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# ПОЛИМЕРНЫЕ НОСИТЕЛИ ДЛЯ ИММОБИЛИЗАЦИИ ФЕРМЕНТОВ В СИНТЕЗЕ БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ

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***В работе представлен синтез биокатализаторов на основе пероксидазы корня хрена, иммобилизованной на коммерчески доступные полимерные носители: сверхсшитый полистирол марки MN-100 и Sepabeads EC-HA. Иммобилизация осуществлялась путем ковалентной сшивки фермента с носителем при помощи глутарового альдегида. Глутаровый альдегид способен образовывать основания Шиффа, которые обеспечивают прочную связь между ферментом и носителем. Установлено, что оптимальное количество глутарового альдегида для ковалентного связывания HRP с поверхностью коммерческого носителя составляет 0,2 г/л. Представленные в работе биокатализаторы пероксидаза/MN-100 и пероксидаза/Sepabeads EC-HA проявили хорошую активность в процессе окисления 2-метилнафтола до 2-метил-1,4-нафтогидрохинона (витамин К4). Биокатализатор на основе MN-100 показал более высокую активность по сравнению с биокатализатором Sepabeads EC-HA, что, вероятно, связано с различной структурой поверхности исходных полимерных подложек. Образцы сохраняли свою активность при повторных применениях в десяти повторных случаях. Высокая стабильность биокатализаторов пероксидаза/MN-100 и пероксидаза/Sepabeads EC-HA объясняется высокой сорбционной способностью коммерческих полимерных носителей MN-100 и Sepabeads EC HA и образованием прочных ковалентных связей между ферментом и носителем. Были также определены оптимальные условия окисления 2-метилнафтола до 2-метил-1,4-нафтогидрохинона с использованием синтезированных биокаталитических систем. В работе подобраны оптимальные условия для окисления предложенного субстрата: температура 40 °С и pH 7,2. В качестве окислителя был использован недорогой и экологически безопасный пероксид водорода. Представленные результаты, несомненно, внесут положительный вклад в развитие химической и фармацевтической промышленности.***

**Ключевые слова:** Ферменты, иммобилизация, полимерные носители, окисление 2-метил-1-нафтола, витамин К4, возможность повторного использования

# POLYMERIC SUPPORTS FOR ENZYMES IMMOBILIZATION IN SYNTHESIS OF BIOLOGICALLY ACTIVE COMPOUNDS

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***The report presents the synthesis of biocatalysts based on horseradish peroxidase immobilized on commercially available polymeric supports: hyper cross-linked polystyrene MN-100 and Sepabeads EC-HA. The immobilization was carried out by covalent crosslinking of the enzyme with the support using glutaraldehyde. The optimal amount of glutaraldehyde for covalent binding of HRP was found to be 0.2 g/l. The peroxidase/MN-100 and peroxidase/Sepabeads EC-HA biocatalysts presented in the work showed good activity in the oxidation of 2-methylnaphthol to 2 methyl-1,4-naphthohydroquinone (vitamin K4). The biocatalyst based on MN-100 showed higher activity compared to the biocatalyst based on Sepabeads EC-HA, which is likely due to the different surface structure of the original polymer supports. The samples retained their activity in ten consecutive reuses. The high reusability of peroxidase/MN-100 and peroxidase/Sepabeads EC-HA is explained by the high sorption ability of commercial polymer supports MN-100 and Sepabeads EC-HA and the formation of strong covalent bonds between the enzyme and the support. The optimal conditions for the oxidation of 2-methylnaphthol to 2-methyl-1,4-naphthohydroquinone using synthesized biocatalytic systems were also selected. The temperature of 40 °C and pH 7.2 were found to be optimal for the oxidation of the proposed substrate. The presented results will undoubtedly make a positive contribution to the development of the chemical and pharmaceutical industry.***

**Key words:** enzymes, immobilization, polymer supports, oxidation of 2-methyl-1-naphthol, vitamin K4, reusability

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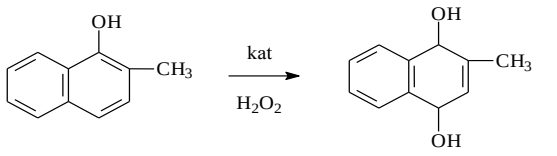
## INTRODUCTION

Currently, one of the most studied enzymes used in biocatalysis is horseradish peroxidase (HRP; EC 1.11.1.7), a representative of the oxidoreductase class. Particular attention is paid to HRP due to its high activity and selectivity, high resistance to inhibitory substances, high efficiency in a wide range of reaction conditions [1-6]. However, like most other HRP enzymes, it has disadvantages such as low stability, short life, high price and difficulty in reuse, which limits the commercial use of HRP. To eliminate such shortcomings in biocatalysis, enzyme immobilization on various support is used, which allows improving their activity and stability, use enzymes in more severe conditions and separate the biocatalyst from the reaction products [7-9].

Macro- and microporous polymers with a large internal surface and the ability to swell in a liquid medium can be used as promising supports for the sorption immobilization of enzymes [7, 10-12]. In this report, biocatalytic systems based on HRP immobilized on commercial polymer supports with functional amino groups (hypercross-linked polystyrene of MN-100 (MN 100) and Sepabeads EC-HA) were synthesized. The first support is a three-dimensional polymer network consisting of benzene rings crosslinked by methylene bridges [13] with a hierarchical structure containing all types of pores and a specific surface area of 1100 $m^2/g$. Sepabeads EC-HA is a polymeric support based on a rigid methacrylic matrix. The specific surface of this type of support is characterized by a macroporous structure and a low surface area (80 $m^2/g$), compared with MN-100. Both supports possess high mechanical and chemical stability and can be promising supports for enzymes, since there are functional amino groups on the surface [12, 14-15]. The biocatalytic properties of the synthesized systems were evaluated in the oxidation reaction of 2-methyl-1-naphthol using hydrogen peroxide as an oxidizing agent:




2-methyl-1-naphtol 2-methyl-1,4-naphtohydroquinor

This reaction can serve as an alternative method for the synthesis of vitamin K4 (2-methyl-1,4-naphthohydroquinone).

## EXPERIMENTAL PART

### *Materials*

The following materials were used in the study: Macronet MN-100 hyper cross-linked polystyrene (MN-100) (Purolite Int., Great Britain), Sepabeads EC-HA (Resindion SRL, Italy), $H_2O_2$ (35%, Reakhim,

Russia), HRP (RZ > 2.0 , 150 Un./mg, Sigma Aldrich, USA), glutaraldehyde (~25% in water, Fluka, Switzerland), Phosphate buffer ($KH_2PO_4$ (Nevareactive, Russia), $Na_2HPO_4$ (Nevareactive, Russia)) , Pyrogallol (Nevareactive, Russia), 2-methyl-1-naphthol, 2-methyl-1,4-naphthoquinone (Sigma Aldrich, USA).

*Synthesis of biocatalysts*

Polymer supports MN-100 and Sepabeads EC-HA containing amino functional groups were initially modified with glutaraldehyde (GA). For this, 1 g of the support was stirred for 24 h with 50 ml of GA of various concentrations (from 0.1 to 0.4 g/l). The modified support was filtered and washed several times with water to pH of 7.0. The resulting support was stirred for 6 hours with 0.15 g HRP in 10 ml of phosphate buffer. Ready samples of biocatalysts were filtered off, washed and dried under vacuum at 25 °C. Scheme of the biocatalyst synthesis is shown in Fig. 1.

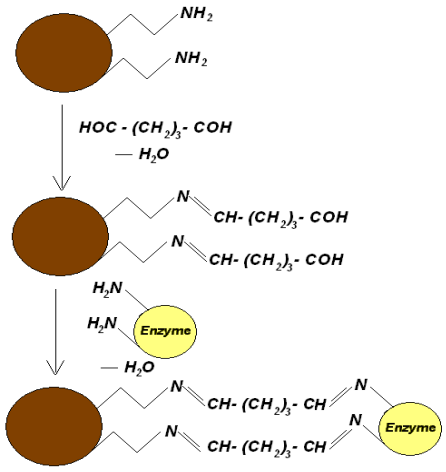



Fig. 1. Scheme of the biocatalyst synthesis by the horseradish peroxidase immobilization on a polymer support

Рис. 1. Схема синтеза биокатализатора иммобилизацией пероксидазы хрена на полимерной подложке

To calculate the degree of immobilization, the values of the activity of native HRP, immobilized HRP, and the filtrate containing residues of an immobilized enzyme were determined. HRP activity was determined by the intensity of purpurogallin formation in the reaction of pyrogallol oxidation with hydrogen peroxide. The intensity of the formation of purpurogallin was measured spectrophotometrically at a wavelength of 420 nm.

The degree of immobilization of HRP was calculated as the ratio of the activity of the immobilized enzyme to the difference between the activities of the native enzyme and the filtrate.

The amount of HRP attached was evaluated determining the immobilization coefficient (IC) [16]:

$$IC = (C_0\text{-}C_1)/C_0 \cdot 100\%, \quad (1)$$

where $C_0$ is the initial HRP activity and $C_1$ is the HRP activity in the filtrate after the immobilization and the biocatalyst separation.

*Oxidation of 2-methyl-1-naphthol*

The oxidation reaction was carried out in a thermostatic glass reactor. The biocatalyst (0.15 g) and an aqueous solution of a substrate of a given concentration (30 ml) were loaded in a reactor. The feed rate of hydrogen peroxide was controlled by a pump. The required pH value was maintained using phosphate buffer. Samples of the reaction mixture were periodically taken for analysis. At the end of each experiment, the catalyst was separated by filtration.

The analysis of the reaction mixture was performed by high performance liquid chromatography (HPLC). Ultimate 3000 (Dionex) HPLC system equipped with an ultraviolet detector, an API-2000 mass spectrometer (Applied Biosystems), Luna C18 analytical column (7 μm) with a theoretical number of plates of 40,000 and a size of 150×4 mm was used in the analysis. The solution of acetonitrile: water in a ratio of 50:50 was used as the mobile phase. The eluent flow rate was 0.5 ml/min at 7 MPa and 30 °C. Detection was carried out by a UV detector at a wavelength of 254 nm. Additional products and intermediate reaction products were not found in the reaction mixture, which can be explained by the high substrate specificity of HRP.

The activity of biocatalysts was evaluated by the conversion of 2-methyl-1-naphthol in the catalytic reaction of its oxidation with hydrogen peroxide. The values of the relative rate correspond to 20% conversion of 2-methyl-1-naphthol (W20%, g/l×s×g (cat)).

## RESULTS AND DISCUSSION

*HRP immobilization on MN-100 and Sepabeads EC-HA*

Commercial polymeric supports of MN-100 and Sepabeads EC-HA initially have functional amino groups [12, 17-18], which are necessary for covalent immobilization of HRP. To modify their surface, a crosslinking agent (linker) was applied. In this investigation, the linker is glutaraldehyde, which is widely used to immobilize enzymes. Crosslinking of the enzyme with the support using this linker proceeds through the formation of a Schiff base. Aldehyde groups located at the ends of a symmetric crosslinking agent, reacting with amino groups, form azomethine bonds:

$$\underset{\text{Chitosan}}{R\text{-}NH_2} + \underset{\text{Glutaraldehyde}}{HOC\text{-}(CH_2)_3\text{-}COH} + \underset{\text{Enzyme}}{NH_2\text{-}E} \longrightarrow$$

$$\longrightarrow \underbrace{R\text{-}N=CH\text{-}}_{\text{Azomethine bond}}(CH_2)_3\underbrace{\text{-}CH=N\text{-}E}_{\text{Azomethine bond}}$$

The advantage of covalent immobilization of enzymes using this method lies in its simplicity [19-20].

To assess the influence of linker concentration on the activity of immobilized HRP, a series of experiments was conducted to optimize the concentration of glutaraldehyde. For this, samples of SPS supports MN-100 and Sepabeads EC-HA were treated with a GA solution of various concentrations (0.1, 0.2, 0.3, 0.4 g/L).

According to the data obtained, the value of the relative rate of 2-methylnaphthol oxidation was calculated depending on the concentration of glutaraldehyde (Table). Conditions: C(kat) = 5 g/l, C(2-methyl-1-naphthol) = 55 g/l, t = 35 °C, pH 6.0, $C(H_2O_2)$ = 0.2 mol/l.

***Table***

**Dependence the relative rate on the glutaraldehyde concentration**

***Таблица.* Зависимость относительной скорости от концентрации глутаральдегида**

| C(GA), g/l | W 20%, g(methylnaphthol)/(g(cat)·s) | |
|---|---|---|
| | MN-100 | Sepabeads EC-HA |
| 0.1 | 0.175 | 0.140 |
| 0.2 | 0.233 | 0.175 |
| 0.3 | 0.175 | 0.140 |
| 0.4 | 0.127 | 0.116 |

Table shows that the highest oxidation relative rate of 2-methyl-1-naphthol for HRP/MN-100 (0.233) and HRP/Sepabeads EC-HA (0.175) is observed in the samples treated with glutaraldehyde of 0.2 g/L concentration. Moreover, the samples based on HRP immobilized on MN-100 showed the highest activity. It was found that the degree of immobilization for HRP/MN-100 is 20%, while for HRP/Sepabeads EC-HA the one is 17%. Further experiments were performed using Sepabeads EC-HA and MN-100 samples treated with 0.2 g/l sol rum glutaraldehyde.

*Effect of pH on the oxidation of 2-methyl-1-naphthol*

To study the influence of pH on the oxidation process of 2-methyl-1-naphthol, the process was carried out at varying pH from 4.2 to 9.2 (Fig. 2). The reaction was carried out under the following conditions: C(kat) = 5 g/l, C(2-methyl-1-naphthol) = 55 g/l, t = 35 °C, $C(H_2O_2)$ = 0.2 mol/l.

The maximum value of the relative rate (0.26 and 0.175 g (2-methylnaphthol)/(g(cat)·s)) is observed at pH 7.2 for the HRP/MN-100 and HRP/Sepabeads EC-HA biocatalytic systems, accordingly. At pH values below 6 and above 8 the oxidation rate decreases slightly. The pH 7.2, which provides the highest activity of biocatalysts, was used in all further experiments.

*Effect of reaction temperature*

To determine the temperature dependence of the process.

To assess the temperature influence on the activities of the biocatalysts, experiments were carried out a temperature varying from 20 to 50 °C in increments of 5 °C (C(kat) = 5 g/l, C(2-methyl-1-naphthol) = 55 g/l, pH 7.2, $C(H_2O_2)$ = 0.2 mol/l). Fig. 3 shows that the activity of biocatalytic systems increases to a temperature of 40 °C (0.26 and 0.175 g (2-methylnaphthol)/(g(cat)·s)), after which the relative rate decreases (to 0.09 and 0.16 g (2-methylnaphthol/(g(cat)·s) for HRP/MN-100 and HRP/Sepabeads EC-HA samples, accordingly). It may be associated with the denaturation of the protein molecule of the enzyme at high temperatures [21].

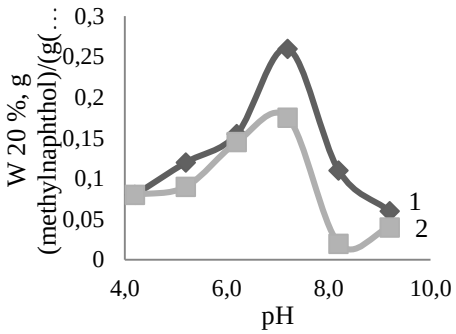



Fig. 2. Dependence the relative rate on the pH: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

Рис. 2. Зависимость относительной скорости от pH: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

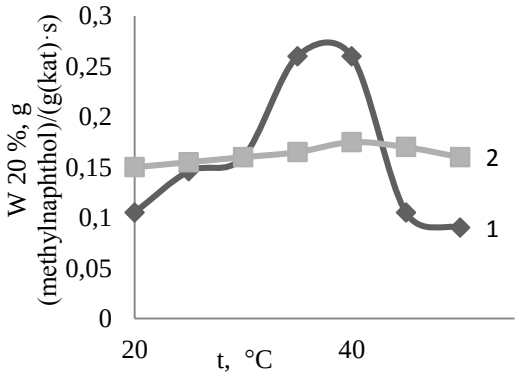



Fig. 3. Dependence the relative rate on the temperature: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

Рис. 3. Зависимость относительной скорости от температуры: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

A temperature of 40 °C was chosen for all the further experiments. It should be noted that the HRP Sepabeads EC-HA biocatalyst proved to be more stable at 45 and 50 °C, losing about 10-15% of activity with the temperature increase. At the same time, the HRP/MN-100 biocatalyst lost more than 60% of its activity. This difference in stability as a function of tem-

perature is probably related to the structure of the polymer systems and the different configuration of the enzyme on their surface.

*Biocatalyst stability in repeated experiments*

To test stability of biocatalysts in the repeated use, we carried out ten successive experiments of the 2-methylnaphthol oxidation in the optimal conditions (C(kat) = 5 g/l, C(2-methyl-1-naphthol) = 55 g/l, t = 40 °C, pH 7.2, $C(H_2O_2)$ = 0.2 mol/l).

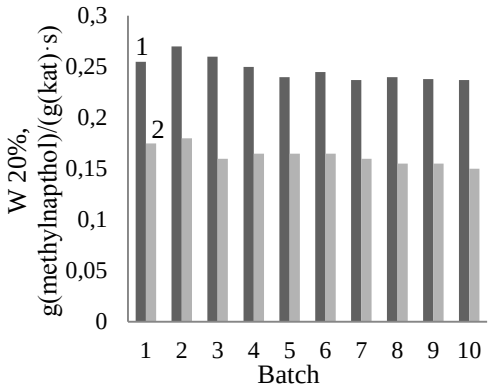



Fig. 4. Stability of immobilized HRP in ten cycles: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

Рис. 4. Стабильность иммобилизованной HRP за десять циклов: 1) HRP / MN-100; 2) HRP / Sepabeads EC-HA

Fig. 4 shows that the activity of HRP/MN-100 and HRP/Sepabeads EC-HA remains practically unchanged for ten consecutive cycles. Such reusability may be due to the stability of the covalent bond of the enzyme with commercial supports MN-100 and Sepabeads EC-HA. From all the presented experiments, it can be seen that the HRP/MN-100 biocatalyst is more active than HRP/Sepabeads EC-HA, which is probably due to the different surface structure of the initial polymer supports.

## CONCLUSION

The biocatalytic systems described in this report based on HRP immobilized on commercial supports MN-100 and Sepabeads EC-HA showed good activity in the process of the oxidation of 2-methylnaphthol to 2-methyl-1,4-naphthohydroquinone (vitamin K4). Moreover, the biocatalyst based on MN-100 showed higher activity compared to the biocatalyst based on Sepabeads EC-HA, which is likely due to the different surface structure of the original polymer supports. It should also be noted that the samples retained their activity in the repeated using in ten cycles. The high reusability of HRP/MN-100 and HRP/Sepabeads EC-HA is explained by the high sorption ability of commercial polymer supports MN-100 and Sepabeads EC-HA and the formation of strong covalent bonds between the enzyme and the support. The synthesized biocatalysts can be successfully used in the processes of obtaining biologically active compounds, as alternative environmentally friendly catalysts for the synthesis of vitamin K4. The presented results will undoubtedly make a positive contribution to the development of the chemical and pharmaceutical industry.

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