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IMMOBILIZATION OF TYRAMINE-HRP ONTO TYRAMIDE-CARBOXYMETHYL CELLULOSE MATRIX FOR WASTEWATER TREATMENT

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Abstract

Horseradish peroxidase (HRP, E.C. 1.11.1.7) catalyzes oxidation of aqueous aromatic compounds using hydrogen peroxide. Enzymatic treatment methods for phenol removal from wastewaters has become an efficient and environmentally friendly alternative for the traditional methods. Carboxymethyl cellulose (CMC) derivative with tyramine attached via amide bond to carboxyl groups has been chosen as carrier for immobilization. In effort to overcome the main disadvantage of entrapment immobilization method, loss of enzyme activity due to washing out from the carrier, HRP was modified in a reductive amination reaction and tyramine was bound to the enzyme. Immobilization of tyramine-HRP onto tyramide-carboxymethyl cellulose carrier was carried in an emulsion polymerization reaction that produced carboxymethyl cellulose microbeads. The highest specific activity of the obtained biocatalyst was 0.227 U/ml and after 48h of storage 0.197 U/ml. Immobilized tyramine-HRP retained 87% of activity after 48 h. Immobilized HRP is a suitable candidate for wastewater treatment.

Keywords: immobilization, HRP, carboxymethyl cellulose, tyramine, phenol removal

INTRODUCTION

Aromatic compounds that are found in wastewaters, like phenols and its derivatives, are dangerous for the environment due to their toxicity and suspected carcinogenicity [1]. Methods for their removal such as microbial degradation, chemical oxidation, activated carbon adsorption and solvent extraction, have been thoroughly investigated. Some disadvantages of the traditional methods like low efficiency, extreme reaction conditions, high cost or generation of even more toxic products could be overcome using enzymatic treatment [2]. Peroxidases, like horseradish peroxidase (HRP) or soybean peroxidase (SBP), have been successfully used for enzymatic removal of phenolic compounds from aqueous solutions [3]. Advantages of enzyme-based treatment methods are high specificity and low energy requirements. Enzymes operate over wide range of conditions and have minimal environmental impact [4].

HRP catalyzes oxidation of aromatic compounds in the presence of hydrogen peroxide producing free radicals that polymerize to produce high molecular weight polymers with low

solubility and easy to separate from the solution [4]. Although enzymes proved to be efficient biocatalysts, their wider application is limited due to high cost of their purification and inability to retrieve them from the solution after treatment. Enzyme immobilization onto solid carriers like cellulose provides some improved characteristics like stability, reusability, improved performance in organic solvents, pH tolerance, heat stability and selectivity [5]. Cellulose, as constituent of the primary plant cell wall, is a common biopolymer and an excellent source of renewable polymeric material. Like all natural polymers it is nontoxic, biocompatible and biodegradable, therefore an ideal carrier for enzyme immobilization [6,7]. A major drawback to entrapment method of enzyme immobilization is a so called "leaking" (or washing out) of the enzyme from the carrier followed by reduced specific activity [8]. Carboxymethyl cellulose (CMC), a low cost, water-soluble cellulose derivative, has been conjugated with tyramine using carbodiimide activation reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in the presence of N-Hydroxysulfosuccinimide (NHS) [9]. Obtained tyramide-CMC was suitable material for hydrogel formation through enzyme catalyzed covalent crosslinking and an ideal carrier for HRP immobilization.

In our previous work we immobilized peroxidase onto tyramine modified natural polysaccharides like alginate and pectin. In this work our goal was to reduce enzyme leakage from the carrier so we used a similar procedure to modify the enzyme and immobilized tyramine modified HRP onto tyramide-carboxymethyl cellulose polymer synthesized as previously described by Ogushi *et al.* [9]. HRP was first oxidized with sodium periodate and tyramine was covalently bound to a glycosidic side chain. Specific activity was measured 1 h, 24 h and 48 h after immobilization, in order to determine the stability and enzyme leakage from the matrix.

MATERIALS AND METHODS

Material

Horseradih peroxidase, carboxymethyl cellulose, glucose oxidase (160 U/mg), triton X-100 (t-octylphenoxypolyethoxyethanol), tyramine hydrochloride, N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), mineral oil and span 80 (sorbitan monooleate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium cyanoborohydride was obtaineed from Fluka (Buchs, Switzerland). TRIS was obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany). Glucose was purchased from Zorka Pharma (Šabac, Serbia).

Modification of HRP

Horseradish peroxidase was dissolved in 50 mM sodium carbonate buffer pH 8.0 to a final concentration of 0.5 mg/ml. Two hundred microlitres of 50 mM sodium periodate was added into 2 ml of buffered HRP solution and incubated in the dark for 6 h at 4 °C with occasional shaking. Reaction was stopped by adding 20 μl of 99% glycerol, left for another 30 min at 4 °C and afterwards dialyzed against 50 mM sodium carbonate buffer pH 8.0. After dialysis, obtained HRP sample volume was measured and tyramine hydrochloride in the same buffer was added to final concentration of 50 mM and incubated for 2 h at 25 °C. Sodium

cyanoborohydride was added to final concentration of 1 mg/ml and left for 1 h at +4 °C in the dark. Solution was dialyzed against 100 mM sodium phosphate buffer pH 7.0 with minimum 3 solution changes. Finally, solution volume was measured to calculate modified enzyme concentration and absorbance of native and modified enzyme was recorded at 260 nm and 280 nm on UV-VIS spectrophotometer (Shimadzu Corporation UV-2501PC, Japan).

Immobilization of tyramine-HRP

Tyramide-carboxymethyl cellulose solution in 0.1 M Tris HCl buffer pH 7.0 was mixed with 0.1 U of glucose oxidase, 0.5 U of tyramine-HRP and 0.13 M glucose. Polymerization reaction started as soon as glucose was added so a total of 300 μl containing 2 % (w/v) tyramide-CMC was quickly poured into 600 μl of light mineral oil with 3 % Span 80 and stirred for 10 minutes on magnetic stirrer. Reaction was stoped using 1 ml of 0.5 % Triton X-100 in 5 % $CaCl_2$ solution and stirred for another 5 minutes. Immobilized tyramine-HRP onto CMC microbeads was rinsed 3 times with Triton X-100 and 3 times with $CaCl_2$ in 10 mM Tris HCl buffer pH 7.0 and stored at 4 °C.

Enzyme activity assay

Specific activity of tyramine-HRP immobilized inside tyramide-CMC microbeads was measured by adding 150 μl of 60 % (v/v) of immobilized enzyme suspension into 3 ml of 13 mM pyrogalol containing 0.1 U glucose oxidase and 0.1 M glucose. Solution was constantly stirred during reaction and absorbance was measured after 10 min at 420 nm.

RESULTS AND DISCUSSION

In effort to minimize leaking of the enzyme from the carrier after immobilization, HRP was modified to introduce tyramine functional group into glycosidic side chain. HRP is folded as a single polypeptide chain, that is glycosylated at eight specific sites [7]. Peroxidase was oxidized with sodium periodate and subsequently modified with tyramine in a reductive amination reaction. Absorbances at 260 nm and 280 nm were recorded to confirm the modification (Table 1).

Table 1 *Absorbance of 0.33 mg/ml HRP and tyramine-HRP at 260 nm and 280 nm*

Wavelenghts	$A_{260\ nm}$	$A_{280\ nm}$
HRP	0.264	0.278
Tyramine-HRP	0.247	0.292

Increased absorbance at 280 nm of tyramine-HRP compared to the native enzyme originates from tyramine since its absorbance maximum is at 275 nm [9].

Tyramine-HRP immobilization was carried out in emulsion polymerization reaction resulting in carboxymethyl cellulose microbeads formation. Tyramide-carboxymethyl cellulose was enzymatically crosslinked and HRP was covalently immobilized onto hydrogel microbeads through phenol moieties. Instead of using hydrogen peroxide, it was gradually generated in glucose oxidase reaction with glucose as substrate. Obtained specific activity of immobilized tyramine-HRP after 1, 24 and 48 h is presented in Figure 1.

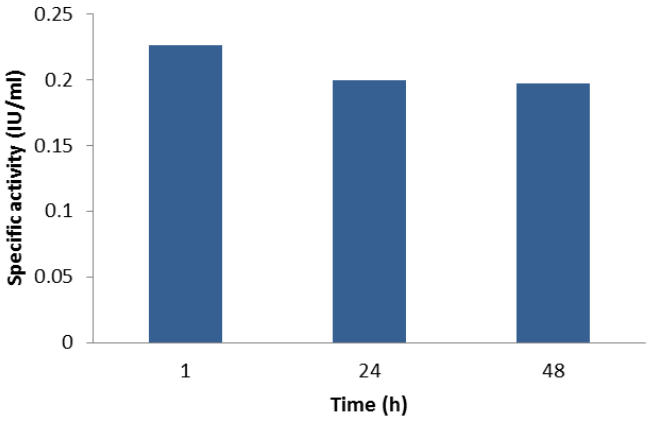

Figure 1 *Specific activity (U/ml) of immobilized tyramine-HRP versus time (h)*

Specific activity of the obtained immobilized enzyme was 0.227 U/ml immediately after immobilization. Recorded activity 24 and 48 h after immobilization, 0.200 U/ml and 0.197 U/ml respectively, showed that enzyme leakage from the carrier was reduced to 13 %.

CONCLUSION

This study showed that modified tyramine-HRP was successfully immobilized onto carboxymethyl cellulose carrier. With introduction of tyramine on the glycosidic side chain of HRP the enzyme was attached covalently to the matrix in a peroxidase catalyzed oxidation of phenolic groups in the presence of internally delivered hydrogen peroxide. Retained specific activity after 48h of storage was 87 % of the initial 0.227 U/ml. Therefore, leaking of the enzyme from the matrix has been significantly reduced due to stronger, covalent attachment of HRP inside crosslinked hydrogel polymeric network. Obtained biocatalyst has great potential for applications in wastewater treatment and phenol removal from polluted waters.

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