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RECOMBINANT HORSERADISH PEROXIDASE FOR ANALYTICAL APPLICATIONS

A. M. EGOROV[1, 2], V. G. GRIGORENKO[1], I. P. ANDREEVA[1, 3], M. YU. RUBTSOVA[1]

[1]Laboratory of Enzyme Engineering, Chemical Department of Lomonosov Moscow State University, Moscow, Russia
[2]Microbiology Dept., Russian Medical Academy of Postgraduate Education, Moscow, Russia
[3]Immunotek of MSU, Moscow, Russia

E-mail: vitaly.grigorenko@gmail.com

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The article deals with prospects of using recombinant horseradish peroxidase in analytical biochemistry and biotechnology. Problems of recombinant horseradish peroxidase cloning in different expression systems, possible approaches to their solution, advantages of recombinant recombinant horseradish peroxidase and recombinant horseradish peroxidase-fusion proteins for immunoassays are considered. Possibility for development of mediatorless bienzyme biosensor for peroxide and metabolites, yielding hydrogen peroxide during their transformations, based on co-adsorption of recombinant horseradish peroxidase and the appropriate oxidase was demonstrated. The possibility to produce a fully active recombinant conjugate of recombinant horseradish peroxidase with human heart-type fatty acid binding protein, which may be used in competitive immunoassay for clinical diagnosis of acute myocardial infarction, and recombinant conjugates (N- and C-terminus) of recombinant horseradish peroxidase with Fab-fragments of the antibody against atrazine, which may be applied for atrazine pesticides detection, are demonstrated for the first time.

Key words: recombinant horseradish peroxidase, fusion proteins, immunoassays, mediatorless bienzyme biosensor, Fab-fragments of antibody, heart-type fatty acid-binding protein, atrazine pesticides.

Horseradish peroxidase isoenzyme C (HRP, EC 1.11.1.7), a heme- and Ca^{2+}-containing glycoprotein, is a member of the superfamily of plant peroxidases [1–3] that are able to utilize hydrogen peroxide to catalyze the one electron oxidation of a wide variety of organic and inorganic substrates. Whereas plant peroxidases find interest for applications in various biotechnological processes, like bleaching and degradation of organic compounds (e.g. phenols and lignin), HRP specifically is used in analytical biochemistry and biotechnology as a marker enzyme for antibodies, DNA and low molecular mass analytes. Broad substrate specificity and high catalytic activity and stability determined the world-wide application of HRP in bio- and immunosensors, chemiluminescent, fluorescent and electrochemical detection systems, DNA microarrays and biochips with HRP-based colorimetric detection [4–6, 14, 56].

Progress made in peroxidase gene heterologous expression opened up the prospect to study structure-function relationships by means of genetic engineering. The baculovirus expression system allowed production of recombinant HRP in soluble, active and glycosylated form [7], however, this system is laborious and not as wide spread as expression in *E. coli*. Expression of HRP in yeast indicated only very low expression of active enzyme [8].

Up to date for the production of wild-type as well as mutant recombinant HRP the *E. coli* expression system is mostly used [9, 10]. Recombinant HRP forms inclusion bodies containing only traces of heme if expressed in bacterial cytoplasm. Multistep refolding and reactivation of recombinant apo-peroxidase with the prosthetic heme group is complex as the protein contains four disulfide bonds and in addition must bind two Ca^{2+}-ions per molecule. Moreover, the plant-derived enzyme contains 18% carbohydrates via 8 glycosylation sites. Crystal structure of recombinant HRP has been solved, revealing the presence of two domains formed by a total of ten α-helices [11].

Among of the factors leading to the formation of inclusion bodies upon expression of

cysteine-rich proteins is the reduction potential of *E. coli* cytoplasm, preventing correct formation of disulfide bonds [12]. One solution to this problem was fusion of such a protein to a signal peptide for secretion into the bacterial periplasm. After translocation the signal peptide is cleaved off and a correctly folded protein with disulfide bonds can be formed under the oxidizing conditions of this compartment. This approach has been successfully applied for the production of soluble active recombinant HRP, though with rather low yield [13].

In biotechnological applications, however, a more effective and reliable way for production of recombinant HRP is desirable. Introduction of a C-terminal His-tag facilitated renaturation and purification of recombinant peroxidase from inclusion bodies. Moreover, by addition of a His-tag to the recombinant peroxidase it was envisaged to facilitate downstream processing, in particular of the dilute solutions obtained after the refolding procedure. The high yield and high specific activity obtained with the optimized protocol enables to produce sufficient recombinant enzyme for the development of biosensors in which electrons are directly transferred from the electrode to the immobilized peroxidase [4, 6] or in which the sensitivity of the enhanced chemiluminescence reaction is increased [14]. In particular, His-tag recombinant HRP co-adsorbed with corresponding oxidases producing H_2O_2 can be considered as promising for future multienzyme biosensor development [4, 5].

Principal possibility of the development of a mediatorless bienzyme biosensor for peroxide and metabolites, yielding hydrogen peroxide during their transformations, based on co-adsorption and cooperation of HRPhis, capable of efficient direct electron transfer, and the appropiate oxidase, e.g., LysOx, was demonstrated. Amperometric bienzyme biosensor for the determination of L-lysine based on LysOx and HRP containing 6-His tag at the C-terminus physically co-immobilized on the surface of polycrystalline gold electrodes is shown to be simple in manufacturing and operation, sufficiently effective and highly reproducible. Efficient direct electron transfer between gold electrodes and immobilized His-tag HRP makes it possible a mediatorless detection of hydrogen peroxide that is released during the enzymatic oxidation of L-lysine, thus decreasing the number of components in the system used for the detection [4–6].

Genetic engineering approach offers new opportunities for broad application of recombinant HRP to design highlysensitive immunobiosensors of a new generation, based on the recombinant DNA technology.

Survey on recombinant conjugates for analytical application

Horseradish peroxidase is a key marker enzyme for immunodiagnostics. Enzyme immunoassays for the detection and quantitative analysis of various substances are based on coupling of marker enzymes like HRP with antigens or antibodies. However, all the chemical conjugation methods result in partial inactivation of the enzyme and heterogeneity of the conjugates, which in turn influence specificity and sensitivity of the assays. With the advance of genetic engineering it became clear that genetic in frame fusions of antigens/antibodies and enzymes would provide many of the desirable features of conjugates for use in immunoassays, in particular homogeneity, 1:1 stoichiometry, reproducibility and ease of production [15].

Early fusion proteins contained the bacterial enzymes β-galactosidase [16–18] or alkaline phosphatase [19–21], which can be easily expressed in *E. coli*. In addition to these enzymes, bioluminescent or fluorescent marker proteins such as aequorin or green fluorescent protein [22, 23] have been used as fusion partners for a model octapeptide. Genetic fusion was also employed to construct conjugates with protein A [24] or an in vitro biotinylated polypeptide tag for β-galactosidase [25]. The genetic approach is particularly attractive for fusions to small peptides with numerous functional groups, which are difficult to control [26], or with human proteins which often are not easily available [27]. Whereas *β*-galactosidase is solubly expressed in the cytosplasm, the disulfide-containing alkaline phosphatase is secreted into the periplasm, thus also broadening the spectrum of fusions to disulfide-containing proteins [28, 29]. The drawback of the lower specific activity of the bacterial alkaline phosphatase in comparison to the calf intestinal alkaline phosphatase routinely used in chemical conjugations could be partly overcome by using a genetically engineered mutant of the bacterial enzyme with increased activity [30]. Recombinant conjugates of antibodies with alkaline phosphatase [31–35], luciferase [36], and peroxidase *Arthromyces ramosus* [37] were obtained earlier.

A principal problem associated with β-galactosidase or alkaline phosphatase fusions is their tetrameric and dimeric structure, respectively, which likely leads to an increased apparent affinity (avidity) of a conjugate in comparison to the free antigen. This is not desirable for the development of competitive immunoassays. On the other hand, horseradish peroxidase, which is very popular for preparation of enzyme-conjugates [38], can only be expressed in *E. coli* in the form of inclusion bodies. The yield of refolded and reconstituted (with heme) recombinant peroxidase used to be rather low [9, 10], which has so far precluded the use of this enzyme in the genetic fusion approach. Progress in heterologous expression in *E. coli* and reactivation of recombinant HRP carrying a C-terminal oligohistidine tag (HRPhis) [13] opened the prospect of producing a recombinant conjugate of HRPhis with a marker enzyme for application in immunoassays.

Recombinant conjugates of HRP with protein antigens

Earlier we've exemplified that a fusion protein of peroxidase and human heart fatty acid binding protein (H-FABP) can be used as a recombinant tracer in immunoassays for detection of H-FABP [39]. This small (15 *kDa*) cytosolic protein is a member of a protein family specialized in transport of fatty acids and is highly abundant in heart muscle (0.52 mg/g heart tissue). Its rapid release into the circulation and its good tissue specificity make it an ideal early marker for clinical diagnosis of acute myocardial infarction [27, 40].

A fusion protein comprising HRP and human heart-type fatty acid-binding protein (H-FABP) was constructed by introducing the coding part of the human H-FABP cDNA into the *Xma*III site of the expression vector for horseradish peroxidase, *pETHRPhis* [13], just in front of the 6xHis tag (Fig. 1).

Recombinant HRP-FABPhis conjugate has the same Soret band absorption with a maximum at 403 nm as native peroxidase and recombinant HRPhis, indicating that the Fe^{3+} co-ordination by heme as well as proximal and distal histidines is not affected. These data indicate that C-terminal extension of the recombinant peroxidase with the 15 kDa human H-FABP has no drastic influence on the activity of the recombinant conjugate, which values corresponds to that for recombinant HRPhis alone and the plant-derived enzyme (1000 U/mg against ABTS substrate)

Fig. 1. **Cloning scheme (*A*) and spatial model (*B*) of HRP-FABP conjugate**

[13]. At the same time the recombinant conjugate bound fatty acids as shown qualitatively by a gel elution assay with radioactive oleic acid (data not shown) and was recognized by a sandwich ELISA with two monoclonal antibodies [27] indicating the structural integrity of the FABP part.

The competitive assay format with its reduced number of incubations can be successfully employed to develop fast immunoassays. Solid phase immunoassay is based on competition of free H-FABP with HRP-FABP conjugate for binding sites on microtiter plates coated with polyclonal antibodies against H-FABP. A conjugate of plant peroxidase and human H-FABP has been synthesized based on periodate oxidation of oligosaccharides [41].

The typical calibration curves shown in Fig. 2 demonstrate the wide measuring range (3 orders of magnitude of H-FABP concentration) and the good detection limit — 1,5 ng H-FABP per ml of sample (0.05 ng/well). The intraassay variation coefficient was between 4 and 8% [39]. The detection limit of this new immunoassay is similar to that observed with sandwich-type ELISAs using monoclonal antibodies against human H-FABP (0.02–0.1 ng/well) [27, 40]. Interestingly, at higher concentrations H-FABP competed better with the recombinant conjugate than with the chemically prepared conjugate for binding to the polyclonal capture antibodies. One explanation could be the presence of chemically prepared conjugate consisting of HRP with more than one H-FABP attached, which would exhibit a higher apparent affinity due to multipoint binding to the immobilized antibodies.

To challenge the competitive ELISA we analyzed a set of plasma samples periodically withdrawn from one patient with diagnosis of AMI over a period of 24 h after admission to the hospital (Fig. 3) [39]. The values for

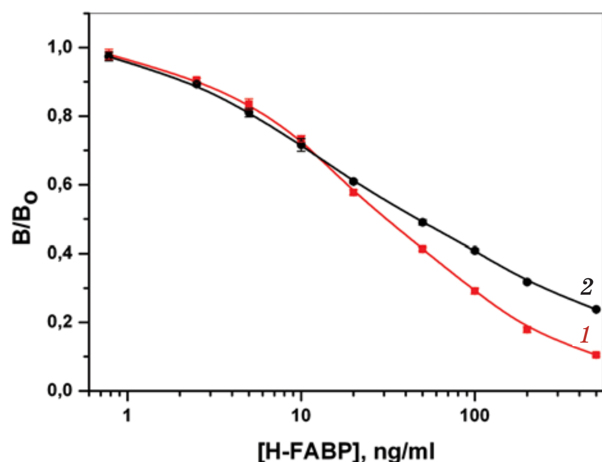

Fig. 2. **Calibration curves for the competitive immunoassay. Competition of *1* — recombinant (-●-) or *2* — chemically prepared conjugate (-■-) with H-FABP**

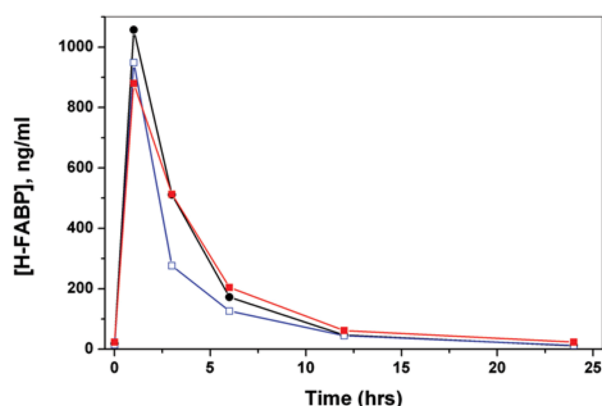

Fig. 3. **Comparison of FABP plasma concentration, obtained with different immunoassay formats. Profile of patient No. 14: -●-sandwich ELISA, -□- electrochemical immunosensor, -■- competitve ELISA**

H-FABP concentrations in the plasma samples assayed with the competitive ELISA exhibited good correlation with those obtained by the reference sandwich ELISA and the developed EUROCARDI immunosensor [27, 40].

Thus we have opened up for the first time the possibility to reproducibly produce a recombinant conjugate of a protein antigen with horseradish peroxidase as marker enzyme for use as tracer in competitive immunoassays [39]. The applicability of this genetically engineered fusion protein with a defined 1:1 stoichiometry for a clinically relevant analyte, the human heart fatty acid binding protein, has been shown with plasma from a patient after myocardial infarction. Our approach paves the way for broad application of the popular peroxidase marker enzyme in competitive immunoassays employing genetically engineered conjugates. We have already extended the concept by preparation of a recombinant conjugate of peroxidase and human myoglobin, another analyte important for early detection of myocardial infarction.

Recombinant conjugates of HRP with (Fab) antibodies fragments

The functional expression of the recombinant conjugate of HRP and antibody fragments in *E. coli* is associated with a number of difficulties, since there is no post-translational glycosylation of proteins in *E. coli* cells, resulting in low solubility and aggregation of the resulting protein. This problem can be solved by replacing the expression system. For instance, it has been shown that methylotrophic yeast *Pichia pastoris* is a more suitable medium for antibody expression than *E. coli* cells [42, 43].

HRP [44] and antibody fragments [45] were successfully expressed individually in *P. pastoris* cells, both in the single-stranded form scFv [46, 47] and in a Fab form [48]. Moreover, certain immune conjugates have also been created using this expression system [49–51]. It has been demonstrated that gene expression in the *Pichia pastoris* system in the secreted form considerably simplifies the scaling of the process for biochemical applications [52].

Progress in functional secreted expression of HRP and antibodies in *Pichia pastoris* [44, 53] open the prospect to produce recombinant conjugates of *HRP* with antibodies for application in immunoassays. However, the production of recombinant conjugates is an appreciably complicated task, since it remains thus far impossible to reliably predict the structure of the desired conjugate; hence, loss of the functional activity of both the marker enzyme and antigen is possible, due to the incorrect folding of two components.

General versatile expression system for recombinant conjugates of peroxidase with *Fab* fragments of antibodies production has been elaborated based on *pPICZalfa* vector and *X33 P.pastoris* strain (*Invitrogen*). These systems provide secreted, methanol-inducible expression in cultural medium two types of conjugates where the peroxidase part genetically fused to *N*- or *C*-terminal part of variable heavy chain of antibody via short flexible linker sequences (*Gly$_4$Ser*) (Fig. 4) [54].

To exemplify the applicability of this approach for the first time we have produced set of conjugates of peroxidase with *Fab* against atrazine pesticides (Fig. 4).

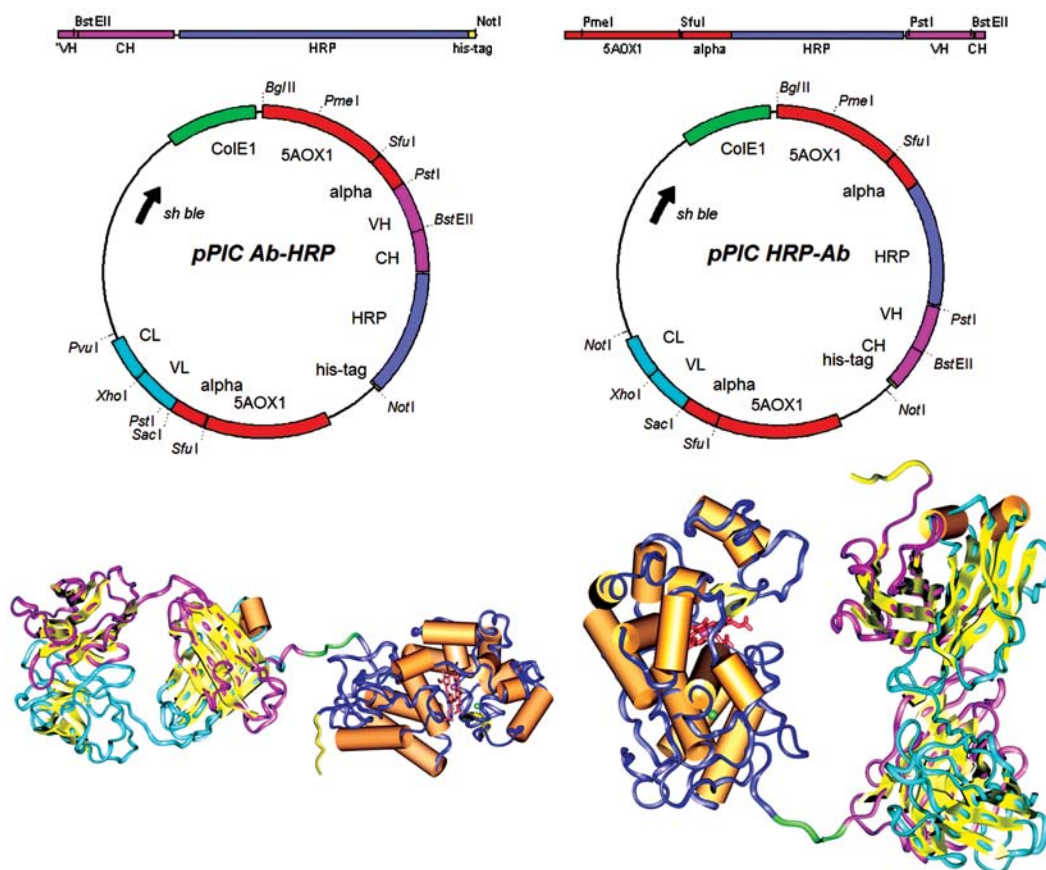

Fig. 4. **Cloning schemes and spatial models of recombinant conjugates: Fab-HRP and HRP-Fab (left and right panel respectively)**

The developed expression vectors allow simple recloning of any variable heavy (*PstI/BstEII* sites) and light (*BamHI/XhoI* sites) chains, thus providing general vectors for recombinant conjugates of peroxidase with antibodies production in *P. pastoris*.

The total yield of recombinant conjugates was approximately 3–10 mg per 1 L of the *P. pastoris* culture supernatant. A relatively low yield of secreted conjugates correlates with the yield upon expression of the HRP gene only [54]. We believe that one of the factors that have a negative effect on the yield of the secreted product is the excessive glycosylation of the peroxidase component of the conjugate, which is typical of *P. pastoris* cells. In order to verify this hypothesis, it may be reasonable to remove all N-glycosylation sites in HRP or replace HRP with another reporter protein, such as EGFP.

In order to confirm the antigen-binding activity of recombinant conjugates, we selected the scheme of indirect competitive single-stage ELISA carried out on the wells with an immobilized atrazine–BSA conjugate. The data obtained attest to the presence of both catalytic and antibody activity in all forms. However, the low activity of the HRP-Fab in comparison with the C-terminal conjugate Fab-HRP may attest to the fact that the mutual spatial arrangement of two components of the chimeric protein in this case results in a decrease in the catalytic activity of peroxidase. Typical calibration curve (Fig. 5) allows one to determine the atrazine concentration over a wide range, from 0.1 to 50 ng/ml; the variation coefficient being no higher than 8%. IC_{50} is equal to 3 ng/ml, which agrees well with the results of atrazine determination by a two-stage ELISA procedure using recombinant Fab fragments of the same antibody K411B [54] and with the data on the single-stranded mini-antibody (scFv) obtained earlier in *E. coli* [53, 55]. Thus, the recombinant conjugates of peroxidase with Fab fragments of antibody against atrazine obtained in the present study possess functional activity and can be used to determine atrazine via ELISA.

We have for the first time demonstrated the possibility to produce a fully active recombinant conjugate of a protein antigen with horseradish peroxidase as marker enzyme for

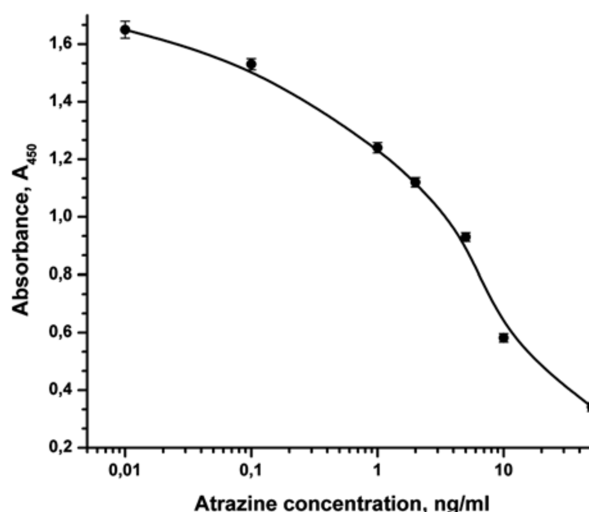

Fig. 5. **Calibration curve for atrazine determination in competitive ELISA with recombinant conjugate of Fab-HRP**

use as tracer in competitive immunoassay for a clinically relevant analyte. We have already extended the concept by preparation of a recombinant conjugate of peroxidase and human myoglobin, another analyte important for early detection of myocardial infarction.

The possibility of using a recombinant, functionally active HRP (as a marker enzyme) conjugated with Fab fragments of the antibody against atrazine was shown for the first time. Recombinant conjugates were obtained in which the Fab fragment of an antibody is bound both to the N- and the C- terminus of peroxidase. Both these variants manifest immunological and catalytic activity.

Thus successful genetic engineering towards horseradish peroxidase opens the new opportunities of using this traditional marker enzyme for analytical and biomedical application.

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ВИКОРИСТАННЯ РЕКОМБІНАНТНОЇ ПЕРОКСИДАЗИ ХРОНУ ДЛЯ АНАЛІТИЧНИХ МЕТОДІВ

О. М. Єгоров[1, 2]
В. Г. Григоренко[1]
І. П. Андреєва[1, 3]
М. Ю. Рубцова[1]

[1]Лабораторія інженерних ферментів,
Хімічний факультет МДУ
ім. М. В. Ломоносова, Москва, Росія
[2]Відділ мікробіології,
Російська медична академія
післядипломної освіти,
Москва, Росія
[3]Immunotek МДУ,
Москва, Росія

E-mail: vitaly.grigorenko@gmail.com

Статтю присвячено перспективам застосування рекомбінантної пероксидази хрону в аналітичній біохімії та біотехнології. Розглянуто проблеми клонування пероксидази хрону в різних експресійних системах, можливі підходи до їх вирішення, а також переваги використання в імуноаналізі рекомбінантної пероксидази хрону і злитих протеїнів на її основі. Показано принципову можливість створення безмедіаторного біензимного біосенсора для виявлення пероксиду водню і метаболітів, що утворюють його під час трансформації, на основі коадсорбованих рекомбінантної пероксидази хрону та відповідної оксидази. Уперше показано можливість одержання функціонально активного рекомбінантного кон'югату пероксидази хрону із серцевим протеїном людини, що зв'язує жирні кислоти, який може бути застосовано в конкурентному імуноаналізі для діагностики інфаркту міокарда, а також N- і C-кінцевих рекомбінантних кон'югатів пероксидази хрону із Fab-фрагментами антитіл проти атразину — для виявлення пестициду атразину.

Ключові слова: рекомбінантна пероксидаза хрону, злиті протеїни, імуноаналіз, безмедіаторний біензимний біосенсор, Fab-фрагменти антитіл, серцевий протеїн, що зв'язує жирні кислоти, пестицид атразин.

ИСПОЛЬЗОВАНИЕ РЕКОМБИНАНТНОЙ ПЕРОКСИДАЗЫ ХРЕНА ДЛЯ АНАЛИТИЧЕСКИХ МЕТОДОВ

А. М. Егоров[1, 2]
В. Г. Григоренко[1]
И. П. Андреева[1, 3]
М. Ю. Рубцова[1]

[1]Лаборатория инженерных ферментов,
Химический факультет МГУ
им. М. В. Ломоносова,
Москва, Россия
[2]Отдел микробиологии,
Российская медицинская академия
последипломного образования,
Москва, Россия
[3]Immunotek МГУ,
Москва, Россия

E-mail: vitaly.grigorenko@gmail.com

Статья посвящена перспективам применения рекомбинантной пероксидазы хрена в аналитической биохимии и биотехнологии. Обсуждаются проблемы клонирования пероксидазы хрена в различных экспрессионных системах, возможные подходы к их решению, а также преимущества использования в иммуноанализе рекомбинантной пероксидазы хрена и слитых протеинов на ее основе. Показана принципиальная возможность создания безмедиаторного биэнзимного биосенсора для выявления пероксида водорода и метаболитов, образующих его при трансформации, на основе коадсорбированных рекомбинантной пероксидазы хрена и соответствующей оксидазы. Впервые показана возможность получения функционально активного рекомбинантного конъюгата пероксидазы хрена с сердечным протеином человека, связывающим жирные кислоты, который может быть применен в конкурентном иммуноанализе для диагностики инфаркта миокарда, а также N- и C-концевых рекомбинантных конъюгатов пероксидазы хрена с Fab-фрагментами антител против атразина — для выявления пестицида атразина.

Ключевые слова: рекомбинантная пероксидаза хрена, слитые протеины, иммуноанализ, безмедиаторный биэнзимный биосенсор, Fab-фрагменты антител, сердечный протеин, связывающий жирные кислоты, пестицид атразин.