BEHAVIOR OF ENHANCED CHEMILUMINESENCE PEROXIDASE-CATALYZED PEROXIDATION OF LUMINOL IN THE SYSTEM OF SURFACTANT–WATER–ORGANIC SOLVENT

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This work includes some observations of the chemiluminescent reaction behavior in the system surfactant (Triton X-100 and Aerosol OT) -water-organic solvent (octane and hexane). The HRP-chemiluminescent activity was detected without the surfactant or using low surfactant concentration (less than 0.01 M). The greatest activity was observed applying the surfactant at 0.001 M. It was demonstrated that in the system surfactant-water-organic phase chemiluminescent reaction shown the HRP superactivity only at low substrate and enhancer concentration. To detect the chemiluminescent intensity, the hydroperoxide concentration was significantly decreased comparing to water-contained system. At the same time the chemiluminescent signal was more stable in these systems than in the aqueous system. The hydration ratio ($W_0 = [H_2O]/[surfactant]$) corresponding to the greatest activity was 2222 and 2778 for 0.001 M Triton X-100 and AOT contained systems in octane, respectively. These observations support the view that the model different from reversed micelle model must be proposed to describe the HRP-chemiluminescent reaction behavior in the organic phase in the presence of surfactant and water.

Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) is a very popular enzyme both for fundamental research and practical applications [1, 2]. The discovery of enhanced substrates for HRP catalyzed chemiluminescence has led to the development of commercial kits [2–4]. The HRP-catalyzed enhanced chemiluminescence provides a sound basis for an assay of enzymatically generated H_2O_2, an enzyme immunoassay and DNA dot-hydrolization assay [1, 2]. The high selectivity, the simplicity and the extreme sensitivity of the chemiluminescent methods explain the success of its recent utilization [1].

Enhanced chemiluminescence (ECL) describes the phenomenon of the light output increase in the reaction of oxidation of luminol catalyzed by horseradish peroxidase in the presence of certain phenolic compounds. However, the exact mechanism of enhancement is still unresolved [1–6]. Chemiluminescence is generated when a chemical reaction gives an electronically excited product, which emits radiation to convert back to the resting state. In the case of luminol oxidation catalyzed by peroxidase, the decay of aminophthalate dianion provides light emission ($\lambda = 425$ nm) [1, 2].

In this work we presented some observations related to the behavior of enhanced chemiluminescent reaction (ECL-reaction) in nonconventional media contained surfactant-water-organic solvent. The present study has done in order to reveal possible peculiarities of the HRP-catalyzed peroxidation of such substrate as luminol in organic media.

Materials and Methods

HRP (type IV A, 1100 U/mg), Trizma, hydrochloric acid, 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol), *para*-iodophenol, EDTA, hydrogen peroxide, NaOH, Tween-20, Brij-96, Triton X-100 and sodium bis(2-ethylhexyl)sulfosuccinate, also known as Aerosol OT (AOT) were purchased from Sigma Chemical Co. Chemiluminescence intensity was measured using an EMILITE EL 1003 portable luminometer (Russia).

The stock solutions of luminol (1.2 mM) and *para*-iodophenol (0.8 mM) were prepared in 1 M NaOH solution (50 μl) and 0.1 M Tris-HCl buffer, pH 8.5 using bidistilled water. The *para*-iodophenol was used as an enhancer. The stock solutions were kept in amber flasks at 4°C.

To study the ECL-reaction in the water contained system, the measurements of chemiluminescent intensity were performed in 0.1 M Tris-HCl buffer containing 2 mM of EDTA, pH 8.5. Aliquots of luminol, *para*-iodophenol and H_2O_2 were added to a cuvette to a final volume of 1.01 ml. The reaction was initiated by addition of 10 μl of enzyme solution (5 U/ml). The ECL intensity profiles were recorded and the maximum intensity (I_{max}) was used to plot the graphs. To select optimal conditions for light emission concentrations of luminol, *para*-iodophenol and H_2O_2 were varied over the range of 0.01–0.1 mM, 0.01–0.04 mM and 0.001–0.075%, respectively. Each measurement was in triplicate, as well as every one of the assays.

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To study ECL- reaction in systems of surfactant-water-organic phase, nonionic and anionic surfactants (Triton X-100 and AOT) and organic solvents (hexane and octane) differing in the number of carbon atoms were applied. First, the surfactants (0.1, 0.01, 0.005, and 0.001 M) was dissolved in each organic solvent. ECL-reaction was carried out in the same manner for each of these solutions: surfactant solution (1 ml) was placed in a luminometer cuvette, then 10 μl of enzyme solution (11 U/ml) and 10 μl of H_2O_2 were added in the same cuvette. The reaction mixture was shaken for 30 s at 250 rpm until it became uniform. After 30 s more, the solution of 0.2 mM luminol and 0.3 mM *para*-iodophenol (20 μl) was added. The value of the light intensity emitted during the ECL-reaction was determined rapidly after shaking of the reaction mixture during 7–10 s. Then, 10 μl of the same 0.1 M Tris-HCl buffer containing 2 mM of EDTA, pH 8.5, were added. The reaction mixture was shaken every time before the intensity measurement. Buffer addition was repeated thirty times more.

Results and Discussion

We examined the behavior of ECL-reaction catalyzed by peroxidase, which is well defined, can be solubilized in organic solvents in the presence of surfactants (both anionic and nonionic) which normally form reversed micellar environment [7, 8]. The protein is hosted in a water pool and is shielded from the bulk organic phase. The peroxidase is a versatile model to study the protein behavior in surfactant-containing organic phase. The critical question this work addresses herein is: what is the condition to carry out the ECL-reaction catalyzed by peroxidase in the surfactant–water–organic solvent containing system.

Using the technique described for obtaining of reversed micellar system in the earlier studies of HRP-catalyzed reaction monitored spectrophotometrically [7–9], we could not detect the chemiluminescence in the enhanced reaction with luminol and *para*-iodophenol. As is showh from Figs. 1 and 2 demonstrate, the HRP-chemiluminescent activity was detected either without the surfactant (Triton X-100 and AOT) or using low surfactant concentration (no more than 0.01 M). In the both cases (with nonionic and ionic surfactant) the major activity was observed applying the surfactant at 0.001 M that is significantly less then it commonly was used in the reversed micelles. This peculiarity of behavior of the HRP-catalyzed ECL-reaction of luminol peroxidation may be explain by its more sensibility to surfactant concentration than the reactions with other spectrophotometrically detected substrates described in the literature data [7–9]. Moreover, in the presence of 0.01 M of anionic surfactant AOT the intensity was not detected (Fig. 2) whereas in the presence of the same concentration of nonionic surfactant Triton X-100 the chemiluminescence was observed (Fig. 1). In the both case, the greater activity was detected in octane-contained system than in the presence of hexane as organic solvent. These effects may be related to the difference in the surfactant structures and solvent hydrophobicity.

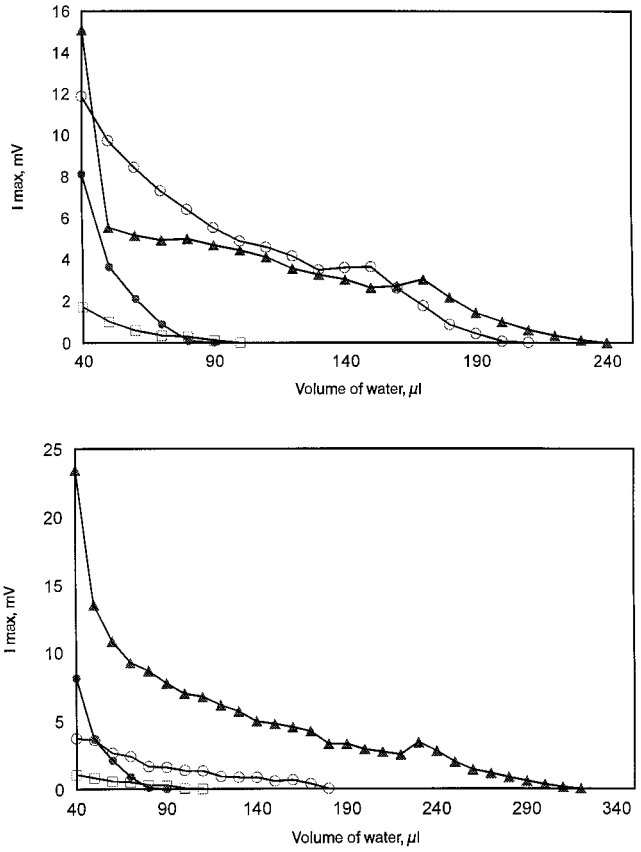

Fig. 1. Effect of water content on the HRP activity in the ECL-reaction of luminol peroxidation: Top, in hexane-contained system and Bottom, in octane-contained system, without surfactant (Dot) and in the presence of different concentrations of Triton X-100: (Triangle), 0.001 M; (Circle), 0.005 M, and (Square), 0.01 M.

Figures 1 and 2 show, the catalytic activity in the luminol-contained system can be easily regulated by water content variation (that in the reversed micellar systems reflected the micelle inner cavity size changes). As for many other enzymes, a bell-shaped curve obtained for HRP in the presence of different concentrations of AOT is characterized by the maximum at the dependence (Fig. 2) whereas in Triton X-100-contained system the decreasing curves without maximum were obtained at the same water content (Fig. 1).

Expressing water content of the system in terms of the hydration ratio ($W_0 = [H_2O]/[\text{surfactant}]$), it can be seen that the values of W_0 corresponded to major activity on the HRP-catalyzed ECL-reaction of luminol peroxidation increase when the AOT and Triton X-100 concentration is decreased (Table 1). For example, the hydration degree (W_0) corresponding to the major activity for 0.001 M Triton and AOT-contained systems in the presence of octane, was 2222 and 2778 (Table 1), respectively. So great values of W_0 were not correlated with characteristics of reversed micelle systems which normally characterized by optimum W_0 no greater than 40. In reversed micelles the optimum hydration degree corresponding to maximum enzyme activity is observed when the size of the inner cavity of a micelle is equal to that of the protein [8]. In the studying system the water content is so great comparing with the surfactant content that it is probable insufficient to form the reversed micelles.

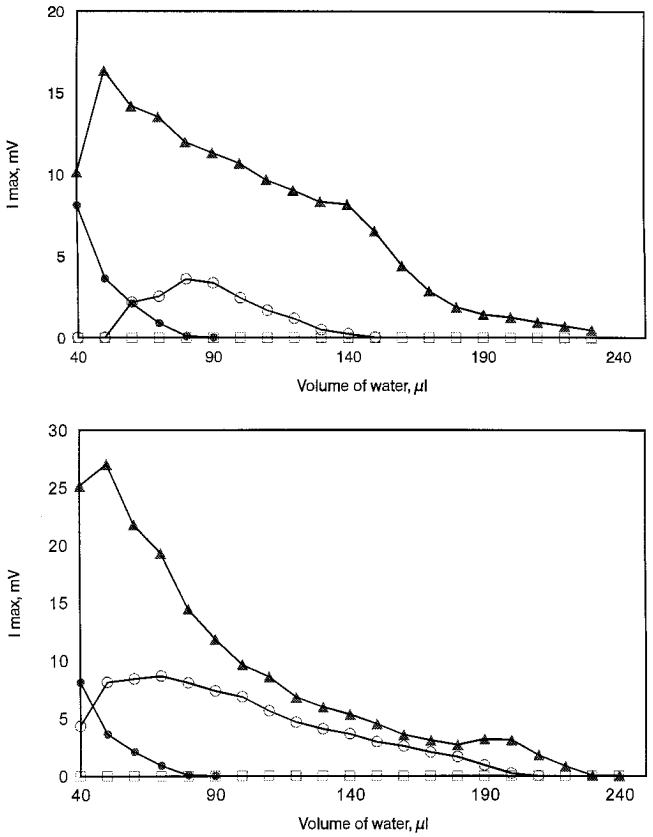

Fig. 2. Effect of water content on the HRP activity in the ECL-reaction of luminol peroxidation: Top, in hexane-contained system and Bottom, in octane-contained system, without surfactant (Dot) and in the presence of different concentrations of AOT: (Triangle), 0.001 M; (Circle), 0.005 M, and (Square), 0.01 M.

These observations support the view that the model different from reversed micelle model must be proposed to describe the HRP-chemiluminescent reaction behavior in the organic phase in the presence of surfactant and water.

Table 1

Comparison of hydration degrees ($W_0 = [H_2O]/[surfactant]$) determined in the surfactant–water–organic solvent containing system for HRP-catalyzed ECL-reaction of luminol peroxidation

Surfactant, M	Optimum W_0	
	(in hexane)	(in octane)
AOT (0.001 M)	2778	2778
AOT (0.005 M)	889	778
Triton X-100 (0.001 M)	2222	2222
Triton X-100 (0.005 M)	444	444
Triton X-100 (0.01 M)	222	222

Early it was demonstrated that complexes of a glycoprotein as peroxidase from horseradish and soybean could be extracted from water solution into isooactane containing low AOT concentration (0.02 M) in the absence of reversed micelles [10–12]. To explain the protein extraction Matsuura et al. [13] speculated the existence of complexation between the surfactant and protein using the model differed to reversed micelle model. They termed this phenomenon as hydrophobic ion-paring. It is probable that the same explication may be applied to describe the behavior of the HRP-catalyzed ECL-reaction of luminol peroxidation in the surfactant-water-organic solvent containing system.

The limitation of the ECL sensitivity is associated with a lag period time [14]. This phenomenon is connected with a delay in light emission after mixing of all reactants (hydrogen peroxide, luminol, enhancer and HRP). Since the lag time phenomenon affects the HRP-induced chemiluminescence only at low HRP concentrations and makes the dependence of light output on HRP concentration nonlinear [15]. In this work the maximum ECL intensity has studied as a function of the enzyme concentration. The obtained curves were nonlinear in water medium as well as in surfactant–water–organic solvent-contained system.

Table 2

Comparison of some characteristics of HRP-catalyzed ECL-reaction of luminol peroxidation in water and surfactant-water-organic solvent systems

Description of the system	Substrate concentration, mM	$K_{m\ app}$, mM	$I_{max\ app}/K_{m\ app}$, mV/M
Water-contained system ([E] = 5 U/ml)	$[H_2O_2] = 4.4$		
Parameters for luminol	[PIP] = 0.03	0.15	1.6×10^6
	0.04	0.15	1.6×10^6
Parameters for *para*-iodophenol (PIP)	[Luminol] = 0.01	0.0082	2.5×10^6
	0.025	0.0029	2.5×10^6
Parameters for H_2O_2	[PIP] = 0.03	1.3	7.8×10^4
	[Luminol] = 0.02		
AOT-water-hexane contained system ([E] = 11 U/ml)	$[H_2O_2] = 0.22$		
Parameters for luminol	[PIP] = 0.003	0.00037	8.0×10^7
	0.004	0.00079	8.2×10^7
Parameters for *para*-iodophenol (PIP)	[Luminol] = 0.002	0.0078	7.6×10^6
	0.0025	0.019	7.9×10^6
Parameters for H_2O_2	[PIP] = 0.003	0.05	1.1×10^3
	[Luminol] = 0.002		
AOT-water-octane contained system ([E] = 11 U/ml)	$[H_2O_2] = 0.22$		
Parameters for luminol	[PIP] = 0.003	0.00016	3.3×10^7
	0.004	0.00047	3.3×10^7
Parameters for *para*-iodophenol (PIP)	[Luminol] = 0.002	0.026	6.8×10^6
	0.0025	0.14	6.8×10^6
Parameters for H_2O_2	[PIP] = 0.003	0.13	9.5×10^2
	[Luminol] = 0.002		

According to the results of some authors [14, 15], the kinetics of enhanced HRP reaction is affected by reagent purity. A remarkable lag period was observed for the water-contained systems with the enzyme concentration less than 22.5 ng/ml and in organic solvent-contained systems with the enzyme concentration less than 45 ng/ml. According to these results it was proposed to use the enzyme concentration of 45 ng/ml (5 U/ml) in water medium and 100 ng/ml (11 U/ml) in organic-contained medium (Table 2). There was virtually no lag period in light emission from the systems that contained this enzyme concentration.

Different authors report the existence of phenomenon of significant increase of HRP-activity (superactivity) in reverse micelles that was observed using different substrates and spectrophotometric detection [7–9]. HRP contains haem as a prostetic group participating in catalysis, protein chain and carbohydrate part which corresponds to 20% of enzyme molecular weight [1, 6]. The revealed phenomenon can be attributed to the HRP ability to interact with micellar matrix due to carbohydrate residues [9].

We demonstrated that in the system surfactant-water-organic phase chemiluminescent reaction reflected the HRP superactivity only under using of low substrate and enhancer concentration (at 10 times lower than in water medium). The chemiluminescent intensity was not detected in the aqueous system when so low luminol and *para*-iodophenol concentrations (Table 2) were applied that demonstrate the phenomenon of enzyme superactivity.

Moreover, to detect the chemiluminescent intensity, the hydroperoxide concentration was decreased significantly (at 20 times) comparing with water-contained system (Table 2). The ECL intensity was reduced when the concentration of this cosubstrate was more than 4.4 mM in water medium and than 0.22 mM in surfactant-water-organic solvent system. Meanwhile, HRP Compound-III can be formed by the oxidation of HRP Compound-II by an excess of H_2O_2 or by the reaction of native HRP with superoxide anion. The HRP Compound-III is less reactive than Compounds I and II [16] and it could be a reason for the decrease in maximum ECL intensity in both cases.

Like for many other substrates studied early in water medium by means of spectrophotometric and chemiluminescent assays [7–9], a bell-shaped curves were obtained for HRP in the presence of different concentrations of luminol, hydroperoxide and *para*-iodophenol in the system surfactant–water–organic solvent. So, during this study it was found that the intensity of ECL-reaction of luminol peroxidation catalyzed by HRP in the system surfactant-water-organic solvent strongly depended on substrates and enhancer concentration: the effect of inhibition of the ECL-reaction by substrates and enhancer was observed whereas as various authors reported [7–9], in the reversed micelle system applied in the study of HRP-activity using some spectrophotometrically monitoring substrates, the inhibitory effect was disappeared. The luminol, hydroperoxide and *para*-iodophenol concentrations that did not provoke the inhibition of the reaction were significantly lower that in the water-contained system (Table 2). The increasing parts of obtained in the present study curves described the maximum ECL intensity ($I_{\max}$) as the function of concentrations of luminol and *para*-iodophenol, are in accordance with the Michaelis–Menten equation. A series of parallel lines were obtained in Lineweaver-Burk coordinates in water medium and in the surfactant–water–organic solvent systems. It can signify that the reaction of luminol peroxidation catalyzed by HRP, in both case (water and organic mediums) is described by Ping-Pong mechanism as it was reported early [2, 5, 17]. The tangent values of these lines give the constant value of $I_{\max\ app}/K_{m\ app}$ ratio (Table 2) due to a simultaneous change in $I_{\max\ app}$ and $K_{m\ app}$. In the organic solvent-contained systems the decrease of $K_{m\ app}$ was observed for both substrates of enzyme but not for enhancer (Table 2).

Moreover, in this study was demonstrated that the chemiluminescent signal detected in the surfactant-water-organic solvent contained system was more stable than in the water-contained system. More specifically, the signal detected in the aqueous system decrease rapider than the signal observed in the surfactant-contained system. As it was reported early [18, 19], the enzyme inactivation is the main reason for light decay in the course of the reaction. The loss of enzyme activity can be partially explained by nonspecific interaction of radical species with protein globule. In water medium the addition of bovine serum albumin provided almost complete protection of peroxidase from inactivation [18]. Concerning the enzyme stability in the surfactant contained system, the effect to improve the peroxidase stability in the course of the reaction was observed.

In conclusion, we point out that this work illustrates some differences in the behavior of HRP-catalyzed reaction of luminol peroxidation in surfactant–water–organic solvent-contained system to compare with the responses in water-contained system. To explain the revealed differences more studies must be effectuated.

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