A Low, Particle-Sized, Nonporous Support for Enzyme Immobilization: Uniform Poly(glycidyl methacrylate) Latex Particles

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ABSTRACT: The uniform and nonporous poly(glycidyl methacrylate) (poly(GMA)) latex particles, 1.7 μm in size, were first tried as a support in enzyme immobilization. For this purpose, α-chymotrypsin (CT) was selected as the model enzyme. The low particle size and nonporous character of the selected support allowed to carry out the enzyme–subtrate interaction on a sufficiently large surface area (3.36 m^2/g) and in the absence of intraparticular diffusion limitations. This property is particularly important when the immobilized CT is used for the substrates with high molecular weights (i.e., proteins). The latex particles were synthesized by dispersion polymerization of GMA. The reactive character of poly(GMA) allowed the direct attachment of primary amine groups onto the particles. Confocal laser scanning microscopy (CLSM) showed that primary amine groups were preferentially located on the particle's surface. Hence, the selected enzyme, CT was immobilized on the surface of nonporous particles via glutaraldehyde activation. For CT-immobilized poly(GMA) particles, the maximum activity (r_m) and Michealis constant (K_m) were found to be 17.2 μmol/mg CT min and 121.6 μm, respectively. No significant loss was observed in the activity of immobilized CT, during the course of experiments. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 818–824, 2006

Key words: enzyme immobilization; α-chymotrypsin; glycidyl methacrylate; dispersion polymerization; monodisperse particles; latex; affinity HPLC

INTRODUCTION

Various types of polymeric materials have been tried as support in the immobilization of α-chymotrypsin (CT). The kinetics of immobilized enzyme was investigated in a packed-bed reactor.[1,2] The polydisperse spherical particles, produced by suspension polymerization in the size range of 100–1000 μm, were mostly used for CT immobilization.[2–9] The modeling of mass transfer with enzymatic reaction was performed in a continuous reactor packed with a CT-immobilized porous Sepharose 4B particles.[2] As soluble carriers for CT immobilization, the graft copolymers, comprising a hydrophobic backbone and the side chains containing hydrophilic polymers (i.e., based on acrylic acid or 2-hydroxyethyl methacrylate), were also examined.[10,11] Temperature-sensitive polymers were the support materials recently tried for CT immobilization.[12–14] The isolations of protease inhibitors and anti-CT antibodies are the characteristic affinity chromatography applications performed with the packing materials, including immobilized CT.[15–18] The separation media obtained by the covalent immobilization CT on silica-based packing materials were able to resolve stereochemically some dipeptides and amino acids in HPLC.[19,20]

To obtain sufficient surface area, both for enzyme immobilization and the interaction between immobilized enzyme and substrate, the porous forms of large, polydisperse particles are usually preferred. In such a case, the intraparticular mass-transfer limitation is one of the significant problems, leading to a decrease in the activity of the immobilized enzyme. Hence, a low, particle-sized support material, serving sufficient surface area in the nonporous form, can be considered as a better alternative to the porous particles with large size. The uniform latex particles smaller than or ~1 μm in size are suitable materials having sufficiently large surface area for enzyme immobilization. In the past 5 years, various dispersion polymerization protocols were developed for the synthesis of uniform and nonporous poly(glycidyl methacrylate) (poly(GMA)) latex particles, ~1 μm in size.[21–24] The reactive character of their epoxy groups allows easier immobilization of enzymes on these particles. In our study, the nonporous, uniform poly(GMA) microspheres, 1.7 μm in size, were obtained by dispersion polymerization. Poly(GMA) latex particles were first used as a low-

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sized, nonporous support, serving large surface area without intraparticular diffusion resistance, to carry out the enzymatic reaction. The enzyme CT was covalently attached onto the particle surface via amination and glutaraldehyde activation. The activity behavior of poly(GMA) latex particles, carrying CT on their surface, was investigated.

EXPERIMENTAL

Materials

The monomer, glycidyl methacrylate (GMA) was supplied from Aldrich Chem. Co., and used without further purification. Absolute ethanol (Merck, AG, Darmstadt, Germany) was used as solvent in the dispersion polymerization. The initiator, 2,2′-azobisizo-butyronitrile (AIBN, BDH Chem. Ltd., Poole, England) was recrystallized from methanol. Ethanol (technical grade, 96% v/v) was supplied from Birpa A.S., Turkey. The stabilizer, poly(acrylic acid) (PAA; M_v, 1.2 $\times 10^4$) was prepared by the solution polymerization of acrylic acid.[25,26] The aqueous ammonia solution and the glutaraldehyde solution (25% w/w) were supplied from Aldrich Chem. Co. The enzyme CT and the synthetic substrate benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma Chemical Co., USA. Distilled–deionized water was used in the polymerization and enzymatic activity experiments.

Synthesis of poly(GMA) particles

Poly(GMA) microspheres were synthesized by dispersion polymerization. Typically, GMA (3 mL) was dissolved in ethanol (30 mL), containing AIBN (0.06 g) and PAA (0.12 g) at room temperature. The pyrex reactor was purged with bubbling nitrogen for 2 min and then sealed. The polymerization was conducted at 70°C, with a shaking rate of 120 cpm for 24 h. The particles were cleaned by successive centrifugation–decantation by using ethanol and water. Finally, poly(GMA) microspheres were redispersed in distilled water. The average size and size distribution of poly(GMA) particles were determined by scanning electron microscope, according to the method described previously.[27,28]

Amination of poly(GMA) particles

The amination reaction was performed according to the literature.[23] The aqueous dispersion of cleaned poly(GMA) particles (1 g) was added onto aqueous ammonia solution (10 mL; 25% w/w). The new dispersion was kept at 50°C for 6 h for the conversion of epoxypropyl groups into the primary amine form. Following amination, the particles were washed with water, until neutral pH was obtained in the washing solution. The nitrogen content of the aminated particles was determined by elemental analysis.

FITC labeling and CLSM examination

A common protocol described in the literature was used for the labeling of aminated particles with FITC.[29] The aminated particles (0.25 g) were dispersed in a mixture of acetone (0.2*M*, pH 9) and a bicarbonate buffer (10 mL; acetone/buffer, 1/9 v/v), containing freshly dissolved FITC (1.0 mg/mL). The labeling was performed at room temperature in darkness for 6 h, in the presence of magnetic stirring at 200 rpm. As a control material containing no amine groups, poly-(methyl methacrylate) (poly(MMA)) latex particles, 2 μm in size, were selected. The same labeling protocol was also applied for the poly(MMA) particles. To remove physically adsorbed FITC, both particles were extensively washed with acetone:water (1:9 v/v) by a centrifugation–decantation protocol and finally redispersed in distilled–deionized water. The surface and internal structure of poly(GMA) particles labeled with FITC were investigated by confocal laser scanning microscope (CLSM, Leica SP2, Heidelberg, Germany), equipped with He-Ne laser. The CLSM images were taken both in three-dimensional form and in the form of optical section by performing z-scan. These images were obtained by fixing the excitation wavelength at 488 nm.

Glutaraldehyde activation

The aminated particles (~0.1 g) were dispersed in a borate buffer (pH 8.0; 10 mL), containing GA (2% w/w). The reaction was performed under magnetic stirring for 30 min, at room temperature. The activated particles were extensively washed with borate buffer at pH 8.

Enzyme immobilization

GA activated particles (0.1 g) were dispersed in a borate buffer (pH 7.8; 10 mL) at 4°C, containing CT at a concentration ranging between 10 and 100 μg/mL. The immobilization was conducted at 4°C for 24 h, with a stirring rate of 250 rpm. The particles were isolated by centrifugation and washed several times with cold borate buffer. Finally, CT-immobilized poly(GMA) particles were redispersed in borate buffer and stored in the refrigerator until use.

Enzymatic activity experiments

The activity of immobilized CT was determined in a shaking, batch reactor. Typically, CT-carrying mono-

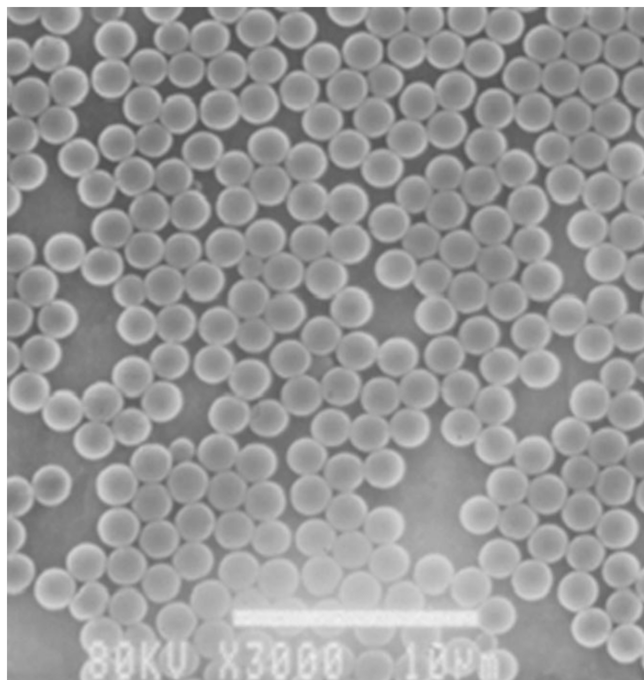

Figure 1 SEM photograph showing the monodispersity of poly(GMA) particles. Magnification: ×3000.

disperse particles (0.05 g) were dispersed in a reaction medium (20 mL; pH 7.8), comprising of an aqueous borate buffer (95% v/v) and ethanol (5%), and including 400 μM BTEE. The enzymatic activity runs were conducted at 25°C, with a shaking rate of 120 cpm. The activity of immobilized CT was measured, based on the formation of benzoyl-L-tyrosine (BT) by the hydrolysis of BTEE. A similar analysis protocol was also used in the literature and in our previous studies.[30,31] In the analysis, the absorbance increase, originated from BT formation, was followed by a UV–vis spectrophotometer (Shimadzu, Japan). For this purpose, the samples were taken from the reaction medium at different times. Each sample was centrifuged and the supernatant was isolated. Following the filtration of the supernatant, the absorbance was measured at 258 nm. The activity of immobilized CT (R, μM BTEE min^{-1}) was defined, based on the following expression:[30,31]

$$R = S_0[(dA\ /\ dt)\ /\ (A_1 - A_0)] \qquad (1)$$

where S_0 is the initial BTEE concentration (μM) in the reaction medium and dA/dt (min^{-1}) is the derivative of the absorbance with respect to time. This value was calculated using the absorbances of the samples taken in the initial course of the reaction. A_0 and A_f are the initial and final absorbance values of the reaction medium at 258 nm, respectively.

RESULTS AND DISCUSSION

Particle characterization

A SEM photograph showing the monodispersity of poly(GMA) microspheres is given in Figure 1. The average size and the coefficient of variation (CV) for size distribution, calculated based on this photograph, were 1.7 μm and 4.1%, respectively. The specific surface area of the poly(GMA) particles was calculated as 3.36 m^2/g. On the basis of this value, we thought that a satisfactory activity with the immobilized enzyme could be obtained, without observing an internal mass-transfer resistance during the enzyme–substrate interaction.

No change was observed in the spherical form of poly(GMA) particles by the reaction route applied for CT immobilization. In the first stage of this route, the epoxypropyl groups of the particles were converted into amine groups. Following amination, the nitrogen content of poly(GMA) particles was determined as 4.5% w/w by the elemental analysis. The theoretical N content of fully aminated poly(GMA) is 8.8% w/w. This value showed that ~51% w/w of GMA in the particle structure was converted into the amine-carrying form.

To have an idea about the distribution of amine groups in the particles, aminated particles were labeled with FITC. The labeled particles were imaged by CLSM. Three-dimensional CLSM image of the labeled particles, showing both the particle surface and the optical section at particle midplane, is given in Figure 2. As seen here, the labeled particles were observed in

Figure 2 A three-dimensional CLSM image showing both surface and optical section at midplane of the FITC-labeled aminated poly(GMA) particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

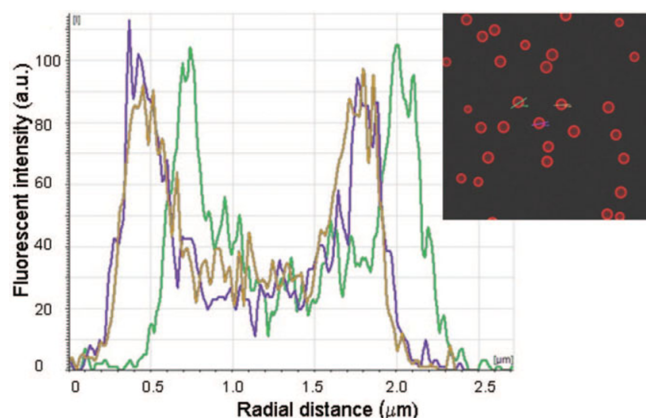

Figure 3 The optical section and the intensity profile at midplane of the FITC-labeled aminated poly(GMA) particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the fluorescent form. This view indicated that FITC was covalently attached onto the aminated poly(GMA) particles. Note that the same labeling protocol was also applied onto the poly(MMA) latex particles (2.0 μm in size) as a control material, and as expected, no photograph, including fluorescent microspheres, could be obtained, since no FITC was attached to the poly(MMA).

On the other hand, the fluorescent intensity distribution on the optical section was not homogeneous (Fig. 2). To observe the optical section at the midplane of the particles more clearly, a z-scan was performed in the aqueous dispersion of FITC-labeled aminated poly(GMA) particles. The photograph of the optical section at particle midplane is given in Figure 3, together with the fluorescent intensity profile generated in the radial direction. Note that the fluorescent intensity at a certain radial distance is directly proportional to the fluorescent probe (i.e., FITC) concentration at the same point. The concentration of bound FITC at a certain radial distance should be considered as a measure of the concentration of primary amine groups. Thus, one can have an idea about the distribution of primary amine groups in the particle structure by the evaluation of fluorescent intensity profile. As seen in Figure 3, a core–shell structure was observed in the optical section obtained in the middle of the particles. So, the intensity profile of the marked particles exhibited a maximum on the particle surface and a minimum nearly at the center of the optical section. This view indicated that the bound FITC—also the primary amine—concentration was significantly higher in the shell region, with respect to the inner core. In other words, only the surface region of the particles could be effectively aminated by the experimental procedure followed in our study. Because FITC should normally diffuse in the radial direction and react with all primary amine groups in the particles, the labeling was performed in the presence of acetone.[28]

The volume fraction of fluorescent shell in the whole particle was calculated as 0.578, by using the radial length of the fluorescent region and the radius of particle in Figure 3. Note that the elemental analysis indicated that 51% of poly(GMA) was converted to the primary amine-carrying form by the amination reaction. One can conclude that there is a rough consistency between these values, when the fluorescent intensity is considered as a measure of primary amine concentration.

Another optical section was also obtained on the particle surface by performing z-scan in the upward direction (not shown). The intensity profile on the surface was completely different than that of the internal part. In other words, the fluorescent intensity on the particle surface did not change by the position. This finding is attributed to the fact that FITC—and also primary amine—concentration was constant on the particle surface.

Similar structural properties to those given in Figure 3 were also observed for the poly(GMA) particles obtained with different initiators and stabilizers by the dispersion polymerization.[28] The dominant localization of primary amine groups on the particle surface also involves the immobilization of CT in the same layer. Hence, CT-immobilized aminated poly(GMA) particles can be used as a biocatalyst in the absence of intraparticular diffusion limitations. This property is particularly important when the immobilized CT is used for the substrates with high molecular weights (i.e., proteins).

The amine-carrying particles were then reacted by GA to have reactive aldehyde groups on the particle surface. GA-activated poly(GMA) microspheres were interacted with CT in buffer solution at pH 7.8. The variation of enzymatic activity of immobilized CT with the CT concentration in the enzyme-binding medium is given in Figure 4. The effect of CT concentration on the amount of immobilized CT equivalent to the free enzyme was also given in the same figure. As expected, the activity of immobilized enzyme increased with the increasing CT concentration in the immobilization medium. However, this increase was not proportional to the CT concentration. In other words, although the CT concentration was increased 10-fold, the increase obtained in the activity of immobilized enzyme was approximately twofold. This result should be probably explained by the conformational change of the enzyme molecules during the immobilization and multiattachment of CT molecules onto the aldehyde functionalized microspheres. It should be noted that the maximum amount of immobilized CT equivalent to free enzyme was 180 μg/g dry particles. Of course, the actual amount of immobilized CT should be higher than this value.

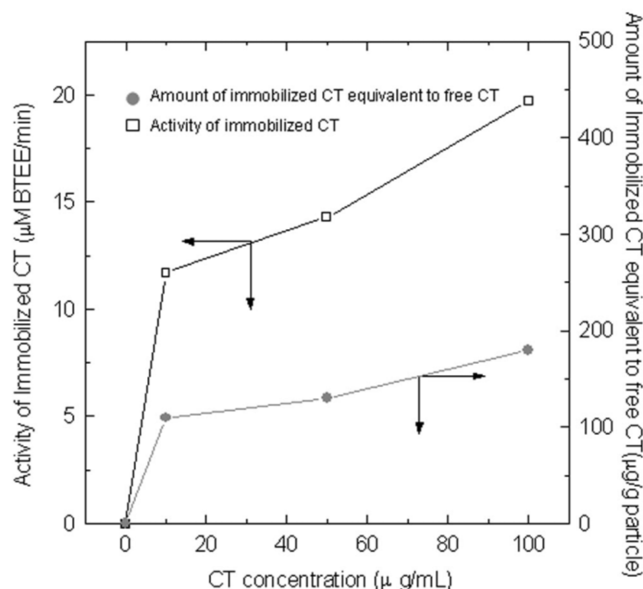

Figure 4 The effect of CT concentration in the immobilization medium on the activity of immobilized CT and the amount of immobilized CT equivalent to free enzyme. Activity test conditions: batch reactor volume, 20 mL; 95% (v/v) phosphate buffer–ethanol (5% (v/v)); poly(GMA) particles, 0.05 g (pH 7.8; 25°C); initial BTEE concentration, 400 μm.

Enzymatic activity experiments

Poly(GMA) particles, with the enzyme loading of 110 μg CT/g (i.e., the particles prepared with the CT concentration of 10 mg/mL), were used in the activity runs. To test the operational stability of immobilized CT, 10 successive activity runs were carried out in 1 day. Each run was continued for 1 h. Hence, the operational stability was defined as the stability for multiuse in 1 day. The variation of relative activity with the run number is given in Figure 5. Here, the relative activity was defined as the ratio of enzymatic activity in any run to that observed in the first run. As seen in Figure 5, a relatively sharp activity decrease was observed in the first five runs. This decrease should be related to the removal of weakly bound enzyme molecules from the surface of poly(GMA) particles, during the interaction of immobilized CT with BTEE. This case is a typical behavior observed in our previous studies on the immobilization of CT.[9,30,31] The stability of CT immobilized within k-carrageenan beads was tested by Belyaeva et al.[8] They observed ~15% activity decrease after seven successive uses.

For testing of storage stability, one activity run was done per day in a total period of 10 days. Between the successive runs, CT-immobilized poly(GMA) particles were stored at 4°C, in a medium at pH 7.8, including 95% v/v borate buffer and 5% v/v ethanol. The variation of relative activity with the run number is given

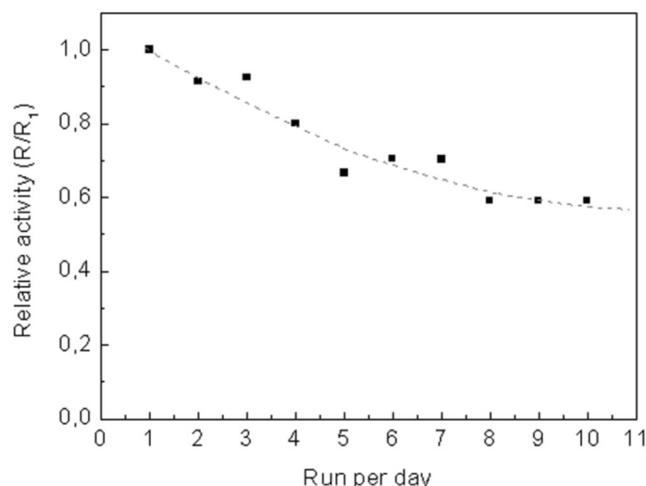

Figure 5 The graph showing the relative activity change for multiuse in one day. Activity test conditions: batch reactor volume, 20 mL; 95% (v/v) phosphate buffer–ethanol (5% (v/v)); poly(GMA) particles, 0.05 g (pH 7.8, 25°C); initial BTEE concentration, 400 μm.

in Figure 6. In this case, a marked decrease in the relative activity of immobilized CT occurred only after the first run. By the second run, the relative activities approximately corresponding to half of the initial activity were obtained. A similar behavior was also observed in the storage stability of CT entrapped within Ca-alginate gel beads tested in a period of 15 days.[6]

The effect of initial BTEE concentration on the activity of immobilized CT is shown in Figure 7. As expected, the activity increased with the increasing BTEE concentration and reached a plateau after the initial BTEE concentrations were higher than 200 μm. The kinetic constants of immobilized CT were determined, according to the Michealis–Menten model by

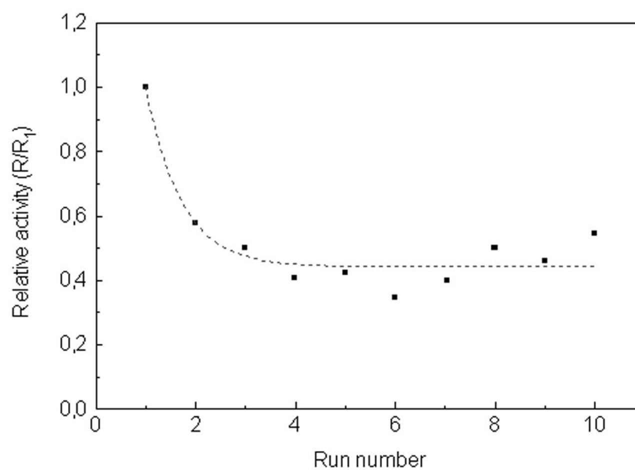

Figure 6 The graph showing the storage stability of immobilized CT. One run was carried out per day for a total period of 10 days. Activity test conditions are the same with those in Figure 5.

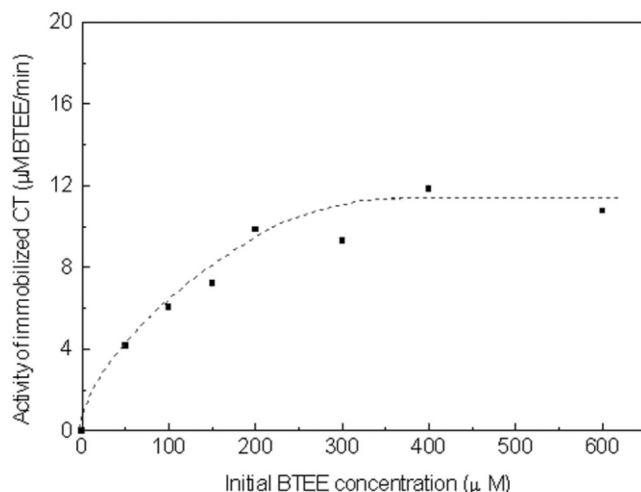

Figure 7 The effect of initial BTEE concentration on the activity of immobilized CT. Activity test conditions: batch reactor volume, 20 mL; 95% (v/v) phosphate buffer–ethanol (5% (v/v)); poly(GMA) particles, 0.05 g (pH 7.8; 25°C).

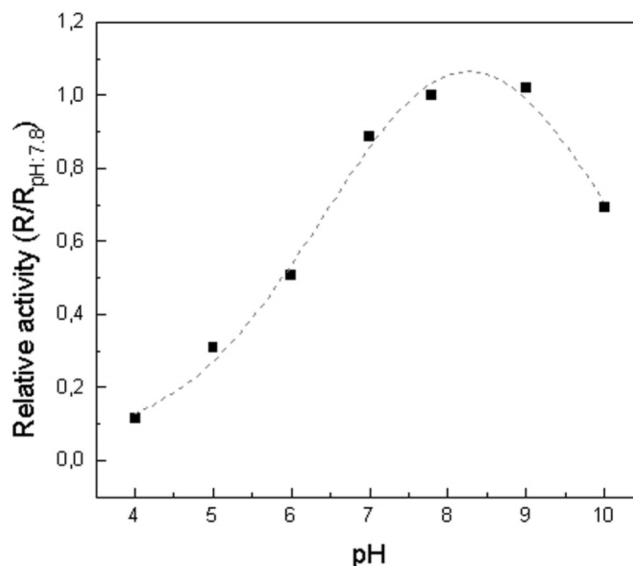

Figure 8 The effect of pH on the activity of immobilized CT. Activity test conditions: batch reactor volume, 20 mL; 95% (v/v) phosphate buffer–ethanol (5% (v/v)); poly(GMA) particles, 0.05 g, 25°C; initial BTEE concentration, 400 μm.

using the data in Figure 6. For CT-immobilized poly(GMA) particles, the maximum activity (r_m) and Michealis constant (K_m) were found to be 17.2 μmol BTEE/mg CT min and 121.6 μm, respectively. In our previous studies, the maximum activity and Michealis constant of the free enzyme were determined as 44.0 μmol BTEE/mg CT min and 63.06 μm, respectively.[30,31] The CLSM image clearly showed the localization of primary amine groups on the surface of nonporous particles. This case involves the immobilization of CT onto the particle surface. Hence, no significant limitation should exist for the intraparticular diffusion of substrate. For this reason, lower maximum activity and higher K_m of immobilized CT with respect to free enzyme should be related to the conformational changes occurring during the covalent immobilization. CT was covalently immobilized onto the Sepharose 4B beads by Clark and Bailey.[2] In their work, approximately two- or threefold increase in K_m and r_m values, ranging between 15 and 60% of the free enzyme, were found depending upon the immobilization conditions.[2] In the study performed with CT-immobilized Teflon particles, the r_m values obtained for free and immobilized CT were 0.45 and 0.34 μmol BTEE/min, respectively.[6] These comparisons indicated that the kinetic constants of our system were satisfactory.

The effect of pH on the activity of immobilized CT is given in Figure 8. Here, the relative activity was defined as the ratio of activity at any pH to the activity at pH 7.8. The relative activity of free CT was particularly lower in the basic pH. Immobilized CT exhibited maximum activity at the same pH with the free enzyme (i.e., pH 7.8).

The effect of temperature on the activity of immobilized CT is given in Figure 9. Here, the relative activity was defined as the ratio of activity at any temperature to the maximum activity. As seen here, a sharper activity profile providing the maximum activity at 40°C was obtained with the free enzyme. In some of the studies on CT immobilization, the maximum activity of the immobilized enzyme was ob-

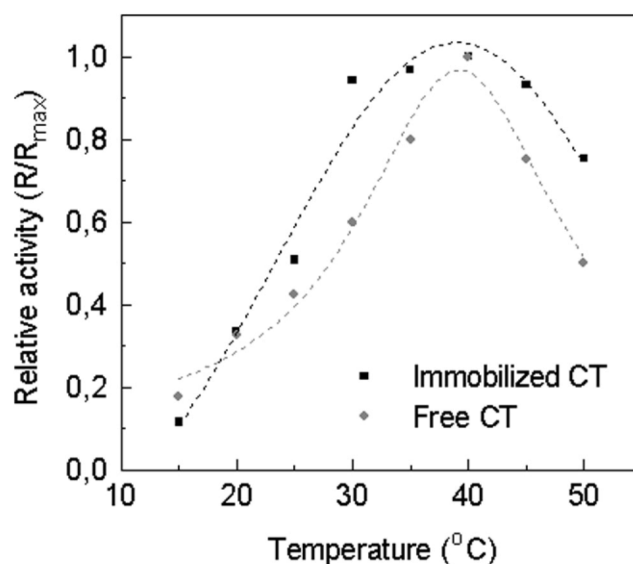

Figure 9 The effect of temperature on the activity of immobilized CT. Activity test conditions: batch reactor volume, 20 mL; 95% (v/v) phosphate buffer–ethanol (5% (v/v)); poly(GMA) particles, 0.05 g, pH 7.8; initial BTEE concentration 400 μm.

served at the same temperature with the free one or at slightly higher temperatures with respect to the free one.[9,10] In our case, the maximum activity was also observed at 40°C. Hence, one concludes that no significant temperature shift for the maximum activity occurred by the immobilization. As expected, immobilized CT exhibited higher relative activities with respect to the free one at most of the temperatures studied.

CONCLUSIONS

In this study, CT was covalently immobilized onto the uniform and nonporous poly(GMA) particles, 1.7 μm in size. The immobilized CT exhibited a satisfactory performance in the activity tests performed in a batch fashion. These results indicated that uniform and nonporous poly(GMA) latex particles were a suitable new support for CT immobilization.

On the other hand, both the support (i.e., poly(GMA)) and the enzyme (i.e., CT) selected in our study are important in the affinity HPLC applications. Poly(GMA) based nonporous particles, $\sim$2 μm in size, were used as packing material for the purification of cellular proteins, DNA and IgG by affinity HPLC.[32–34] Besides, the enantiomeric separations performed with CT-linked column materials showed that activity behavior of immobilized CT played an important role on the chromatographic separation in HPLC.[19,20,35]

Based on these applications, CT-immobilized uniform, nonporous poly(GMA) microspheres can be evaluated as a potential support for the affinity HPLC applications, particularly performed with the short columns or cartridges.

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