Luminol Enhanced Chemiluminescent Assay of Indole-3-Acetic Acid

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Chemiluminescence (CL) from horseradish peroxidase catalyzed aerobic oxidation of indole-3-acetic acid (IAA) increased markedly in the presence of luminol. The luminol enhanced CL was applied to the determination of IAA. The detection limit of IAA was 6.0×10^{-6} M in the presence of luminol, and was improved a factor of ten times compared to that in conventional spectrophotometric method.

Indole-3-acetic acid (IAA) plays an important role as a natural phytohormone with many growth regulatory function. The concentration of IAA in plants is controlled by peroxidase catalyzed oxidation of IAA with oxygen. The aerobic oxidation of IAA results in a low level of chemiluminescence (CL).[1] The appearance of the CL was explained on the basis of the oxidation cleavage of the 2,3-double bond of the indole ring.[1] In order to enhance the weak CL, xanthene dyes such as eosin Y were added to the horseradish peroxidase (HRP)/IAA/O_2 system.[2] In addition, the light emission increased markedly in the presence of fluorescein compared to that of eosin Y.[3] However, no reports have been found on the determination of IAA based on the enhanced CL reaction.

In the course of our studies on enhancing the light emission from the HRP/IAA/O_2 system, we have found that the rate of the light emission and the maximum light emission increased remarkably in the presence of luminol compared to that of fluorescein. The luminol-enhanced CL can thus be applied to the determination of IAA.

HRP (type VI) and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. IAA, luminol, fluorescein and eosin Y were obtained from Kanto Chemical Co. A CL reagent solution containing 2.0×10^{-7} M (1M =1 mol dm^{-3}) HRP and 5.0×10^{-5} M luminol and an IAA solution were prepared in 50 mM phosphate buffer solution (pH 7.4). The concentration of HRP and SOD were determined spectrophotometrically with an ε_{403} value of 1.02×10^{5} M^{-1} cm^{-1} and an ε_{265} value of 1.59×10^{4} M^{-1} cm^{-1}, respectively.[4,5]

The general CL experimental procedure consisted in pipetting a 500-μl portion of the CL reagent solution into a glass-cuvette in the CL detector (TD-3A; Tohoku Denshi Sangyo Co. Ltd.). Next, a 500-μl portion of an IAA solution was added into the cuvette using an injector. Thus the oxidation of IAA with HRP was initiated and the light output was counted with the CL detector. The resultant photocurrent was converted to a voltage, whose value was displayed on a chart recorder. A maximum light emission is referred to as a CL intensity. All CL measurements were made at 25 °C . The CL spectrum was observed by measurements with a fluorescence spectrophotometer (F-2000; Hitachi Co. Ltd.) while running it with the excitation light off during the measurements. The concentration of dissolved oxygen was measured by using a portable oxygen meter (DO-11P; TOA Electronics Ltd.) equipped with an oxygen electrode (OE-2102).

The light emission was observed during the HRP-catalyzed aerobic oxidation of IAA in the absence of the enhancer. A 500-μl portion of 2.0×10^{-3} M IAA solution was added to a 500-μl portion of 2.0×10^{-7} M HRP solution. Typical CL response curve is shown in Figure 1. The very weak light emission was observed from the start of the reaction. In order to confirm the enhanced effect of xanthene dyes on the light emission, we measured the CL response curve by adding a 2.0×10^{-3} M IAA solution into the CL reagent solutions containing 1.0×10^{-3} M eosin Y or 1.0×10^{-3} M fluorescein. Typical CL response curves are shown in Figure 1. The CL intensities increased in the presence of eosin Y and fluorescein compared to that in water alone. The CL enhancement was eminent in the presence of fluorescein.

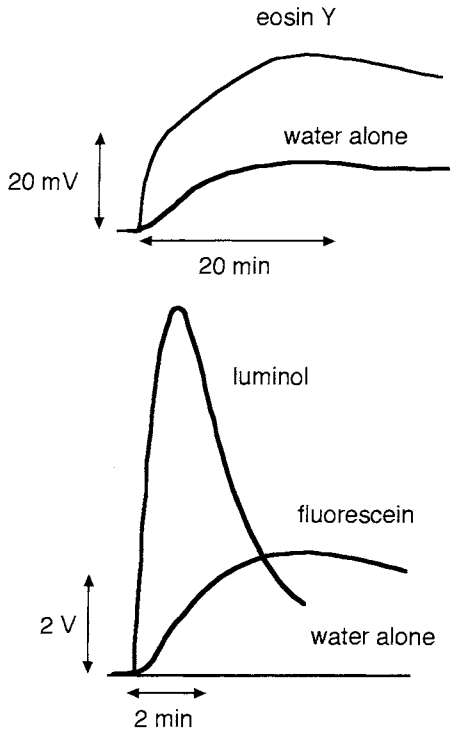

Figure 1. Typical CL response curves from HRP-catalyzed IAA in the presence of eosin Y, fluoresein and luminol. HRP: 2.0×10^{-7} M, IAA: 2.0×10^{-3} M, eosin Y: 1.0×10^{-3} M, fluoresein: 1.0×10^{-3} M, luminol: 5.0×10^{-5} M.

Next, the effect of luminol on the light emission was investigated according to the procedure. As can be seen in Figure 1, the rate of the light emission and the maximum light emission in the presence of luminol increased remarkably compared to those of the xanthene dyes. The relative CL intensity is defined as the ratio of the CL intensity in the presence of the enhancers to that in water alone. The relative CL intensities in the presence of luminol, fluorescein and eosin Y were 320, 150 and 1.8, respectively. Therefore, luminol was most effective with respect to the ability to enhance the CL from the HRP/IAA/O_2 system.

The dependence of pH on the CL intensity in the presence of

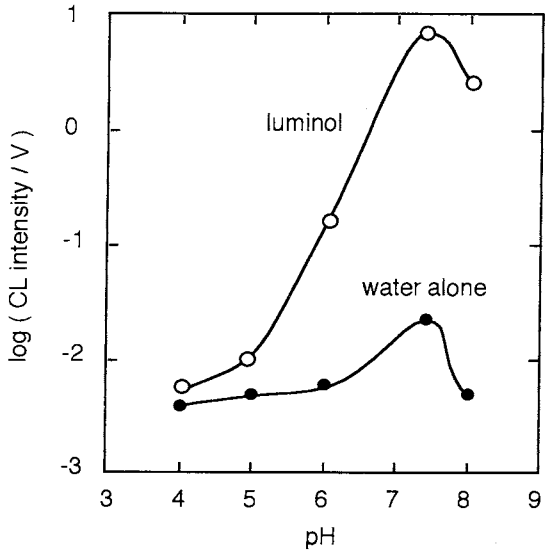

Figure 2. Effect of pH on the CL intensity. HRP: 2.0×10^{-7} M, IAA: 2.0×10^{-3} M, luminol: 5.0×10^{-5} M.

luminol was examined over the pH range from 4.0 to 8.0. Figure 2 shows the influence of pH on the CL intensity in water alone and in the presence of luminol. The CL intensity exhibited a maximum at pH 7.5 both in water alone and in the presence of luminol. The enhanced luminol CL was observed in the same pH range in which the HRP-catalyzed oxidation of IAA proceeds.

In order to investigate the emissive species, the CL sepctrum was measured according to the procedure. The CL sepctrum corresponded to that of luminol in which the light emission was maximum at 420 nm. On the other hand, no CL appeared by adding the luminol solution to the HRP solution in the absence of IAA. Next, we measured the concentration of dissolved oxygen during the CL reaction according to the procedure, in which a 2.0×10^{-3} M IAA solution was injectd to the CL reagent solution. The concentration of dissolved oxygen decreased with an increase in the reaction time. This result suggests that oxygen species could be concerned with the CL reaction. We then examined the effect of super oxide on the CL intensity in the presence of SOD. The CL intensity decreased with an increase in the SOD concentration. Therefore, the luminol-enhanced CL from the HRP/IAA/O_2 system could be explained by taking into account those results obtained. That is, IAA as a hydrogen donor reacts first with HRP to produce IAA radical.[1] Next, dissolved oxygen reacts with IAA radical to form super oxide. Luminol reacts with super oxide to yield endoperoxide. The endoperoxide decomposes to yield an electronically exited 3-aminophthalate dianion which returns to a ground state to emit light.[6]

In subsequent studies, the optimum conditions for the quantification of IAA were determined by measuring the CL intensities, so as to be maximal under optimum conditions. The dependence of the CL intensity upon HRP concentration was examined in the range 2.0×10^{-8} - 2.0×10^{-6} M. The CL intensity increased with increasing HRP concentration up to 2.0×10^{-7} M, after which it leveled off with increasing HRP concentration. Thus, the optimum HRP concentration was determined to be 2.0×10^{-7} M. The effect of luminol concentration was tested in the range 1.0×10^{-6} - 1.0×10^{-4} M. The CL intensity exhibited a broard maximum at 2.0×10^{-5} M. The optimum luminol concentration was thus chosen to be 2.0×10^{-5} M.

Analytical calibration curve was prepared under the optimum experimental conditions. Logarithmic calibration curve of IAA was linear over the range from the detection limit of 6.6×10^{-6} M up to 5.0×10^{-4} M with a slope of 0.84. The detection limit for IAA was defined as the concentration of IAA that produced the CL intensity equal to three folds of the blank intensity counted in the mixture containing no IAA. The detection limit of the present CL method for IAA is improved by factors of ten compared to that of conventional spectrophotometric method.[7]

In conclusion, luminol is superior to xanthene dyes such as eosin Y and fluorescein in the CL enhancement in HRP-catalyzed oxidation of IAA. Based on this finding, a sensitive CL assay of IAA has been developed. Further studies on the mechanism of the luminol enhanced CL from the HRP/IAA/O_2 system are underway.

References and Notes

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