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29th International Conference Ecological Truth & Environmental Research

EcoTER'22

Proceedings

Editor
Prof. Dr Snežana Šerbula

21-24 June 2022, Hotel Sunce, Sokobanja, Serbia

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COVALENT IMMOBILIZATION OF HORSERADISH PEROXIDASE ON NOVEL MACROPOROUS POLY(GMA-CO-EGDMA) FOR PHENOL REMOVAL

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Abstract

For the purpose of immobilization, one of the most commonly used enzymes is horseradish peroxidase (HRP). Different carriers can be used as supports for the immobilization of HRP: alginate, pectin, magnetic-beads, macroporous copolymers, silicas etc. Covalent binding of an enzyme to the carrier leads to the formation of strong linkage, thus preventing the enzyme leakage. Macroporous copolymers with different porous characteristics were used for the immobilization of horseradish peroxidase by employing periodate and glutaraldehyde method. Five and 25 mg of HRP were immobilized per gram of the copolymer. Increasing the amount of added enzyme leads to the increase of specific activity of immobilized enzyme. Copolymer with the pore diameter of 297 nm showed the most promising results in terms of specific activity. Immobilized enzymes can be used for the removal of phenolic compounds from waste effluents.

Keywords: macroporous copolymer, periodate immobilization, horseradish peroxidase, dispersion polymerization, phenol removal

INTRODUCTION

Horseradish peroxidase (HRP, E.C.1.11.1.7) is one of the most frequently used plant peroxidases. Inactivation of the enzyme by its own substrate during long-term use is a major issue, which directly affects enzyme activity and its operational stability [1]. Immobilization of HRP on different carriers enables the repeated use of enzyme and enhances its properties [2,3]. Various support materials can be used as immobilizing agents (natural polymers such as alginate, pectin, chitosan, magnetic-beads [1,2,4–6], silica [7] and polyacrylamide gels [8]).

The immobilization of horseradish peroxidase can be acquired by using different methods: covalent binding, entrapment, adsorption and cross-linking. Covalent binding provides strong linkage between the enzyme and carrier, thus preventing the enzyme leakage from the support [9]. Macroporous copolymers have been widely used as carriers in covalent immobilization reactions [3]. A wide application of these supports relies on the presence of epoxide groups that can be easily transformed into more reactive groups such as amino, keto, carboxyl or hydroxyl.

Porosity of macroporous carriers is considered as one of the most important parameters that directly affects the activity and stability of immobilized enzymes [10]. This property can be controlled during the suspension copolymerization reaction by a careful choice of cross-linking agent as well as the type and amount of inert components [11,12].

Peroxidase immobilized onto macroporous carriers can be widely used in different areas, such as development of biosensors [13,14], decolorization of textile dyes [15], removal of phenolic compounds from wastewaters etc. [1,2]. The removal of phenols with immobilized HRP from waste effluents is of major interest due to high toxicity of phenol like compounds even at low concentrations and its great abundance in watercourses worldwide.

MATERIALS AND METHODS

Materials

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 1-dodecanol, cyclohexanol, horseradish peroxidase (150–250 U/mg), pyrogallol used as a substrate for peroxidase oxidation reaction, glutaraldehyde (25% solution in water) and sodium periodate were purchased from Sigma Aldrich (St. Louise, Mo, USA). Ethylenediamine was obtained from Merck (Kenilworth, New Jersey, USA). Hydrogen peroxide was purchased from AppliChem GmbH (Darmstadt, Germany).

Copolymer Preparation and Amination

A continuous phase consisting of 2.78 wt% PVP (Mw = 24 000 g/mol) in ethanol was heated to 70 °C. A monomer phase (5.0 g of both the monomer GMA and cross-linking agent EGDMA (GMA/EGDMA = 60/40)), initiator (0.05 g of AIBN) and inert phase (2.25 g of 1-dodecanol and 2.25 g of cyclohexanol) was added to the continuous phase and stirred for 6 h. The obtained copolymer was washed 5 times with ethanol and dried at room temperature. After the amination of epoxide groups with ethylenediamine, the polymer particles were washed first with ethanol and subsequently with water until the pH value of the filtrate was 6. The samples were dried in the oven at 50 °C.

Copolymer Characterization

The pore size distributions of the synthetized copolymers poly(GMA-co-EGDMA) were determined by a mercury porosimetry (Carlo Erba 2000, software Milestone 200). Scanning electron microscope (Tescan FE-SEM Mira 3 XMU) was employed to characterize the morphology of poly(GMA-co-EGDMA).

Horseradish Peroxidase Immobilization by Glutaraldehyde Method

Aminated copolymers were first deaerated for 10 min in sodium phosphate buffer pH 8 (0.1 mol/L) and rinsed twice with the same buffer. Prepared copolymers were incubated for 2h in a glutaraldehyde solution. Subsequently, the copolymers were incubated with different amounts of horseradish peroxidase (5 and 25 mg/g). After the incubation, copolymers were rinsed twice with sodium phosphate buffer pH 7 (0.1 mol/L), resuspended in the same buffer and stored at 4 °C until further use.

Horseradish Peroxidase Immobilization by Periodate Method

Sodium periodate solution (50 mmol/L) in sodium acetate buffer pH 5 was used for the oxidation of horseradish peroxidase. Oxidized HRP was dialyzed overnight against sodium acetate buffer pH 5. The aminated copolymers were first deaerated in sodium phosphate buffer pH 7 (0.1 mol/L), rinsed with the same buffer and subsequently, incubated with different amounts of oxidized HRP (5 and 25 mg/g) for 48 h. Copolymers with immobilized enzyme were rinsed with sodium phosphate buffer pH 7 (0.1 mol/L) and stored in the same buffer at 4 °C until further use.

Enzyme Activity Studies

In order to determine peroxidase activity, pyrogallol and hydrogen peroxide (H_2O_2) were used as substrates. In the most common assay, 10 µL of the enzyme dilution from the washings and 10 µL of H_2O_2 (9.7 mmol/L) were introduced into 1 mL of the pyrogallol solution (13 mmol/L) in sodium phosphate buffer pH 7. Absorbance was measured for 3 min at 420 nm using UV–VIS spectrophotometer (Shimadzu Corporation UV-2501PC, Japan). To measure the activity of the immobilized enzyme, 9.0 mg of the copolymer with immobilized HRP and 30 µL of H_2O_2 were introduced into 3 mL of pyrogallol. Every 60 s aliquots were taken out from the mixture, filtrated and the absorbance at 420 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produces 1mg of purpurogallin in 20 s at 20 °C.

RESULTS AND DISCUSSION

Dispersion polymerization was used for the synthesis of macroporous poly(GMA-co-EGDMA) copolymers. The spherical nature of particles with diameter of around 1.5 µm is presented in Figure 1.

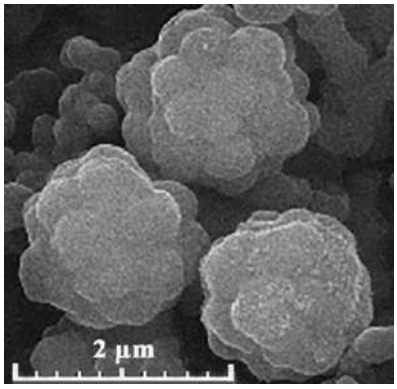

Figure 1 *SEM image of poly(GMA-co-EGDMA) copolymer*

Various studies have shown that the porosity of particles obtained by suspension copolymerization method is greatly affected by the amount of added cross-linking agent and polarity of used solvent [11,12]. An increase in the water content leads to decrease in the average pore diameter (Table 1).

Table 1 *Porous properties of copolymer samples*

Sample ID	ZP2	ZP5	ZP12
GMA:EGDMA (wt:wt%)	60:40	60:40	60:40
Ethanol:water (mL:mL)	30:15	20:25	15:30
Average pore diameter (nm)	460	297	235

The immobilization of horseradish peroxidase was performed via two different methods: glutaraldehyde and periodate method. Periodate method involves covalent binding to a copolymer surface through carbohydrate moiety located on the enzyme molecule. The other method used for this study (glutaraldehyde method) implies binding of an enzyme to a glutaraldehyde-activated carrier via amino groups presented on the surface of the protein (Figure 2).

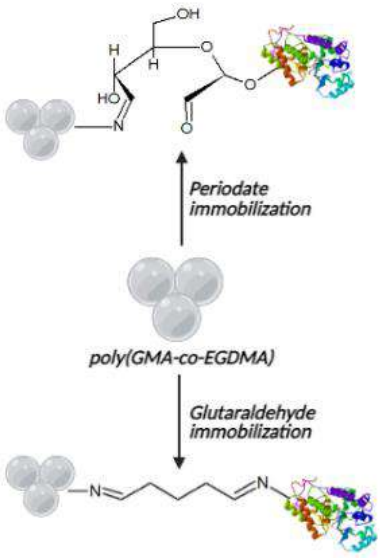

Figure 2 *Covalent immobilization of horseradish peroxidase onto poly(GMA-co-EGDMA) copolymer*

Different amounts of HRP were added per gram of the copolymer (5 and 25 mg/g) and specific activity of the immobilized enzyme was calculated. Results presented in Figure 3a show that specific activity increases with the increase in the amount of added enzyme per gram of the copolymer, regardless of the method used. Copolymer labelled as ZP5 with the pore diameter of 297 nm showed the most promising results. Immobilization of the enzyme by periodate method provides higher specific activities than immobilization by the glutaraldehyde method, under the same conditions (the same copolymer and the same amount of immobilized peroxidase) (Figure 3b). This is probably a consequence of preserved structure of enzyme active site which resulted from the oxidation of carbohydrate moiety.

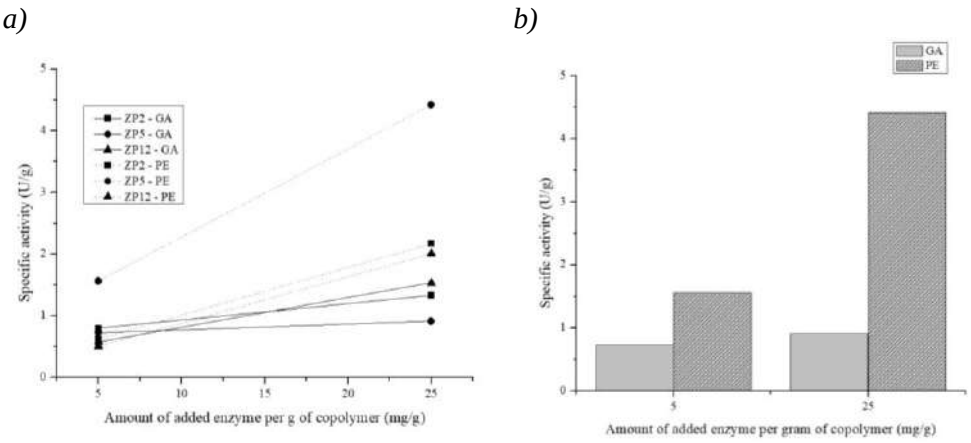

Figure 3 *a) Effect of the amount of added enzyme on the specific activity of immobilized HRP; b) Differences in specific activities of enzymes immobilized by periodate and glutaraldehyde method*

CONCLUSION

Macroporous copolymers were successfully synthesized by the dispersion polymerization. Periodate and glutaraldehyde methods were used for the immobilization of horseradish peroxidase. Obtained results showed that the increase in the amount of added enzyme per gram of the copolymer leads to the increase in the specific activity of immobilized enzyme. When two methods for HRP immobilization were compared, more promising results were obtained by the periodate method. Horseradish peroxidase immobilized onto macroporous copolymers can be used for the removal of phenolic compounds.

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