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Polydisulfides of Substituted Phenols as Effective Protectors of Peroxidase against Inactivation by Ultrasonic Cavitation

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Abstract—Kinetics of inactivation of horseradish peroxidase (HP) induced by low-frequency ultrasonic (US) treatment (27 kHz) with the specific power of 60 W/cm^2 were studied in phosphate (pH 7.4) and acetate (pH 5.2) buffers within the temperature range of 36.0 to 50.0°C and characterized by effective first-order rate constants of US inactivation k_{in}(us) in min^{-1}. Values of k_{in}(us) depend on the specific ultrasonic power within the range of 20-60 W/cm^2, on the concentration of HP, and on pH and temperature of the solutions. The activation energy of US inactivation of HP is 9.4 kcal/mole. Scavengers of HO$^\bullet$ radicals, mannitol and dimethylformamide, significantly inhibit the US inactivation of HP at 36.0°C, whereas micromolar concentrations of polydisulfide of gallic acid (poly(DSG)) and of poly(2-aminodisulfide-4-nitrophenol) (poly(ADSNP)) virtually completely suppress the US inactivation of peroxidase at the ultrasonic power of 60 W/cm^2 on the sonication of the enzyme solutions for more than 1 h at pH 5.2. Various complexes of poly(DSG) with human serum albumin effectively protect HP against the US inactivation in phosphate buffer (pH 7.4). The findings unambiguously confirm a free radical mechanism of the US inactivation of HP in aqueous solutions. Polydisulfides of substituted phenols are very effective protectors of peroxidase against inactivation caused by US cavitation.

Key words: horseradish peroxidase, ultrasonic inactivation, inactivation kinetics, phenol polydisulfides, polydisulfide of gallic acid, poly(2-aminodisulfide-4-nitrophenol), free radicals

Wide use of ultrasound (US) in physiology, diagnosis, and therapy motivates studies on its effect on biologically important objects on the molecular level [1-3]. Most of the numerous works in this field are descriptive [1-3]: as a rule, the authors suggest that ultrasound induces changes in proteins and enzymes. Among the many studies on the destruction of enzyme in US fields designed to elucidate the inactivation mechanisms, studies on US inactivation of α-chymotrypsin [4], penicillin amidase [5, 6], lysozyme [7], and of the polyenzyme system of cellulase complex [8] should be especially mentioned. Among studies on US inactivation, studies on proteolytic enzymes (chymotrypsin, pepsin, trypsin) are of special interest because these enzymes are thought to play an important role in the US-induced destruction of fibrin clots, as recently shown for streptokinase [9].

A critical review of data on US-induced destruction of enzymes and proteins in aqueous solutions has defined the conditions required for the inactivation. These include: 1) the presence of cavitation in a solution exposed to ultrasound; 2) the dilution of enzyme (protein) solutions because in concentrated solutions US inactivation often fails to occur or is rather slow [1, 4]; 3) the presence of air (or oxygen) in the solution, because without air or in the atmosphere of inert gases many proteins are resistant to US exposure; 4) the formation in the cavitation field and passage into the solvent of active radicals (HO$^\bullet$, $O_2^{-\bullet}$, $HO_2^\bullet$), H$^\bullet$ atoms, or radicals of added organic cosolvents (CCl_4, $CHCl_3$, etc.) [1-4]. So far, little attention has been given to assessment of the contribution of active radicals to inactivation of proteins and enzymes during US-induced destruction, on one hand, and of the energy of the environmental reorganization and of sound effects in capillaries to inactivation kinetics, on the other.

Abbreviations: US) ultrasound; DMF) dimethylformamide; PBS) 0.01 M phosphate buffer (pH 7.4) supplemented with 0.15 M NaCl; poly(ADSNP)) poly(2-aminodisulfide-4-nitrophenol); poly(DSG)) polydisulfide of gallic acid; HP) horseradish peroxidase; HSA) human serum albumin; TMB) 3,3′,5,5′-tetramethylbenzidine.

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Clearly, other factors in the cavitation zone in aqueous solutions can also contribute to the inactivation and denaturation of proteins [2, 3].

In the present study, peroxidase was chosen because it is known to have carbohydrate fragments that protect the enzyme against active radicals [10, 11], and the US inactivation of peroxidase is thus especially interesting. On the other hand, peroxidase, catalase, and other heme-containing enzymes have been repeatedly shown to be suicidal biocatalysts which loose their activities during the enzymatic reactions because their active sites are attacked by oxygen-containing radicals ($HO^{\bullet}$, $O_2^{\bullet-}$, $HO_2^{\bullet}$) produced during homolytic degradation of peroxidase and catalase peroxide complexes [10, 12, 13], i.e., the carbohydrate fragments of peroxidase fail even to partially protect the enzyme against the active radicals. In the US cavitation field in aqueous solutions, $HO^{\bullet}$, $O_2^{\bullet-}$, and $HO_2^{\bullet}$ radicals are inevitably produced [2, 3]. Therefore, the behavior of peroxidases in a US cavitation field and the mechanism of their possible inactivation during exposure to ultrasound are very interesting.

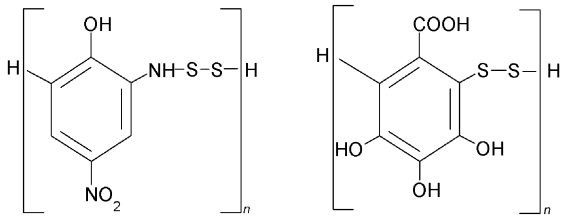
A problem of importance in practice is the prevention of inactivation of peroxidase (and other enzymes) during the US treatment of their solutions. The solution of this problem is especially important when ultrasound is used therapeutically and in cardiology [9] when the protection of proteolytic enzymes responsible for hydrolysis of fibrin clots against the ultrasound, which destroys thrombi in blood vessels, is imperatively needed.

The purpose of this work was to study the inactivation kinetics of horseradish peroxidase in aqueous solutions exposed to low-frequency ultrasound (27 kHz) in dependence on the specific power of the ultrasound, the solution pH, and the enzyme concentration, and also to prevent the inactivation of peroxidase in the US cavitation field with highly efficient antioxidants of the new generation, polydisulfides of substituted phenols, which have been successfully used as inhibitors of many free radical reactions in biochemical processes [14-17].

MATERIALS AND METHODS

Horseradish peroxidase (HP, EC 1.11.1.7) of type A with RZ 2.5 was from Biolar (Olaine, Latvia). The enzyme concentration was determined spectrophotometrically using the molar absorption coefficient in the Soret band maximum (403 nm), ε_{403} = 102,000 $M^{-1}\cdot cm^{-1}$ [18]. Diluted perhydrol (Reakhim, Russia) was used as an oxidizer; the H_2O_2 concentration was determined spectrophotometrically using the molar extinction coefficient ε_{230} = 72.1 $M^{-1}\cdot cm^{-1}$ [19]. 3,3′,5,5′-Tetramethylbenzidine (TMB, Serva, Germany) and guaiacol (Reakhim, Russia) were used as reducing substrates of HP. Human serum albumin (HSA, Reanal, Hungary) was used without additional purification; dimethylformamide (DMF) (Reakhim, Russia) was used immediately after distillation. All other reagents were from Reakhim (Russia).

Inhibitors (antioxidants). Mannitol (Reakhim) and DMF were used as scavengers for $HO^{\bullet}$ radicals. Poly(2-aminodisulfide-4-nitrophenol) (poly(ADSNP)) with an average molecular weight of ~1400 daltons (~7 monomer units) was synthesized as described in [20]. Polydisulfide of gallic acid (poly(DSG)) with an average molecular weight of ~1760 daltons contained 7-8 monomer units ($HOOC\text{-}C_6(OH)_3\text{-}S\text{-}S\text{-}$). Poly(DSG) was synthesized as described in [21]. The UV spectrum of an aqueous solution of poly(DSG) has two absorption maxima: at 215 nm (ε_{215} = 421,000 $M^{-1}\cdot cm^{-1}$) and at 266 nm (ε_{266} = 126,000 $M^{-1}\cdot cm^{-1}$) [22]. The structural formulas of poly(ADSNP) and of poly(DSG) are presented below:



Ultrasonic treatment of peroxidase solutions. The dissolved peroxidase was sonicated with a Tekhnosonic ultrasonic generator (Bauman Moscow High Technical University, Moscow) equipped with a piezoelectric transformer and a titanium waveguide, with the working frequency of 27 kHz. The specific power (intensity) of the ultrasound was varied within the range 20-60 W/cm^2 that corresponds to the amplitude oscillations of the waveguide end-wall within the range of 25.6-57.0 μm. The US treatment of HP was performed in phosphate buffer (or in PBS) (pH 7.4) or in 0.01 M acetate buffer (pH 5.2). The enzyme solution (10 ml) in a glass conic tube was thermostatted and treated with ultrasound for different times at in the range 36.2-50.0°C. The concentration of HP was usually 10 nM, or 30 nM in studies on the effect of radical scavengers. During the sonication of peroxidase solutions, aliquots were taken to determine the residual activity of the HP at the same temperature as the US treatment of the enzyme solution.

The HP activity before and during the sonication was determined in PBS (0.01 M phosphate buffer with 0.15 M NaCl, pH 5.8-7.4), 0.01 M acetate buffer (pH 3.9-5.3), and 0.01 M phosphate citrate buffer (pH 3.5). The mixture analyzed (1.3-1.5 ml) was placed into a cuvette of a KFK.3 photometer (Russia) equipped with a cuvette section and with a digital indication of the absorption. In typical experiments, the activity of HP was determined by the oxidation rate of TMB or of guaiacol [23]. In the first case the mixture under analysis contained 10% DMF (vol. %), 1 mM H_2O_2, and 0.3-1 mM TMB, or its dihydrochloride TMB·2 HCl, and in the second case the mixture contained 0.33 mM H_2O_2 and 22 mM guaiacol. The recording of changes in the mixture absorption was started 5 sec after the addition to the solution of the HP-con-

taining aliquot and was continued for 1-2 min. The light absorption was recorded at wavelengths of 655 and 470 nm corresponding to the absorption maxima of oxidation products of TMB and guaiacol, respectively. By the initial linear regions of the kinetic dependences A_{655} and A_{470}, the initial rates of TMB and guaiacol oxidation were calculated in $M \cdot sec^{-1}$ using the absorption coefficients of the substrate oxidation products equal to 39,000 [18] and 26,600 $M^{-1} \cdot cm^{-1}$, respectively.

To characterize the US inactivation of HP during the initial stages of its sonolysis, the first-order rate constants k_{in}(us) in min^{-1} were used; these constants were determined from the semilogarithmic anamorphoses of kinetic curves of decrease in the activity of HP during its sonication (see Fig. 1); the activity of HP was expressed either in terms of the initial rate of the reaction, v_o, or in terms of absorption of TMB or guaiacol oxidation products at the certain moment, A_{655} or A_{470}.

RESULTS AND DISCUSSION

Features of kinetics of peroxidase inactivation by US cavitation. Figure 1 presents semilogarithmic anamorphoses of kinetic curves of HP (10 nM) inactivation in PBS (pH 7.4) during the US treatment (27 kHz, 60 W/cm^2) within the temperature range 36.2-50.0°C and also the linearization of the kinetic curve of thermal inactivation at 50.0°C under the same conditions. The thermal inactivation of HP at temperatures from 36.0 to 45.7°C was insignificant and was not taken into account when the total inactivation of the enzyme in the ultrasonic field was analyzed. Figure 1 shows that the first-order equation describes only the initial stages of HP inactivation to small degrees of the enzyme transformation. At greater degrees of transformation, an autoacceleration of the HP inactivation was found at temperatures from 36.2 to 45.7°C, and the process was slightly decelerated at 50.0°C (curve *4*).

The effective rate constants of US-induced HP inactivation were calculated for each temperature from the initial linear parts of the dependences in Fig. 1, and the temperature dependences of these constants in Arrhenius coordinates are presented in Fig. 2. From the data presented in Fig. 2, the effective value of activation energy (E_{act}) of US destruction of HP was found to be 9.4 kcal/mole. The resulting E_{act} is significantly less than the E_{act} values for the thermal inactivation of HP, which change within the range of 47.4-62.4 kcal/mole depending on the environment [24]. Thus, the activation barrier of the US inactivation of peroxidase is much lower that its value during the thermal inactivation, and this suggests different mechanisms of the loss in the HP activity during these two processes.

Figure 3 presents dependences of the effective rate constants k_{in}(us) on the specific power of the ultrasound during the treatment of HP solutions: in PBS (pH 7.4), k_{in}(us) increased monotonically with an increase in I from 23 to 60 W/cm^2, whereas in acetate buffer (pH 5.2) k_{in}(us) sharply increased at the ultrasound power ~60 W/cm^2, i.e., the inactivation of HP strongly depended on the environmental pH. Therefore, the effect of pH on the

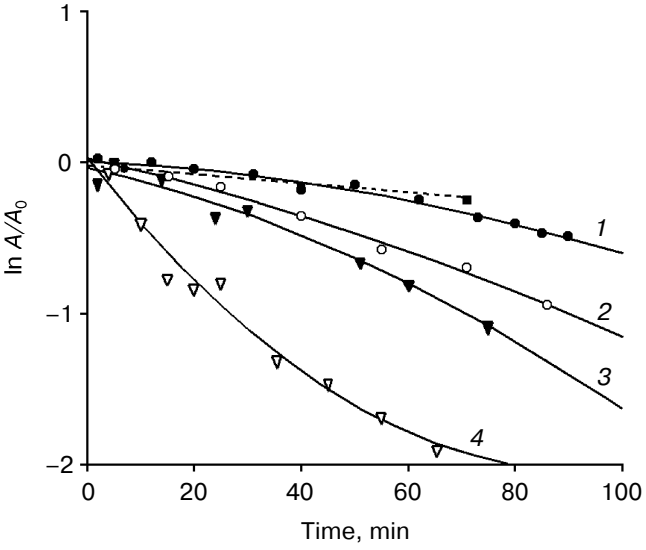


Fig. 1. Semilogarithmic anamorphoses of kinetic curves of decrease in the activity of peroxidase (10 nM) during the US treatment (27 kHz, 60 W/cm^2) of its solution in PBS (pH 7.4) at different temperatures: 36.0 (*1*), 39.7 (*2*), 45.7 (*3*), and 50.0°C (*4*); the dotted line describes the decrease in the HP activity at 50.0°C without sonication of its solution.

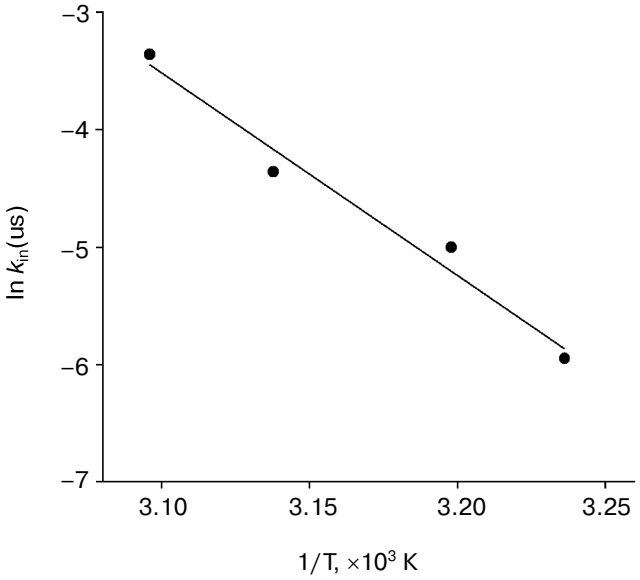


Fig. 2. Temperature dependence of effective rate constants of US inactivation of peroxidase k_{in}(us) in Arrhenius coordinates (10 nM HP, 27 kHz, 60 W/cm^2, PBS (pH 7.4)).

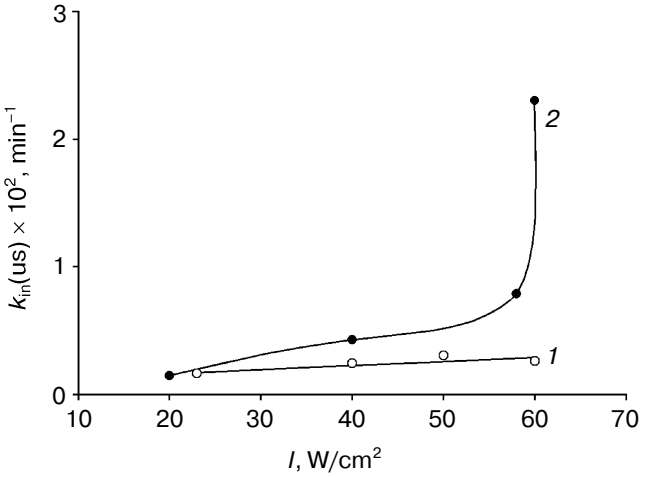


Fig. 3. Dependence of effective rate constants of US inactivation of HP (10 nM) at 36.0°C in PBS (pH 7.4) (*1*) and in 0.01 M acetate buffer (pH 5.2) (*2*) on the specific power of ultrasound (*I*).

k_{in}(us) value was studied in detail at the maximum ultrasound power of 60 W/cm^2. For all values of pH, the constants of US inactivation of the enzyme were calculated from the semilogarithmic anamorphoses of kinetic curves of decrease in the HP activity. The pH dependences of these constants are presented in Fig. 4: dependence *2* shows that k_{in}(us) increased in direct proportion to the hydrogen ion concentration within the pH range from 4.0 to 7.4. Histidine, which is an axial ligand of the heme iron [10, 11], plays a key role in the peroxidase activity. According to statistical data, the pK_a of histidine varies from 5.6 to 7.0 [25], but the pK_a of imidazole with its nitrogen coordinated with the heme iron is 6.04 [26]. The heme distal region in HP is coordinated by the amino acid sequence Phe-His-Asp-Cys-Phe-Val [11] which in addition to histidine also includes two phenylalanine and one cysteine residues: each of these easily react with HO$^\bullet$ radicals, the rate constants being $4.6 \cdot 10^9$, $6.5 \cdot 10^9$, and $1.4 \cdot 10^{10}$ $M^{-1} \cdot sec^{-1}$ for histidine, phenylalanine, and cysteine, respectively [27]. Thus, the HO$^\bullet$ radicals generated in the US cavitation field can attack at high rates the functionally important amino acid residues of the HP active site and decrease the catalytic function of the enzyme.

The transformation of amino acid residues coordinating the heme distal region in HP is indirectly supported by the low value of the activation energy of HP inactivation in the US cavitation field: this energy is only 9.4 kcal/mole (by data of Fig. 2). Under comparable conditions of sonication, E_{act} of a decrease in the functional activity of soybean urease was 16.6 kcal/mole [28]. These values of E_{act} of decreases in the activities of two enzymes on sonication of their solutions under similar conditions reflect the attack of HO$^\bullet$ radicals on different amino acid residues in the active sites of the two enzymes. The barrier of 9.4 kcal/mole characterizes the attack of radicals on aromatic His and Phe and, possibly, on Cys in the active site of HP, while the barrier of 16.6 kcal/mole characterizes the attack on the more stable bonds $-CH_2-$ and NH_2 in the Asp and Glu residues in urease molecule; these residues can be ligands for nickel ions in the active site and maintain the quaternary structure of the urease homohexamer. Actually, the rate constants of reactions of HO$^\bullet$ radicals with Asp ($4.9 \cdot 10^7$) and with Glu ($2.3 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$) [27] are two-to-three orders of magnitude lower than the rate constants of HO$^\bullet$ reactions with His, Phe, and Cys; so it is not surprising that E_{act} of the US inactivation of HP is significantly lower than in the case of urease. Our findings for urease [28] and for HP in the present work strongly confirm the concept of the authors of works [4, 5] that the ultrasonic treatment of enzymes is promising as a sensitive test for their stability and for the role of their active sites.

As mentioned, the US inactivation of proteins and enzymes strongly depends on their concentrations in solutions. Therefore, we studied the dependence of k_{in}(us) at 36°C on the initial HP concentration in 0.01 M acetate buffer on sonication of the system (27 kHz, 60 W/cm^2). Figure 5 presents the dependence of k_{in}(us) on the initial HP concentration, and this dependence shows that concentrating the HP monomer promotes its stabilization in the US cavitation field.

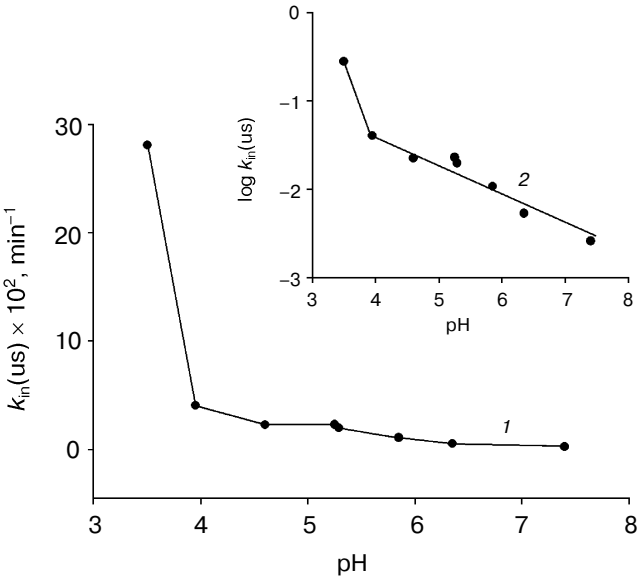


Fig. 4. Dependence of effective rate constants of US inactivation of HP (10 nM) at 36.0°C on the environmental pH (*1*) and the logarithmic transformation of this dependence (*2*) (27 kHz, 60 W/cm^2, 0.01 M phosphate-citrate buffer (pH 3.5); 0.01 M acetate buffer (pH 3.9-5.3); 0.01 M phosphate buffer (pH 5.8-7.4)).

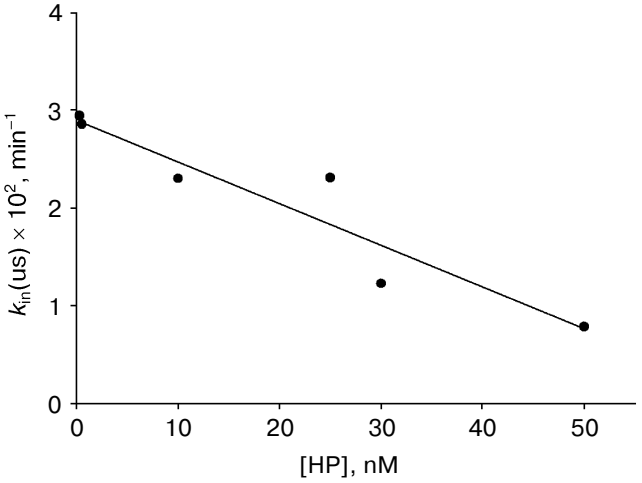


Fig. 5. Dependence of effective rate constant of US inactivation of HP at 36.0°C in 0.01 M acetate buffer (pH 5.2) on the enzyme concentration (27 kHz, 60 W/cm^2).

Inhibition of US inactivation of peroxidase with polydisulfides of substituted phenols and with scavengers of HO$^\bullet$ radicals. The main particles produced in the US cavitation field are free the radicals HO$^\bullet$, $O_2^{\bullet-}$, $HO_2^\bullet$ and the atom H$^\bullet$ which in the presence of O_2 is converted to $HO_2^\bullet$ radical with rate constant ~10^{10} $M^{-1}\cdot sec^{-1}$, i.e., with collision frequency [27]. The high reactivity of the HO$^\bullet$ radical is well known and has been confirmed by the rate constant values of its reactions with various amino acid residues. The superoxide anion and the $HO_2^\bullet$ radical can also react with peroxidase with the rate constants $1.6\cdot10^6$ $M^{-1}\cdot sec^{-1}$ (pH 7.0-8.8) and $2.2\cdot10^8$ $M^{-1}\cdot sec^{-1}$ [27]. Note that in an acidic environment $O_2^{\bullet-}$ is protonated and exists as the $HO_2^\bullet$ radical, with pK_a = 4.5 [29]. Thus, the main oxygen-containing radicals which attack peroxidase in the US cavitation field are HO$^\bullet$, $O_2^{\bullet-}$, and $HO_2^\bullet$. We recently found that the new generation antioxidants, polydisulfides of substituted phenols [14-17], are the most effective inhibitors of free radical biochemical reactions; the antiradical activity of these antioxidants are many times higher than the activities of their monomer units and are due to an "intramolecular" synergism of the effect [17]. We have used poly(DSG) and poly(ADSNP) as possible protectors of peroxidase against the US inactivation.

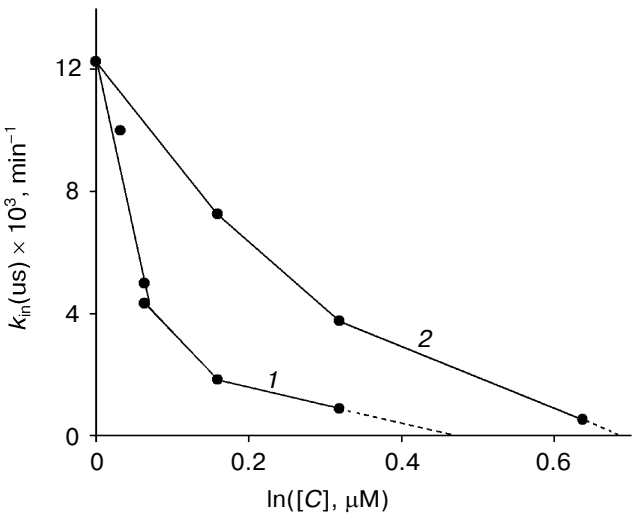


Fig. 6. Dependence of effective rate constants of US inactivation of HP (30 nM) on concentrations (*C*) of protectors poly(DSG) (*1*) and poly(ADSNP) (*2*) (36.0°C, 27 kHz, 60 W/cm^2, 0.01 M acetate buffer (pH 5.2)).

The US treatment (27 kHz, 60 W/cm^2) of 10 ml of 0.01 M acetate buffer (pH 5.2) containing 1 mM poly(DSG) at 36.0°C for 120 min did not induce changes in the absorption spectrum of poly(DSG), i.e., no noticeable expenditure of the inhibitor occurred under these conditions. However, in PBS (pH 7.4) a decrease in the poly(DSG) absorption was recorded within 90 min in the band maximum at the wavelength of 215 nm, corresponding to the poly(DSG) oxidation rate of $3.9\cdot10^{-9}$ M·min^{-1}. Therefore, poly(DSG) was used only in 0.01 M acetate buffer (pH 5.2).

Solutions of 30 nM HP in 0.01 M acetate buffer were sonicated at 36.0°C in the absence of inhibitors and in the presence of their increasing concentrations. In all cases, the k_{in}(us) values were determined from semilogarithmic anamorphoses of kinetic curves describing the decrease in the HP activity. Figure 6 presents the dependences of k_{in}(us) effective values on concentrations of poly(DSG) (*1*) and of poly(ADSNP) (*2*). Figure 6 shows that very low concentrations of both polydisulfides strongly inhibits the US inactivation of HP and can completely prevent it at the concentration of poly(DSG) ~ 0.47 μM and of poly(ADSNP) ~ 0.70 μM as seen from the extrapolation of the dependences (*1*) and (*2*) in the coordinates k_{in}(us)–[poly(DSG)], [poly(ADSNP)].

Dependences *1* and *2* in Fig. 6 show that poly(DSG) protects HP against inactivation in the US cavitation field more efficiently than poly(ADSNP), and this is in good agreement with the effectiveness of these antioxidants during the oxidation of TMB by peroxidase [30]. The complete inhibition of US inactivation of HP with both polydisulfides unambiguously confirms the free radical mechanism of the decrease in the enzyme activity in the cavitation field generated by low-frequency ultrasound in 0.01 M acetate buffer (pH 5.2).

In 0.01 M phosphate buffer (pH 7.4) it is more advantageous to use not poly(DSG) as it is but its complex with serum albumin, because HSA was shown in our laboratory to stabilize poly(DSG) and thus to prevent oxidation of the antioxidant at pH > 7.0 [31]. The HSA–poly(DSG) complexes with component ratios of 1 : 3 and 1 : 6 were used as stabilizers and in 0.01 M phosphate buffer (pH 7.4) they decreased the extent of US inactivation from 44.4 to 11.8% on the 60-min US treatment of HP solution (30 nM). The table compares the data on inactivation of HP exposed to

Extent of peroxidase inactivation (%) during its US treatment (27 kHz, 60 W/cm^2) for 60 min under varied conditions at 36.0°C (HP + poly(DSG)) and at 39.8°C (HP + HSA–poly(DSG))

System	Total concentration of protein, nM	Poly(DSG), μM	pH	Extent of HP inactivation for 60 min, %
HP	30.0	0	5.2	61.7
HP + poly(DSG)	30.0	0.06	5.2	41.8
HP + poly(DSG)	30.0	0.16	5.2	23.2
HP + poly(DSG)	30.0	0.32	5.2	5.0
HP	30.0	0	7.4	44.4
HP + HSA	87.9	0	7.4	36.8
HP + HSA–poly(DSG) (1 : 3)	87.9	0.17	7.4	18.7
HP + HSA–poly(DSG) (1 : 6)	87.9	0.35	7.4	11.8

ultrasound for 60 min in 0.01 M acetate buffer (pH 5.2) in the presence of poly(DSG) and in 0.01 M phosphate buffer (pH 7.4) in the presence of HSA–poly(DSG) complexes of different ratio. The table shows that the effects of poly(DSG) and of its complexes with HSA are quite comparable, i.e., at pH > 7.0 the HSA–poly(DSG) complex is recommended to be used as the HP stabilizer instead of the polydisulfide itself.

It has been mentioned that the radicals $HO^{\bullet}$, $O_2^{\bullet-}$, and $HO_2^{\bullet}$ can destroy HP in a cavitation field. To assess the contribution of each of them to the HP inactivation during its US treatment, the enzyme was inactivated with ultrasound in the presence of known scavengers of $HO^{\bullet}$ radicals, mannitol and DMF. An analog of mannitol D(+), maltose, reacts with $HO^{\bullet}$ radicals with the rate constant of $2.3 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$, and dimethylformamide at pH 5.5 reacts with the rate constant of $1.7 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$ [27]. At 36.0°C the $HO_2^{\bullet}$ radicals did not react with mannitol and DMF. Figure 7 presents the effect of increasing mannitol concentrations on the k_{in}(us) during the treatment of HP solution (30 nM) in 0.01 M acetate buffer (pH 5.2). Even the highest concentrations of mannitol (up to 50 mM) failed to completely prevent the US inactivation, the extent of which was 60%. Figure 8 compares in semilogarithmic coordinates the inactivation of HP in the presence of DMF (0.25% or 31.2 mM) and without it: DMF stabi-

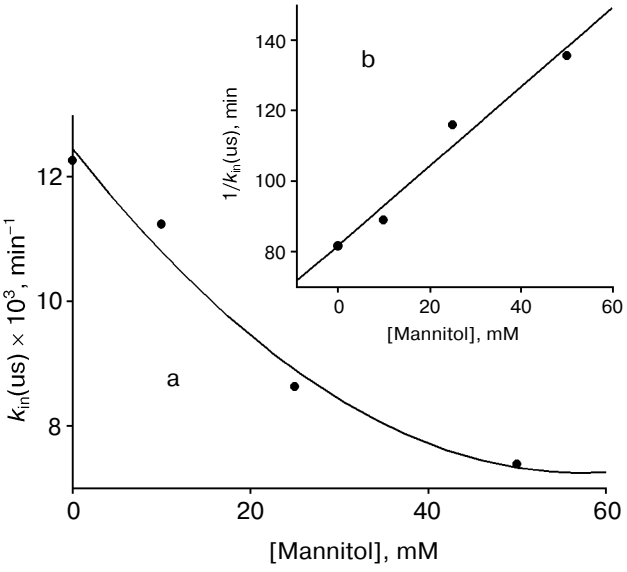


Fig. 7. Dependence of effective rate constants of US inactivation of HP (30 nM) (a) and of its inverse value (b) on the concentration of mannitol in 0.01 M acetate buffer (pH 5.2) (36.0°C, 27 kHz, 60 W/cm^2).

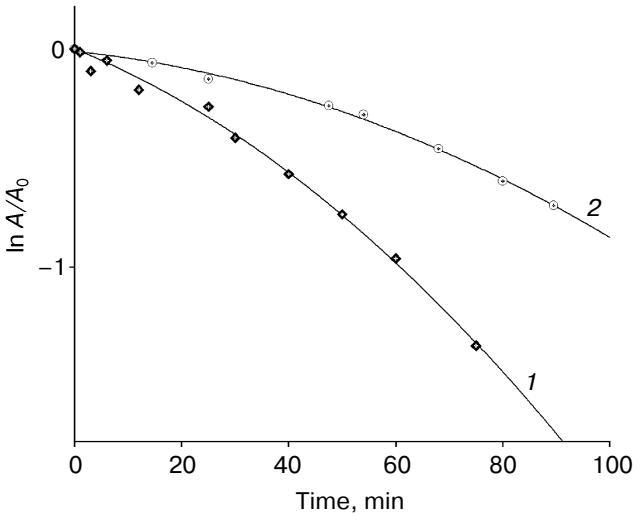


Fig. 8. Semilogarithmic anamorphoses of kinetic curves of decrease in the activity of HP (30 nM) on US treatment of its solution in 0.01 M acetate buffer (pH 5.2) in the absence (*1*) and in the presence of 0.25% DMF (vol. %) (*2*) (36.0°C, 27 kHz, 60 W/cm^2).

lizes HP and protects the enzyme against the inactivation, but a further increase in the content of DMF is undesirable because of its own destructive effect on HP [32].

The incomplete prevention by mannitol of the US inactivation of HP could be due to two causes. First, the $HO^{\bullet}$ radicals are responsible for only part of the inactivation of HP; the enzyme is also inactivated by the $O_2^{\bullet-}$ and $HO_2^{\bullet}$ radicals. Second, mannitol is present in the solvent space but cannot penetrate to the active site region through the hydrophobic channel; thus, the radicals easily attack amino acids in the active site of HP and thus deactivate the enzyme.

Thus, the treatment of peroxidase solutions by low-frequency ultrasound results in enzyme inactivation depending on the enzyme concentration, solution pH, and the ultrasound specific power. In the initial stages, the US inactivation of peroxidase fits the first-order equation with respect to the HP concentration; however, at later stages the inactivation is accelerated at temperatures within the range of 36.0-45.7°C. The effect of the $HO^{\bullet}$ radical scavengers mannitol and dimethylformamide and of the highly effective new generation antioxidants, polydisulfides of substituted phenols, unambiguously confirm the free radical mechanism of peroxidase inactivation. Low concentrations of phenol polydisulfides provide complete protection of peroxidase activity during its treatment with the low-frequency ultrasound for an hour or more. The US treatment of HP can be used as a test for resistance of the enzyme active center, confirming the concept of Berezin, Klibanov, and Martinek [4, 5].

The present work has shown that low concentrations of polydisulfides of substituted phenols can completely prevent US-induced inactivation of peroxidase in aqueous solutions during an extended period sonication.

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