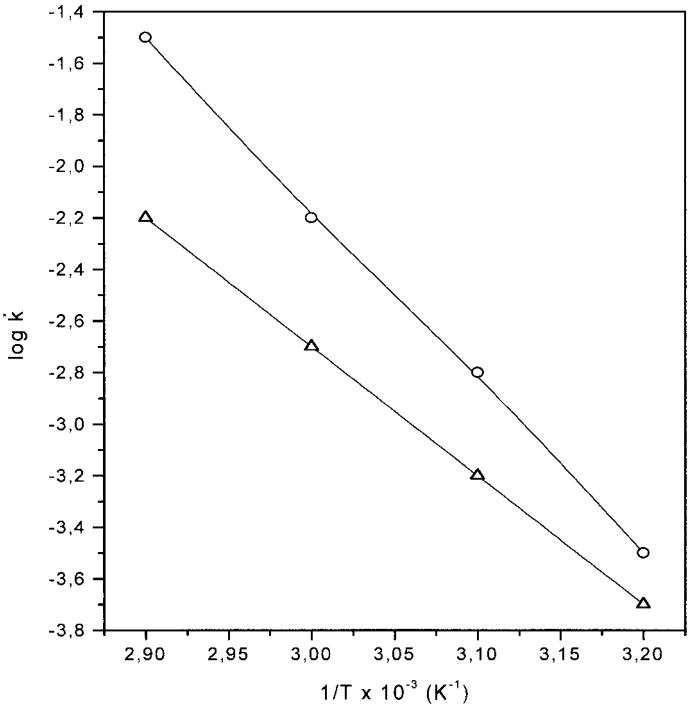


Figure 11 Arrhenius plot for the heat inactivation of (○) free and (△) immobilized invertase. Sucrose concentration: 2.0*M* in sodium acetate buffer (50 m*M,* pH 5.0).

spectively. The kinetic constants for the hydrolysis of sucrose by free and immobilized invertase for different VP-*co*-DMAM grafting degrees are given in Figure 9.

In all cases, the increase or decrease in the K_M values with the grafting degree may be attributed to diffusional effects. The formation of an enzyme–substrate complex may be more difficult with the immobilized invertase due to the porous structure of the grafted PP, which causes the substrate to pass with difficulty through the pores.

Scanning electron micrographs of PP and PP-*g*-(VP-*co*-DMAM) are given in Figure 10. Both the grafted and ungrafted PP was found to present porous structures without a significant morphological alteration after the grafting process. The presence of the porous structures on the PP graft copolymer may result in a decrease of the reaction rate due to the increased concentration of the product within the pores that may not be removed after successive measurements and, consequently, an increase in K_M.

The thermodynamic parameters for both the soluble and immobilized enzymes were determined using the Arrhenius plot, $\log k^* = -E_a/2.303 + C$ (Fig. 11) and the Eyring relation $\Delta G^* = RT \ln(k^*h/k\mathrm{T})$, where k, h, and R are the Boltzmann, Planck, and gas constants, respectively. The enthalpy (ΔH^*) and entropy (ΔS^*) of activation (Table I) were determined from $\Delta H^* = \Delta U^* - RT$ and $\Delta S^* = (\Delta H^* = \Delta G^*)/T$.[40]

The activation energies (E_a) for soluble and immobilized invertase in the temperature range of 25–70°C and substrate concentration of 50 m*M* in acetate buffer were determined and are shown in Figure 11. For the activation energies of 126.4 and 95.7 kJ/mol for the free and immobilized invertase, respectively, which are evaluated using the Arrhenius plot (Fig. 11), there is good agreement.

The observed diminution on the activation energy with the invertase immobilization probably

Table I Activation Parameters for the Irreversible Heat Inactivation of Free and Immobilized Invertase

System	ΔG^* (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* (kJ/mol)
Free invertase	−93.8	150.7	0.359
Immobilized invertase	−97.1	127.8	0.203

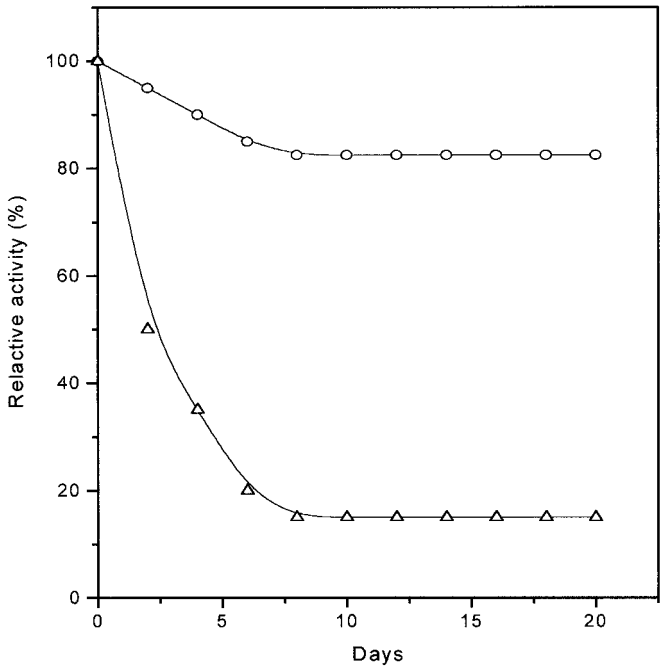


Figure 12 Storage stability of (△) soluble and (○) immobilized invertase at 4°C in sodium acetate buffer (pH 5.0; 50 m*M*).

may be due to a minor dependence of the rate constant of the hydrolysis sucrose to the temperature. Activation parameters for the heat inactivation of immobilized invertase are summarized in Table I. Kinetic stability indices (ΔG^*, ΔH^*, ΔS^*) will be expected to be indicative of enzyme conformational stability with respect to some irreversible inactivation processes.[41]

Significant changes of the ΔG^* values, the apparent Gibbs free energy for irreversible inactivation of enzymes, after invertase immobilization were not observed (Table I). Because ΔG^* values are often considered a poor measure of enzyme tertiary structure stability, other kinetic stability indices, for example, ΔH^* and ΔS^*, are likely to be a function for enzyme unfolding.[42]

The diminution of the ΔS^* values (Table I) for immobilized invertase (0.203 kJ/mol) is probably due to the more ordered protein unfolding compared with the free invertase (0.359 kJ/mol). The decrease of the ΔH^* value for immobilized invertase (127.8 kJ/mol) relative to the free enzyme (150.7 kJ/mol) (Table I) may be due to an alteration of its tertiary structure of the insoluble enzyme.

Important parameters when considering immobilized enzymes are the lifetime, durability, and storage stability of the system. The PP spheres containing the immobilized invertase were kept at 4°C in an acetate buffer (50 m*M*, pH 5) when not in use. The results in Figure 12 show that 85% of the initial activity was maintained for the immobilized system, whereas the soluble system retained 20% of the initial activity within the same period.

CONCLUSIONS

This study showed that a nonionic water-soluble polymer VP-*co*-DMAM can be used successfully, after grafting onto PP, for the immobilization of invertase. The immobilization of invertase onto PP-*g*-(VP-*co*-DMAM) matrices provides some advantages such as ease of incorporation of the invertase onto the graft copolymers and fast response to a substrate. The excellent mechanical strength of polymers with immobilized enzyme facilitates the use of the support in bioreactors.

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