CHEMISTRY LETTERS, pp. 887-890, 1992.

Fluorescein Chemiluminescence-Delay Method for the Determination of Ultratrace Amounts of Copper(II)

Tamio KAMIDATE,* Isao KUNIYA, Tadashi SEGAWA, and Hiroto WATANABE
Faculty of Engineering, Hokkaido University, Kita-ku, Sapporo 060

A delayed chemiluminescence(CL) was observed in the copper(II)-catalyzed oxidation of cysteamine with oxygen in the presence of fluorescein(FL) and horseradish peroxidase. The delayed CL reaction of FL was applied to the determination of Cu(II). The delay time was correlated linearly with Cu(II) concentration over the range from 5.0×10^{-9} M to 1.0×10^{-6} M.

The copper(II)-catalyzed oxidation of thiol by oxygen was focused on a model reaction for elucidating the mechanism of the biological oxidation with copper-bearing enzymes.[1,2] In the catalytic oxidation, hydrogen peroxide(H_2O_2) was shown to be produced as an intermediate from oxygen. The H_2O_2 is usually measured by spectrophotometric method with $TiCl_4$.[3] On the other hand, we previously proposed the horseradish peroxidase(HRP) catalyzed-fluorescein(FL) chemiluminescence(CL) method for the determination of trace amounts of H_2O_2.[4]

In the course of our studies on the application of the FL CL method to the detection of H_2O_2 formed during the catalytic reaction, we have found that a CL flash suddenly appeared after a certain dark period from the initiation of the Cu(II)-catalyzed oxidation of cysteamine ($H_2NCH_2CH_2SH$) with oxygen. Thus, a delay time from reaction initiation to a flash of CL was observed. The delay time decreased with an increase in Cu(II) concentration. Based on this finding, we propose a highly sensitive CL-delay method for the determination of Cu(II).

The recommended procedure for the determination of Cu(II) is as follows: 1 cm^3 of the solution containing Cu(II), 0.5 cm^3 of 1.5×10^{-6} M (= mol dm^{-3}) HRP solution and 1 cm^3 of 1.5×10^{-3} M FL solution are placed together in a 10-cm^3 glass vial in a luminometer. The HRP and FL solutions were prepared with Carmody's buffer solution(pH 8.5). Next, 0.5 cm^3 of 6.0×10^{-3} M cysteamine solution was injected into the vial. Thus the CL-delay reaction was initiated and the light emission was

detected by a photomutiplier tube. Bubbling of oxygen at the rate of 45 cm^3 min^{-1} and vigorous agitation by a magnetic stirrer were continued during the reaction. The resultant photocurrent was converted to a voltage and displayed on a chart recorder. All measurements were made at room temperature. The maximum light emission was referred to as the CL intensity. A time period from reaction initiation to reaction time to reach a maximal CL intensity was defined as a delay time.

The Cu(II)-catalyzed oxydation of cysteamine with oxygen was carried out in the presence of HRP and FL according to the recommended procedure in which a 1.0×10^{-6} M solution of Cu(II) was used. Typical CL response curve is shown in Fig.1. A CL flash suddenly appeared after a dark period of 5 min from the start of the reaction, thus indicating that HRP was ineffective as a catalyst for the FL CL reaction during the dark period. This result may be interpreted by taking into acount the HRP-catalyzed oxidation of cysteamine with oxygen, since HRP catalyzes the oxidation of such thiols as cysteine and reduced-glutatione with oxygen.[5,6]

We then examined the HRP-catalyzed oxidation of cysteamine with oxygen according to the recommended procedure except that the buffer solution was employed in place of a Cu(II) solution. The concentration of cysteamine consumed during the catalytic reaction was determined spectrophotometrically with 2,2'-dithiobis(5-nitropyridine).[7] In addition, the

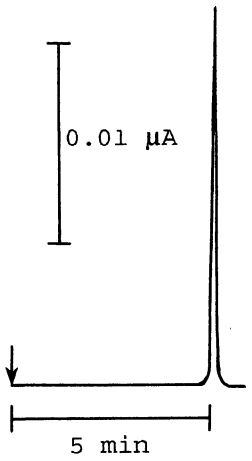

Fig.1. Typical responce curve for delayed Chemiluminescence of fluorescein.
[Cu(II)]=1.0×10^{-6} M,
[cysteamine]=6.0×10^{-3} M,
[HRP]=1.5×10^{-6} M, [FL]=1.5×10^{-3} M,
At the arrow cysteamine was added.

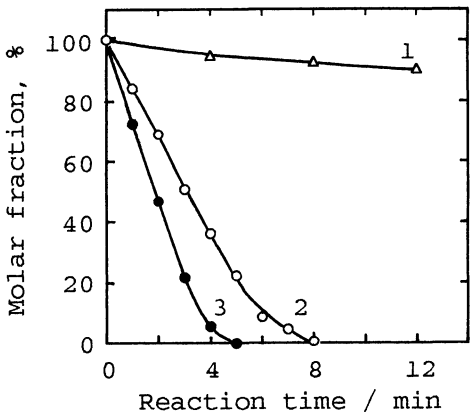

Fig.2. Molar fraction of cysteamine unoxidized to disulfide.

[Cu(II)]=1.0×10^{-6} M,
[cysteamine]=6.0×10^{-3} M,
[HRP]=1.5×10^{-6} M,
1.HRP, 2.Cu(II), 3.HRP + Cu(II).

same experiments as above were carried out by using Cu(II) alone or by the mixture of Cu(II) and HRP. The time course for cysteamine comsumption is shown in Fig.2. No autoxidation of cysteamine with oxygen occurs under same condition. Consequently, the result shown in Fig.2 indicates the progress of the HRP-catalyzed oxidation of cysteamine with oxygen. However, the oxidation rate by HRP was remarkably slower than that by Cu(II). On the other hand, the oxidation rate by the combined use of Cu(II) with HRP increased compared to that by Cu(II) 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. Therefore, the appearence of the delay time could be explained as follows. The catalytic oxidation of cysteamine(RSH) by both Cu(II) and HRP proceeds preferentially, resulting in the formation of H_2O_2.

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

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

$$FL + H_2O_2 \xrightarrow{HRP} FL^* \longrightarrow FL + h\nu$$

Next, we examined the effect of the HRP and FL concentration on the delay time and the CL intensity. The effect of HRP concentration was tested in the range 1.0×10^{-6} - 1.2×10^{-5} M. The CL intensity increased to a maximal value at 1.5×10^{-6} M of HRP, after which the CL intensity decreased rapidly with increasing HRP concentration. The delay time was decreased gradually with an increase in the HRP concentration. The dependence on the FL concentration was investigated in the range 1.0×10^{-3} M - 6.0×10^{-3} M. The CL intensity increased with increasing FL concentration up to 1.5×10^{-3} M and levelled off at higher concentrations. The delay time was decreased as the concentration increased above 1.0×10^{-3} M. In the determination of Cu(II), the optimum conditions was chosen by taking into account the CL intensity so as to be maximal under optimum conditions. Thus, the optimum HRP and FL concentration were chosen to be 1.5×10^{-6} M and 1.5×10^{-3} M, respectively.

The dependence of the CL intensity and delay time upon the Cu(II) concentration was examined. The results are shown in Fig.3. A plot of delay time vs. log Cu(II) concentration gave a straight line over the range from the determination limit of 5.0×10^{-9} M up to 1.0×10^{-6} M. The determination limit was taken as being the concentration of Cu(II) that produced an error of more than 5% in the relative standard deviation of the delay time in five successive experiments. The relative standard

deviation was 2.8% at 1.0×10^{-7} M of Cu(II). On the other hand, a logarithmic curve between Cu(II) concentration and the CL intensity was not linear in the same Cu(II) concentration range. Thus, the analytical caribration curve based on the delay time was more apporopriate for the determination of Cu(II). The sensitivity of the proposed method is comparable to that of the uranine sentitized CL method for Cu(II) which is being accepted to be highly sensitive method.[8]

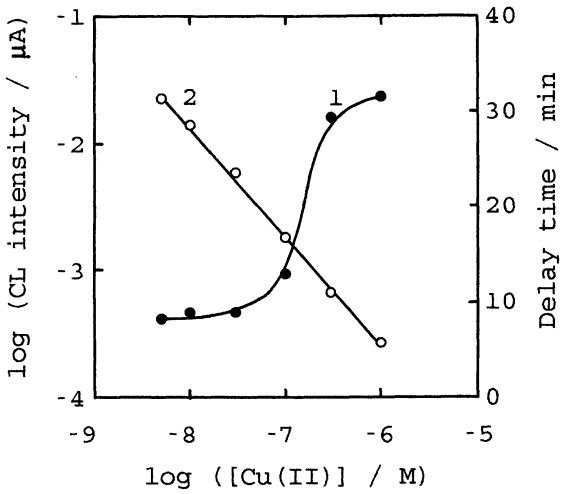

Fig.3. Dependence of CL intensity and delay time upon Cu(II) concentration.

[cysteamine]=6.0×10^{-3} M, [HRP]=1.5×10^{-6} M, [FL]=1.5×10^{-3} M, 1.CL intensity, 2.Delay time.

In conclusion, the FL CL-delay reaction catalyzed by HRP is potentially useful for the new indicator reaction for the determination of Cu(II) at sub-ppb levels. The high sensitivity in the present method is achieved by the improvement of S/N ratio, because Cu(II) can be determined without suffering from background signals observed at the initiation of CL reaction in a conventional CL method. Further studies on the mechanism of the delayed CL reaction and the selectivity of the proposed method are under way.

References

1) J.Zwart, J.H.M.C.Van Wolput, and D.C.Koningsberger, J.Mol.Catal., 12, 85 (1981).
2) A.Hanaki and H.Kamide, Bull.Chem.Soc.Jpn., 56, 2065 (1983).
3) H.Pobiner, Anal.Chem., 33, 1423 (1961).
4) T.Segawa, T.Kamidate, and H.Watanabe, Anal.Sci., 6, 763 (1990).
5) M.H.G.Medeiros, H.Wefers, and H.Sies, J Free Radicals Biol.Med., 3, 107 (1987).
6) L.S.Harman, C.Mottley, and R.P.Mason, J.Biol.Chem., 259, 5606 (1984).
7) A.Swaitditat and C.C.Tseu, Anal.Biochem., 45, 349 (1972).
8) X.Z.Wu, M.Yamada, T.Hobo, and S.Suzuki, Anal.Chem., 61, 1505 (1989).

(Received February 28, 1992)