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Simultaneous Determination of Cysteine and Glutathione via Use of Time-Resolved Luminol Chemiluminescence Method

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Hydrogen peroxide produced successively from the copper(II)-catalyzed oxidation of both cysteine (CySH) and glutathione (GSH) was detected using arthromyces ramosus peroxidase (ARP)-catalyzed luminol chemiluminescence (CL) reaction. Two peaks appeared in the CL responce curve. The former peak was due to CySH while that of the latter to GSH. The time-resolved luminol CL signals were applied for the simultaneous determination of CySH and GSH.

Thiol compounds such as cysteine (CySH) and glutathione (GSH) play a diverse and physiologically important role in living cells. Thus, many analytical methods for thiol compounds have been developed based upon use of colorimetric and fluoromeric reactions.[1,2] However, it is usually very difficult to determine more than two thiols simultaneously using such chemical reactions.

In the copper(II)-catalyzed oxidation of thiols with oxygen, hydrogen peroxide (H_2O_2) is produced as an intermediate from oxygen.[3] The rate of formation of H_2O_2 was dependent upon the thiol reactant. Due to the fact that the rate of formation of the H_2O_2 produced from the catalytic oxidation of CySH is different than that from GSH, the peroxidase (POD)-catalyzed luminol CL can be employed for the detection of H_2O_2 produced successively during the course of the catalytic oxidation of these two thiols. The CL responce curve was time-resolved into two peaks corresponding to CySH and GSH only when arthromyces ramosus peroxidase (ARP) was used as a POD. The time-resolved CL reaction system can thus be applied for the simultaneous determination of CySH and GSH.

ARP, horseradish peroxidase (HRP) and lactoperoxidase (LPO) were used as a POD. All of the solutions used were prepared with Carmody's buffer solution (pH=9.0) containing 0.2 M (= mol dm^{-3}) boric acid, 0.05 M citric acid and 0.1 M tertiary sodium phosphate. The general CL experimental procedure was as follows: 1 cm^3 of the solution containing POD and luminol

was placed in a glass cuvette in the luminometer. The solution was saturated with oxygen by bubbling of oxygen. Then, each 1 cm^3 of Cu(II) solution and the solution containing both CySH and GSH was injected respectively into the cuvette. The latter injection initiated the CL reaction and the light emission was detected using a photomultiplier tube. The resultant light emission was converted to a current and displayed on an integrator. The area under the CL peak was defined as the CL intensity. Bubbling of oxygen at 45 cm^3 min^{-1} and vigorous agitation by a magnetic stirrer were performed in each run. All measurements were made at 25 °C.

The Cu(II)-catalyzed oxidation of CySH and GSH with oxygen was carried out in the presence of POD and luminol. Typical CL responce curves are shown in Fig.1. In the case of ARP, two peaks appeared in the CL response curve. The first, nearly instantaneous peak reached its maximal height within 10 s and then rapidly disappeared. The maximal height of the second peak occurred after about 2 min. The first and second peak heights increased with increasing the CySH and GSH concentrations, respectively. Therefore, the first and second CL responses reflect the formation rates of H_2O_2 generated successively in the catalytic oxidation of CySH and GSH, respectively. In contrast, no time-resolved CL was observed in the presence of HRP and LPO. HRP caused a CL delay. A CL flash suddenly appeared after a dark period of about 12 min from the injection of the thiols solution. When LPO was used, the CL emission appeared immediately after the injection of the thiols solution.

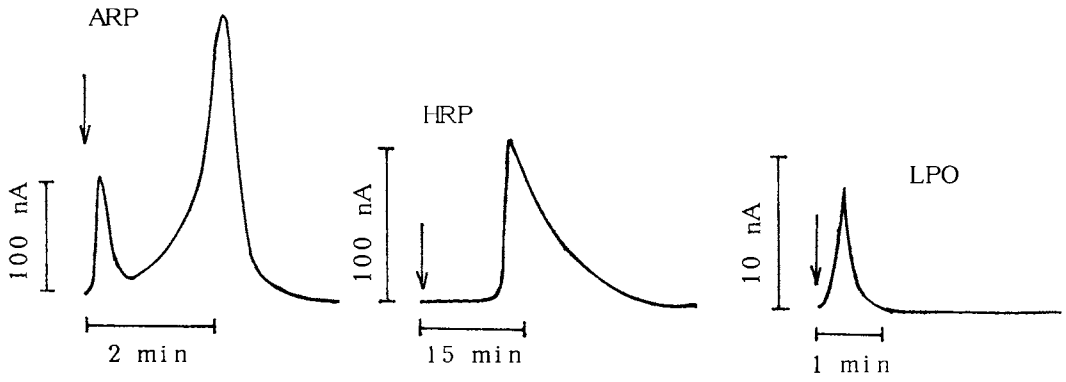

Fig.1. Typical chemiluminescence response curves.
[Cu(II)]=5.0×10^{-6} M, [CySH]=5.0×10^{-5} M, [GSH]=1.0×10^{-3} M,
[POD]=5.0×10^{-7} M, [luminol]=1.0×10^{-4} M,
At the arrow the thiols solution was injected.

The difference of CL response curves between ARP and HRP could be due to the different rates of formation of luminol radicals produced in the enzymatic cycle. Antioxidants, such as GSH, can effectively act as a quenching agent for radicals.[4] Consequently, the luminol radicals formed could be reduced by the thiols, thereby preventing oxidation of luminol radicals to excited aminophthalate and subsequent light emission. In the case of HRP, the formation rate of luminol radicals may be smaller than the disappearence rate of luminol radicals with CySH and GSH. This would result in the appearence of a delayed CL as long as any CySH and GSH are present. The formation rate of luminol radicals in ARP-induced luminol oxidation was remarkably higher than that observed for the HRP-induced reaction.[5] Although the rate constants for reaction of luminol radicals with either CySH or GSH are not known, the rate of luminol radical formation may be higher than its disappearence rate due to reaction with CySH or GSH. This would result in the appearance of a CL signal at the initiation of the reaction. Therefore, the CL intensity-time profile in the presence of ARP will reflect the difference of the formation rates of H_2O_2 between the catalytic oxidation of CySH and that of GSH.

When LPO was used, light emission was observed immediately after the injection of the thiols solution. Measurement of the absorption spectra of LPO as a function of reaction time revealed that the absorbance in the Soret spectra decreased with time. Therefore, these results are probably attributable to the decomposition of LPO.

The results shown in Fig.1 indicate that the most promising POD is ARP for the simultaneous determination of CySH and GSH. The effect of pH on the CL responce curve in the presence of ARP was determined. Time-resolved CL was observed in the 8.5 - 10 pH range. The first peak height increased with increasing pH, while the second peak height exhibited a broad peak at pH 9.0. The time required to reach a maximal peak height for the first peak was constant. However, for the second peak, it increased with an increase in pH. Thus, a pH of 9.0 was selected for the recommended procedure.

The influence of the CySH concentration on the CL responce curve was next investigated. Typical CL response curves are shown in Fig.2. The first peak increased in height with increasing CySH concentration. The effect of the GSH concentration was also examined. The second peak height increased with an increase in the GSH concentration (results not shown). The times required to achieve maximal peak heights for the first and second peaks increased gradually with increasing CySH and GSH concentration, respectively.

Analytical calibration curves for CySH and GSH were prepared under the following conditions: a 1.0×10^{-6} M Cu(II) solution and solution containing 1.0×10^{-7} M ARP and 1.0×10^{-4} M luminol were injected into the solution containing 1.0×10^{-6} M - 5.0×10^{-5} M CySH and 5.0×10^{-5} M GSH or solution containing 1.0×10^{-6} M - 5.0×10^{-5} M GSH and 5.0×10^{-5} M CySH, respectively. Logarithmic calibration curves of CySH and GSH were linear over the range 1.0×10^{-6} M and 3.0×10^{-6} M to 5.0×10^{-5} M with slopes of 1.81 and 1.64, respectively. Within these concentration ranges, the mutual interference on the CL intensity between CySH and GSH was less than 3%. The relative standard deviation of five successive experiments was 2.5% at 5.0×10^{-6} M of CySH and 2.8% at 1.0×10^{-5} M of GSH.

In conclusion, the ARP-catalyzed luminol CL is potentially useful as a reaction for the simultaneous determination of CySH and GSH. Further studies on the mechanism of the time-resolved luminol CL reaction are underway.

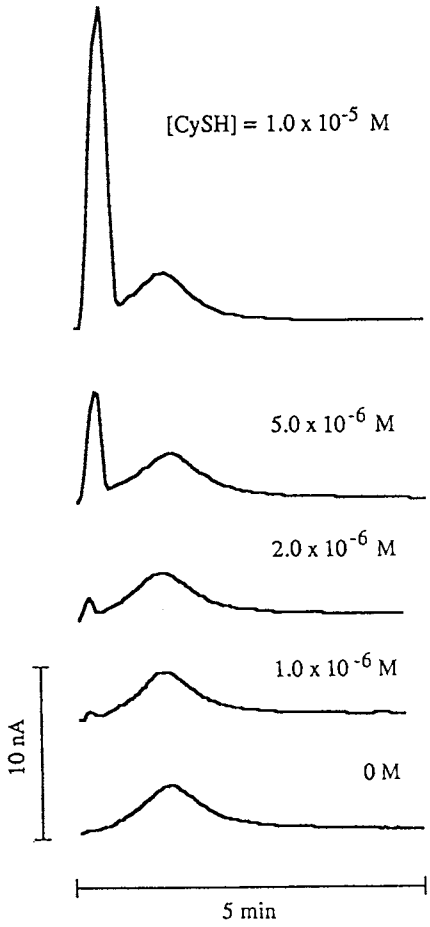

Fig.2. Effect of cysteine concentration on time-resolved CL response curve.
[Cu(II)]=1.0×10^{-6} M,
[GSH]=1.0×10^{-5} M,
[ARP]=1.0×10^{-7} M,
[luminol]=1.0×10^{-4} M.

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