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Enzymatic Determination of Some Pharmaceuticals in Direct and Reversed Sodium Dodecyl Sulfate Micelles

A. V. Kireiko, I. A. Veselova, and T. N. Shekhovtsova

Analytical Chemistry Department

e-mail: toshta@mail.ru

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Abstract—The properties of horseradish peroxidase in sodium dodecyl sulfate (DDS) reversed micelles in benzene–pentanol–water solutions are studied. The potential of the analytical application of direct and reversed DDS micelles is demonstrated using newly developed methods for the determination of peroxidase substrates (hydrogen peroxide and cystein), inhibitor (sulfanylamide), and activator (imidazole) via the oxidation of *o*-dianisidine (*o*-D) with hydrogen peroxide.

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Carrying out enzymatic reactions in solutions with high concentrations of an organic solvent or in nonaqueous solutions is an important problem of contemporary bioanalytical chemistry. The use of organic solutions to carry out enzymatic processes can substantially widen the range of determinable compounds to include water-insoluble or low-soluble substrates and effectors (inhibitors and activators) of enzymes; this will also make it possible to analyze water-insoluble objects, to improve the metrological characteristics of existing methods, and to increase the selectivity of the determination of biologically active compounds via concentrating.

Conserving the activity of enzymes in nonaqueous solutions is a challenge. An analysis of the literature showed that the catalytic activity of an enzyme in apolar organic solvents is conserved when the enzyme is enclosed (solubilized) into the cavity of an inverted micelle [1–4], which is generated using surfactants.

Horseradish peroxidase is one of the most widely used and well studied (in particular, by us) enzymes. This biocatalyst is distinguished by its high activity, stability in aqueous solutions, specificity to hydrogen peroxide (the major substrate), and sensitivity to many inorganic and organic effectors. Peroxidase was used to develop many processes for the determination of biologically active compounds (which are peroxidase substrates, inhibitors, or activators) [5–7]. However, many compounds capable of acting as second substrates, as well as peroxidase effectors, are incompletely soluble in water, and some substances, e.g., oils, are hydrophobic. In this context, there is a need for methods of enzymatic determination of peroxidase effectors and substrates in organic and aqueous–organic solutions.

Only few works concerned the analytical application of peroxidase enclosed in reversed micelles of sodium di-2-ethylhexylsulfosuccinate (AOT), an anionic surfactant, in an apolar organic solvent [8, 9]. Other surfactants have not yet been used for solubilizing peroxidase in chemical analysis.

The purpose of this work was to demonstrate the pertinence of carrying out peroxidase-catalyzed indicator reactions in the presence of another anionic surfactant, namely sodium dodecyl sulfate (DDS), and enclosing peroxidase into DDS reversed micelles. We worked out procedures for the determination of pharmaceuticals that differently affect the enzymatic process, namely, hydrogen peroxide (the major substrate), cystein (the second substrate), imidazole (an activator), and sulfanylamide (an inhibitor).

EXPERIMENTAL

Reagents

A lyophilized horseradish peroxidase sample (E.C. 1.11.1.7, from Merck) with a specific activity of 192 IU/mg was used. A peroxidase solution with the concentration $n \times 10^{-6}$ mol/L was prepared by dissolving a weighed sample in a 0.1 M phosphate buffer solution (pH 7.0). The concentration of the peroxidase solution was determined spectrophotometrically ($\varepsilon = 9.4 \times 10^4$ L/(mol cm), $l = 1$ cm) at 403 nm [10]. The solid peroxidase and the solution were stored in a refrigerator at 4°C.

Sodium tetraborate (reagent grade), boric acid (high purity grade), ammonium hydrogenphosphate (high purity grade), and ammonium dihydrogenphosphate (high purity grade) were used to prepare buffer solutions; all chemicals were purchased from Reakhim (Russia). Buffered solutions were prepared as described in [11].

For experiments in reversed micelles, benzene (from Reakhim), pentan-1-ol (from Acros-Organics),

and DDS (Helikon, Russia) were used. Benzene was purified by distillation. Aqueous solutions of DDS were prepared by dissolving an exact DDS aliquot in water with continuous stirring on a magnetic stirrer for 15–20 min.

The exact concentration of aqueous hydrogen peroxide (high purity grade, Reakhim) was determined permanganometrically [12]. A solution of *o*-dianisidine (*o*-D; from Sigma) was prepared in rectified ethanol. Solutions of cystein (IREA-2000, Moscow), imidazole (Serva, France), and sulfanylamide (pharmaceutical grade, from Lumi, Russia) were prepared from exact weights and water; sulfanylamide was heated on a water bath. Deionized water purified on a Millipore setup was used to prepare all aqueous solutions.

Instruments

The rate of the *o*-D oxidation indicator reaction was monitored spectrophotometrically as the increase in the absorbance (*A*) of the reaction products; it was characterized by the slope ($\tan\alpha$) of the starting linear fragment of the rate curve in the absorbance–time coordinates. A Shimadzu UV-2201 spectrophotometer was used to record the absorbance of solutions and the absorption spectra of reaction products.

The optimum wavelength for observing the pileup of the products of the peroxidase-catalyzed oxidation of *o*-D (440 nm) and the molar absorptivity (ε = 10700 L/(mol cm)) were determined in DDS reversed micelles with the degree of hydration equal to 27.

Indicator Reaction of o-D Oxidation by Hydrogen Peroxide in Buffered Aqueous Solution

To a glass test tube with a ground stopper, the following chemicals were added in sequence:

(1) the required amount of a 0.1 M phosphate buffer (pH 5.6) for hydrogen peroxide, sulfanylamide, or cystein determinations, or 0.1 M borate buffer solution (pH 8.0) for imidazole determinations;

(2) 3 mM peroxidase solution (0.1 mL) or, for imidazole determinations, a 9 nM solution;

(3) 6 mM *o*-D solution (0.1 mL) for the indicator reaction without effectors or for hydrogen peroxide, cystein, or imidazole determinations or the same amount of a 0.6 mM *o*-D solution for sulfanylamide determinations; and

(4) 7.5 mM hydrogen peroxide (0.1 mL).

In working out the hydrogen peroxide determination procedure, the hydrogen peroxide concentrations in the reaction mixture was varied in the range 0.1–25 μmol/L. The total volume of the reaction mixture was 3 mL. After hydrogen peroxide was added, the solution was stirred and a timer was switched on at the same time. The solution was transferred to a quartz cell (l = 1 cm), and either the absorption spectrum of reaction products was recorded after some time, or the absorbance of the reaction mixture was recorded over time at λ = 460 nm (ε = 31000 L/(mol cm) [13]).

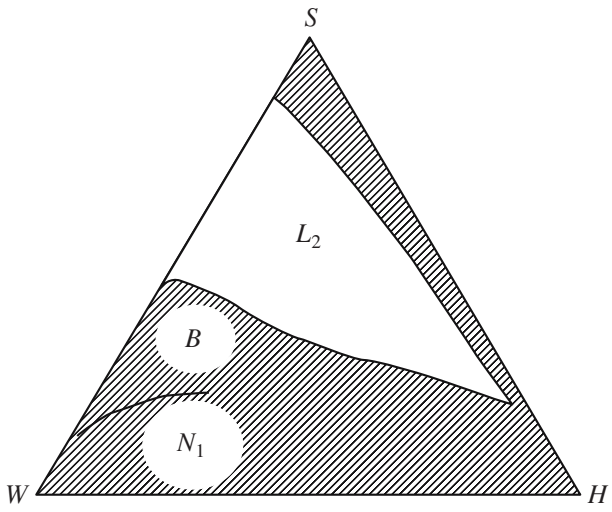

Fig. 1. Phase diagram of the benzene (H)–pentanol + DDS (S)–water (W) system (L_2, the reversed microemulsion field) [14].

The indicator reaction in the presence of DDS was carried out as described above with the only difference that, after peroxide was added, a 30 mM DDS solution (0.1 mL) was added to the reaction mixture and the absorbance was recorded at λ = 395 nm.

In the presence of effectors or the second peroxidase substrate, the reaction was carried out by the above-described procedure with the only difference that, after the enzyme was added, 50 μL of an aqueous solution of an effector (imidazole or sulfanylamide) or the second substrate (cystein) with the required concentration was added. In the presence of DDS direct micelles, the investigation procedure differed in that addition of the enzyme was followed by addition of a 30 mM DDS solution (0.1 mL) and, then the effector solution was added.

Preparation of a DDS Reversed Micellar Solution and the Indicator Reaction in This Solution

The optimum parameters for preparing a DDS micellar solution in an organic solvent were determined from the phase diagram of the four-component system and from experiments (Fig. 1 [14]). A certain DDS sample was dissolved in a magnetically stirred mixture of a 0.02 M citrate buffer solution (pH 5.0; 4.35 mL), pentan-1-ol (7.35 mL), and benzene (1.0 mL). The indicator reaction in the micellar solution was carried out as follows. To a glass test tube with a ground stopper, added were an organic solution (2.85 ml) of DDS, 6 nM enzyme solution (50 μL), 0.02 M citrate buffer solution (25 μL, pH 5.0) or an aqueous effector solution (50 μL), and 0.06 M *o*-D solution (50 μL; for sulfanylamide determinations, the *o*-D concentration was

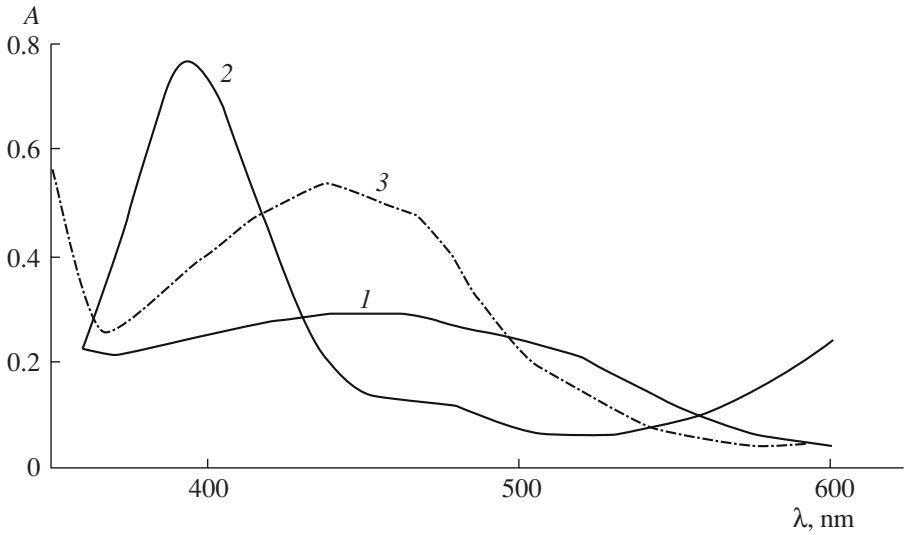

Fig. 2. Absorption spectra for the product of the peroxidase-catalyzed *o*-D oxidation with hydrogen peroxide measured (*1*, *2*) 2 min and (*3*) 3 min after the reaction started in various media: (*1*) 0.1 M phosphate buffer solution (pH 5.6), (*2*) 0.1 M phosphate buffer solution (pH 5.6) in the presence of 1 mmol/L DDS, and (*3*) in DDS reversed micelles.

6 mmol/L), and 0.03 M hydrogen peroxide (25 μL). The mixture was vigorously shaken until it became optically clear. At the moment the aforementioned solutions were combined, a timer was switched on; the mixture was transferred to a cell, and its absorbance was measured every 15 s during 2 min at $\lambda = 440$ nm.

The inhibition effect of sulfanylamide on the catalytic activity of peroxidase (I, %) was calculated from

$$I, \% = 1 - (\tan_{SA}/\tan_0) \times 100,$$

where $\tan_{SA}$ and $\tan_0$ are the slopes of the starting linear fragments of the rate curves of the indicator process with and without sulfanylamide, respectively.

The activation effect of imidazole on the catalytic activity of peroxidase (A, %) was calculated from

$$A, \% = \{(\tan_{Im}/\tan_0) - 1\} \times 100,$$

where $\tan_{Im}$ and $\tan_0$ are the slopes of the starting linear fragments of the rate curves of the indicator process with and without imidazole, respectively.

RESULTS AND DISCUSSION

Sodium dodecyl sulfate, an anionic surfactant, was chosen to solubilize peroxidase. Sodium dodecyl sulfate has shown a potential for use as the most versatile modifier of electrodes [15] and nanoparticles (e.g., carbon nanotubes) [16, 17] among the surfactants studied: it creates strong, structured, thin surface films of various polyelectrolytes [18], including enzymes [16, 17]. These properties of DDS can in future help to create biosensors on the basis of peroxidase solubilized to DDS micelles, with a high sensitivity, stability, raggedness, and rapid response. Phase diagrams for DDS in water–organic solutions are well known (Fig. 1 [14]).

An anionic surfactant was chosen for the pertinence of immobilizing peroxidase inside a reversed micelle (pI = 7.2 [19]) by means of the electrostatic interactions on the inner side of the micelle. This approach, we think, should ensure the best conditions (the least diffusion hindrance) for the transport of compounds from the organic medium to the enzyme.

We chose the oxidation of *o*-dianisidine by hydrogen peroxide for monitoring the peroxidase activity both in aqueous solution and in reversed micelles. This reaction in aqueous solution is well known: its mechanism is described [20, 21], and we previously elucidated the optimum parameters of this reaction [22].

Before addressing the behavior of peroxidase in reversed micelles, we elucidated how DDS affected the peroxidase catalytic activity in a buffered aqueous solution. Previously [22] it was demonstrated that the chemistry of the peroxide-catalyzed oxidation of *o*-D changed in a phosphate buffer solution (pH 5.5) in the presence of more than 0.1 mmol/L DDS: the substrate was stabilized as a result of the electrostatic interactions between the positively charged intermediate of its oxidation and the anionic surfactant (Fig. 2). In concentrations less than 0.1 mmol/L, DDS does not affect the catalytic activity of the enzyme and the chemistry of the indicator reaction: the absorption spectra of reaction products obtained with and without DDS virtually coincide. In the presence of DDS, the intermediate of *o*-D oxidation ($\lambda_{max} = 395$ nm) is piled-up more rapidly than the main product in the absence of DDS; this fact can help to improve the metrological characteristics of the determination of peroxidase effectors and substrates.

The DDS–pentan-1-ol–benzene–water four-component system (Fig. 1) was chosen for solubilizing perox-

Metrological characteristics of the determination of hydrogen peroxide, cystein, sulfanylamide, and imidazole by the peroxidase-catalyzed oxidation of *o*-dianisidine in various media

Analyte	Medium	DDS	Analyte concentration range, μmol/L	Calibration-curve equation	c_{min}, μmol/L
Hydrogen peroxide	Water	–*	1–25	$y = 0.71x + 0.04$ $y = 1.4x + 0.2$ $y = 4.03x + 0.03$ $y - \tan \times 10^2$; $x - C$, μmol/L	0.4
		+**	0.5–25		0.2
	Reversed micelles		0.1–2.5		0.06
Cystein	Water	–	0.5–50	$y = 23x + 151$ $y = 35x + 230$ $y = 46x + 304$ $y - I$, %; $x - \log(C$, mol/L)	0.3
		+	0.5–50		0.1
	Reversed micelles		0.5–50		0.08
Sulfanylamide	Water	–	1000–10000	$y = 5.5x + 5.1$ $y = 7x + 10$ $y = 15x + 9$ $y - I$, %; $x - C$, mmol/L	300
		+	500–10000		100
	Reversed micelles		100–5000		30
Imidazole	Water	–	20–600	$y = 194x + 968$ $y = 194x + 968$ $y = 323x + 1632$ $y - A$, %; $x - \log(C$, mol/L)	10
		+	20–600		10
	Reversed micelles		10–200		5

Notes: * In the absence of DDS.
** In the presence of DDS.

idase to reversed micelles; this system has not been studied as applied to enzymatic processes. The parameters of the peroxidase-catalzyed oxidation of *o*-D in this system were optimized: the degree of hydration, $W_0 = [H_2O]/[Surfactant]$, was 27; the surfactant concentration was 60% (in a mixture with pentanol); the *o*-D, H_2O_2, and peroxidase concentrations were 1 μmol/L, 0.25 mmol/L, and 0.1 nmol/L, respectively; the buffer solution was 0.02 M citrate buffer; and pH was 5.0.

The rate constants (k_{cat}) were calculated for the enzymatic reaction of *o*-D oxidation in an aqueous solution and in reversed micelles (1500 and 200 s^{-1}, respectively). It is known from the literature [4] that, in the same reaction in AOT reversed micelles, peroxidase is superactive (k_{cat} equals 1400 and 33000 s^{-1} in aqueous solution and reversed micelles, respectively). Our data provide evidence that in DDS reversed microemulsions the rate constant of the peroxidase-catalyzed oxidation of *o*-D remains virtually unchanged; i.e., peroxidase does not manifest superactivity after being solubilized to DDS reversed micelles. A likely reason for this is a partial denaturing of the biocatalyst by high pentanol concentrations (60% in a mixture with DDS). When peroxidase is transferred to DDS reversed microemulsions, its affinity to *o*-D (the reducing substrate) decreases, as in AOT reversed micelles: k_{cat}/Km = 42 and 7 L/(s μmol) in the aqueous and organic phase, respectively. The k_{cat} in hydrogen peroxide and the affinity of the enzyme to this major substrate increase: k_{cat} = 2770 and 3268 s^{-1} and k_{cat}/Km = 5 and 22 L/(s μmol) for aqueous solutions and DDS reversed micelles, respectively. As a result, the indicator reaction in a DDS micellar solution improved the metrological characteristics of hydrogen peroxide determination: the detection limit and the lower determination limit decreased significantly, and the sensitivity coefficient of the calibration curve increased (table).

To compare the analytical characteristics of the determinations of peroxidase effectors in three media (aqueous solution, direct DDS micelles, and reversed DDS micelles), we chose the following pharmaceuticals to be model compounds: cystein, sulfanylamide, and imidazole. In choosing these compounds, we were guided by the importance of their determination and by the fact that they have different effects on peroxidase, being its second substrate (cystein), a competitive inhibitor (sulfanylamide), or a noncompetitive activator (imidazole at pH > 6.5) [23, 4]. We have optimized the determination parameters for all aforementioned compounds and worked out their determination in a buffered aqueous solution in the absence of DDS (at λ = 460 nm), in the presence of 1 mM DDS (at λ = 395 nm), and in reversed DDS micelles (λ = 440 nm) (table). The table makes it clear that both direct and reversed DDS micelles decrease the detection limit and increase the sensitivity (the slope of the calibration curve) for the determination of all aforementioned compounds.

In summary, adding a surfactant to an enzymatic process and carrying out the process in direct or reversed micelles improve the analytical characteristics of determination of the peroxidase substrate and effec-

tors. Therefore, it becomes possible to develop sensitive methods for the determination of slightly water-soluble or water-insoluble components of peroxidase-catalyzed reactions.

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