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Optimization of horseradish peroxidase-catalyzed enhanced chemiluminescence reaction by full factorial design

Marina M. Vdovenko, Alexandra S. Demiyanova, Tamara A. Chemleva, Ivan Yu. Sakharov*

Department of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia

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ABSTRACT

Using a full factorial design the optimization of experimental conditions of enhanced chemiluminescence reaction (ECR) catalyzed by horseradish peroxidase (HRP-C) in the presence of 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) and 4-morpholinopyridine (MORP) as enhancers was performed. The effect of concentrations of SPTZ, hydrogen peroxide, MORP, luminol, and Tris on a ratio of peroxidase-catalyzed CL to background was studied. The use of the full 2^5 factorial design instead of "one-variable-a time" method allowed to increase the sensitivity of HRP-C determination 2355 fold without a change of detection limit. The obtained results open up very promising perspectives for using HRP-C-catalyzed ECR to improve the sensitivity of chemiluminescent enzyme immunoassay.

1. Introduction

Enzyme immunoassay is one of the widely used methods in current analytical practice [1,2]. In this assay an isozyme *c* of horseradish peroxidase (HRP-C, EC 1.1.11.7) is commonly used as a label of immunoreagents. Multiple detection methods for enzyme activity of peroxidase-labeled immunoreagents are applied, including colorimetry, fluorimetry and chemiluminescence (CL). CL detection is markedly more sensitive than other methods [3,4]. This method is based on the enzymatic oxidation of luminol by peroxides in the presence of peroxidases under mild alkaline conditions. Luminol oxidation leads to the formation of an 3-aminophthalate ion in an excited state, which emits light when returning to the ground state. The emission spectrum shows a maximum at 425 nm [5].

Since peroxidases are poor catalysts in luminol oxidation [6], certain compounds known as enhancers are added to the substrate mixture to increase CL intensity. Previously the mechanism of enhanced chemiluminescence reaction (ECR), where luminol and an enhancer are oxidized simultaneously, was described in [7,8].

At the first step of ECR the enhancer molecule, which is a better substrate for HRP than luminol, is oxidized by hydrogen peroxide in the presence of HRP according to "ping-pong" mechanism (Eqs. (1)–(3)):

$$E + H_2O_2 \rightarrow EI + H_2O \quad (1)$$

$$EI + SH \rightarrow EII + S^{\bullet} \quad (2)$$

$$EII + SH \rightarrow E + S^{\bullet} + H_2O \quad (3)$$

where SH – enhancer, $S^{\bullet}$ – radical product of one-electron oxidation of enhancer, E is the ferric enzyme (resting state), EI and EII – compound I and compound II, the oxidized intermediates of peroxidase, which are by two and one oxidation equivalents above the resting state, respectively.

Then, the formed radical product ($S^{\bullet}$), using its oxidative potential, reacts with luminol molecule (Eq. (4)). This chemical process is complex and is not clear so far. However, it is well known that the final product of this reaction is also 3-aminophthalate (Eq. (4)).

$$S^{\bullet} + \text{luminol} \rightarrow SH + \text{3-aminophthalate} + N_2 + h\nu$$

Therefore, the enhancers play a role of mediators in the peroxidase catalysis [9], and their presence in the reaction solution does not affect chemical nature of the final product, but increases CL intensity due to their higher reactivity towards Compound I and Compound II than that of luminol.

Although a number of compounds was successfully used in the enhancement of peroxidase-induced CL [10–12] presently the most effective enhancer is 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) (Fig. 1) [13]. This compound can markedly increase CL

Abbreviations: HRP-C, isozyme *c* of horseradish peroxidase; SbP, soybean peroxidase; CL, chemiluminescence; ECR, enhanced chemiluminescence reaction; SPTZ, sodium 3-(10′-phenothiazinyl)propane-1-sulfonate; MORP, 4-morpholinopyridine; RLU, relative luminescence units.

* Corresponding author. Fax: +7 495 9395417.
E-mail address: sakharovivan@gmail.com (I.Yu. Sakharov).

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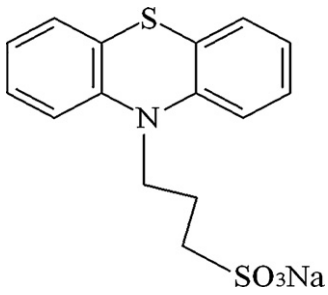

Fig. 1. Chemical structure of sodium 3-(10′-phenothiazinyl)propane-1-sulfonate.

induced by HRP-C [14]. Furthermore, it was shown that an introduction of 4-morpholinopyridine (MORP), 4-dimethylaminopyridine and 4-pyrrolidinopyridine (Fig. 2) to a reaction mixture containing luminol, hydrogen peroxide and SPTZ resulted in the increase of enhancing ability of SPTZ and, in turn, increased CL intensity [14]. The use of SPTZ and MORP as primary and secondary enhancers, respectively, allowed developing the sensitive chemiluminescent method for determination of different plant peroxidases [15,16]. Later, ECR with employment of above mentioned enhancers was successfully used in development of ultra-sensitive immunochemical methods for the determination of human thyroglobulin and ochratoxin A [17,18].

Although the sensitivity of CL detection of plant peroxidases in the presence of SPTZ and MORP was high, the peroxidase activity was determined under experimental conditions defined previously as optimal by an "one-variable-a time" method. Taking into consideration that ECR is a complex multistep reaction, in which a lot of substances take place or may affect its efficiency, there is a high probability that the "one-variable-a time" method has not allowed determining the most favorable conditions for the performance of the reaction of interest.

In this paper we describe the use of a full factorial design for the optimization of the experimental conditions of the enzymatic oxidation of luminol by hydrogen peroxide in the presence of SPTZ and MORP as enhancers and HRP-C as biocatalyst. The obtained results demonstrated that the use of this method allowed a significant improvement of the sensitivity of chemiluminescent determination of HRP-C activity.

2. Experimental

2.1. Reagents and materials

Horseradish peroxidase (isoenzyme *c*, RZ 3.0) was purchased from Sigma (USA) and used without further purification. Sodium 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ, a purity of 99.9%) was prepared as described in Ref. [14]. 4-Morpholinopyridine (MORP) was from Aldrich (USA); luminol and Tris were from Sigma (USA), H_2O_2 (30%) from ChimMed (Russia). The concentration of HRP-C was measured by using $\varepsilon_{402} = 102{,}000\ M^{-1}\ cm^{-1}$ [19]. The

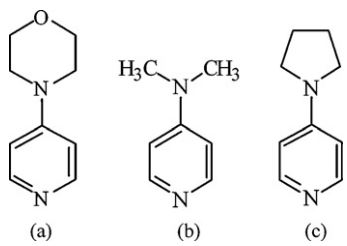

Fig. 2. Chemical structures of 4-morpholinopyridine (a), 4-dimethylaminopyridine (b) and 4-pyrrolidinopyridine (c).

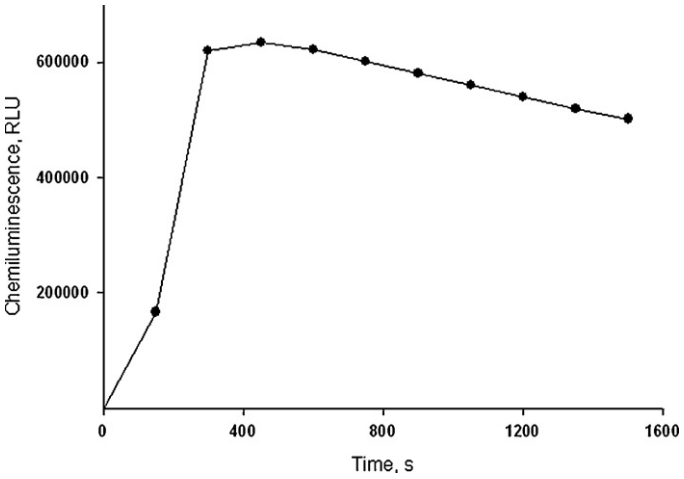

Fig. 3. Kinetic curve of HRP-C-induced chemiluminescence in the presence of SPTZ and MORP determined under the conditions optimized by the factorial design: [HRP-C] = 7.5 pM; [luminol] = 0.17 mM; [H_2O_2] = 1.75 mM; [SPTZ] = 2.1 mM; [MORP] = 8.75 mM; 80 mM Tris buffer, pH 8.3.

H_2O_2 concentration was determined by monitoring A_{240}, using $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$ [20]. The required dilutions of H_2O_2 were prepared daily.

2.2. Chemiluminescent determination of peroxidase activity

HRP-C activity was assayed as follows: 0.6–3.0 mM hydrogen peroxide, 0.05–0.65 mM luminol, 0.4–3.6 mM SPTZ and 0.01–15 mM MORPH were dissolved in 200 μl of 20–80 mM Tris buffer, pH 8.3 in wells of black polystyrene plates (MaxiSorp, NUNC, Denmark) for chemiluminescent enzyme immunoassay. Then the enzymatic reaction was initiated by adding 50 μl of peroxidase solution in Tris buffer at relevant pH and concentration. Chemiluminescence kinetics was measured for 25 min at room temperature on a luminometer 3100 (Anthos, Austria). The experimental data of the decay of HRP-C-induced CL in the presence of enhancers were analyzed by a first-order reaction rate equation (Eq. (5)).

$$\ln(I_t/I_{max}) = -k_{decay}t, \quad (5)$$

where I_{max} and I_t are CL intensities measured 7.5 min after the initiation of luminol oxidation and at moment t, respectively; k_{decay} is a decay constant. The measurement of CL was carried out in 7.5 min, because the kinetic study demonstrated that at this time CL intensity showed a maximum (Fig. 3). The chemiluminescent signal in the absence of the enzyme was taken as background value. The light intensity was expressed in relative chemiluminescence units (RLU).

2.3. Experimental design

Full two-level (2^5) factorial design was used to optimize the experimental conditions of performance of ECR catalyzed by HRP-C. The effect of five factors such as concentrations of luminol, hydrogen peroxide, SPTZ, MORP, and Tris was evaluated. The used concentrations of variables are presented in Table 1. The efficiency of ECR performance was evaluated as a ratio of peroxidase-catalyzed CL to background. The obtained results were analyzed using Statgraphics XV.I.

3. Results and discussion

To optimize the experimental conditions of performance of ECR catalyzed by HRP-C we used the full factorial design. Previously

Table 1
The variable values used in the optimization of experimental conditions of ERC catalyzed by HRP-C.

Variables	Levels		
	Low (−1)	Centered (0)	High (+1)
X_1 – [SPTZ], mM	0.4	2.0	3.6
X_2 – [H_2O_2], mM	0.6	1.8	3.0
X_3 – [MORPH], mM	0.01	7.5	15
X_4 – [luminol], mM	0.05	0.35	0.65
X_5 – [Tris], mM	20	50	80

this approach was successfully applied to solve various tasks connected with the optimization of values of some variables carrying out a minimum of experiments [21,22]. In our work the concentrations of luminol, hydrogen peroxide, SPTZ, MORP, and Tris in the reaction medium were selected as independent. The used values of variables are presented in Table 1. Preliminary the values of these variables were determined using "one-variable-a time" method. A ratio of CL intensity formed upon the enzymatic reaction to background was used to estimate the efficiency of ECR performance.

The results of CL determinations at different experiment conditions are presented in Table 2. The analysis of the obtained results was performed using Statgraphics XV.I that allowed a determination of the dependence of a ratio of CL intensity formed upon the enzymatic reaction to background (Y) upon the concentrations of SPTZ (X_1), hydrogen peroxide (X_2), MORP (X_3), luminol (X_4), and Tris (X_5) (Eq. (6)).

$$Y = 127,711 + 3623X_1 - 2638X_2 + 14,042X_3 - 8682X_4 + 8491X_5 - 32,276X_1^2 + 4966X_1X_2 + 2675X_1X_3 + 2622X_1X_4 + 1514X_1X_5 - 30,880X_2^2 - 494X_2X_3 + 20X_2X_4 + 31X_2X_5 - 66,823X_3^2 - 5318X_3X_4 + 5364X_3X_5 - 6573X_4^2 + 1181X_4X_5 + 23,180X_5^2 \quad (6)$$

Eq. (6) allowed a calculation of a combination of concentrations of the reacting substances to obtain the maximum value of light intensity. The calculated conditions were the following: 80 mM Tris, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM MORP, and 1.75 mM H_2O_2. It should be noted that under these conditions the background value was extremely low (2–3 RLU) that is the promising fact to develop highly sensitive analytical devices.

Under the optimized conditions the dependence of CL on the HRP-C concentration was determined (Fig. 4). We compared the obtained dependence of CL with similar ones determined under the experimental conditions previously reported as optimal for ECR in the presence of SPTZ and MORP catalyzed by HRP-C (125 mM Tris, pH 9.0, containing 5 mM luminol, 1.5 mM SPTZ, 1.5 mM MORP, and 4 mM H_2O_2 [14] and soybean peroxidase (50 mM Tris, pH 8.3, containing 0.75 mM luminol, 1 mM SPTZ, 1 mM MORP, and 0.5 mM H_2O_2 [15]. The comparison showed that the value of detection limit (0.25 pM) did not affect the conditions of the ECR performance (Fig. 4). At the same time, the optimization of the experimental conditions improved markedly the sensitivity of ECR (bias of the curve at the concentration of HRP-C higher than a detection limit value). So, whereas the bias of the curve 3 (the reaction conditions was determined by "one-variable-a time" method) was 52 RLU/pM of HRP-C, after the optimization of the reaction conditions by the factorial design this value (curve 1) was 122,470 RLU/pM of HRP-C, i.e. the sensitivity was improved in 2355 times. Therefore, the use of full factorial design instead of "one-variable-a time" method allowed a significant improvement of the sensitivity of HRP-C determination without any change of detection limit.

Table 2
Full factorial design used in the optimization of experimental conditions of ECR catalyzed by HRP-C.

No	Variable level					Ratio of HRP-C-induced CL to background
	X_1	X_2	X_3	X_4	X_5	
Main block						
1	−1	−1	−1	−1	−1	19,577
2	+1	−1	−1	−1	−1	39
3	−1	+1	−1	−1	−1	8253
4	+1	+1	−1	−1	−1	37
5	−1	−1	+1	−1	−1	47,920
6	+1	−1	+1	−1	−1	25,602
7	−1	+1	+1	−1	−1	13,970
8	+1	+1	+1	−1	−1	33,735
9	−1	−1	−1	+1	−1	858
10	+1	−1	−1	+1	−1	20
11	−1	+1	−1	+1	−1	94
12	+1	+1	−1	+1	−1	2
13	−1	−1	+1	+1	−1	995
14	+1	−1	+1	+1	−1	17
15	−1	+1	+1	+1	−1	8
16	+1	+1	+1	+1	−1	5
17	−1	−1	−1	−1	+1	17,565
18	+1	−1	−1	−1	+1	57
19	−1	+1	−1	−1	+1	1661
20	+1	+1	−1	−1	+1	90
21	−1	−1	+1	−1	+1	61,492
22	+1	−1	+1	−1	+1	26,544
23	−1	+1	+1	−1	+1	33,963
24	+1	+1	+1	−1	+1	68,352
25	−1	−1	−1	+1	+1	2787
26	+1	−1	−1	+1	+1	53
27	−1	+1	−1	+1	+1	304
28	+1	+1	−1	+1	+1	14
29	−1	−1	+1	+1	+1	27,049
30	+1	−1	+1	+1	+1	38,460
31	−1	+1	+1	+1	+1	2107
32	+1	+1	+1	+1	+1	29,613
Additional block						
33	−1	0	0	0	0	24,620
34	+1	0	0	0	0	163,785
35	0	−1	0	0	0	102,042
36	0	+1	0	0	0	89,155
37	0	0	−1	0	0	142
38	0	0	+1	0	0	119,170
39	0	0	0	−1	0	139,269
40	0	0	0	+1	0	100,542
41	0	0	0	0	−1	84,796
42	0	0	0	0	+1	214,524
43	0	0	0	0	0	132,635

We also demonstrated that the conditions for HRP-C-catalyzed ECR determined in this work were more preferable in comparison with the conditions previously reported for soybean peroxidase. The performance of ECR at the conditions optimized by the factorial design increased the sensitivity 2.8 fold. The detection limit value was unchanged.

One of advantages of use of SPTZ/MORP as enhancers in ECR was shown to be the formation of long-term CL signal [14,15]. As seen in Fig. 3, the change of the experimental conditions as a result of their optimization did not affect the kinetics of HRP-C-induced CL. At the first step the CL intensity increased, reaching its maximum, and then quenched slowly. The CL decay obeyed a first-order kinetics with a decay constant (k_{decay}) of 2.0×10^{-4} s^{-1}. The low rate of CL quenching is characteristic of application of SPTZ/MORP as enhancers in contrary to commonly used *p*-iodophenol, in the presence of which quick quenching of CL is observed [23].

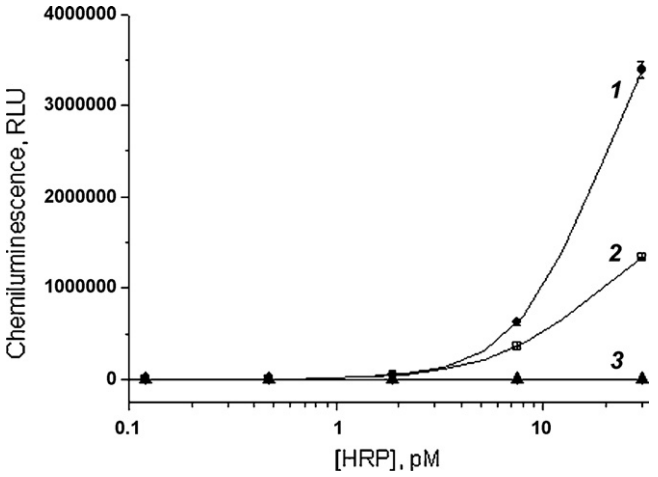

Fig. 4. Effect of HRP-C concentration on light output from luminol/H_2O_2 substrates in the presence of SPTZ and MORP evaluated under following conditions: (1) [luminol] = 0.17 mM; [H_2O_2] = 1.75 mM; [SPTZ] = 2.1 mM; [MORP] = 8.75 mM; 80 mM Tris buffer, pH 8.3, (2) [luminol] = 0.75 mM; [H_2O_2] = 0.5 mM; [SPTZ] = 1.0 mM; [MORP] = 1.0 mM; 50 mM Tris buffer, pH 8.3, and (3) [luminol] = 5 mM, [H_2O_2] = 4.0 mM; [SPTZ] = 1.5 mM; [MORP] = 1.5 mM; 125 mM Tris, pH 9.0. Chemiluminescence intensity was recorded 7.5 min after the start of the reaction. Each point represents the mean of triplicates. Vertical bars indicate ±S.D. about the mean.

4. Conclusions

In enhanced CL reaction a lot of substances (luminol, hydrogen peroxide, SPTZ, MORP, HRP-C, Tris) take place. It creates severe difficulties to optimize experimental conditions of this reaction. Replacement of an "one-variable-a time" method traditionally used in the literature with a full factorial design used in the present paper allowed an improvement of the sensitivity of HRP-C determination in 2355 times without any change of detection limit value. The obtained results open up very promising perspectives for using HRP-C-catalyzed ECR to develop enzyme immunoassay kits with improved sensitivity.

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