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PREPARATION OF CROSSLINKED TYRAMINE-ALGINATE HYDROGEL USING EDC/NHS WITH SELF-IMMOBILIZED HRP

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Abstract

Alginate is a natural polymer present in the cell wall of brown algae. Due to its many advantages, it has been used extensively in the food industry, pharmacy, and biomedicine. To enhance properties, such as stability and biodegradability, alginate is often chemically crosslinked. In this study, alginate was crosslinked using N-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and tyramine hydrochloride. Horseradish peroxidase was self-immobilized within hydrogel microbeads during the polymerization reaction. The glucose oxidase/glucose system generates H_2O_2 internally, which can prevent the detrimental effect of excess peroxide. A small amount of leaking enzyme shows potential for longer storage and reuse.

Keywords: alginate, horseradish peroxidase, chemical modification, immobilization

INTRODUCTION

Alginic acid is a natural biopolymer derived from brown algae. It is widely used as an immobilization matrix for cells, enzymes and drugs. The main advantages of alginate compared to other biopolymers are the low cost of extraction and the great potential for modification [1]. Covalent and enzymatic crosslinking is usually a method of choice to modify the mechanical properties of alginate and obtain hydrogel with high mechanical stability [2]. Hydrogels are polymeric networks with the ability to absorb large amounts of water while maintaining a 3-dimensional structure [3,4]. Horseradish peroxidase (HRP) is one of the most studied peroxidases, isolated from plants and a common model system for enzyme immobilization [5]. Immobilization of HRP onto insoluble carriers enables researchers to increase its performance, preserve its catalytic activities through several cycles, prolong its storage time, etc. [6]. HRP immobilizates can be used in wastewater remediation (removal of phenolic compounds and azo dyes), syntheses of organic compounds, as biosensors, etc. [7,8].

The aim of this study was to chemically modify alginate in a way that it can easily form hydrogel with self-immobilized HRP.

MATERIALS AND METHODS

Chemical modification of alginate

1% (w/v) alginate solution in water was prepared by dissolution of 0.25 g of sodium alginate in 25 mL distilled water. Another solution in water was made by dissolving 2.25 g tyramine hydrochloride, 0.14 g NHS (N-hydroxysuccinimide), and 0.13 g EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) in 25 mL of distilled water. Solutions were mixed and placed under constant stirring for 24 hours. After that time, the reaction mixture was dialyzed against distilled water. When dialysis was over, the solution was frozen and vacuum dried and obtained powder was stored at room temperature.

Formation of hydrogel microbeads and HRP immobilization

Dry powder of tyramine-coupled alginate was dissolved in 50 mM Tris buffer pH 7.0 at a final concentration of 2% in 0.3 mL mixture volume. 0.5 U of HRP and 3 U of glucose oxidase (GOx) were added. The polymerization reaction started with the addition of glucose solution in a final concentration of 133 mM and the mixture was rapidly poured into 0.6 mL Light Mineral Oil with 3% Span 80 detergent. The mixture was left for 15 min on a magnetic stirrer and after that time the reaction was stopped by the addition of 1 mL of 0.5% Triton X-100 in water with 0.5 mM H_2O_2. Stirring was continued for 5 minutes, and HRP-immobilized microbeads were collected and washed 4-5 times to remove any unbound enzyme as well as detergents. Supernatants were kept for the determination of unbound activity. Microbeads were stored in Tris buffer with 5mM $CaCl_2$ and left in the fridge for 24 hours.

Enzyme activity assay

HRP activity was measured in hydrogel microbeads for a specific activity and in supernatants after washing beads for unbound activity. Pyrogallol and H_2O_2 were used as substrates and absorbance was measured at 420 nm. The activity of immobilized HRP was determined with 100 μL of microbeads suspension (10%) in 3 mL of 13 mM pyrogallol solution after the addition of 30 μL of 0.97 M H_2O_2. The reaction was monitored for 15 min under constant stirring and aliquots were taken every 5 minutes. Specific activity was calculated per volume of hydrogel (U/mL). The leaching of the enzyme from tyramine-coupled alginate was measured after 24 hours. 1 unit (U) of HRP activity was defined as the amount of enzyme that produces 1 mg of purpurogallin in 20 s at 20 °C.

RESULTS AND DISCUSSION

Tyramine moieties were introduced within alginate chains, which have been confirmed with increased UV absorbance (from 260 to 280 nm) (Table 1).

Table 1 *Absorbance (at 260 nm and 280 nm) of 0.1% (w/v) polysaccharide in water*

Wavelength (nm)	260	280
Alginate	0.125	0.112
Tyramine-alginate	1.443	1.700

Modified alginate formed hydrogel microbeads in a peroxidase – induced emulsion polymerization reaction, with HRP encapsulated. Slow and continuous release of hydrogen peroxide (with glucose as a substrate for glucose oxidase) could prevent HRP substrate inactivation. The results of HRP immobilization are shown in Table 2. Obtained specific activity (Sp) of microbeads was 0.17 U/mL of suspension. Enzyme leaching after 24 hours was 0.004 U, which shows potential of the immobilizate for storage and repeated uses.

Table 2 *Immobilization parameters (specific activity, unbound activity and enzyme leaching) for HRP in tyramine-alginate microbeads*

Parameters	Sp (U/mL)	Unbound activity (U)	Leaching (U)
Immobilized HRP microbeads	0.171	0.067	0.004

CONCLUSION

This study presented modified tyramine-alginate hydrogel with EDC/NHS as the crosslinking agent. HRP was successfully self-immobilized within hydrogel microbeads. GOx with added glucose serves as an internal generator of H_2O_2, a substrate for immobilized peroxidase. The immobilized system can be stored in the fridge and reused. Further experiments are planned focused on optimization of the immobilization technique and testing the immobilized enzyme. With the introduction of tyramine onto the polymeric chains of alginate, novel charged groups within the hydrogel polymer could increase the binding of enzymes and other biomolecules, while also contributing to water solubility and changing the mechanical properties of the hydrogel. That is a favorable effect for different applications, such as drug delivery or tissue engineering.

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