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ARTICLES

Tobacco Peroxidase as a New Reagent for Amperometric Biosensors[1]

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Abstract—The results of testing a new enzyme, anionic tobacco peroxidase (TOP), in various amperometric biosensors are summarized. The biochemical and electrochemical properties of the enzyme are briefly characterized. As compared to the commonly used cationic peroxidase from horseradish roots, TOP exhibits a wider optimum stability pH range, higher stability to inactivation with hydrogen peroxide, and higher efficiency in direct electron-transfer processes. The enzyme immobilized by adsorption on graphite is effective in determining aminophenols and aromatic diamines under flow conditions with a detection limit of 10 nM. Upon immobilization on graphite by incorporation into a gel of a redox-active polymer (crosslinked polyvinylimidazole with osmium 4,4'-dimethylbipyridinium chloride), TOP exhibited sensitivity and stability comparable to those of horseradish peroxidase and a wider linearity range. Upon immobilization on a self-assembled thiol monolayer at a gold electrode, TOP was much superior to horseradish peroxidase in the sensitivity of determining hydrogen peroxide, regardless of the charge of the monolayer. Prospects for the further use of the native enzyme and its genetically engineered unglycosylated form are considered.

Heme-containing peroxidases are widespread in nature; they play an important role in the protective mechanisms of plants and animals. These enzymes are of both fundamental and considerable practical interest. Horseradish peroxidase (HRP, EC 1.11.1.7) is the most popular enzyme from this class; this peroxidase is commercially produced on a large scale. This enzyme is most commonly used in enzyme immunoassay; as a rule, it is detected by spectrophotometry or using enhanced chemiluminescence reactions. In the past 10–15 years, the use of HRP in various electrochemical biosensors has been actively studied on a global scale. A considerable advantage of the electrochemical detection of peroxidase activity over luminescence and spectrophotometric techniques consists in the possibility of designing so-called reagentless biosensors, in which the electrode acts as an electron-donor substrate. This design is responsible for either the repeated use of the biosensor without additional reagents or its use under continuous-flow conditions for the monitoring of industrial fermentation processes and food and water quality control.

Along with the obvious requirement of high operational stability under analytical conditions, the reagentless sensors impose additional special requirements on the enzyme. For example, if a biosensor is designed for determining hydrogen peroxide (or a compound that generates hydrogen peroxide by enzymatic oxidation), the protein moiety of the enzyme should come in maximum contact with the electrode surface. In naturally occurring plant peroxidases, carbohydrate residues constitute a considerable fraction (from 10 to 50%) of the molecular weight; these residues can affect the tight contact of the active center of the enzyme with the electrode. In this case, peroxidase with a minimum degree of glycosylation or a genetically engineered unglycosylated enzyme is an optimum. If a biosensor is designed for the detection of aromatic peroxidase substrates, the main criterion is the catalytic activity of the enzyme toward a given analyte; this catalytic activity depends on the catalytic properties of the chosen peroxidase.

The results of studies on the use of the most available native HRP in biosensors demonstrated that this is not an optimum enzyme for several reasons. First, HRP immobilized on an electrode exhibits low operational stability because of inactivation by hydrogen peroxide. Second, the carbohydrate moiety of the enzyme (25% of the total molecular weight) prevents effective direct electron transfer between the active center and the electrode. Third, the substrate specificity profile does not cover the entire range of compounds that should be determined in actual practice. In this context, an active search of alternative peroxidases is currently in

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progress for the development of new sensitive methods for determining an increasing number of analytes.

In addition to peroxidase from horseradish roots, native peroxidase from soybean is commercially manufactured; however, it is available only as immunoconjugates (Pierce). Genetically engineered unglycosylated peroxidase from horseradish and native peroxidases from tobacco, peanut, yam, and palm are also used in applied scientific research. The main problem for these enzymes is that they are available in only small amounts. In the case of native peroxidases, labor-intensive and sophisticated purification procedures, which are difficult to scale up, should be used because enzyme concentrations in natural sources are low. In the case of the genetically engineered enzyme, active recombinant HRP is prepared by reactivation (refolding) from insoluble inclusion bodies. The efficiency of this process is no higher than 10–20%, and no more than 40–50 mg of an active enzyme in a single operation. We experimentally optimized and scaled up the process of the preparation of recombinant HRP. Because of this, the degree of reactivation in refolding was 50% and the total yield was as high as a few grams.

A procedure for the preparation of weakly glycosylated (2–5%) recombinant anionic tobacco peroxidase (TOP) in gram amounts from transgenic tobacco and tomato plants, which superproduce this enzyme, was also developed in our laboratory [1]. Recently, with the use of TOP gene expression in the cells of *E. coli,* a genetically engineered unglycosylated enzyme was obtained in amounts sufficient for crystallization, other resource-demanding physicochemical investigation techniques, and testing for analytical purposes [2]. Thus, our laboratory is the only world's laboratory capable of producing genetically engineered (recombinant) HRP and recombinant TOP in gram amounts. Therefore, unlike other laboratory-available native peroxidases from exotic plants prepared in a homogeneous form by multistage chromatographic purification, recombinant TOP is, in principle, suitable for commercial manufacture and, consequently, for widespread use.

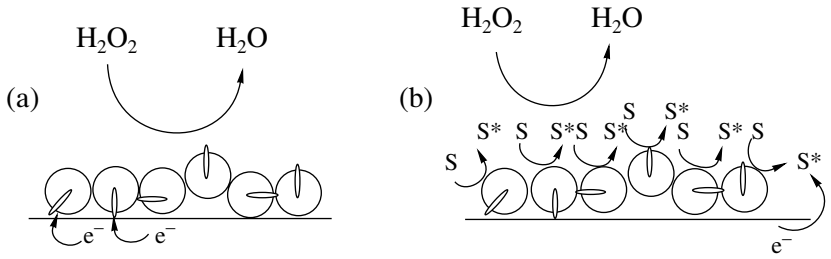
In addition to the fundamental studies of natural glycosylated TOP performed in 1996–1999 [3–6], which demonstrated a number of practically promising properties of this enzyme, the new enzyme was also tested for analytical purposes [7–20].

This paper presents a survey of our results obtained over the past eight years in the use of TOP for analytical purposes, namely, in amperometric biosensors. For comparison, data on native HRP and recombinant HRP, which are the enzymes most commonly used in biosensors, are also given. Unfortunately, a comparative analysis of the results with data obtained by other methods is beyond the scope of this work because of limitations on the length of an article and an enormous body of available published data. A detailed description of the variety of peroxidase biosensors, their operating principles, and trends in the development, as well as a comparison with other analytical techniques, can be found in the reviews [15, 21].

The aim of this review was to demonstrate the advantages of TOP in amperometric biosensors over HRP and hence to attract the attention of analytical chemists to the capabilities of the new enzyme, which is currently available in a genetically engineered form.

Catalytic properties of tobacco peroxidase. TOP and HRP differ dramatically in many parameters. First, the former enzyme is negatively charged in neutral solutions (isoelectric point at pH 3.5). Second, TOP is less glycosylated than HRP (the oligosaccharide content of the native enzyme is 10 against 25% in natural HRP or no more than 3–5% in genetically engineered TOP expressed in transgenic tobacco and tomato) [1]. Third, an unusual characteristic property of the enzyme is its stability in strongly acidic and strongly alkaline solutions [5]. This stability is due to the occurrence of a negatively charged glutamic acid residue at the inlet of the active center. This residue prevents the dissociation of heme from a protein globule, as demonstrated by the directed mutagenesis of TOP (this study was supported by the Russian Foundation for Basic Research, project no. 04-04-48286). Fourth, the distinctive property that differentiates POT from all of the other now described plant peroxidases is the stability of its intermediate with an oxidation number of +2 (so-called compound **I**, EI) and the high activity of an intermediate with an oxidation number of +1 (compound **II**, EII) [1, 4]. In addition to the stability of the enzyme in an alkaline medium, the above property is responsible for the fact that the activity of TOP in a luminescence reaction is higher than that in an enhanced chemiluminescence reaction catalyzed by HRP by an order of magnitude [7]. This property of TOP was clearly demonstrated in studies performed in cooperation with our colleagues from the University of Lund using chemiluminescence enzyme immunoassay for a number of pesticides as an example [8]. The sensitivity of TOP in a luminescence reaction is highly competitive with that of recently isolated palm peroxidase [22]. Fifth, the specific feature of TOP consists in the regulation of its activity by calcium ions in the half reaction of hydrogen peroxide degradation [4]. Finally, the substrate specificity profile of TOP is dramatically different from that of HRP. For example, classical HRP substrates (phenol and its halogen derivatives) are almost not oxidized by TOP [9, 10], whereas positively charged substrates (such as amines, which are poor substrates for HRP) are readily oxidized by TOP. The above properties of the new enzyme formed the basis of testing it in various biosensors.

Principles of the electrochemical detection of peroxidases. The detection of peroxidase immobilized on the surface of an electrode is based on the detection of the electrochemical reduction of an oxidized form of the enzyme (Scheme (1)).



(1)

Scheme 1.

This process occurs by different mechanisms in the absence and in the presence of substrates. In the absence of substrates, the enzyme is reduced by a mechanism of direct electron transfer, when the electrode serves as an electron donor (Scheme (1), a). The native ferric form of peroxidase is oxidized by hydrogen peroxide to an intermediate with an oxidation number of +2 (EI) (reaction (2)). Next, this intermediate is reduced by electrons obtained from the electrode (reaction (3)):

$$E + H_2O_2 \xrightarrow{k_1} EI + H_2O, \quad (2)$$

$$EI + 2e \xrightarrow{k_s} E. \quad (3)$$

The efficiency of direct electron transfer strongly depends on the distance between the active center of the enzyme and the electrode surface. This distance depends on both the size of a protein globule and the orientation of the active center in the binding of the enzyme to the electrode surface. Scheme (1) clearly indicates that not all of the enzyme molecules are oriented by their active centers toward the electrode surface because of the statistical character of immobilization. Therefore, the number of peroxidase molecules that participate in direct electron transfer is always lower than the total number of enzyme molecules arranged at the electrode surface. It is also obvious that, in a comparison between two peroxidases in direct electron transfer, the current intensity is higher in the case of an enzyme with a lower molecular weight because the smaller size of a protein globule in this enzyme shortens the distance between the active center and the electrode surface at the same orientation.

So-called mediated electron transfer occurs in the presence of substrates. This process is much more effective than direct electron transfer, and mediated electron transfer completely replaces direct electron transfer at saturation substrate concentrations (Scheme (1), b). A predominant number of peroxidase substrates are one-electron donors; therefore, the enzyme is reduced in two steps (reactions (4) and (5)) through the formation of an additional intermediate with an oxidation number of +1 (EII). The rate of reduction (k_3) of this intermediate is limiting for the overall process. Next, the oxidized substrate species S* is nonenzymatically reduced at the electrode to the parent substrate S (reaction (6)):

$$EI + S \xrightarrow{k_2} EII + S^{\cdot}, \quad (4)$$

$$EII + S \xrightarrow{k_3} E + S^{\cdot}, \quad (5)$$

$$2S^{\cdot} + 2e \xrightarrow{k_{s,m}} 2S. \quad (6)$$

The efficiency of direct electron transfer depends on the catalytic activity of a particular peroxidase in the half reaction of hydrogen peroxide degradation to water (k_1) and on the efficiency of direct electron transfer from the electrode to the peroxidase molecule (k_s). The efficiency of mediated electron transfer and its predominance over direct electron transfer are governed by the activity of the enzyme toward the substrate (constants k_2 and k_3). Peroxidases from different sources differ in all of these three parameters, particularly, in activity profiles toward various natural and artificial electron donors. Readily oxidizable or good peroxidase substrates are those in which the constant k_3 is of the order of 10^5 M^{-1} s^{-1} or higher.

Electrochemical properties of tobacco peroxidase. To study the electrochemical properties of electrode-immobilized TOP, we used a method proposed by Ruzgas *et al.* [11]. The method is based on studying the dependence of the kinetic component of current on the concentrations of hydrogen peroxide and an electron-donor substrate. This is an electrochemical analog of a classical method of steady-state enzyme kinetics. Technically, this experiment can be performed either at a rotating disk electrode [11] or under flow conditions [12]. The developed approach is based on a comparison between direct electron transfer and mediated electron transfer at the same electrode.

Figure 1 shows the dependence of kinetic current on hydrogen peroxide concentration in double inverse coordinates for native TOP in the absence and in the presence of a mediator (direct electron transfer and mediated electron transfer modes, respectively) [11]. The rate constants of the elementary half reactions of immobilized peroxidase oxidation by hydrogen peroxide (Eq. (2)) (constants k_1 and k_1^* for mediated electron transfer and direct electron transfer modes, respectively) can be evaluated from the slope of straight lines (Fig. 1), and the rate constants of enzyme reduction (constants k_3 and k_s for mediated electron transfer and direct electron transfer modes, respectively) can be

found from the intercepts. Note that the constant k_1 does not depend on the nature of the substrate used in the second half reaction. The fraction of enzyme molecules that participate in direct transfer can be determined from the k_1^*/k_1 ratio.

We used this approach for the comparative characterization of four native peroxidases from plants [13–15], as well as a number of recombinant forms of unglycosylated HRP prepared by expression in the cells of *E. coli* [11, 15, 16]. Table 1 summarizes the experimental results. Data given in Table 1 allowed us to draw the following interesting conclusions on the operation of peroxidases in biosensors in a direct electron transfer mode: (1) Anionic peroxidases from tobacco and potato, which are glycosylated to a much lesser extent than cationic peroxidases from horseradish and peanut, exhibited better contact with the electrode and, consequently, higher efficiencies in direct electron transfer (68 and 91% in anionic peroxidases against 44 and 48% in cationic species), which are comparable to that of deglycosylated recombinant HRP (63%). (2) The efficiencies of direct transfer (constant k_s) in all of the native peroxidases varied within an order of magnitude, and an appropriate orientation of the immobilized enzyme at the electrode is a crucial parameter of direct electron transfer. (3) A comparison between data for native and recombinant HRPs indicates that the deglycosylation of the enzyme significantly improved all of the characteristics of an enzyme electrode under conditions of both direct electron transfer and mediated electron transfer. Thus, the preparation of unglycosylated recombinant TOP in our laboratory [2] is of undoubted interest for its use in biosensors.

Immobilization of tobacco peroxidase on a modified gold electrode. An analysis of data given in Table 1 suggests that TOP is much more active than HRP in the reaction of direct electron transfer. As mentioned above, the rate of direct electron transfer is controlled by both an appropriate orientation of the active center of the enzyme with respect to the electrode surface and the distance between the hemin and the electrode. This distance can be controlled by properly choosing the immobilization method. In the case of peroxidase immobilization by physical adsorption on a graphite electrode, we can only guess which the structure of the enzyme layer is because of the heterogeneity of the electrode surface. The use of self-assembled thiol monolayers (SAMs) at the surface of a gold electrode allowed us both to control distance from the electrode surface by a thiol chain length and to orient the enzyme using the mixtures of thiols that provide specific recognition or binding (Fig. 2). TOP was studied in an amperometric biosensor with a SAM at a gold electrode [17] on two different types of surfaces: positively and negatively charged (Fig. 3). In both cases, TOP was more effective than HRP in direct electron transfer. In the case of the negatively charged SAM, the sensitivity of TOP or HRP was 23 or 14 μA per mole of hydrogen peroxide, respectively (Fig. 3a), although a direct oppo-

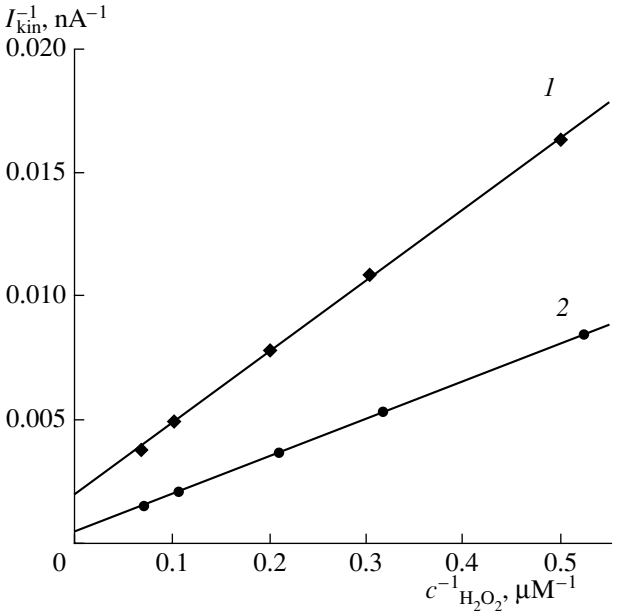


Fig. 1. Dependence of kinetic current on the concentration of hydrogen peroxide in double inverse coordinates for native HRP (*1*) in the absence and (*2*) in the presence of a mediator (60 μM *p*-cresol) [11]. The kinetic current was obtained by extrapolating the current as a function of the square root of electrode rotation rate to the axis of ordinates (the Koutecky–Levich coordinates).

Table 1. Kinetic characteristics of peroxidases immobilized on a rotating graphite electrode ($n = 3$; $P = 0.95$)

Enzyme form	Direct electron transfer, %	$k_1 \times 10^{-5}$, M^{-1} s^{-1}	k_s, s^{-1}	$k_3 \times 10^{-5}$, M^{-1} s^{-1}
Native HRP [11]	48 ± 4	1.3 ± 0.2	1.9 ± 0.3	0.38 ± 0.16
Recombinant HRP [16]	63 ± 7	2.8 ± 0.6	7.6 ± 2.5	1.40 ± 0.70
Peanut peroxidase [13]	44 ± 7	1.3 ± 0.3	1.3 ± 0.3	0.50 ± 0.33
Potato peroxidase [13]	91 ± 6	2.8 ± 0.8	4.8 ± 2.3	3.50 ± 1.80
TOP [13]	68 ± 3	0.53 ± 0.03	2.6 ± 1.0	0.19 ± 0.07

Notes: Measurement conditions: 0.1 M phosphate--sodium buffer solution, pH 7.0. Mediator: 60 μM *p*-cresol. The concentrations of enzymes were calculated assuming a monolayer coverage [11].

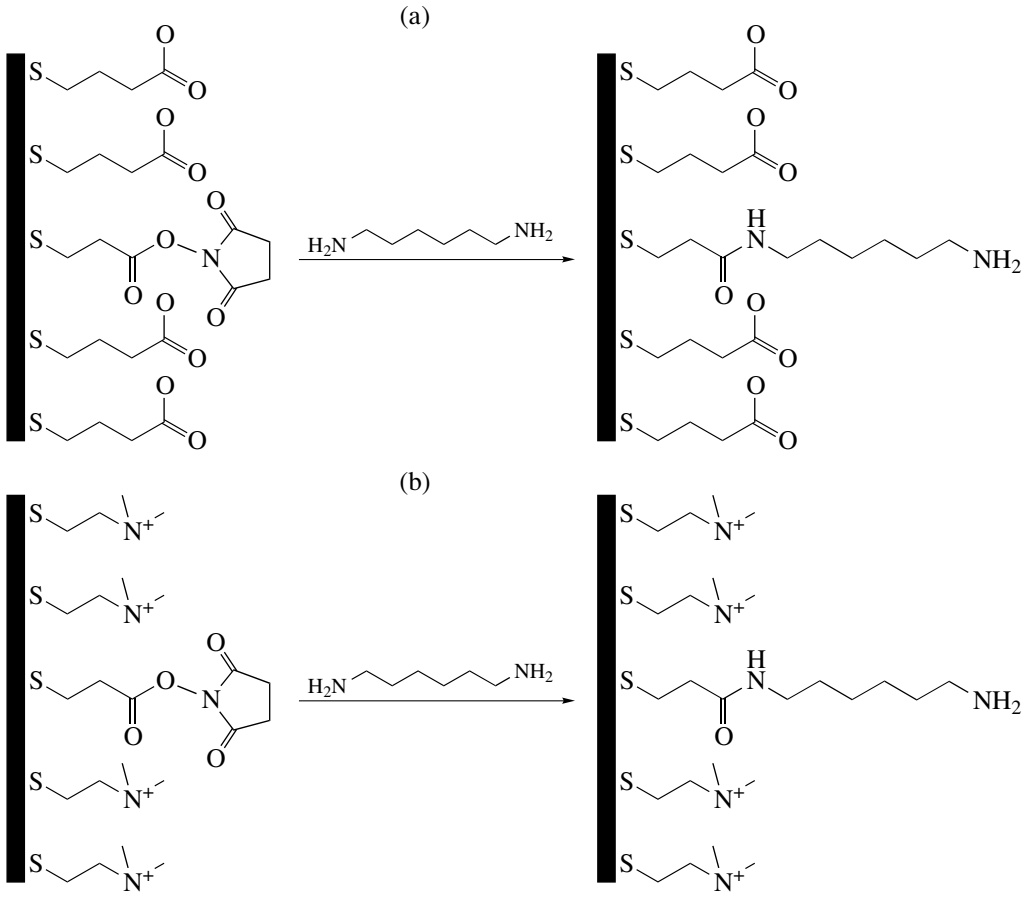


Fig. 2. Structure of self-assembled thiol monolayers used for the immobilization of peroxidases [11]: (a) a negatively charged monolayer formed by 3,3'-dithiodipropionic acid di(N-succinimidyl ester) in a mixture (19 : 1) with 3-carboxypropyl disulfide; (b) a positively charged SAM prepared usinga mixture (19 : 1) of 3,3'-dithiodipropionic acid di(N-succinimidyl ester) and hexamethylcystamine. The subsequent addition of a spacer (1,6-diaminohexane) removed steric hindrances on adsorption and additionally ordered the enzyme monolayer.

site might be hypothesized based on the total charges of the enzymes (negative in TOP and positive in HRP). As expected, negatively charged TOP on the positively charged SAM was more sensitive by almost an order of magnitude: 179 μA per mole of hydrogen peroxide. In the case of HRP immobilized on the positively charged SAM, analysis was impossible because the signal was on a background level for an inert protein (BSA) (Fig. 3b). The negatively charged SAM exhibited a minimum background current; this suggests a dense monolayer on the electrode surface and is of considerable practical interest. In this case, it is likely that the higher sensitivity of TOP is a consequence of its lower glycosylation than that of HRP and, hence, a higher accessibility of the active center to direct electron transfer.

Determination of aminophenols with the use of tobacco peroxidase. Table 1 summarizes the kinetic characteristics of peroxidases from various sources immobilized on a rotating graphite electrode [18]. As follows from Table 1, TOP exhibited a minimum rate constant of reaction with hydrogen peroxide (k_1), as compared with enzymes from other sources. By this we meant that, in the absence of a mediator, TOP gives a smaller signal than the other peroxidases. On the other hand, the appearance of a good mediator dramatically increases the signal. Mediated electron transfer on the addition of a good substrate can form the basis for the quantitative determination of this compound [18].

The screening for phenols and amines [18] demonstrated that, after attaining a steady current under flow conditions in the absence of a substrate, the addition of these substrate mediators to TOP resulted in a current spike (Fig. 4a), whose value is related to the concentration of the substrate added by the Michaelis–Menten equation (Fig. 4b) [18]. A minimum detection limit (about 10 nM) was obtained in the case of *o*-aminophenol, *o*-phenylenediamine, and *p*-phenylenediamine (Table 2). This value is at least five times lower than the corresponding values for HRP and peanut peroxidase.

Data given in Table 2 also demonstrate the opportunities provided by the use of a set of consecutive peroxidase biosensors for the identification of aromatic

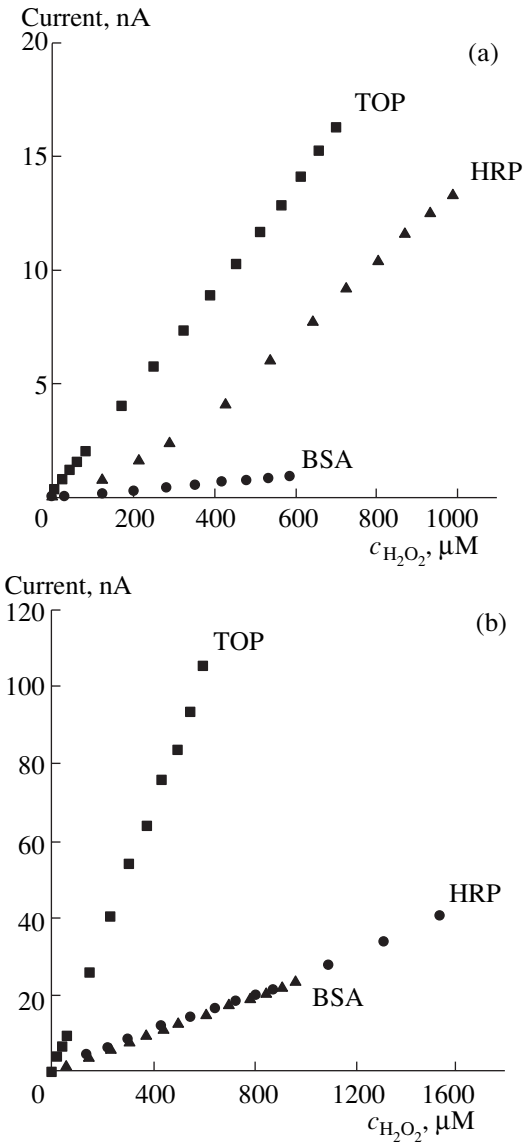


Fig. 3. Calibration graphs for determining hydrogen peroxide with the use of (■) TOP and (●) HRP at a gold electrode with a (a) negatively or (b) positively charged SAM [17]. As a reference, (▲) BSA was used. 10 mM phosphate buffer solution with pH 7.0; potential of +50 mV.

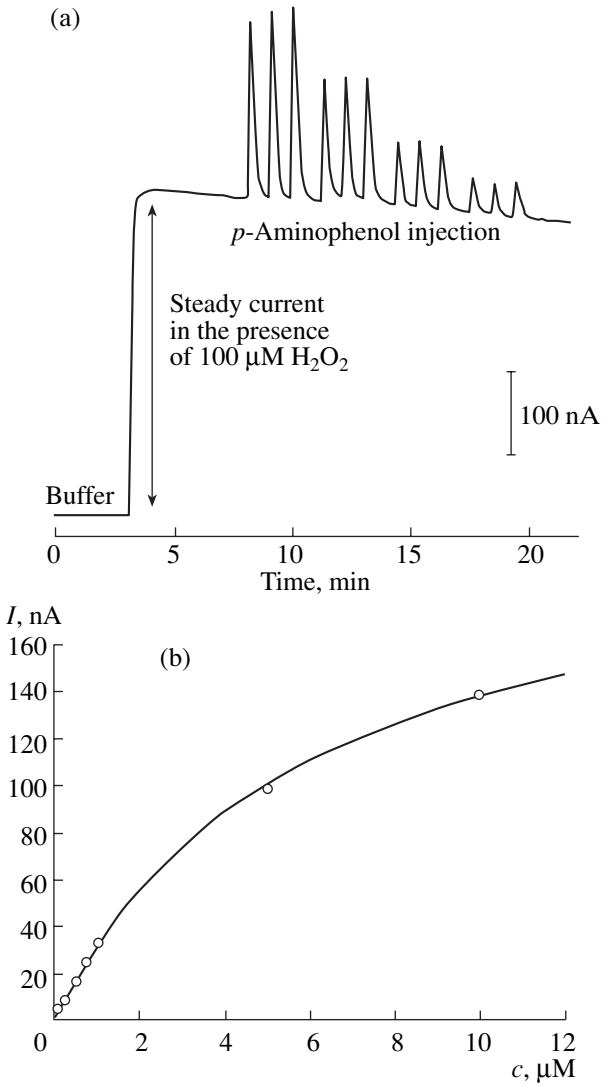


Fig. 4. Determination of aromatic phenols and amines in a flow-injection cell using HRP immobilized on a graphite electrode [18]: (a) shape of primary experimental data; (b) approximation by the Michaelis–Menten equation for *p*-aminophenol. Hydrogen peroxide concentration of 100 μM.

amines and phenols because the ratios of signals in biosensors with different peroxidases (signal profiles) are specific for each individual chemical compound (on condition that the enzymes are saturated with respect to substrates, i.e., in a linearity range of the concentration dependence, which is shown in Fig. 4b for *p*-aminophenol).

Effect of calcium on the characteristics of a biosensor based on tobacco peroxidase. Upon the addition of calcium cations to solution, the TOP molecule undergoes a conformation transition that results in a lower accessibility of the active center of the enzyme to both hydrogen peroxide and substrates [14]. In the presence of 50 mM calcium chloride, the amount of the enzyme active in direct electron transfer decreased by 16%. The rate constant k_s of direct electron transfer and the rate constant k_3 for *p*-cresol decreased by 30%, and the rate constant k_3 for pyrocatechol decreased by 50% [14]. The effect of calcium was synergetic to a decrease in pH. This effect was likely due to surface saturation with positive charges, which compensated the repulsion of many negatively charged groups at the enzyme surface, including glutamic acid residue 141 at the entrance of the enzyme active center [14]. Figure 5a demonstrates the effect of calcium on the efficiency of direct transfer; in this case, the observed decrease in the

Table 2. Comparative characterization of procedures for determining phenols and aromatic amines with the use of biosensors based on tobacco, horseradish, and peanut peroxidases [18] ($n = 3$, $P = 0.95$)

Compound	Sensitivity, nA per μM			Detection limit, μM		
	HRP	TOP	peanut peroxidase	HRP	TOP	peanut peroxidase
Phenol	2.0 ± 0.1	0.76 ± 0.08	1.4 ± 0.1	3.6 ± 0.5	10.1 ± 0.8	7.0 ± 0.6
Pyrocatechol	8.1 ± 0.2	21 ± 1	8.0 ± 0.2	1.3 ± 0.1	0.45 ± 0.05	0.75 ± 0.05
Aniline	1.1 ± 0.1	0.64 ± 0.09	0.62 ± 0.09	6.5 ± 0.5	10.0 ± 0.7	10.0 ± 0.7
o-Aminophenol	34.5 ± 1.5	521 ± 15	443 ± 15	0.10 ± 0.01	0.010 ± 0.001	0.010 ± 0.001
p-Aminophenol	37.0 ± 1.5	214 ± 10	20 ± 1	0.10 ± 0.01	0.040 ± 0.005	0.250 ± 0.01
o-Phenylenediamine	132 ± 10	960 ± 20	115 ± 6	0.050 ± 0.004	0.010 ± 0.001	0.050 ± 0.004
m-Phenylenediamine	10.0 ± 0.8	6.4 ± 0.5	10.0 ± 0.7	1.2 ± 0.1	3.0 ± 0.6	0.75 ± 0.05
p-Phenylenediamine	74 ± 4	860 ± 20	147 ± 7	0.050 ± 0.004	0.010 ± 0.001	0.050 ± 0.005

Table 3. Comparison between biosensors for determining hydrogen peroxide based on direct and mediated electron transfer for horseradish and tobacco peroxidases [19]

Electrode type and mode	Enzyme	Maximum current (V_{max}), μA	Sensitivity, μA per mM	Linearity range, μM	Detection limit, nM
Simple adsorption, direct electron transfer	HRP	12.6	24.7	0.5–130	71
	TOP	27.7	21.0	0.5–240	136
Coimmobilization in a hydrogel with a mediator, mediated electron transfer	HRP	67	169	0.5–70	38
	TOP	96	139	0.5–200	81

current was proportional to the concentration of calcium added (Fig. 5b). The detection limit of calcium was about 5 mM, which is high compared to that of commonly used analytical methods. However, the occurrence of calcium-induced effects can serve as an additional characteristic of the state of the immobilized enzyme at the electrode, namely, the mobility of its active center (see below).

Electrochemical properties of tobacco peroxidase upon immobilization by incorporating into a redox-active gel. As noted above, peroxidases are much more active in mediated electron transfer than in direct electron transfer. Therefore, to improve the sensitivity of the detection of hydrogen peroxide, an enzyme and a mediator are simultaneously immobilized on an electrode. Hydrogels are considered most promising for the combined immobilization of an enzyme and a mediator. Hydrogels provide an opportunity to use an optimally charged matrix for an enzyme chosen and exhibit a good permeability to hydrogen peroxide. Osmium complexes were found to be good peroxidase mediators. These complexes were used previously for a comparative study of TOP and HRP [19, 20]. The enzymes were immobilized in a hydrophilic polymer (polyvinylimidazole containing osmium dimethylbipyridinium chloride) using a crosslinking reagent (diglycidyl ether of polyethylene glycol) [19]. As expected, TOP lost sensitivity to calcium ions in the concentration range 5–500 mM upon immobilization in a polycationic redox gel [19]. This could be due to the mobility of a protein globule being lost upon enzyme immobilization in a gel matrix because of strong fixation by crosslinks. On the other hand, the enzyme microenvironment in a cationic matrix can induce the same conformation transition in the enzyme molecule as in the case of the saturation of solution with calcium cations.

The principle of the detection of hydrogen peroxide in amperometric biosensors with peroxidase immobilized in combination with a mediator is analogous to that shown in Fig. 4a. After the establishment of a steady current, the injection of a sample containing H_2O_2 into the flow caused a current spike, whose value was proportional to the concentration of hydrogen peroxide. Table 3 gives a comparative analysis of the results of determining H_2O_2 with the use of HRP and TOP upon their coimmobilization with a mediator in a hydrogel (mediated electron transfer mode) or ordinary adsorption in the absence of mediators (direct electron transfer mode) [19]. Data in Table 3 indicate that mediated electron transfer was more efficient than direct electron transfer by almost an order of magnitude in both of the peroxidases. The main electrochemical parameters of both enzymes are very similar, except for the linearity range. In both cases, the linearity range of TOP was greater than that of HRP by a factor of 2 to 3.

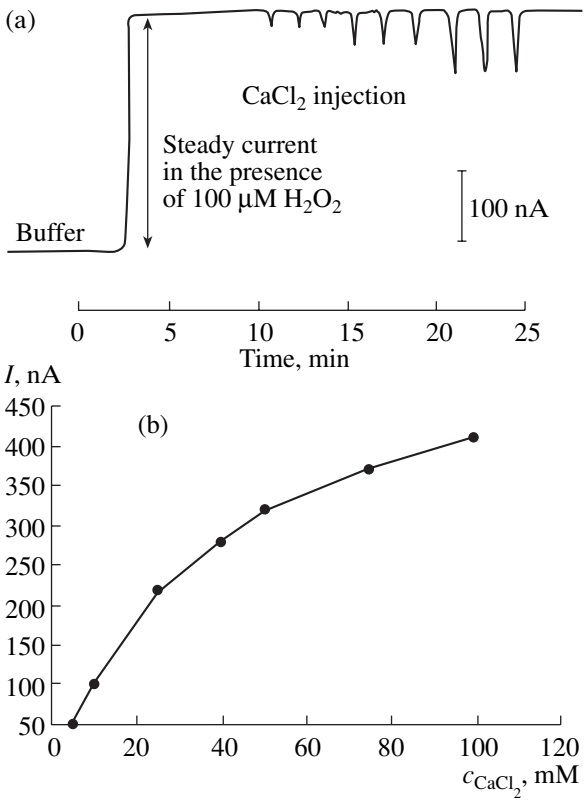


Fig. 5. Effect of calcium cations on steady current in a flow-injection system with a constant concentration of hydrogen peroxide (100 μM) [14]: (a) a decrease in the current upon injection of calcium solutions with different concentrations; (b) approximation of the hyperbolic dependence of a decrease in the current on the concentration of calcium added.

This is a consequence of a lower rate constant k_1 in TOP with respect to hydrogen peroxide (Table 1); because of this, the apparent Michaelis constant has a greater value, which is responsible for a wide linearity range.

Prospects for the use of genetically engineered tobacco peroxidase. Thus, the tests of TOP in various amperometric biosensors demonstrated that the new enzyme is superior to HRP in a number of parameters; this enzyme is of great interest to the development of new analytical methods. A further improvement in parameters and the design of new biosensors based on TOP can be implemented usinga recently prepared unglycosylated recombinant form of the enzyme [2]. In addition to the designs of biosensors considered in this survey, genetically engineered TOP can be used in amperometric flow immunosensors based on a so-called P-chip (peroxidase chip) principle [23] and enzyme tunneling [24]. The operating principle of these sensors consists in the combined immobilization of recombinant peroxidase and a monoclonal antibody to a particular analyte (for example, the herbicide Simazin) on a gold electrode. The determination of Simazin is performed in its competition with the conjugate of Simazin and glucose oxidase for binding with antibodies at the electrode. In the binding of the conjugate with the antibodies at the electrode, glucose oxidase is responsible for the formation of hydrogen peroxide near peroxidase (enzyme tunneling). The sensitivity of an immunosensor based on recombinant HRP for Simazin was 0.1 ng/L, which is better than the sensitivity of traditional ELISA by four orders of magnitude [24].

The use of genetically engineered TOP extended the linearity range of amperometric biosensors of this kind and improved their stability because the new enzyme greatly surpasses recombinant HRP in stability. We found in preliminary experiments on the mutagenesis of the negative glutamic acid residue at the entrance of TOP enzyme active center on the phenylalanine residue, which simulates the active center of HRP, that this residue is responsible for the stability of the enzyme in storage under extreme conditions.

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REFERENCES

1. Gazaryan, I.G. and Lagrimin, L.M., *Phytochemistry*, 1996, vol. 41, no. 4, p. 1029.
2. Khushpul'yan, D.M., Savitskii, P.A., Rozhkova, A.M., Chubar', T.A., Fechina, V.A., Sakharov, I.Yu., Lagrimini, L.M., Tishkov, V.I., and Gazaryan, I.G., *Biokhimiya*, 2003, vol. 68, no. 11, p. 1480 [*Biochemistry* (Moscow) (Engl. Transl.), vol. 68, no. 11, p. 1189].
3. Gazaryan, I.G., Lagrimini, L.M., Ashby, G.A., and Thorneley, R.N.F., *Biochem. J.*, 1996, vol. 313, no. 2, p. 841.
4. Gazarian, I.G., Ashby, G.A., Thorneley, R.N.F., and Lagrimini, L.M., *Appl. Biochem. Biotechnol.*, 1996, vol. 61, nos. 1–2, p. 1.
5. Gazaryan, I.G.., Lagrimini, L.M., George, S.J., and Thorneley, R.N.F., *Biochem. J.*, 1996, vol. 320, no. 3, p. 369.
6. Gazarian, I.G. and Lagrimini, L.M., *Biophys. Chem.*, 1998, vol. 72, no. 3, p. 231.
7. Gazaryan, I.G., Rubtsova, M.Yu., Kapeliuch, Yu.L., Rodriguez-Lopez, J.N., Lagrimini, L.M., and Thorneley, R.N.F., *Photochem. Photobiol.,* 1998, vol. 67, no. 1, p. 106.
8. Dzgoev, A., Gazaryan, I.G., Lagrimini, L.M., Ramanathan, K., and Danielsson, B., *Anal. Chem.*, 1999, vol. 71, no. 22, p. 5258.
9. Gazaryan, I.G., Loginov, D.B., Lialulin, A.L., and Shekhovtsova, T.N., *Anal. Lett.*, 1994, vol. 27, no. 15, p. 2917.

10. Khushpul'yan, D.M., Fechina, V.A., Kazakov, S.V., Sakharov, I.Yu., and Gazaryan, I.G., *Biokhimiya*, 2003, vol. 68, no. 9, p. 1231 [*Biochemistry* (Moscow) (Engl. Transl.), vol. 68, no. 9, p. 1006].

11. Ruzgas, T., Gorton, L., Emneus, J., and Marko-Varga, G., *Electroanal. Chem.*, 1995, vol. 391, nos. 1–2, p. 41.

12. Lindgren, A., Munteanu, F.-D., Gazaryan, I.G., Ruzgas, T., and Gorton, L., *J. Electroanal. Chem.,* 1998, vol. 458, no. 1, p. 113.

13. Lindgren, A., Ruzgas, T., Gorton, L., Csoregi, E., Ardila, G.B., Sakharov, I.Yu., and Gazaryan, I.G., *Biosens. Bioelectron.*, 2000, vol. 15, nos. 9–10, p. 491.

14. Munteanu, F.D., Gorton, L., Lindgren, A., Ruzgas, T., Emneus, J., Csoregi, E., Gazaryan, I.G., Ouporov, I.V., Mareeva, E.A., and Lagrimini, L.M., *Appl. Biochem. Biotechnol.*, 2000, vol. 88, nos. 1–3, p. 321.

15. Gorton, L., Lindgren, A., Larsson, T., Munteanu, F-D., Ruzgas, T., and Gazaryan, I., *Anal. Chim. Acta*, 1999, vol. 400, no. 1, p. 91.

16. Lindgren, A., Tanaka, M., Ruzgas, T., Gorton, L., Gazaryan, I.G., Ishimori, K., and Morishima, I., *Electrochem. Commun.,* 1999, vol. 1, no. 5, p. 171.

17. Gaspar, S., Zimmermann, H., Gazaryan, I.G., Csoregi, E., and Schuhmann, W., *Electroanalysis*, 2001, vol. 13, no. 4, p. 284.

18. Munteanu, F.-D., Lindgren, A., Emneus, J., Gorton, L., Ruzgas, T., Csoregi, E., Ciucu, A., van Huystee, R.B., Gazaryan, I.G., and Lagrimini, L.M., *Anal. Chem.*, 1998, vol. 70, no. 13, p. 2596.

19. Gaspar, S., Popescu, I.C., Gazaryan, I.G., Bautista, A.G., Sakharov, I.Yu., Mattiasson, B., and Csoregi, E., *Electrochim. Acta,* 2000, vol. 46, no. 2, p. 255.

20. Gaspar, S., Habermuller, K., Csoregi, E., and Schuhmann, W., *Sen. Actuators,* vol. 72, no. 1, p. 63.

21. Gorton, L., Csoregi, E., Ruzgas, T., Gazaryan, I.G., and Marko-Varga, G., in *Methods in Biotechnology*, vol. 6: *Enzymes and Microbial Sensors: Techniques and Protocols,* Mulchandani, A. and Rogeers, K.R., Eds., New York: Humana, 1998, p. 93.

22. Sakharov, I.Yu., *Biokhimiya,* 2004, vol. 69, no. 8, p. 1013 [*Biochemistry* (Moscow) (Engl. Transl.), vol. 69, no. 8, p. 823].

23. Ferapontova, E.E., Grigorenko, V.G., and Egorov, A.M., *Biokhimiya*, 2001, vol. 66, no. 8, p. 1026 [*Biochemistry* (Moscow) (Engl. Transl.), vol. 66, no. 8, p. 832].

24. Zeravik, J., Ruzgas, T., and Franek, M., *Biosens. Bioelectron.*, 2003, vol. 18, no. 11, p. 1321.