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Investigation of the Properties of Immobilized Horseradish Peroxidase on Magnetic Particles

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Summary: Magnetic particles coated with copolymer of acrylamide and acrylonitrile have been prepared. Those particles were used as a matrix for a covalent binding of peroxidase. The periodic-oxidized enzyme was bound to the matrix by immobilization procedure at 4°C for 18 hours. The immobilized enzyme showed relative activity of 86%. The following results were obtained for pH and optimum temperature of the immobilized enzyme – 7.0 and 30°C, respectively. The analysis of the kinetic parameters of the immobilized enzyme showed values of V_{max} – 0.0517 $M.10^{-6}$/sec and K_m – $2.3x10^{-4}$ M.

Key words: horseradish peroxidase, magnetic particle, covalent immobilization

1. INTRODUCTION

Magnetic nanoparticles modified with organic molecules have been widely used for biotechnological and biomedical applications because their properties can be magnetically controlled by applying an external magnetic field [1]. They offer a high potential for numerous biomedical applications, such as cell separation [2], purification of nucleic acid [3], hyperthermia [4] and immunosensors [5].

Magnetic nanoparticles as immobilization materials have the following advantages: (1) more specific surface area obtained for the binding of larger amounts of biomolecules; (2) lower mass transfer resistance; (3) selective separation of the immobilized biomolecules from a reaction mixture on application of a magnetic field. Among these materials, Fe_3O_4 magnetic nanoparticles are the most commonly studied. Fe_3O_4 magnetic nanoparticles have good biocompatibility, strong superparamagnetism, low toxicity, and an

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easy preparation process, and their use in biosensors has already shown attractive prospects [5].

Horseradish peroxidase (HRP) is an important peroxidase that contains heme, which is the protein active site with the resting state of the heme iron, Fe(III), as prosthetic group. It can catalyze the H_2O_2 dependent one-electron oxidation of a great variety of substrates, and has been commonly employed to construct H_2O_2 biosensors [6]. Peroxidase is the most frequently used enzyme for the construction of immunosensors. Successful immobilization of horseradish peroxidase on modified magnetic particles and their employment in the amperometric biosensors are mentioned in [6, 7].

The objectives of this study were to investigate the properties of immobilized horseradish peroxidase on magnetic particle coated with copolymer of acrylamide and acrylonitrile.

2. MATERIALS AND METHODS

Obtaining magnetic particle coated with copolymer of acrylamide and acrylonitrile

The copolymerization of acrylonitrile and acrylamide occurs by a free radical mechanism with an initial stage of initiation resulting in free radicals. $FeSO_4$, NaS_2O_5 and $(NH_4)_2S_2O_8$ are used as initiators. The following reagents were added to obtain copolymer containing 20% acrylamide: H_2O – 174 ml, acrylonitrile – 12 g, acrylamide – 3 g, NaS_2O_5 – 0.125 g, $FeSO_4$ – 0.125 g, $(NH_4)_2S_2O_8$ – 0.125 g. Copolymerization takes 2 hours at room temperature under constant stirring. The copolymer is filtered and profusely flushed with water in buchner funnel, and finally with methanol. It is dried in a vacuum oven at 50^0C. The dried polymer is dissolved in dimethyilformamide and then using a syringe it is poured drop by drop in a solution of ethanol and water (1 : 4) and surfactant. The spherical particles thus formed have an average diameter of ~ 1 mm. The water contents of the carrier thus formed was determined to be – 80%. The spherical particles with magnetic properties were obtained by producing a 10% solution of copolymer containing 5% magnetite.

Method for oxidation of peroxidase
Horseradish peroxidase (HRP, EC 1.11.17) was obtained from Fluka. The oxidation of carbohydrate moieties of enzyme with periodic acid (0.04 mM in 0.05 mM acetate buffer, pH 5.0) was performed according to Zaborsky and Ogletree [8]. The unreacted periodic acid was removed with 0.025 – ethylene glycol. The oxidized enzymes were dialyzed against 50 mM phosphate buffer with pH 6.0 for 18 h.

Immobilization of HRP on magnetic particle
The immobilization of HRP was performed in the following manner: 20 ml of oxidized dialytic solution of peroxidase was added to 1.0 g of absolutely dry particles. The process was implemented by constant stirring with a magnetic stirrer for 18 hours at t = 4°C, in dark.

Enzyme activity assay
The activity of peroxidase was determined using o-dianisidine. The increase in absorbance at 460 nm was measured using a Spekol 11 spectrophotometer (Zeiss, Jena, Germany). One unit of peroxidase activity was defined as that amount of enzyme which caused the decomposition of 1 μmol of hydrogen peroxide per 1 min at 25°C and at pH 7.0.

Protein assay
The total protein contents of the immobilized cells was determined by the modified method of Lowry according to Schacterlee et al. [9] using bovine serum albumin (Sigma Co) as a standard protein.

Determination of the pH and temperature optima
The residual activities of soluble and immobilized enzymes were determined in 0,01mM phosphate buffer (for the range of pH 5.0 – 7.5) at 25°C and for the temperature range of 25 – 40°C at pH 6.0.

Determination of Michaelis' constants
The Michaelis' constants, K_m, of native and immobilized enzymes were determined at increasing substrate concentrations in 0.01 mM phosphate buffer at the optimal pH (0.05 – 1.5 mM hydrogen peroxide). The Km values were calculated using OriginPro (OriginLab Data analysis and graphing software).

3. RESULTS AND DISCUSSION

The copolymer obtained according to the methodological part has a spherical shape with magnetic properties and average diameter of 0.8 mm (Fig.1) The magnetic particles have a high magnetic stability which is a necessary condition to obtain the desired shape and size relevant to the requirements of the experiment. The mechanical robustness of the particles was preserved throughout the process.

The magnetic particles coated with copolymer were used as a matrix for covalent binding of the peroxidase.

Fig.1 Picture of received magnetic particle

The results from the investigations on the activity of the free and immobilized peroxidase at pH = 6 are shown in Table 1.

Table 1. Investigation on the activity of the free and immobilized enzyme

Enzyme	Specific activity U/mg	Relative activity %	Amount of bound protein mg/g dry carrier
Soluble enzyme	210	-	-
Immobilized enzyme	181	86	0,613

It can be seen from the table that the enzyme preserves its high relative activity.

The pH optima of free and immobilized peroxidase were performed following the methodological part and are given in Fig. 2. When examining the effects related to the pH optimum the effect of the matrices on the enzyme should be considered. In this case the pH optimum increases to 7.0. This change occurs due to the irregular distribution of the protons caused by the positive or negative loads of the employed carrier as well as the presence of diffusion limitations. In this case it can be assumed that the magnetic particles affect the enzyme molecules.

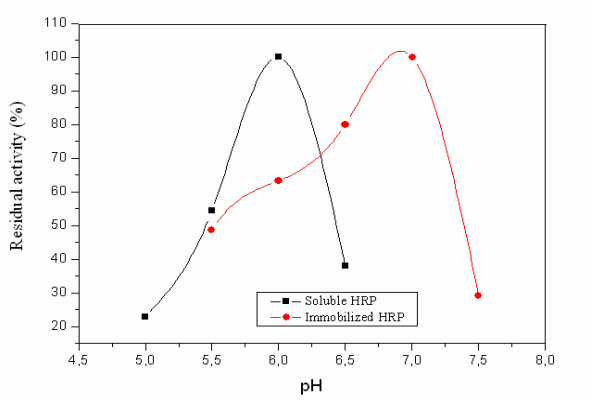

Fig. 2 Effect of pH on the activity of soluble and immobilised peroxidase

The experiments on the temperature optimum also showed a shift of the optimum of the immobilized enzyme at 30°C (Fig. 3).

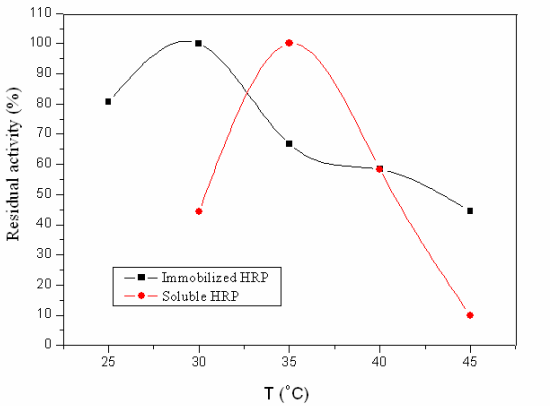

Fig. 3 Effect of temperature on soluble and immobilised peroxidase

The immobilization of enzymes to different carriers usually leads to a change in their kinetic behaviour characterized by different constants, the most important being the Michaelis constant (Km).

Table 2 shows a comparison between the kinetic parameters of soluble and immobilized peroxidase and on magnetic particles.

Table 2 Kinetic parameters of soluble and immobilized peroxidase

Enzyme	Km [M]	Vm [M.10^{-6}/sec]	R^2
Soluble enzyme	1.19x10^{-3}	-	-
Immobilized enzyme	2.3x10^{-4}	0.0517	0.99885

In an investigation similar to ours, the following values for Km of free and immobilized peroxidise, respectively 5.5 and 3.6 mM were obtained in immobilization on chitosan. The results from this investigation show that the immobilized enzyme has a greater affinity to the substrate compared to the free enzyme [10].

4. CONCLUSIONS

Magnetic particles coated with copolymer of acrylamide and acrylonitrile were successfully obtained. The peroxidase immobilized on these particles preserves its high relative activity. It was established that the catalytic parameters of the immobilized peroxidase change as follows: t_{opt} – 30°C, pH_{opt} – 7 и Km – $2.3x10^{-4}$. Based on the results obtained from the covalent immobilization of peroxidase to magnetic particles coated with copolymer of acrylamide and acrylonitrile we can outline the steps in construction of a biosensor for mycotoxins.

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