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THE EFFECT OF ULTRASOUND ON HEME ENZYMES IN AQUEOUS SOLUTION

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The effect of cavitating 22 kHz ultrasound on aqueous solutions of hydrogen peroxide-consuming enzymes, catalase and peroxidases, both plant (horseradish peroxidase) and animal (lactoperoxidase) was studied. Catalase did not undergo inactivation during sonication, whereas activity of peroxidases decreased with increased duration of sonication. It is suggested, basing on the absorption spectra, that some conformational changes occur in peroxidases upon sonolysis. It is concluded from the experiments with free radical scavengers that partial enzyme inactivation and modification has not a chemical but a mechanical basis.

Keywords: Catalase; Hydroxyl radicals; Horseradish peroxidase; Lactoperoxidase; Ultrasound

INTRODUCTION

When water containing small gas bubbles is exposed to ultrasound above a certain intensity threshold, acoustic cavitation occurs. Implosion and fragmentation of collapsing bubbles lead to a high increase of temperature and pressure inside bubbles (of the order of several thousands degrees K and hundreds of atmospheres) which result in the thermal dissociation of water into hydroxyl radicals and hydrogen atoms. In the presence of dissolved air, dissociation of O_2 and N_2 in cavitation bubbles also occurs:

$$H_2O\,)) \rightarrow H^{\bullet} + {}^{\bullet}OH \quad (1)$$

$$O_2\,)) \rightarrow 2O \quad (2)$$

$$N_2\,)) \rightarrow 2N. \quad (3)$$

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Inside the bubble or in the liquid shell surrounding the cavity these radicals and atoms recombine or react with gases and water vapor present, which leads to the formation of such products as H_2O_2, H_2, HNO_3 and HNO_2 in the medium.[1,2]

Only about 10% of the radicals generated from water vapor in the cavitation bubbles reach the liquid phase.[3] They can then react with solutes to give products similar to those found in aqueous radiation chemistry.[2] Besides chemical effects produced by ultrasound, via products of water sonolysis, a mechanical degradation of macrostructures in sonicated solution is well known.[4,5]

The increasing application of ultrasound in medicine as well as in preparative biochemistry has caused increased interest in its biological and biochemical side effects. The role of free radicals and of mechanical effects induced by ultrasound in DNA, degradation, inactivation of enzymes, lipid peroxidation, and cell killing, has been recently reviewed by Riesz and Kondo.[2]

There are, however, conflicting contributions on enzyme inactivation induced by ultrasound. Some authors suggest mechanical mechanisms of inactivation[6,7] whereas others argue that ultrasonic inactivation of enzymes is due to chemical reactions.[8,9]

In this work we have investigated the effect of cavitating ultrasound on aqueous solutions of hydrogen peroxide scavenging enzymes: catalase and heme peroxidases. These enzymes contain ferric heme in the active site. The mechanisms of reactions catalyzed by these enzymes are given below:

$$\text{Cat} + H_2O_2 \rightarrow \text{compound I} \quad (4)$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Cat} \quad (5)$$

$$\text{Per} + H_2O_2 \rightarrow \text{compound I} \quad (6)$$

$$\text{Compound I} + AH_2 \rightarrow \text{compound II} \quad (7)$$

$$\text{Compound II} + AH_2 \rightarrow \text{Per} \quad (8)$$

where: Cat – catalase, Per – peroxidase, compound I is ferryl [Fe(IV) = O] π-radical cation, compound II is ferryl derivative of enzyme and AH_2 is the molecule undergoing oxidation.

MATERIALS AND METHODS

Bovine-liver catalase (twice crystallized), RZ = 0.8, horseradish peroxidase type VI A (HRP), RZ = 3.0, lactoperoxidase (LPO) from bovine milk,

RZ = 0.8 and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Sigma. Extinction coefficients of $3.24 \times 10^5\,M^{-1}\,cm^{-1}$ at 405 nm,[10] $1.02 \times 10^5\,M^{-1}\,cm^{-1}$ at 403 nm,[11] and $1.12 \times 10^5\,M^{-1}\,cm^{-1}$ at 412 nm[12] were used for spectrophotometric determinations of catalase, HRP and LPO concentrations, respectively. The activity of catalase was determined by the method of Beers and Sizer.[13] The activity of HRP and LPO was assayed using 2,2′-azino-bis[ethyl-benzothiazoline-(6)-sulphonic acid] (ABTS).[14] A concentration of hydrogen peroxide formed in sonicated water was determined by the peroxidase method.[15] Hydroxyl radicals formed in sonicated water were scavenged by ABTS to form a stable radical cation measured spectrophotometrically at 414 nm.[16] Superoxide radical anions formed in sonicated water were scavenged by nitro blue tetrazolium (NBT^{2+}) to form monoformazan (MF^+), measured spectrophotometrically at 530 nm.[17] Water from the MilliQ Plus (Millipore) was used throughout. Phosphate buffer (10 mM) was used in all measurements to maintain pH = 7.0.

A commercial 22 kHz sound generator (Techpan UD-20, Poland, horn diameter 20 mm) was used. 10 ml of solution was irradiated in a beaker (inner diameter, 30 mm), the distance between the horn and the bottom of the vessel being 10 mm. The intensity of the sound was determined calorimetrically and found to be $15\,W/cm^2$. In order to avoid heating of the probes, sonication periods were 10 s and the reaction vessel was cooled with ice water.

In experiments, where deaeration of the sonicated solution was needed, the solution was first bubbled with argon and the sonolysis was carried out in a glove bag under an argon atmosphere.

Spectrophotometric measurements were done on a Hewlett-Packard 8452A diode-array spectrophotometer.

RESULTS

Products from Water Ultrasonic Irradiation

The linear dependence of $ABTS^{+\bullet}$ radical cation formation on sonication time has been observed (data not shown). In the presence of 0.1 M of t-butanol, a scavenger of $^\bullet OH$ radicals, $ABTS^{+\bullet}$ radical cation does not form during the sonolysis of ABTS aqueous solution. This suggests that $^\bullet OH$ radicals are solely responsible for the sonolytic oxidation of ABTS in aqueous solution to produce $ABTS^{+\bullet}$ radical cation. Thus ABTS was

used to monitor the formation of $^{\bullet}OH$ radicals by ultrasound. The rate of $ABTS^{+\bullet}$ formation depended on ABTS concentration and a saturation level was achieved at 50 mM of ABTS. Therefore at this ABTS concentration all hydroxyl radicals that escape from cavitation bubbles become scavenged. The rate of $^{\bullet}OH$ radical formation during sonolysis has been found to be $0.28\,\mu M\,s^{-1}$.

Hydroperoxyl radical ($HO_2^{\bullet}$) is formed in air-saturated sonicated water in the following reaction:

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet}. \tag{9}$$

It can also form in cavitation bubbles of oxygen-free sonicated water in the following reactions:

$$2^{\bullet}OH \rightarrow O + H_2O, \tag{10}$$

$$2O \rightarrow O_2, \tag{11}$$

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet}. \tag{12}$$

$HO_2^{\bullet}$ and its conjugate base $O_2^{-\bullet}$ exist in aqueous solution in a pH-dependent equilibrium:[18]

$$HO_2^{\bullet} \Leftrightarrow O_2^{-\bullet} + H^+ \quad K = 1.6 \times 10^{-5} M$$

thus, at pH = 7.0 superoxide radical anion is the dominant species. 1.0 mM of NBT (in the presence of 0.1 M of t-butanol) appeared sufficient to scavenge all $O_2^{-\bullet}$ generated in sonicated water. The rate of superoxide formation during sonolysis of water has been found to be $0.25\,\mu M\,s^{-1}$.

The rate of hydrogen peroxide formation during water sonolysis has been found to be $0.31\,\mu M\,s^{-1}$.

It has been reported that the rate of nitrous acid formation during air-saturated water sonolysis is the same as that of H_2O_2 formation, however the rate of nitric acid formation under such conditions is about 3.5 times slower.[1]

Activity of Enzymes after Sonication

Figure 1 shows the effect of ultrasonic irradiation on the activity of catalase, HRP and LPO. Catalase appears resistant to ultrasound under our conditions of sonication, whereas the activity of HRP and LPO decreases with the duration of sonication. We have found that catalase remains

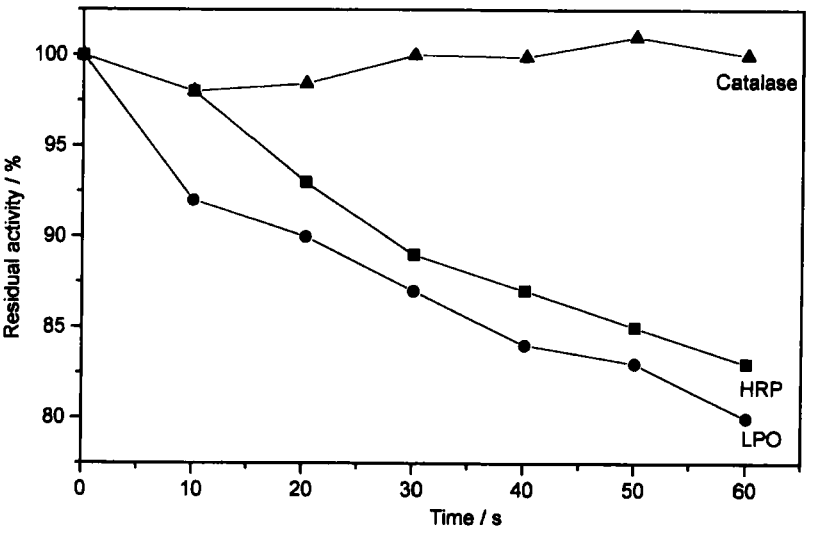

FIGURE 1 Residual enzyme activity versus sonication time. Concentration of enzymes: [catalase] = 2.0 μM; [HRP] = 4.0 μM and [LPO] = 2.5 μM. Points are the mean of at least three determinations.

resistant to ultrasound generated in our system even after dilution down to 2×10^{-8} M. Neither the change of sonicated volume of the catalase solution (from 20 ml to 7.5 ml) nor the depth of insertion of the resonator into the solution has influence on catalase resistance to ultrasound. On the other hand, we have found that the extent of inactivation of HRP or LPO depends upon the initial enzyme concentration: the lower the concentration, the higher the inactivation extent. HRP retained its full activity after 1 min of sonication when it was sonicated at a concentration exceeding 10 μM.

We have checked that the activity-loss profile of HRP or LPO does not change, in the range of the experimental error, when they are sonicated in the presence of t-butanol (0.1 M) which, being added in such concentration, scavenges all hydroxyl radicals in the bulk solution. Hence, it seems that hydroxyl radicals produced sonolytically in our system are not able to inactivate the investigated enzymes.

It is known that superoxide reacts with heme peroxidases to form so called compound III.[19] This compound is catalytically inactive. Compound III of HRP or LPO is stable for several minutes at room temperature.[19] Its decomposition causes partial enzyme inactivation.[20] We have measured the activity loss of HRP or LPO in the presence of 5×10^{-6} M of SOD, and

have found that scavenging of superoxide by SOD has no effect on ultrasound-induced inactivation of investigated enzymes. It should be noted here that this amount of SOD sonicated for 120 s in the presence of 1×10^{-3} M of NBT did reduce the formation of MF^{+} by a half. Activity changes observed after the sonolysis of catalase, HRP or LPO, as carried out in an atmosphere of argon, were the same as in the presence of air. Hence we suggest that under our reaction conditions enzyme inactivation has a mechanical rather than a chemical basis.

Absorption Spectra of Sonicated Enzymes

The absorption spectra of catalase taken after different sonication times (up to 1 min.) show no changes in the intensity either at the Söret band (380–450 nm) or in the UV region (280 nm) (data not shown).

The absorption spectra of HRP taken after different sonication time are shown on Figure 2. The spectrum taken after 10 s of sonication is characteristic for compound I formed in reaction (6). After longer sonication time, species having an absorption spectrum at 420 nm, characteristic for compound II of HRP, appears. The addition of 10^{-4} M of ascorbic acid caused the recovery of absorption spectrum to that of native enzyme

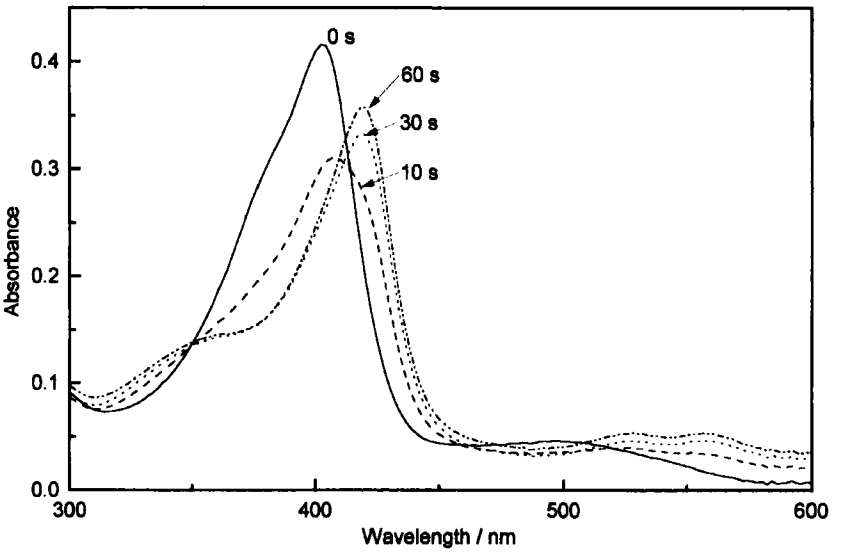

FIGURE 2 Representative absorption spectra of 4 μM HRP solution measured after different sonication time.

(reaction 8). When HRP was sonicated in the presence of 0.1 M of t-butanol ($^{\bullet}OH$ scavenger), 5×10^{-6} M of SOD ($O_2^{-\bullet}$ scavenger), or under an Ar atmosphere the compound II was formed as well. This means that under our experimental conditions, neither superoxide nor protein radical which could have been formed due to $^{\bullet}OH$ reaction, can reduce compound I to compound II. Such reactions have been, however, observed in pulse radiolysis studies.[21,22] The reaction of compound I with nitrite anion[23] could also not account for compound II production, as in the Ar-saturated solution, where nitrite and nitrate anions were absent, the rate of compound II formation was exactly the same as in the air-saturated solution. We have also checked that the half-life time of the spontaneous reduction of compound I to compound II of non-modified enzyme is about 10 min., i.e more than ten-fold longer than the half-life time of the sonolytically-induced conversion of compound I to compound II. Hence we suggest that sonication of HRP solution causes conformational changes that facilitate electron transfer from an internal donor to the porphyrin ring of HRP.

Figure 3 shows absorption spectra of LPO taken after different sonication times. It can be seen that already after 10 s of sonication, the species having absorption maximum at 430 nm is present, while an increase of absorbance in the UV region after prolonged sonication was detected. Compound I of LPO in the form of a π-radical cation is very unstable and

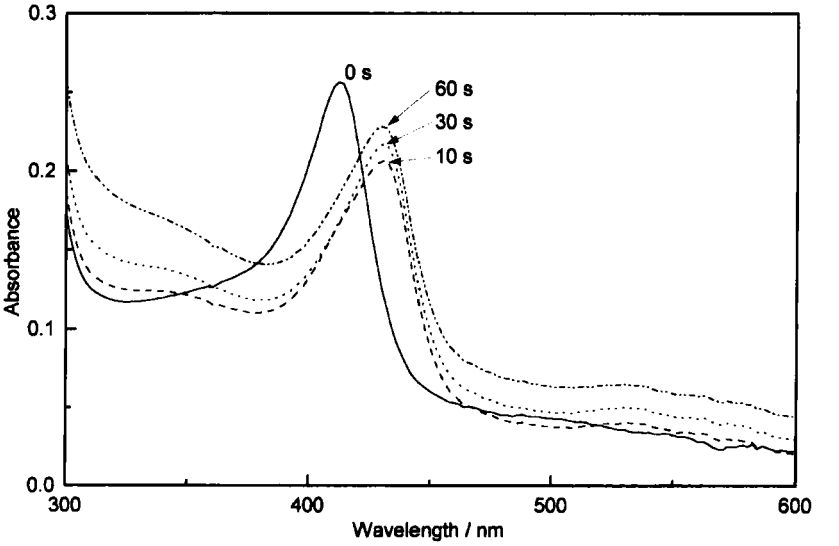

FIGURE 3 Representative absorption spectra of 2.5 μM LPO solution measured after different sonication time.

can be observed only on a millisecond time scale. It converts to its second form with the unpaired electron on the protein part. The absorption spectra of this "second" form of LPO compound I and of LPO compound II with the maximum at 430 nm in the Söret region are indistinguishable.[24] An absorbance increase in the UV region can be related to the unfolding of the enzyme molecule and the exposure of its aromatic amino acids.[25] Among the investigated enzymes, LPO is the only one in which sonolytically induced changes of the absorption spectrum in the UV region remained after its recovery to the ferric state (the absorbances of native HRP or LPO and their compounds II are almost identical in the region of 270–300 nm). Like in the case of HRP, the absorption spectra of sonicated LPO solution were the same when t-butanol or SOD were added to the sonicated solution or when the sonication was carried out under argon.

DISCUSSION

We have found that catalase does not undergo inactivation during sonication under our experimental conditions. Coakley *et al.*[8] and Kashkooli *et al.*[6] have also found that the activity of catalase remains unchanged after 20 kHz ultrasound irradiation. On the other hand Sarrach and Siefke[9] have found that after 10 min of sonication of 0.1 μM catalase solution, i.e. at the same enzyme concentration as used by Coakley *et al.*[8] the enzyme retains about 80% of its activity. From the observation of the decrease of bleaching of nitrosodimethylaniline (NDA), an •OH scavenger, sonicated in the presence of catalase, they have concluded that •OH radicals are responsible for the sonolytic destruction of catalase. It is quite possible that the differences between the results obtained by Sarrah and Siefke [9] and by other authors,[6,8] including results presented here, are due to the fact that catalase used by Sarrah and Siefke[9] has been obtained from other source (Reanal) than catalase used by other authors[6,8] and by us (Sigma).

We have shown that although free radicals do form in our system during ultrasound irradiation, the investigated peroxidases are not inactivated by hydroxyl or superoxide radicals. We have shown earlier that although hydroxyl radicals react with heme enzymes with a diffusion controlled rate constant,[22,26] only a small fraction of radiolytically generated •OH radicals inactivate catalase, HRP or LPO.[27,28] Also bearing in mind that the rate constant of the reaction of heme peroxidases with superoxide is at least one order of magnitude lower than the rate constant of their reaction with H_2O_2,[29] it might supposed that, under our reaction conditions, compound III formation would be rather unfavorable. The third product of water

sonolysis, hydrogen peroxide, is a substrate for heme enzymes and at such concentrations that are obtainable during sonolysis it could not be the inactivating agent for investigated enzymes.

Taking into account that inactivation profiles, and conformational changes induced by ultrasound in HRP or LPO are the same in the presence and in the absence of free radical scavengers, it is concluded that using 20 kHz sound intensity, i.e. under such irradiation conditions which are often applied in experiments in which biological cells are to be ruptured, partial enzyme inactivation has not a chemical but a mechanical origin.

Acknowledgements

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