Chemiluminescent Determination of Peroxidase with Purpurogallin Carboxylic Acid

Tadashi SEGAWA, Takahiro OOIZUMI, Taichi MATSUBARA, Tamio KAMIDATE and Hiroto WATANABE

Faculty of Engineering, Hokkaido University, Sapporo 060, Japan

Purpurogallin carboxylic acid (PurA) was proposed as a highly sensitive chemiluminescent (CL) reagent for the detection of peroxidase. The blank signal of PurA was lower than that of its mother moiety, purpurogallin, thus resulting in a decrease in the detection limit of peroxidase. The calibration curves of horseradish peroxidase and *Arthromyces ramosus* peroxidase with PurA were linear over the ranges from a detection limit of 20 fmol to 5.0 pmol and 10 fmol to 5.0 pmol, respectively. PurA CL was less subject to interference from diverse albumin than was luminol CL.

Keywords Chemiluminescence, polyphenol, peroxidase

Peroxidase (EC 1.11.1.7; POD) is widely used for a non-radioactive label in an enzyme immunoassay and a DNA dot-hybridization assay.¹⁻³ Of PODs, horseradish POD (HRP) is accepted to be a versatile label for the assays, because HRP is easily available. HRP has been determined by both a colorimetric method and a fluorometric method.^{1,2} Recently, a chemiluminescent (CL) method for HRP detection has been noted in view of its high sensitivity and wide dynamic range. For this purpose, luminol^{4,5} and polyphenolic compounds, such as pyrogallol (PG)⁶⁻⁸ and purpurogallin (Pur)⁹, are used. In luminol CL, an excellent enhancer, such as *p*-iodophenol, was found.^{10,11} Subsequently, many assay kits for biological substances with the enhanced-luminol CL have been developed.¹²

These days, ultra-weak light emission can easily be detected by a photomultiplier tube (PMT). Hence, in a CL analysis using a PMT, a strongly emissive CL reagent is not necessary if the CL signal of a sample solution and that of a blank solution are clearly distinctive. We should thus pay attention to the lower blank CL for developing a sensitive CL method.

In HRP-catalyzed luminol CL, the blank CL signals are significant, because of impurities in luminol. Kricka *et al.*, attempted the recrystallization of luminol for reducing the blank CL.¹³ However, their effort was unsuccessful, since the blank CL signal still remained considerable. On the other hand, no reports have been found in an attempt to decrease the blank CL in the HRP-catalyzed polyphenol CL.

During the course of our studies on POD-catalyzed polyphenol CL we have found that carboxyl derivatives of PG and Pur were a good choice for decreasing the blank CL. Hence, in the present study we tested the carboxyl derivatives of Pur for the sensitive CL determination of PODs.

Experimental

Apparatus

All of the CL measurements were made with a CL detector (TD-3A; Tohoku Denshi Sangyo Co., Ltd.) equipped with an automatic injector (Model 500, Nichiryo Co., Ltd.). The absorption spectra and the absorbance were recorded on a spectrometer (U-2000; Hitachi Co., Ltd.)

Reagents

Figure 1 lists the polyphenol compounds tested. 7-Carboxy-2,3,4,6-tetrahydroxy-5*H*-benzocyclohepten-5-one (PurA) was synthesized according to a procedure of Crow *et al.*¹⁴ Aqueous solutions of PG (15.5 g in 160 cm³) and potassium iodate (27.1 g in 330 cm³) were added simultaneously with stirring and ice-cooling to a 200 cm³ portion of an aqueous solution containing 21.0 g of gallic acid (GA) and 11.5 g of sodium hydrogen carbonate within 23 min. After stirring for an additional 15 min the mixture was acidified with 12 M (1 M=1 mol dm⁻³) hydrochloric acid (10 cm³), set overnight and filtered. The filtrate was then extracted with diethylether (1 dm³). The ethereal solution was dried, and an orange powder of PurA was obtained.

All other reagents were used as purchased without any further purification. HRP-Type VI was obtained from Sigma Chemical Co. *Arthromyces ramosus* POD (ARP) was obtained from Suntory Ltd. All of the aqueous solutions were prepared with water purified using a Millipore Milli-Q Labo system.

An H₂O₂ solution was prepared by diluting a 31% H₂O₂ solution with a 0.10 M 2-morpholinoethanesulfonic acid (MES) buffer solution adjusted to pH 7.0.

Fig. 1 Polyphenol compounds tested.

Procedure

A 0.10 cm³ portion of a POD solution and a 0.40 cm³ portion of a CL reagent solution were introduced into a glass cuvette (22 mm i.d.×20 mm), which was subsequently placed in the holder of the CL counter. A 0.50 cm³ portion of the H₂O₂ solution was then added into the cuvette using the injector. The CL reaction was thus initiated, and the CL emission was monitored as a function of time with a PMT. The resultant photocurrent was converted to a voltage, whose value was displayed on a chart recorder. The CL intensity was defined as the voltage at which the CL emission was maximum.

All of the reagent concentrations in the figure captions are final values.

Results and Discussion

CL response of PG and Pur

Typical CL response curves of PG and Pur were measured by using a 0.30 pmol HRP solution. The results are shown in Fig. 2. When PG was used, light emission occurred several seconds after the start of the CL reaction (Fig. 2, curve 1). The intensities of the light emission gradually increased, reaching a maximum value, and then decreased. On the other hand, when Pur was used, CL appeared instantaneously after the initiation of the reaction (Fig. 2, curve 2). The CL emission reached a maximum value after about 1 min, and then decreased. The blank CL response curves of PG and Pur are also shown in Fig. 1, curves 3 and 4. Pur gave a higher CL intensity and blank CL than did PG.

Recently, the luminol CL method has been modified by the use of POD from different origins such as fungus¹⁵, for improving the sensitivity. However, the use of POD is only limited to HRP as a catalyst in the polyphenol CL reaction.

We then examined the polyphenol CL by using

Fig. 2 Typical CL response curves of PG and Pur. HRP=0.30 pmol; [H₂O₂]=3.0×10⁻² M; pH 7.0, curves 1 and 3, PG=1.2×10⁻² M; curves 2 and 4, Pur=6.0×10⁻⁴ M. Curves 3 and 4 are the blank CL in the absence of HRP.

Table 1 CL intensity with several polyphenols

	Blank/mV	HRP/mV	ARP/mV
PG	25	78	60
Pur	45	131	180
GA	3	3	65
Pur A	19	243	250

POD=0.10 pmol; [GA]=1.2×10⁻² M; [H₂O₂]=3.0×10⁻² M; [Pur]=6.0×10⁻⁴ M; [PG]=1.0×10⁻² M; [Pur A]=5.0×10⁻⁴ M.

0.10 pmol POD solutions of HRP and ARP. In addition, the CL reaction was made by using water in place of the POD solution. Table 1 summarized the CL intensities of PG and Pur for the blank and PODs (line 1 and 2). ARP gave results similar to those in HRP.

The blank CL intensity of Pur was greater than that of PG. Since the detection limit of POD is commonly defined as moles of POD yielding a CL intensity equal to two- or three-times the blank CL intensity, the decrease in the blank CL emission provides a good means for increasing the sensitivity for POD. Then, the mechanism of PG-CL and Pur-CL was investigated.

CL mechanism of PG and Pur

The speculated CL mechanisms of PG and Pur are shown in Fig. 3.^{6,16,17} Polyphenol CL is believed to emit light from singlet oxygen, which is produced during the oxidation of the polyphenolic compound. In PG-CL, PG is oxidized with POD and H₂O₂ to convert into Pur radical (reaction (1)). The Pur radical is then oxidized by H₂O₂ without POD; then, singlet oxygen is produced (reaction (2)). Light emission occurs when the singlet oxygen returns to its ground triplet state (reaction (3)). Meanwhile, in the Pur-CL, Pur is oxidized with POD and H₂O₂ to form the Pur radical (reaction (1')). CL is emitted in a manner similar to the PG-CL (reactions (2) and (3)). In the presence of a trace amount of POD, the rate-limiting step could be the oxidation of PG with HRP

Fig. 3 Reaction scheme of PG and Pur. PG: Eqs. (1), (2) and (3), Pur: Eqs. (1'), (2) and (3).

and H_2O_2 in the PG-CL. On the other hand, the rate-limiting step of Pur-CL could be the oxidation of the Pur radical with H_2O_2 . This assumption is supported by the fact that PG-CL was not observed from the initiation of the reaction (Fig. 2 curve 1). This fact can be explained in terms of the slow production of the Pur radical in PG-CL (reaction (1)), compared with Pur-CL (reaction (1')).

Since blank CL emission is accompanied by the non-catalytic oxidation with H_2O_2 , a CL reagent which is hardly subject to oxidation gives rise to a low blank CL. In general, the oxidation potential of a phenolic compound increases due to the introduction of an electron-withdrawing substituent, such as a carboxyl group;¹⁸ thus, phenolic compounds possessing a carboxyl group could be hard to oxidize. For example, the oxidation potential of GA is at least 160 mV higher than that of PG over the pH range from 3 to 10.¹⁹

CL responses of carboxy derivatives of PG and Pur

The effects of the carboxyl group on the CL intensity and the blank CL intensity were investigated by using PG and GA. Table 1 also lists the CL intensity of GA (line 3). The blank CL intensity of GA was much less than that of PG. The increase in the oxidation potential may be reflected on the decrease in the blank CL intensity. In POD-catalyzed CL, GA did not chemiluminesce with 0.10 pmol HRP. On the other hand, the ARP-catalyzed CL of GA and PG gave similar CL intensities. The results of ARP indicate that the carboxyl substituent is effective for a depression of the blank CL.

From these results we should choose a CL reagent which satisfies the dual properties of emitting strong CL and of giving a low blank CL. In this respect, the carboxyl derivative of Pur (PurA) has a potentially higher sensitivity than does Pur. Thus, PurA was synthesized, and the CL intensities of a blank and a 0.10 pmol POD solution were observed. The results are also listed in Table 1 (line 4). The CL intensity of PurA was greater than that of Pur, whereas the blank CL intensity of PurA was less than that of Pur, even PG.

Fig. 4 Calibration curves for POD with PurA. Curve 1, HRP; curve 2, ARP; $[\text{PurA}] = 5.0 \times 10^{-4} \text{ M}$; $[\text{H}_2\text{O}_2] = 3.0 \times 10^{-2} \text{ M}$; pH 7.0.

CL detection of ARP and HRP with PurA

The optimum conditions for the determination of ARP with PurA were investigated. The effect of the pH on the CL intensity was examined in the pH range from 6.0 to 8.0. The CL intensity was maximum at pH 7.5. However, the blank CL intensity increased along with an increase in the pH, and greatly increased above pH 7.0. A buffer of pH 7.0 was therefore chosen for the following studies.

The variation in the CL intensity with the PurA concentration was investigated over a PurA concentration range from $1.0 \times 10^{-4} \text{ M}$ to $1.0 \times 10^{-3} \text{ M}$. The CL intensity increased along with an increase in the PurA concentration, and reached almost a constant value above $5.0 \times 10^{-4} \text{ M}$ PurA. Meanwhile, the blank CL intensity increased together with increasing the PurA concentration. A PurA concentration of 5.0×10^{-4} was employed so that the ratio of the CL intensity to the blank CL intensity (S/B ratio) was maximum.

The influence of the H_2O_2 concentration on the CL intensity was examined over the H_2O_2 concentration range from $5.0 \times 10^{-3} \text{ M}$ to $5.0 \times 10^{-2} \text{ M}$. The CL intensity and the blank CL intensity were increased along with an increase in the H_2O_2 concentration. At $3.0 \times 10^{-2} \text{ M}$ H_2O_2 , the S/B ratio was maximum. The optimum H_2O_2 concentration was $3.0 \times 10^{-2} \text{ M}$. HRP gave similar results to those of ARP.

Next, calibration curves for HRP and ARP were prepared under the optimized conditions. The results are shown in Fig. 4. The log-log plots of the CL intensity against the amount of POD in the cuvette were linear over the ranges from a detection limit of 10 fmol to 5.0 pmol for ARP, and 20 fmol to 5.0 pmol for HRP, with the same slope of 0.77. The detection limit was defined as the amount of POD, yielding a CL intensity equal to twice the blank CL intensity. The relative

Table 2 Detection limit of POD with several CL reagent

POD	CL reagent	Detection limit/fmol
HRP	PG	100
	GA	240
	Pur	60
	Pur A	20
	luminol	50
	luminol ^a	1
ARP	PG	100
	GA	30
	Pur	75
	Pur A	10
	luminol	0.1

a. *p*-Iodophenol was used as an enhancer.

standard deviation of the five successive determinations was 3.1% and 3.3% at a level of 0.30 pmol ARP and HRP, respectively.

Table 2 lists the detection limits of POD with polyphenolic CL reagents and luminol. In Table 2, the CL conditions of each CL reagent are optimized as well as that of PurA. Apparently, PurA was the most sensitive polyphenolic CL reagent for POD. In the detection limit of HRP, PurA was comparable to luminol without the use of an enhancer. The use of an enhancer in luminol CL markedly lowered the detection limit. On the other hand, luminol was much superior to PurA for ARP detection. In the CL reaction of polyphenol compounds, the CL intensities were almost independent on the origin of POD, except for GA.

Effect of albumin on CL intensity

In order to confirm the possibility of using PurA for clinical analysis, the CL reaction of PurA was performed by using a 0.30 pmol HRP solution in the presence of 0.016% (w/v) of bovine serum albumin (BSA). The CL response curves along with luminol are shown in Fig. 5. BSA had no significant effect on the CL response of PurA (Fig. 5(a)). On the other hand, BSA markedly increased the blank CL of luminol. This fact indicates that the luminol-CL is interfered by BSA. PurA would be superior to luminol in the CL detection of POD or its conjugates in the presence of proteins.

In conclusion, we have proposed a strategy for the design of a polyphenolic CL reagent for POD. The introduction of a carboxyl group decreased the blank CL intensity. Since PurA CL was less subject to an inhibition of BSA, the PurA method would be more practical for the detection of POD and its conjugates.

References

1. E. Ishikawa, T. Kawai and K. Miyai (eds.), "Kouso Meneki Sokuteihou (Enzyme Immunoassay, in Japanese)", 3rd ed., Igaku-shoin, Tokyo, 1987.

Fig. 5 Effect of bovine serum albumin on the CL response of PurA and luminol. (a) PurA, [PurA]= 5.0×10^{-4} M; $[H_2O_2]=3.0 \times 10^{-2}$ M; pH 7.0, HRP=0.30 pmol. (b) luminol, [luminol]= 2.0×10^{-5} M; $[H_2O_2]=7.5 \times 10^{-4}$ M; pH 9.0, HRP=0.30 pmol. Curve 1, control; curve 2, in the presence of 0.016 wt% bovine serum albumin; B represents a blank CL response.

2. E. Ishikawa, "Kouso Hyousiki-hou (Enzyme Labeling Method, in Japanese)", Gakkai Shuppan Center, Tokyo, 1991.
3. M. Renz and C. Kurz, *Nucl. Acid Res.*, **13**, 745 (1985).
4. H. Arakawa, M. Maeda and A. Tsuji, *Anal. Biochem.*, **97**, 248 (1979).
5. H. Arakawa, M. Maeda and A. Tsuji, *Steroids*, **38**, 453 (1981).
6. R. Nilsson, *Acta Chem. Scand.*, **18**, 389 (1964).
7. M. Halmann, B. Velan and T. Sery, *Appl. Environ. Microbiol.*, **34**, 473 (1977).
8. B. Velan and M. Halmann, *Immunochemistry*, **15**, 331 (1978).
9. M. Halmann, B. Velan, T. Sery and H. Schupper, *Photochem. Photobiol.*, **30**, 165 (1979).
10. G. H. G. Thorpe and L. J. Kricka, *Methods Enzymol.*, **133**, 331 (1986).
11. T. P. Whitehead, G. H. G. Thorpe, T. J. N. Carter, C. Groucutt and L. J. Kricka, *Nature* [London], **305**, 158 (1983).
12. A. Tsuji and T. Kanno (eds.), "Kagakuhaikou Immunoassei (Chemiluminescent Immunoassay, in Japanese)", Life Science, Tokyo, 1992.
13. R. A. W. Stoff and L. J. Kricka, in "Bioluminescence and Chemiluminescence", ed. J. Scholmerich, R. Anderesen, A. Kapp, M. Ernst and W. G. Woods, p. 237, John Wiley, Chichester, 1987.
14. W. D. Crow, R. D. Harworth, *J. Chem. Soc.*, **1951**, 1325.
15. B. B. Kim, V. V. Apisarev and A. M. Egorov, *Anal. Biochem.*, **199**, 1 (1991).
16. D. Slawinska and J. Slawinski, *Anal. Chem.*, **47**, 2101 (1975).
17. N. P. Evmiridis, *Analyst* [London], **112**, 825 (1987).
18. T. Matsuura, "Sanzo Sanka Han-nou (Oxidation Reaction by Oxygen, in Japanese)", p. 181, Maruzen, Tokyo, 1977.
19. D. Dobos, "Electrochemical Data", pp. 310 and 317, Elsevier, New York, 1975.

(Received November 29, 1994)

(Accepted January 27, 1995)