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Covalent immobilization of horseradish peroxidase on macroporous glycidyl methacrylate based copolymer

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Introduction

Macroporous copolymers are often used as carriers for enzyme immobilization and as adsorbents in chromatography. One of the most widely used is copolymer of styrene and divinylbenzene.[1] Copolymers based on glycidyl methacrylate are more suitable for enzyme immobilization since they have epoxy groups that can be easily transformed into hydroxyl, keto, carboxyl or amino groups to facilitate binding of the enzyme for the carrier. Compared to polystyrene they also have higher hydrophilicity which has been proven to have positive influence on protein stability and enzyme activity.[2,3] Porosity of the carrier used in enzyme immobilization is also very important parameter influencing activity and stability of immobilized enzyme.[4] Therefore, precise control of these properties could significantly improve performance of immobilized enzymes.[5]

Horseradish peroxidase (HRP, E.C.1.11.1.7) is the most know peroxidase from plants that can be used for organic synthesis of specialty chemicals like DOPA6 and biphenols,[6] in polymerization reactions during elimination of pollutants such as phenol and aniline from wastewater,[7] and in manufacture of biosensors.[8] The enzyme belongs to the ferroprotoporphyrin group of peroxidases and has limited operational stability due to inactivation by its own substrate. Thus, it is recommended to use HRP in an immobilized form to enable repeated use of the enzyme and enhance its properties such as activity and stability under these extreme operational conditions.[9,10]

Different methods have been used to immobilize HRP on a solid carrier including covalent bonding,[9,11] entrapment in a solid matrix[12,13] or electrodeposition at the electrode surfaces.[14] There are a variety of methods for coupling enzymes to glycidyl methacrylate based copolymers. Glutaraldehyde coupling chemistry is one of the most-used method for enzyme immobilization via amino groups of its exposed amino acids.[9,15] On the other hand, immobilization of glycoproteins like invertase,[16] lipase[2] and others through their carbohydrate moiety seems to be effective and site-specific, bringing about little change in the overall conformation of the enzymes or of their active sites.

In the present work, several macroporous poly(GMA-co-EGDMA) samples with the same chemical composition but different surface characteristic and mean pore size diameter were synthesized and screened for immobilization of horseradish peroxidase by applying two different covalent methods. The immobilization methods were compared with regards to the specific activity and thermal stability. Finely, the best carrier and immobilization method were selected for further use and characterization. The pH profile, kinetic properties and operational stability of immobilized and free HRP were compared.

Results and Discussion

Four different copolymer samples with different mean pore diameters were used SGE-10/4 (44 nm), SGE-10/12 (50 nm), SGE-20/12 (120 nm), SGE-15/16 (200 nm). Particles of the obtained copolymers exhibited spherical shape with diameters in the range of 150–500 μm and surface of these particles was highly porous. Recent studies have revealed that the kinetics and stability of enzymes immobilized on porous carriers are usually modified by diffusion effects, depending strongly on the surface characteristics of carriers used for their immobilization.[4] Thus, in the present study, we wanted to determine which of the copolymer batches were most suitable for horseradish peroxidase (HRP) immobilization by both glutaraldehyde and periodate method previously optimized for hydrolases.[2,4,17] Different amounts of enzyme were added per gram of copolymers and specific activity of immobilized enzyme was determined for all copolymer samples. The results obtained for both methods are shown in Fig. 1.

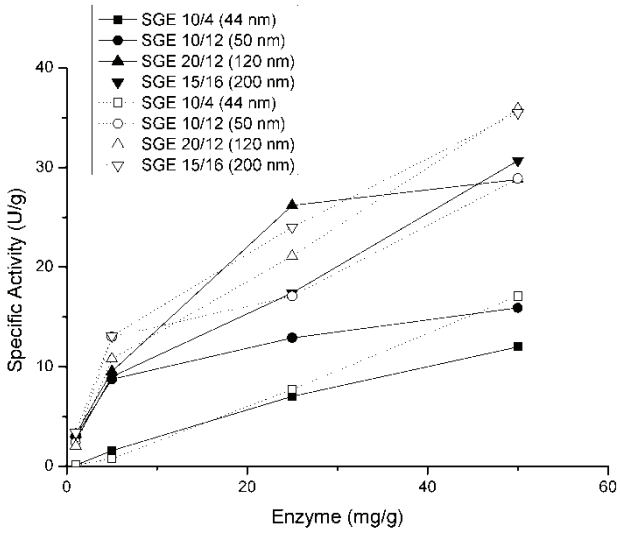

Figure 1. Influence of amount of added enzyme on specific activity of immobilized peroxidase. Open symbols – periodate immobilization, closed symbols – glutaraldehyde immobilization. Copolymer sample used: (□ and ■) SGE-10/4; (○ and ●) SGE-10/12; (Δ and ▲) SGE-20/12 and (∇ and ▼) SGE-15/16.

As expected, the specific activity of immobilized HRP increased as the amount of added enzyme per gram of copolymer increased in all experiments. For all cases studied, the periodate method for HRP immobilization gave in average 1.5 times higher specific activity of the immobilized enzyme compared to the glutaraldehyde method when using the same copolymer and the same amount of added enzyme. Similar results have been obtained previously for lipase from *C. rugosa* using Eupergit C or Sepabeads EC-EP as carriers.[2,3] Surface characteristics of the copolymer were found also to have influence on the specific activity of the immobilized enzyme. For the periodate method, the highest specific activity of around 35 U g^{-1} dry weight of carrier was obtained with the copolymer samples with larger pore size (mean pore diameter 120 and 200 nm), namely SGE 20/12 and SGE 15/16. Similar trend also has been observed for glutaraldehide method, when the specific activity of HRP immobilized on copolymer with 200 nm mean pore diameter was approximately three times higher than that of HRP immobilized on copolymer with 44 nm. Copolymers with a small pore size provided a large total surface area, but the pores appeared to be too small resulting in restricted mass transfer and pore penetration of the enzyme. The observed enhancement in specific activity of carriers with larger pores was conssistent with the study on subtilisin immobilization on different silica carriers which reported that silicas with large mean pore diameter presented higher total and specific activities relative to those with smaller pore sizes.[18] The optimal pore size of carrier appeared to be highly dependent on the particular enzyme and coupling method used. In this work, for HRP (molecular weight 40,000, size of the molecules 5-6 nm) it was found that a 120-200 nm pore size in the carrier microbeads gave the best results that were much higher than recommended value for an efficient enzyme immobilization. Thermal stability of HRP immobilized by two covalent methods has been evaluated and compared the results to the kinetics of deactivation of the free enzyme. For all the cases studied, the immobilized preparations seemed to exhibit increased thermal stability compared with the free enzyme (Fig. 2).

It is apparent that the stability of immobilized HRP obtained by periodate method was higher than that obtained by glutaraldehyde one. HRP that was immobilized onto SGE 20/12 copolymer by periodate method with mean pore diameter of 120 nm showing highest activity and a rather high stability was further characterized.

Both forms of the enzymes exhibited an optimum at pH 7, but immobilized HRP displayed a broadened pH profile relative to the free one. Higher activity of immobilized enzyme was more pronounced at basic pH values, which could be interesting for practical application. The enzyme appeared to follow Michaelis-Menten kinetics and kinetic parameters were determined directly by fitting the experimental data to the kinetic model (data not shown). K_m was determined for pyrogallol when H_2O_2 is maintained at saturating conditions. The value of Michaelis constant for the immobilized enzyme appeared to be 10.8 mM, approximately 5.6 times higher than that of the free enzyme (K_m value for free HRP was 1.93 mM), suggesting decreased accessibility of enzyme to the substrate. V_{max} of the immobilized enzyme was determined to be 54.9 U g^{-1} dry weight of carrier.

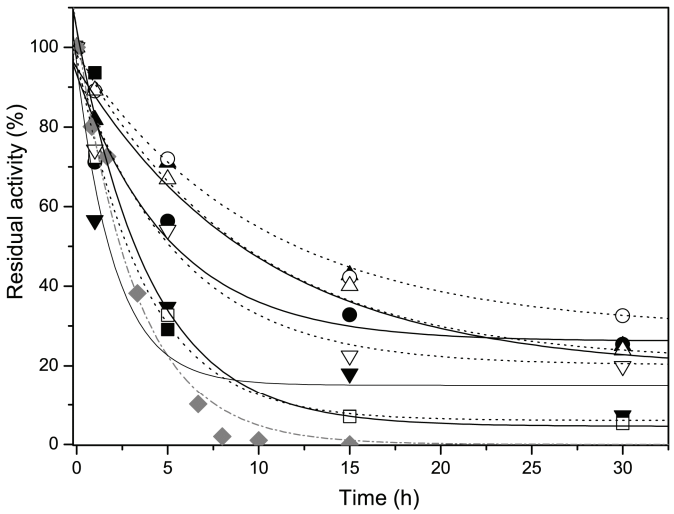

Figure 2. Residual activity of immobilized and free HRP at 65°C versus time. Open symbols – periodate immobilization, closed symbols – glutaraldehyde immobilization, (□) free enzyme. Copolymer sample used: (□ and ■) SGE-10/4; (○ and ●) SGE-10/12; (Δ and ▲) SGE-20/12 and (∇ and ▼) SGE-15/16.

This was in accordance with previous immobilization studies of peroxidases where increase of K_m constant for immobilized enzyme was also observed.[9,11] For instance, 15.6 times higher K_m value for guaiacol has been reported with chitosan immobilized HRP compared to that of free enzyme[19] or K_m value for H_2O_2 was 1.65 times higher in the case of HRP immobilized on the magnetic poly(glycidyl-methacrylate-co-methylmethacrylate compared to the free one.9

In order to test operational stability of immobilized peroxidase during usage the same batch of immobilized enzyme was used in several cycles of pyrogallol oxidation in batch reactor and residual activity of immobilized enzyme was determined after each cycle, Fig. 3.

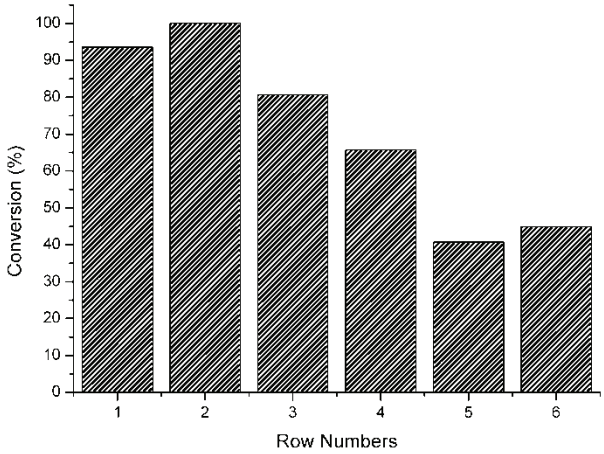

Figure 3. Catalytic activity of HRP immobilized on copolymer SGE-20/12 on repeated batch run.

During first 4 cycles activity was dropping and after that remained constant at 45% of initial activity. Immobilized peroxidase showed good stability under operational conditions and optimization of hydrogen peroxide concentration and delivery to the reaction system could further improve performance of immobilized peroxidase.

Conclusions

In this work HRP was covalent immobilized onto characterized copolymer of glycidyl methacrylate and ethylene glycol dimethacrylate by two covalent methods. The effect of mean pore diameter and surface characteristics of copolymer on enzyme activity and stability has been examined. Overall the enzyme immobilized by periodate method performed substantially better than that immobilized by glutaralde-

hyde. In particular, when HRP was immobilized by periodate method onto copolymer SGE-20/12 with 120 nm mean pore diameter, the biocatalyst showed enhanced specific activity and a rather high thermal stability. The K_m value was determined to be 10.8 mM for the immobilized HRP, approximately 5.6 times higher than that of the free enzyme. Both forms of the enzymes exhibited an optimum at pH 7, but immobilized HRP displayed a broadened pH profile relative to the free one. The operational stability also proved to be satisfactory after five consecutive uses with a residual activity of 45%.

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Kovalentna imobilizacija peroksidaze rena na makroporozni glicidil metakrilatni kopolimer

U ovom radu peroksidaza iz rena (HRP) je kovalentno imobilizovana na kopolimeru glicidil metakrilata i etilenglikol dimetakrilata pomoću dve kovalentne metode. Ispitani su uticaji prečnika pora i površinskih karakteristika kopolimera na enzimsku aktivnost i stabilnost. Enzim imobilizovan perjodatnom metodom je pokazao veću aktivnost i stabilnost u poređenju sa glutaraldehidnom metodom. HRP imobilizovana na kopolimer SGE-20/12 sa prečnikom pora od 120 nm je pokazala najveću specifičnu aktivnost i dobru termostabilnost. K_m vrednost imobilizata je bila 10,8 mM što je oko 5,6 puta više od rastvornog enzima. Obe forme enzima su pokazale optimum na pH 7, ali je imobilizovana HRP pokazala širi pH optimum u odnosu na rastvornu formu. Operaciona stabilnost je bila zadovoljavajuća i nakon pet uzastopnih upotreba zaostala aktivnost je bila 45%.

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