Influence of Polyvinylpyrrolidone on the Hydrophobic Properties of Partially Porous Poly(Styrene–Divinylbenzene) Particles for Biological Applications

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ABSTRACT: For the preparation of polymer particles by free radical, stabilizers are usually present in the reaction mixture to maintain the separation of particles from each other. Polyvinylpyrrolidone (PVP) is one the hydrophilic polymers that has been extensively used as the stabilizer in the polymerization system employing a polar solvent or solvent mixture as the continuous phase. As shown in the present study, the presence of PVP in the later steps could significantly influence the hydrophobic property of partially porous poly(styrene–divinylbenzene) (PS–DVB) particles, prepared by the method of multistep swelling and polymerization involving the use of polymeric porogens. The association of PVP molecules on the particle surface reduced the hydrophobicity and consequently the capability of particles for biological applications. For the reversed-phase liquid chromatography of penicillin G and its enzymatic hydrolysis product, particles prepared without PVP led to an enhancement in both retention and resolution, compared with particles resulting from the use of PVP. Results from lipase immobilization on these particles also showed that the presence of PVP could shield the hydrophobic groups on the particle surface and then reduce the efficiency of enzyme immobilization. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 87: 1818–1824, 2003

Key words: polyvinylpyrrolidone; poly(styrene–divinylbenzene); hydrophobicity; chromatography; enzyme

INTRODUCTION

Porous and crosslinked poly(styrene–divinylbenzene) (PS–DVB) particles have been widely used as stationary phases for size exclusion and ion-exchange chromatography and catalytic supports. Studies on biological applications, for example, protein chromatography and enzyme immobilization, have also been well published. We previously reported a preparation of partially porous PS–DVB particles in the micron-sized range by the method of multistep swelling and polymerization involving the use of polymeric porogens.[1] According to this method, PS seeds were first prepared by dispersion polymerization, expanded in particle size by absorbing styrene and oil-soluble initiator, and then polymerized to form polymeric porogen particles. The newly synthesized PS chains served as the porogens of the PS–DVB particles, resulting from the copolymerization of styrene and divinylbenzene in the swollen polymeric porogen particles. This method is generally derived from the method of activated multistep swelling and polymerization originally proposed by Ugelstad and co-workers.[2] The multiple-step swelling and polymerization method involving the use of polymeric porogens has been employed by Wang et al.[3,4] to prepare monodispersed macroporous PS–DVB beads. However, their procedure involves the use of smaller PS seeds prepared by the emulsifier-free emulsion polymerization method. Conventional methods for preparing porous PS–DVB also include the method of suspension polymerization.[5] In these methods, the copolymerization of styrene and DVB in so-called oil-in-water type suspension usually involves the use of hydrophilic polymer, such as polyvinylpyrrolidone (PVP) or poly(vinyl alcohol–vinyl acetate), as the stabilizer. Water-soluble natural polymers like gums and cellulose ethers have also been used as the stabilizer.[6]

The stabilizer is certainly needed in the dispersion polymerization to yield microsized polymer particles. In the absence of any stabilizer, particle dispersions are not sufficiently stable and may coagulate during their formation.[6] For the preparation of polystyrene by dispersion polymerization in alcohols and other polar solvents, a wide range of polar organic polymers such as PVP, poly(vinyl alcohol), poly(acrylic acid), and cellulose derivatives have been used as the stabilizers.[7–13] The stabilizer used for dispersion polymeriza-

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tion is usually referred to as the steric stabilizer. In the process of multistep swelling and polymerization for the preparation of PS–DVB particles, polymer-based stabilizers were also used. After the polymer particles are swollen by absorption of monomers (styrene and DVB), porogen solvent, and initiator, they are suspended in an aqueous solution containing either polyvinylalcohol[14–16] or PVP[17] as the stabilizer and allowed for an additional polymerization to yield porous PS–DVB. The stabilizer polyvinylalcohol was also used in the step for preparing polymeric porogen particles that yielded porous PS–DVB.[3,4]

The present article describes the influence of PVP on the synthesis and characteristic of partially porous PS–DVB particles. In comparison with totally porous and nonporous particles, the porous structure with large pores near the periphery of the particles will be much more useful and effective for biological applications. Two typical biological applications of PS–DVB, reversed-phase chromatography of biomolecules (antibiotics) and enzyme immobilization are taken as the model systems. The usefulness of particle hydrophobicity is also demonstrated with these applications.

β-Lactam antibiotics have been well analyzed and characterized by reversed-phase high performance liquid chromatography (HPLC) and their reversed-phase chromatographic data are the basic hydrophobicity measures.[18] Therefore, one of the most important β-lactam antibiotics penicillin G and its hydrolyzed product 6-aminopenicillanic acid (6-APA) were taken as the model components for reversed-phase chromatography using the prepared PS–DVB particles as the stationary phase. The hydrophobicity values for 6-APA and penicillin G are determined to be -0.8 and 0.451, respectively. For the study of enzyme immobilization, PS–DVB is employed as the alternative hydrophobic support for lipase immobilization by physical adsorption. Lipase (EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis/synthesis of a wide range of soluble or insoluble carboxylic acid esters and amides. The industrial application of lipase-catalyzed reactions includes the hydrolysis of oils and fats, synthesis of fatty acid esters, and production of intermediates for drug synthesis. Immobilization of lipase on hydrophobic supports by physical adsorption is a very promising method. In addition to the derived support from natural polymer, i.e., octyl-agarose,[19] the hydrophobic polymers that have been used as the supports of lipase immobilization include polypropylene (PP) and polyethylene (PE).[20–24]

The significance of the present article is to discover that the hydrophobic properties of the polymer particles could determine their successes in application. The presence of PVP was found to hurt to some extent the hydrophobic property of porous PS–DVB particles, prepared by the method of multistep swelling and polymerization. The disuse of PVP at the porogen preparation and copolymerization steps did not significantly change the size and porous properties of the resultant particles. The present paper proposes two valuable applications for examining the hydrophobicity of the polymer particles. The experimental results suggest that the control of surface properties is critical in the design of porous materials for biological applications.

MATERIALS AND METHODS

Materials

Styrene, 2,2′-azobisisobutrionitrile (AIBN), benzoyl peroxide (BPO), sodium dodecyl sulfate (SDS), sodium nitrite, cetyl alcohol, ethanol, and all other organic solvents were of reagent grade or higher. Divinylbenzene (80%) was purchased from Fluka and used directly without purification. Polyvinylpyrrolidone (PVP) (K-30) was obtained from BASF. Penicillin G, 6-APA, olive oil emulsion, and lipase from *Candida rugosa* (lyophilized powders containing approximately 20% protein) were obtained from Sigma. Polypropylene (PP) powders (MP1000; particle size, 250–425 μm; pore size, 50–500 nm) were obtained from Akzo (German).

Preparation of PS–DVB particles

A multistep swelling and polymerization method involving the use of a polymeric porogen in the copolymerization of styrene and divinylbenzene was employed, as previously described.[1] To describe the process briefly: monodispersed polystyrene seed particles were first prepared by dispersion polymerization of styrene (10 mL) in ethanol (80 mL), using AIBN (0.091 g) as the initiator, PVP (1.3 g) as the stabilizer, and cetyl alcohol (0.33 g) as the costabilizer. After nitrogen purging, the reaction mixture was shaken (130 rpm) in a water bath of 60°C for 24 h. The resultant seed particles were swollen with a monomer mixture at room temperature and then polymerized in a shaker at 70°C for 24 h, using BPO as the initiator. To the 1 g of PS seeds, the monomer mixture contained styrene (4 or 5 mL), SDS (0.25 g), $NaNO_2$ (0.01 g), BPO (0.25 g or 5%, w/v with respect to styrene), PVP (zero or 1 g), and water (100 mL). When the temperature increased to 70°C, free radicals generated from BPO attacked the styrene molecules and linear PS chains were produced. The particles containing these newly synthesized PS chains are thus called polymeric porogen particles. In the final step, styrene and DVB were both absorbed into polymeric porogen particles, and then copolymerized within the enlarged particles. A solution containing styrene (5 mL), DVB (1 mL), BPO (0.06 g), SDS (0.25 g), $NaNO_2$ (0.01 g), 100 mL water, and 5 mL toluene was prepared, and then mixed with poly-

meric porogen particles (1 g). After swelling for 24 h at room temperature, the copolymerization was carried out in a shaker at 70°C for 24 h. For comparison, some recipes had a certain amount of PVP present in the mixture. The resultant copolymerized particles were washed with methanol and water, and dried in a vacuum oven at room temperature for 12 h. To yield porous particles, the dried particles were extracted with toluene for 24 h using Soxhlet apparatus, and then washed with methanol and water. The specific surface area, pore volume, and pore diameter of the particles were determined with an ASAP 2000 instrument (Micromeritics Instruments), and calculated from the adsorption/desorption isotherm of nitrogen using the Brunauer-Emmett-Teller (BET) equation.

Chromatography of penicillins

The porous PS–DVB particles prepared either in the absence or presence of PVP at later steps were slurry packed into 15 cm × 4.6 mm i.d. stainless-steel columns. Liquid chromatography of penicillin G and its enzymatic hydrolysis product, 6-APA, was carried out by the mode of reversed phase. The mobile phase consisted of a ratio of 0.075*M* sodium phosphate buffer (pH 5) to acetonitrile 8.5 to 1.5, respectively. The chromatographic conditions were as follows: flow rate, 1 mL/min; temperature, 50°C; detector, UV detector with a wavelength of 220 nm.

Lipase immobilization on PS–DVB

PS–DVB particles (20 mg) were wetted with 60 μL of ethanol and then placed on a plate that was covered with 0.1 mL of phosphate buffer (0.1*M*, pH 7.0), containing 5–20 mg lipase. Enzyme was immobilized after removal of ethanol in a vacuum oven at room temperature. The enzymatic activity of the immobilized enzyme was determined using olive oil emulsion as the substrate. Incubation of the substrate with enzyme was at 37°C for 20 min. One unit of lipase activity (U) was defined as the amount of enzyme catalyzing the production of 1 μmol free fatty acid per minute. For a comparison, lipase was also immobilized on the PP powders by following the same procedure.

RESULTS AND DISCUSSION

Evidence of short polymer chains as the porogens

In the production of porogen-containing particles, the polymerization of absorbed styrene in the swollen particles was an oil-in-water type, seeded suspension polymerization. In the polar solution (water), the suspended nonpolar phase consisted of all PS seed particles absorbing both monomer (styrene) and initiator

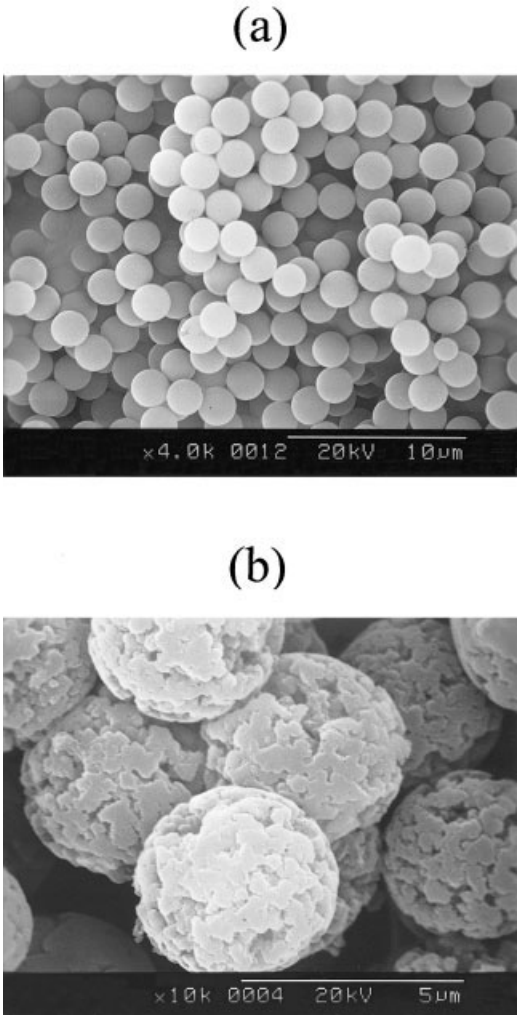

Figure 1 Scanning electron micrographs (4000× and 10,000× magnifications) of (a) the polymeric porogen particles and (b) the partially porous PS–DVB, prepared in the absence of PVP.

(BPO). PVP, SDS, and $NaNO_2$ were used to stabilize the suspended particles. As the temperature increased to 70°C, free radicals were produced from BPO and then the adsorbed styrene polymerized. The resultant compact polymer beads are known as polymeric porogen particles. Based on the measurement using gel permeation chromatography (GPC), the average molecular weight of the polymeric porogen particles, though larger in the particle size, was determined to be much smaller than that of PS seeds. This suggests that the newly produced PS chains in the porogen particles were much more shorter in comparison with the original PS chains in the seeds and susceptible to be dissolved in toluene. These newly produced PS chains were coagulated by van der Waals forces and stayed mainly on the outer layer of the particles. Figure 1(a) shows the scanning electron micrograph (SEM) of the polymeric porogen particles prepared without PVP by the two-phase seeded suspension po-

TABLE I
Specific Surface Area and Pore Diameter of PS–DVB Particles Prepared with Different Polymeric Porogen Preparations and in the Presence or Absence of PVP

PVP present in the copolymerization step (g)[a]	Styrene used in the preparation of porogen (ml)[b]	Specific surface area of PS–DVB (m^2/g)	Pore diameter of PS–DVB (nm)
0	4	17.41	13.7
0	4	16.27	13.8
0	4	16.08	12.2
0	4	13.09	13.2
0	5	18.85	13.6
0	5	22.46	14.5
0	5	20.21	10.9
0.25	5	17.08	13.0
0.5	5	19.08	12.5
0.75	5	13.62	12.2
1.0	5	24.72	11.2
1.0	4	13.28	15.5

[a] Based on per unit gram of polymeric porogen particles used.
[b] Based on one gram of PS seeds used.

lymerization. These particles have a narrow dispersion in particle size, which are very similar to the particles prepared with 1 g of PVP.[1]

The presence of PVP did not influence the growth of polymer particles in the two-phase suspension polymerization. No significant difference in size was observed between particles copolymerized with or without the presence of PVP. Similar results were reported for the preparation of nonporous PS–DVB particles using polyvinylalcohol as the stabilizer.[25] Although the particle size was independent of the use of a stabilizer, it increased with the amount of styrene for the preparation of polymeric porogen particles. The amount of styrene increased from 4 to 5 mL, for example, the average particle diameter increased from 2.1 to 2.2 μm. The weight average molecular weight (M_w) of the polymeric porogen particles decreased with the amount of styrene used in the second step, since the newly produced PS chains were shorter. These shorter PS chains thus served as the porogen that was extracted out with toluene using Soxhlet apparatus after the copolymerization in the third step to form porous PS–DVB particles.

In the second step, the polymerization was a two-phase system, in which surface reagents, such as SDS, stabilized the oil phase that included every swollen polymer particles. Although PVP is necessary in the preparation of PS seeds by the dispersion polymerization, it may not be required in this step. Actually, PVP slightly influenced only the weight average molecular weight of the PS chains in porogen particles. The average molecular weights for polymeric porogen particles prepared with and without 1 g PVP (both shaking at 130 rpm) were determined to be 74,000 and 86,000, respectively. These values, however, are much smaller than the average molecular weight of PS seeds, 180,000.

Influence of PVP on the preparation of porous PS–DVB

The final copolymerization step was also a two-phase suspension polymerization like the preparation of polymeric porogen particles, except that two additional reagents, DVB and toluene, were in the oil phase, which consisted of all polymeric porogen particles. Styrene and DVB were polymerized within the enlarged porogen particles that were swollen by absorption of monomers and oil-soluble initiator. The added toluene dissolved the PS chains in the oil phase, which were mainly produced in the outer layer of polymeric porogen particles. Thus, the formation of PS–DVB structure was mostly in the outer layer where the linear polymeric chains were dissolved in toluene. Porous particles were formed after the polymeric porogen and toluene were removed in the later extraction and washing steps. Using a toluene volume of 5 mL based on per unit gram of polymeric porogen particles (with a particle size of 2.2 μm) and in the absence of PVP, the averaging surface area of PS–DVB particles was 20.5 m^2/g (with a standard deviation of 1.8 m^2/g from 3 experimental runs) and average pore diameter was determined to be 13 nm (see Table I). At the same conditions but with the presence of PVP (from 0.25 to 1 g), the specific surface area was scattered in the range from 13.6 to 24.7 m^2/g with an average of 18.6 m^2/g. These results suggest that the porous property of PS–DVB particles was not significantly influenced by the presence of PVP in the steps for the preparation of polymeric porogen particles and final porous particles. Neither was the particle diameter of PS–DVB particles.

In the final step of preparing porous particles, eliminating the use of PVP could only marginally increase the pore size of the resultant PS–DVB particles. The

particles could be very frangible since many big holes were present on the particle surface as shown in Figure 1(b). Although it was not evident that the pore property was changed by the presence of PVP, the swelling temperature at this copolymerization step could influence the porosity of the resultant PS–DVB particles. The specific surface area was significantly reduced if the swelling temperature was decreased from 30 to 25°C, whether PVP was present or not. In the absence of PVP, the roughness of the particle surface was exhibited, and the porous structure was even present before the solvent extraction. This suggests that PVP could protect to some extent the dissolution of PS chains in the outer layer of particles by toluene and then prevent a portion of the outer layer from eroding away.

The use of PVP in the step of preparing polymeric porogen particles did not change the fact that the yielded porogen particles were nonporous. However, the molecular weight of the newly produced PS chains could significantly influence the porosity of the final PS–DVB particles. As shown in Table I, when 4 mL of styrene were used instead of 5 mL in the step of preparing polymeric porogen, the average specific surface area of the resultant PS–DVB decreased from 20.5 to 15.7 m^2/g (with a standard deviation of 1.8 m^2/g from 4 experimental runs) due to the smaller amount of shorter PS chains produced. However, the averaging pore diameter remained at 13.2 nm. These results were obtained in the absence of PVP in the final copolymerization step. Similar results could be obtained in the presence of PVP. In the preparation of larger PS–DVB beads (7.4 μm) starting from smaller PS seeds (1.0 μm), prepared by the emulsifier-free emulsion polymerization method, Wang et al.[3] found that increasing the proportion of polymeric porogen decreased the specific surface area of porous particles. When 40–60% polymeric porogens are used in the copolymerization step, the specific surface area of the resultant PS–DVB particles can approach 50–57 m^2/g. But when volume percentage of polymeric porogen increases to 80–100%, the specific surface area decreases to around 17–18 m^2/g. The latter is comparable to that obtained in the present work. However, their particles have a higher pore diameter. The median pore diameter ranges from 15 to 48 nm when 20–100% of polymeric porogen are used. These differences between two works may be due to the differences in solvent use and molecular size of polymeric porogen. They used dibutyl phthalate as the solvent and the molecular weight of polymeric porogen was not known.[3]

For the preparation of PS seeds, dispersion polymerizations were carried out in a polar solvent (methanol) and PVP was used as the steric stabilizer. If PVP were also present in the preparation of PS–DVB particles using the two-phase suspension system, the surface of resultant polymer particles would be anchored with PVP. According to the particle formation mechanism proposed by Tseng et al.,[10] PVP molecules were adsorbed by the aggregates of growing polymer chains and finally anchored on the mature particles, in order to stabilize the dispersion of hydrophobic particles in the polar medium. The anchored PVP resulting from possible adsorbing or grafting could not be washed out; the hydrophobicity of the particle surface was thus probably reduced by the presence of this hydrophilic PVP. However, according to a previous study,[26] these anchored PVP could partially release from the particle during the process of chemical modification on the particle surface.

Particles hydrophobicity by means of reversed-phase HPLC

One of the advantages of using multistep swelling and polymerization is that the resulting porous particles are uniformly sized with the diameter in the micron range. Crosslinked PS–DVB particles prepared in the present work are of micron size (4–5 μm) and can be used ideally as the stationary phase of HPLC. Reversed-phase chromatography is a separation method based on the difference of solute distributions in mobile and stationary phases. The latter is always more hydrophobic (i.e., less polar) than the former. Porous PS–DVB particles have been commonly used for reversed-phase chromatography without being derived to introduce C_{18} or other hydrophobic groups, since the aromatic benzene rings in the backbone of polymer chains are hydrophobic. However, PVP anchoring on the particles was found to decrease the hydrophobicity of the particle surface significantly. When the particles were used as the chromatographic support, the nonpolar property of the stationary phase decreased due to the shielding of the aromatic rings by the hydrophilic PVP. As a result, the retention of the solute in the reversed-phase chromatographic column decreased.

Figure 2 shows the separation of a mixture of penicillin G and 6-APA by reversed-phase HPLC using the prepared porous PS–DVB particles as the stationary phase. Two columns of the same geometric size were respectively packed with PS–DVB particles, prepared in both the absence and presence of PVP. Particles from these two different preparations had the same specific surface areas, and columns were operated at the same conditions for comparison. As expected, the more hydrophobic compound penicillin G came out from the column later than 6-APA, since the former was more likely to stay in the hydrophobic stationary phase. As shown in Figure 2, the absence of PVP in the preparation of PS–DVB resulted in the increase of the retentions of penicillin G and 6-APA. Also, penicillin G and 6-APA were well separated

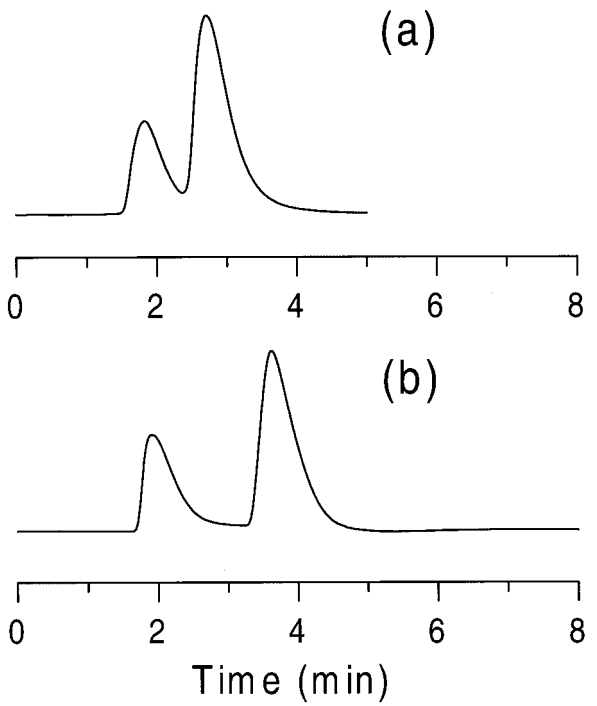

Figure 2 Chromatograph of penicillin G and 6-APA using porous PS–DVB prepared (a) in the presence or (b) in the absence of PVP. Concentrations of both penicillin G and 6-APA in the mixture are 0.5 g/L.

according to their respective hydrophobicities. The increase in retention time for penicillin G was much more significant, resulting in an enhanced resolution of penicillin G and 6-APA. These increases in retention and resolution were attributed to the increase in the hydrophobicity of porous polymer particles when PVP was deleted from use.

Particles hydrophobicity by means of lipase immobilization

Adsorption of lipase on highly hydrophobic supports was proved to be simple and effective. Commercial polymer PP and PE were frequently used as the base materials for immobilization because of their hydrophobic property. According to a previous study,[19] lipases recognize highly hydrophobic groups on the support, at a molecular level, and they become adsorbed through the external areas of the large hydrophobic active centers. The term *open and hyperactivated structure* was used to describe lipase molecules at that adsorption state. Thus, the enzymatic activity of immobilized lipase on the hydrophobic support by adsorption was found much higher than that of soluble lipase even after interfacial activation or in organic medium.[24] The immobilized lipase activity on the prepared particles then could be an indication for the hydrophobic property of particle surface.

As shown in Figure 3, the enzymatic activity of immobilized lipase increased with the amount of lipase in the solution incubated with the particles for immobilization. However, the immobilized activity based on per unit mass of particles could not be increased further at that about 20 mg of enzymes were applied to each type of particles (20 mg). Immobilized lipase on porous PS–DVB prepared without PVP had much higher activity than that on the particles prepared with PVP in the steps for preparing polymeric porogen and final copolymerization. The influence of PVP on the hydrophobicity of the particle surface for lipase immobilization was thus significant. PP powders had the highest specific activity (600 U/g), compared with those on PS–DVB prepared in the absence of PVP (about 350 U/g) and in the presence of PVP. This was because the PP powders had a higher specific surface area (36.2 m^2/g). In term of activity per unit surface area of the particle supports, however, PS–DVB particles prepared without PVP achieved the same immobilized enzyme activity (17 U/m^2) as PP powders did. In contrast to these two supports, the maximum activity of immobilized lipase on particles prepared with PVP was 6 U/m^2 only. The results suggest that if the hydrophobic benzene rings on the porous PS–DVB particles were not hindered by PVP, the polymer was as hydrophobic as PP for lipase immobilization. The enzymatic activity of immobilized lipase on PP obtained in the present work (600 U/g) is comparable to that (725.8 U/g using olive oil as the substrate) of lipase adsorbed on a similar PP powder Accurel EP-100 (particle size 200–400 μm) prewetted with ethanol.[22]

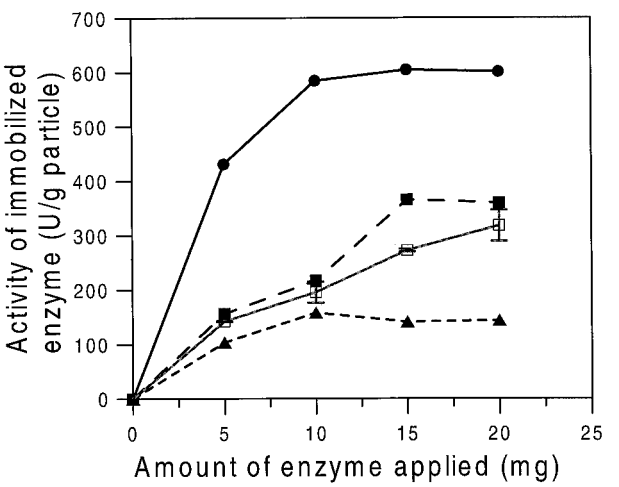

Figure 3 Influence of the amount of applied enzyme on the activity of immobilized enzyme on PP (●) and porous PS–DVB, prepared in the presence (▲) or in the absence of PVP (■, □). For the preparation of porous PS–DVB in the absence of PVP in the later two steps, the polymeric porogen particles were in the size of 2.1 (■) or 2.2 μm (□).

CONCLUSION

Porous PS–DVB particles have a wide range of applications in several biotechnological fields. The multistep swelling and polymerization method is a promising approach to prepare partially porous polymer particles in micron size with a narrow distribution of particle diameter. PVP was used as the stabilizer in the polymerization system employing a polar solvent or aqueous solution as the continuous phase. Results in the present work suggest that the hydrophilic PVP could associate with the resultant polymer particles. Although the mechanism of PVP anchoring on the particle surface needs to be further investigated, the present article demonstrates that anchored PVP on the particle surface could significantly reduce the hydrophobicity of the particles. As shown in the examples of reversed phase chromatography and lipase immobilization, the decrease in hydrophobicity resulted in the reduction of the particle's usefulness. Avoiding the use of PVP at the steps for preparing porogen particles and following copolymerization of styrene and divinylbenzene could maintain the hydrophobicity of PS–DVB particles but without affecting their porosity properties. The present paper suggests that the hydrophobic properties of a polymer material could significantly affect its biological application and should be carefully controlled during the preparation process. Results obtained in the present work provide reasonable explanations and valuable information in designing the partially porous PS–DVB particles.

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