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Peroxidase-Catalyzed Chemiluminescence-Delay of Luminol for Determination of Traces of Copper(II)

Atsushi KATAYAMA, Tamio KAMIDATE,* and Hiroto WATANABE
Faculty of Engineering, Hokkaido University, Kita-ku, Sapporo 060
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Delayed chemiluminescence (CL) was observed in the copper(II)-catalyzed oxidation of cysteamine with oxygen in the presence of horseradish peroxidase (HRP) and luminol. After preferential catalytic oxidation of cysteamine by both Cu(II) and HRP, a HRP-catalyzed luminol CL reaction was subsequently commenced with hydrogen peroxide accumulated from the catalytic oxidation. Thus, a delay time from the reaction initiation to a sharp flash of CL was observed. The HRP-catalyzed CL-delay of luminol was applied to the determination of Cu(II). The delay time was linearly correlated with the Cu(II) concentration over the range from the detection limit of 5.0×10^{-9} to 1.5×10^{-5} M. The detection limit of the present method is a factor of 30 times better than that of the conventional luminol CL method. The relative standard deviation of the delay time in five successive experiments was 3.2% at 1.0×10^{-7} M of Cu(II). The present method was highly sensitive compared to the conventional luminol CL method.

Luminol chemiluminescence (CL) with alkaline hydrogen peroxide (H_2O_2) catalyzed by metal ions has been employed for the highly sensitive determination of trace metal ions.[1-3] However, nonspecificity limits its direct application to analysis of complex samples, since many metal ions catalyze the luminol CL reaction.

We have previously reported the Cu(II)-catalyzed CL-delay of luminol in an alkaline medium for the determination of Cu(II).[4] In the CL-delay reaction, the catalytic oxidation of cysteamine(RSH) by Cu(II) proceeds preferentially, resulting in the formation of H_2O_2 and disulfide (RSSR).

$$2RSH + O_2 \xrightarrow{Cu^{2+}} RSSR + H_2O_2$$

After complete oxidation of cysteamine, the Cu(II)-catalyzed luminol CL reaction is subsequently commenced using the accumulated H_2O_2.

$$\text{luminol} + H_2O_2 \xrightarrow{Cu^{2+}} \text{aminophthalate} + N_2 + H_2O + h\nu$$

Thus, a delay time from the reaction initiation to a sharp flash of CL is observed. The CL-delay reaction has been successfully applied to the selective determination of Cu(II) by measuring the delay time or CL intensities. However, the detection limit was a factor of about 20 times higher than that of the conventional luminol CL method.

In order to improve the sensitivity for Cu(II), we examined the catalytic oxidation of cysteamine by Cu(II) in a mild pH medium in which the amount of H_2O_2 formed during the catalytic oxidation was maximal.[5] Horseradish peroxidase (HRP)-catalyzed luminol CL was applied to the detection of H_2O_2. In the HRP-catalyzed luminol CL, the CL intensity increased with an increase in the H_2O_2 concentration.

$$\text{luminol} + H_2O_2 \xrightarrow{HRP} \text{aminophthalate} + N_2 + H_2O + h\nu$$

In the course of our studies on the application of HRP-catalyzed luminol CL to the detection of H_2O_2, delayed CL of luminol was also observed after a certain dark period from the initiation of the catalytic oxidation of cysteamine by Cu(II). The aim of this work was improve the sensitivity and selectivity for Cu(II) by use of the HRP-catalyzed CL-delay of luminol.

Experimental

Reagents. Horseradish peroxidase (HRP: type VI) was obtained from Sigma Chemical Co. Luminol(5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Kanto Chemical Co. All chemicals used were guaranteed-grade reagents and were used without further purification. A 1.0×10^{-2} M stok solution of luminol was prepared by dissolving the compound in a 0.1 M (1 M=1 mol dm^{-3}) NaOH solution. Working solutions of luminol were prepared by serial dilution with Carmody's buffer solution (pH 8.5). An HRP solution was prepared with Carmody's buffer solution (pH 8.5), and its concentration was determined spectrophotometrically with an ε_{403} value of 90 mM^{-1} cm^{-1}.[6] A cysteamine ($H_2NCH_2CH_2SH$) solution and working solutions of Cu(II) were prepared as described previously.[4] All the solutions used were prepared with water from a Millipore Milli-Q water purification system.

Apparatus. A luminometer constructed in this laboratory and a Hitachi U-2000 type spectrophotometer were employed for the measurements of CL and absorbance, respectively. Information on the CL instrumental systems has been previously described.[4]

Recommended Procedure. A 1.0 cm^3 portion of a Cu(II) solution, a 1.0 cm^3 portion of a 4.5×10^{-6} M HRP solution and a 0.5 cm^3 portion of a 6.0×10^{-5} M luminol solution were added with an Eppendorf pipet into a glass cuvette (22 mm i.d.×20 mm). The whole solution was saturated with oxygen by bubbling. Next, a 0.5 cm^3 portion of a 6.0×10^{-3} M cysteamine solution was injected into the cuvette. The entire solution, thus prepared, was referred to as a final solution. The CL reaction was initiated, and the CL emission was

detected. Bubbling of oxygen at a rate of 50 cm^3 min^{-1} and vigorous agitation by a magnetic stirrer were continued during the reaction. All measurements were made at room temperature. The maximum light emission was referred to as the CL intensity. The time period from the reaction initiation to the time at which CL intensity was maximal was defined as the delay time.

Analytical Procedure for Cysteamine and H_2O_2. The concentration of cysteamine consumed and H_2O_2 formed during the Cu(II)-catalyzed oxidation of cysteamine with oxygen was determined as follows: a 0.1 cm^3 portion of the reaction mixture was pipetted into the cell, into which a 0.1 cm^3 portion of a 1.0×10^{-3} M EDTA solution was added to stop the catalytic reaction. Determinations of cysteamine and H_2O_2 were carried out spectrophotometrically. Cysteamine was determined with 2,2'-dithiobis(5-nitropyridine) by measuring the absorbance at 386 nm,[7] and H_2O_2 with the Fe(II) complex of 1,10-phenanthroline at 510 nm.[8]

Results and Discussion

Delayed CL of Luminol Catalyzed by HRP. The catalytic oxidation of cysteamine was carried out in the presence of HRP and luminol according to the recommended procedure in which the concentration of Cu(II) was in the 1.0×10^{-7}—1.0×10^{-6} M range. Typical CL response curves are shown in Fig. 1. No light emission was detected in the blank which contained no Cu(II). When HRP catalyzes the CL reaction of luminol with H_2O_2 formed from the catalytic oxidation, CL may appear instantaneously and increase gradually with increasing H_2O_2 concentration. However, a CL flash suddenly appeared after a dark period of 6—20 min from the start of the reaction. The delay time increased with an decrease in Cu(II) concentration, whereas the CL intensity increased with an increase in Cu(II) concentration.

The appearance of the delay time could be explained by taking into account the HRP-catalyzed oxidation of cysteamine with oxygen, since HRP catalyzes the oxidation of such thiols as cysteine and reduced-glutathione with oxygen.[9,10] We then examined the oxidation of cysteamine by using a 4.5×10^{-6} M HRP solution according to the recommended procedure, except that the buffer solution was employed in place of a Cu(II) solution and a luminol solution. In addition, the same experiments as above were carried out by using a 5.2×10^{-7} M Cu(II) solution alone or by a mixture of Cu(II) and HRP.

The time course for cysteamine consumption is shown in Fig. 2. No autoxidation of cysteamine with oxygen occured under the same conditions. Consequently, the result in Fig. 2 indicates the progress of the catalytic oxidation of cysteamine by HRP. The oxidation rate by the combined use of Cu(II) with HRP increased compared to that by Cu(II) or HRP alone. The time at which cysteamine was almost completely oxidized was in accordance with the time at the beginning of the CL flash, as shown in Fig. 1 {curve(2)}. Therefore, the appearance of the delay time could be ascribable to the preferential oxidation of cysteamine by both Cu(II) and HRP, resulting in the formation of H_2O_2 and RSSR.

$$2RSH + O_2 \xrightarrow{Cu^{2+},\ HRP} RSSR + H_2O_2$$

After complete oxidation of cysteamine, the HRP-catalyzed luminol CL reaction is subsequently commenced using accumulated H_2O_2 as an oxidizing agent, and a CL emission suddenly appears after a dark period.

$$\text{luminol} + H_2O_2 \xrightarrow{HRP} \text{aminophthalate} + N_2 + H_2O + h\nu$$

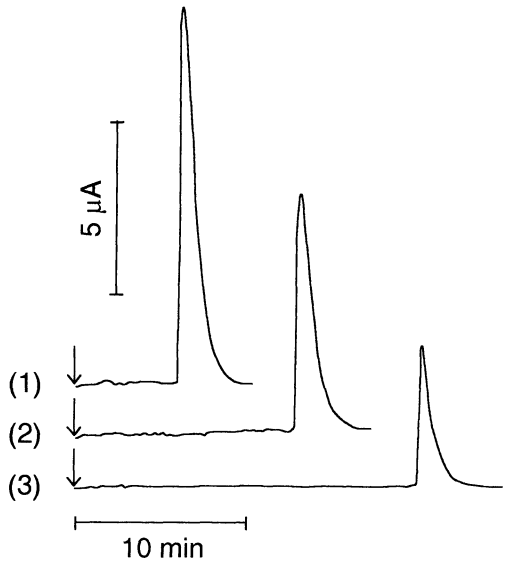

Fig. 1. Typical chemiluminescence response curves. (1) 1.0×10^{-6} M Cu(II), (2) 5.2×10^{-7} M Cu(II), (3) 1.0×10^{-7} M Cu(II). Conditions: 4.5×10^{-6} M HRP, 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine. At the arrow, cysteamine was added.

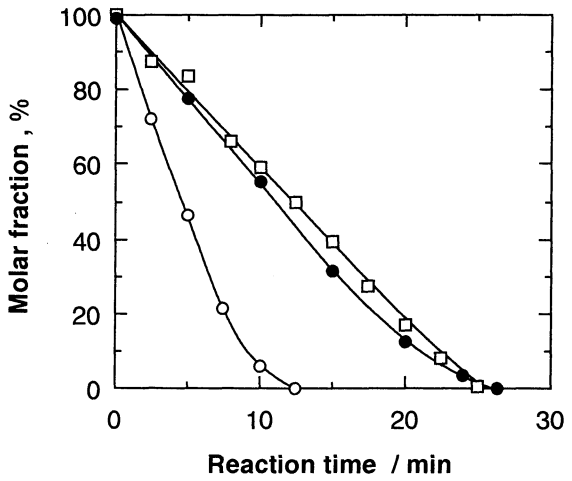

Fig. 2. Molar fraction of cysteamine unoxidized to disulfide. □: HRP, ●: Cu(II), ○: HRP+Cu(II). Conditions: 4.5×10^{-6} M HRP, 5.2×10^{-7} M Cu(II), 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine.

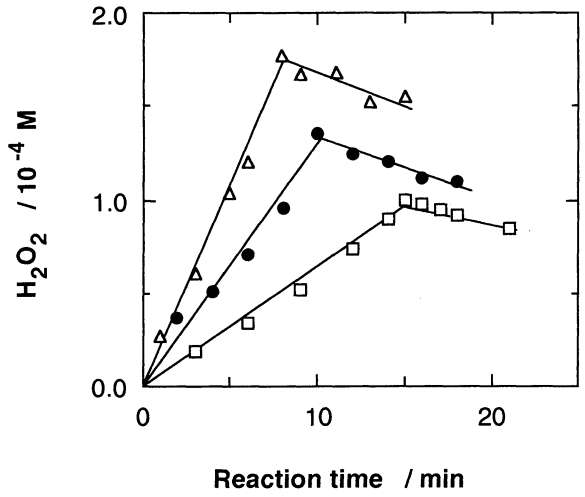

Fig. 3. Concentration of hydrogen peroxide formed during the catalytic oxidation of cysteamine. △: 7.7×10^{-7} M Cu(II), ●: 5.1×10^{-7} M Cu(II), □: 2.6×10^{-7} M Cu(II). Conditions: 4.5×10^{-6} M HRP, 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine.

The delay time and the CL intensity were dependent upon the Cu(II) concentration, as shown in Fig. 1. These results may be interpreted as follows. The delay time is mainly dependent on the oxidation rate of cysteamine, thus decreasing the delay time with an increase in the Cu(II) concentration. This is because the oxidation rate increases with increasing Cu(II) concentration. On the other hand, the magnitude of the CL intensity is probably attributable to the extent of the H_2O_2 formation during cysteamine oxidation. We then determined the amount of H_2O_2 formed during the catalytic oxidation by changing the Cu(II) concentration.

The time course for H_2O_2 formation is shown in Fig. 3. The concentration of H_2O_2 formed increased with increasing Cu(II) concentration. When cysteamine is converted quantitatively to H_2O_2, the reaction yield (mol%) should be 50%, where the reaction yield is defined as a ratio of the amount of H_2O_2 formed to the cysteamine used. However, the reaction yield was 18% at 7.7×10^{-7} M of Cu(II) as is shown in Fig. 3. In addition, the decomposition of H_2O_2 was initiated before a few min from the start of CL emission. These results suggest that a part of the H_2O_2 was consumed as an oxidizing agent for the oxidation of cysteamine.

Optimum Conditions for the Determination of Cu(II). In subsequent studies, the optimum conditions were determined by measuring the CL intensities, so as to be maximal under optimum conditions.

The effect of pH in the final solution was examined in the 8.0—9.5 pH range. Figure 4 shows the influence of pH on the delay time and the CL intensity. The CL intensity exhibited a broad maximum at pH 8.5, whereas the delay time gradually decreased with an increase in pH. The broad maximum of the CL intensity at pH 8.5 is probably attributable to increasing amounts of H_2O_2

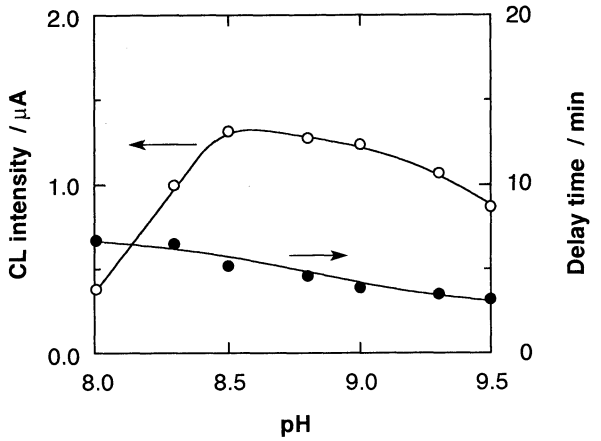

Fig. 4. Effect of pH on chemiluminescence intensity and delay time. ○: CL intensity, ●: delay time. Conditions: 4.5×10^{-6} M HRP, 1.2×10^{-6} M Cu(II), 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine.

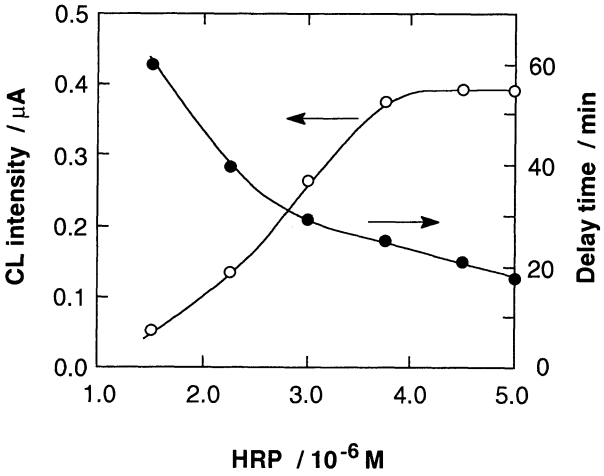

Fig. 5. Effect of HRP concentration on chemiluminescence intensity and delay time. ○: CL intensity, ●: delay time. Conditions: 1.0×10^{-7} M Cu(II), 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine.

used for the luminol CL, since the catalytic oxidation rate of cysteamine by both Cu(II) and HRP was maximal at pH 8.5. Thus, a pH of 8.5 was chosen for the recommended procedure.

The influence of the HRP concentration was investigated in the 1.5×10^{-6}—5.0×10^{-6} M range. The optimization curves for HRP concentrations are shown in Fig. 5. The CL intensity increased with increaing HRP concentration between 1.5×10^{-6} and 3.5×10^{-6} M HRP, after which the CL intensity was constant. The CL intensity-HRP concentration profiles are the same as that shown in the HRP-catalyzed CL of luminol with H_2O_2. On the other hand, the delay time decreased with an increase in the HRP concentration. This was because the oxidation rate of cysteamine increases with increasing HRP concentration. The optimum HRP concentration was thus determined to be 4.5×10^{-6} M.

The parameters next optimized were the luminol and

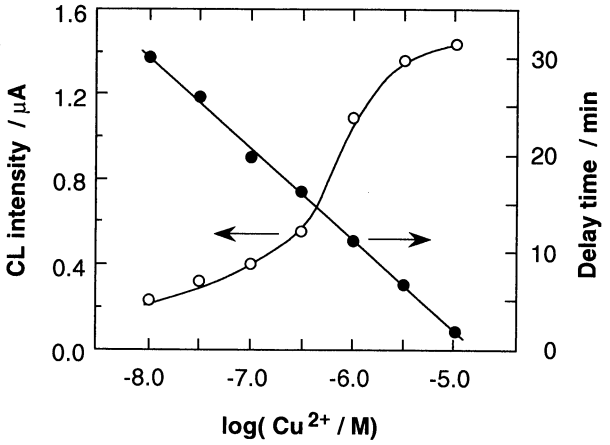

Fig. 6. Dependence of chemiluminescence intensity and delay time upon copper(II) concentration. ○: CL intensity, ●: delay time. Conditions: 4.5×10^{-6} M HRP, 6.0×10^{-5} M luminol, 6.0×10^{-3} M cysteamine.

cysteamine concentrations. The effect of luminol concentration was tested in the 1.0×10^{-5}—1.0×10^{-4} M range. The CL intensity increased to the maximal value at 6.0×10^{-5} M luminol, after which the CL intensity decreased gradually with an increase in the luminol concentration. The delay time was constant in the range tested. The dependence on the cysteamine concentration was investigated in the 1.0×10^{-3}—1.0×10^{-2} M range.The CL intensity increased with increasing cysteamine concentration up to 6.0×10^{-3} M and levelled off at higher concentrations. The delay time decreased when the concentration was above 1.0×10^{-3} M. Thus, the optimum luminol and cysteamine concentrations were chosen to be 6.0×10^{-5} and 6.0×10^{-3} M, respectively.

Analytical Results and Parameters. The denpendence of the CL intensity and delay time upon Cu(II) was examined under optimized conditions. The results are shown in Fig. 6. A log plot of the delay time vs. the Cu(II) concentration gave a straight line over the range 5.0×10^{-9} to 1.0×10^{-5} M. On the other hand, a curve between the Cu(II) concentration and the CL intensity was not linear in the same Cu(II) concentration range. Thus, the analytical calibration curve based on the delay time was more appropriate for the determination of Cu(II). The detection limit of Cu(II) based on the delay time reached as low as 5.0×10^{-9} M, at which the error was more than 5% in the relative standard deviation of the delay time in five successive experiments. The relative standard deviation was 3.2% at 1.0×10^{-7} M Cu(II).

The detection limit of the present method for Cu(II) is a factor of 30- and 600-times better than that of the conventional luminol CL method and the Cu(II)-catalyzed luminol CL-delay method.[4] The sensitivity of the proposed method is comparable to that of the uranine sensitized CL method for Cu(II) which is accepted as a highly sensitive method.[11]

Effect of Metal Ions on Luminol CL-Delay. The influence of metal ions on the delay time was examined according to the recommended procedure in which such metal ions as Cr(III), Mn(II), Fe(III), Co(II), Ni(II), and Zn(II) were added to a 1.0×10^{-7} M Cu(II) solution. The tolerance limit ratio of each metal ion was taken as being the largest amount yielding an error of less than 5% in the delay time.

No interference from Fe(III) was observed even at concentrations 1000 times than that of Cu(II). The amounts of Cr(III) and Zn(II) tolerated were 500 times greater than that of Cu(II), and 10-fold excesses of Co(II), Mn(II), and Ni(II) were tolerated. Therefore, the selectivity for Cu(II) was remarkable compared to that of the conventional luminol CL method in which serious interferences from all the metal ions were observed, even at a level equal to that of Cu(II).[4] In addition, the metal ions were tolerable in greater amounts than those in the Cu(II)-catalyzed luminol CL-delay method based on measuring the delay time.[4] The present method was more selective by a factor of 10—20 than that of the method reported previously. These results suggest that the foreign ions give rise to significant effects on the catalytic activity of Cu(II) for the oxidation of cysteamine in the alkaline medium rather than in the mild pH medinm.

In conclusion, the HRP-catalyzed luminol CL-delay method provides excellent sensitivity and selectivity for the determination of Cu(II). This means that the present method is highly reliable, and hence the method will be promising in the determination of Cu(II). Further studies on the mechanism of the luminol CL-delay reaction and extension to real samples are under way.

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