Magnetic nanoparticles in biocatalysis

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Abstract. The properties of enzymatic systems based on horseradish root peroxidase immobilized on magnetic particles were studied. Magnetic Fe$_3$O$_4$ nanoparticles were synthesized by co-precipitation. Then, horseradish root peroxidase was immobilized on their surface in two ways by covalent crosslinking. For this purposes, in the first case, Fe$_3$O$_4$ was sequentially treated with tetraethoxysilane, 3-minopropyltriethoxysilane, glutaraldehyde and HRP. In the second case, before immobilization of HRP, 3-minopropyltriethoxysilane, glutaraldehyde and HRP were sequentially deposited onto the support surface. The activity of the synthesized biocatalysts was evaluated spectrophotometrically in the oxidation reaction of 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) ammonium with hydrogen peroxide. The kinetic parameters $K_m$ and $V_{\text{max}}$ were also calculated for all types of catalysts, including native HRP. Among all biocatalytic systems, the best values, compared with the native enzyme ($K_m = 4 \text{ mmol/L}$ and $V_{\text{max}} = 12.6 \cdot 10^{-4} \text{ mmol/L/s}$), were obtained for the first type of biocatalyst ($K_m = 5 \text{ mmol/L}$ and $V_{\text{max}} = 2.5 \cdot 10^{-4} \text{ mmol/L/s}$). It was also determined that the optimum pH is 7.2.

1. Introduction

In the past few decades, enzyme immobilization has been widely used in various catalytic processes [1, 2]. However, enzymes cannot be used repeatedly due to difficulties in separation from the reactants and reaction products. The immobilization of enzymes on various carriers helps to solve these problems. Inorganic carriers as adsorbents for enzymes have the advantages, such as, for example, low cost, stability in aqueous media, a wide range of morphological features. In this case, immobilization is often carried out through the formation of covalent bonds between the protein molecule of the enzyme and the carrier, and currently, this method of immobilization is one of the dominant ones [3–7]. A study of the kinetic features of various processes in the presence of immobilized enzymes is an integral part of the study of the mechanisms of reactions [8]. Also, immobilized enzymes, in addition to good stability, are able to exhibit high activity [9].

The immobilization of enzymes in or on insoluble carriers is advantageous for practical use due to its ease of use, ease of isolation of enzymes from the reaction mixture, and the possibility of reuse [10].

Currently, researchers' interest in nanoscale technologies has led to the creation of a huge variety of nanoparticles with biocompatible surfaces for immobilizing enzymes. Many enzymes currently used in biotechnology were covalently immobilized on magnetic nanoparticles using various crosslinking agents [11].

This article synthesized biocatalytic systems based on horseradish root peroxidase (HRP) covalently immobilized on magnetic Fe$_3$O$_4$ nanoparticles. The latter were synthesized by coprecipitation. The use of magnetic Fe$_3$O$_4$ nanoparticles as carriers for enzyme systems is due to the simplicity of separating the synthesized heterogeneous biocatalyst from the reaction mixture using a neodymium magnet [12, 13].
Additional advantages of using nanoparticles can also include their nanoscale, due to which a heterogeneous system can approach a homogeneous one [14]. Glutaraldehyde was used as a crosslinking agent. Crosslinking of the enzyme with the carrier using this linker proceeds with the formation of Schiff bases. Aldehyde groups located at the ends of asymmetric crosslinking agent, reacting with NH2 groups, form azomethine bonds. The advantage of covalent immobilization of enzymes using this method lies in its simplicity [15, 16]. The optimal pH value was also selected for the oxidation of 2,2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) in the presence of synthesized biocatalysts.

2. Materials and methods

2.1. Materials
FeCl₃·6H₂O (99 wt.%), FeSO₄·7H₂O (99 wt.%), NaOH (98 wt.%), tetraethyl orthosilicate (TEOS, 99%), glutaraldehyde solution (GA, 25%), peroxidase (150 ed/mg, Sigma), ethanol (95 wt.%), 3-aminopropyl triethoxysilane (>98 wt.%), peroxidase (Great Britain, RZ > 2.0, act. > 150 u/mg, demineralized, lyophile powder).

2.2. Synthesis of Magnetic Nanoparticles
A solution of a mixture of iron salts, using a burette, was added dropwise to a solution of NaOH (1.5 M), with constant stirring on a magnetic stirrer. The resulting black Fe₃O₄ precipitate was separated from the reaction medium using a neodymium magnet. Subsequently, the precipitate was washed with water to a neutral pH. Then the mixture was dispersed under ultrasound (5 min).

2.3. Synthesis of biocatalytic systems
Two biocatalysts based on immobilized HRP were synthesized in the work. The first was synthesized using 3-aminopropyltriethoxysilane (APTS). To the obtained nanoparticles (0.5 g) were added 100 ml of ethanol, 1 ml of water and 0.2 ml of APTS. The mixture was stirred for 5 hours on a magnetic stirrer. Then the solution was washed several times with distilled water. Modified Fe₃O₄ was activated using glutaraldehyde (GA). For this, 20 ml of water and 1 ml of GA were added to the resulting suspension. The modified and activated carrier was washed several times with phosphate buffer (pH 6.0) and then treated with 20 ml of HRP solution (0.001 g HRP in 20 ml of phosphate buffer (pH 6.0)). The resulting biocatalyst was designated as Fe₃O₄/APTS/GA/HRP.

A second biocatalyst was synthesized using tetraethoxysilane (TEOS). To the resulting nanoparticle mixture (0.5 g), 1 ml of TEOS was added dropwise with mechanical stirring. The resulting suspension was allowed to stir for 3 hours. After that, the resulting solution was washed 5 times with distilled water and ethanol. Then the carrier was modified and activated using APTS, GA, and HRP according to the procedure described above. The resulting biocatalyst was designated as Fe₃O₄/SiO₂/APTS/GA/HRP.

2.4. Kinetic experiments
Kinetic experiments were performed spectrophotometrically. The resulting biocatalytic systems and native HRP were tested in the oxidation reaction of ABTS using hydrogen peroxide. The reaction was observed by increasing the optical density of the ABTS oxidation product (λ = 415 nm). At various initial substrate concentrations (from 0.02 to 0.00125 M), the initial oxidation rate (V₀) was determined. The maximum rate Vₘₐₓ and the Michaelis constant Kₘ were determined using different initial substrate concentrations and fixed concentrations of the enzyme and hydrogen peroxide, since the enzymatic reaction is most fully characterized at the initial time until inhibition by the reaction product occurs [17]. To find the kinetic parameters, the Lineweaver-Burk method was used, according to which a graph of the dependence of 1/V on 1/[S] is constructed. The resulting straight line intersects the ordinate axis at point 1/Vₘₐₓ and the abscissas axis at point 1/Kₘ. The angle of the obtained straight line is determined by Kₘ/Vₘₐₓ.
The amount of catalyst that is used to obtain 1 mg of the target product in 20 s was taken per unit of activity.

To assess the effect of pH, a series of experiments was carried out with different pH values of the phosphate buffer (6.0–7.5).

3. Results and discussion

To determine the kinetic parameters of the Michaelis-Menten equation, experiments were carried out at various initial substrate concentrations. The parameters \( K_m \) and \( V_{\text{max}} \) for all types of biocatalysts, including native peroxidase, were determined according to the Lineweaver-Burke graph presented in Table 1.

**Table 1. Kinetic Parameters.**

| Biocatalyst                  | \( V_{\text{max}} \) \( \times 10^{-4} \), mol/L·s | \( K_m \), mmol/L |
|------------------------------|-----------------------------------------------|-------------------|
| HPR                          | 12.6                                          | 4                 |
| \( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HPR} \) | 2.5                                            | 5                 |
| \( \text{Fe}_3\text{O}_4/\text{APTS/GA/HPR} \)    | 2.0                                            | 6                 |

Table 1 shows that native HRP has the highest affinity for the substrate, since the lowest \( K_m \) value (4 mmol/L) is achieved with it. Also, using a free enzyme, the maximum reaction rate is \( 12.6 \times 10^{-4} \), mmol/L·s. When using \( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HPR} \) as a catalyst, \( K_m \) increased to 5 mmol / L, and \( V_{\text{max}} = 2.5 \times 10^{-4} \), mmol/L·s. The lowest affinity for the substrate was shown by the \( \text{Fe}_3\text{O}_4/\text{APTS/GA/HPR} \) biocatalyst; for it, the \( K_m \) value was 6 mmol/L, with \( V_{\text{max}} = 2.0 \times 10^{-4} \), mmol/L·s. The low kinetic parameters and activity of biocatalysts based on an immobilized enzyme (\( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HRP} \) and \( \text{Fe}_3\text{O}_4/\text{APTS/GA/HPR} \)) compared to the native (HRP) are associated with conformational changes in the HRP protein molecule due to its immobilization and lower the amount of immobilized peroxidase on the media.

With the \( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HRP} \) biocatalyst, which showed greater activity compared to \( \text{Fe}_3\text{O}_4/\text{APTS/GA/HPR} \), experiments were carried out to determine the optimal pH value (figure 1).

![Figure 1](image-url)  
*Figure 1. The dependence of optical density on time with different pH*

Figure 1 shows that the optimal pH is 7.2. At this value, the \( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HRP} \) biocatalyst showed the best results on the oxidation of ABTS.

Experiments were conducted on repeated use of the covalently immobilized \( \text{Fe}_3\text{O}_4/\text{SiO}_2/\text{APTS/GA/HRP} \) system in successive reactions, which showed that the activity of the system...
decreases with each subsequent cycle by no more than 2-3 %, which makes it possible to effectively and repeatedly use it.

4. Conclusion
Both samples of synthesized biocatalysts showed high activity in the oxidation of ABTS. However, a higher catalytic effect, in comparison with the native HRP, was shown by the Fe₃O₄/SiO₂/APTS/GA/HRP sample. The following kinetic parameters were determined for it: \( V_{\text{max}} = 2.5 \times 10^{-4} \text{ mmol/L} \cdot \text{s} \), and \( K_m = 5 \text{ mmol/L} \). These values were slightly worse compared to the native HRP (\( V_{\text{max}} = 12.6 \times 10^{-4} \text{ mmol/L} \cdot \text{s} \), and \( K_m = 4 \text{ mmol/L} \)). However, the enzyme becomes heterogeneous after immobilization, which allows it to be separated from the reaction mixture and reused in subsequent experiments with virtually no loss of activity. The optimum pH of the ABTS oxidation process in the presence of Fe₃O₄/SiO₂/APTS/GA/HRP with hydrogen peroxide was 7.2. The synthesized biocatalysts can be successfully used in the oxidation of phenolic compounds.

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