Notes

Enhancement Effect of 2,6-*O*-Dimethyl- β -cyclodextrin on the Chemiluminescent Detection of β -D-Galactosidase Using a *Cypridina* Luciferin Analog

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Both chemiluminescent immunoassay (CLIA) and chemiluminescent enzyme immunoassay (CLEIA) have been developed as alternatives to radio immunoassay.¹ The former uses chemiluminescent compounds as a label of antigens or antibodies, while the latter uses enzymes as a label for them. Generally, the sensitivity of CLEIA is higher than that of CLIA, since the enzymes amplify signals of antigens. β -D-Galactosidase (β -Gal) is one of enzymes widely used for CLEIA.²⁻⁴ Bronstein *et al.* detected β -Gal at levels below 10^{-13} mol/l at pH12 using 3-(2'-spiroadamantane)-4-methoxy-4-(3''- β -D-galactopyranosyloxyphenyl)-1,2-dioxetane.⁵ Arakawa *et al.* also detected β -Gal at levels of 10^{-14} mol/l (0.2 amol/assay) using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, isoluminol, and microperoxidase.⁶

We have recently developed a chemiluminescent assay for β -Gal by using a novel *Cypridina* luciferin analog, 3-(β -D-galactopyranosyloxy)-6-(4-methoxyphenyl)-2-methylimidazo[1,2-*a*]pyrazine (β -Gal-MCLA), from which galactose is enzymatically removed to produce 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3(7*H*)-one (MCLA). Then it is autoxidized

to emit light (Route A in Fig. 1). β -Gal was detected at a level of 700 amol/assay by this method.⁷ Some improvement of the method is necessary in order to detect β -Gal more sensitively. Interestingly, Toya reported that the light emission obtained from the autoxidation of MCLA was enhanced by an addition of β -cyclodextrins in aqueous solution.⁸ So, the similar enhancement for chemiluminescent assay of β -Gal would be expected by the addition of β -cyclodextrins (Route B in Fig. 1). In this paper, we report the enhancement effect of β -cyclodextrins on the chemiluminescent detection of β -Gal and on the CLEIA for mouse immunoglobulin (Mouse IgG) using β -Gal-MCLA.

Experimental

Materials

β -Gal-MCLA was prepared from MCLA with 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide.⁹ β -Gal (EC 3.2.1.23, from *Escherichia coli*) obtained from Wako Pure Chemical Industries, Ltd., was diluted with sodium

Fig. 1 Reaction scheme of β -Gal-MCLA with β -Gal.

phosphate buffer (50 mM, pH 7.3) containing 1 mM MgCl_2 . The amount of β -Gal was calculated from the absorbance at 280 nm by taking the extinction coefficient and the molecular weight to be $2.09 \text{ g}^{-1} \text{ l cm}^{-1}$ and 540000, respectively.¹⁰ Both Mouse IgG and goat anti-Mouse IgG antibody obtained from Lampire Biological Laboratories (Pennsylvania, USA), were purified by using a protein A chromatography and an affinity chromatography, respectively. Goat anti-Mouse IgG antibody labeled with β -Gal obtained from American Qualex (California, USA), was purified by an affinity chromatography. Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, USA). Microtiter plates were obtained from Nunc (Roskilde, Denmark). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd.

Chemiluminescent detection of β -Gal

A chemiluminescent detection of β -Gal was performed as follows: 25 μl of an enzyme sample and 75 μl of β -Gal-MCLA dimethylsulfoxide solution (100 μM) were added to 650 μl of Tris-HCl buffer (100 mM, pH 8.0) containing 1.2 mM MgCl_2 . The solution was incubated in a glass tube sealed with Parafilm in a thermostatted water bath (BK33, Yamato) at 40°C for 20 min. After incubation, 500 μl of the above mixed solution was placed in the chemiluminescence detector (CLD 100, Tohoku Electronic Ind. Co., Ltd.) and the light intensity was measured at 40°C for 1 min.

Immobilization of goat anti-Mouse IgG antibodies on microtiter plates

One hundred microliter aliquots of goat anti-Mouse IgG antibody (5.0 $\mu\text{g}/\text{ml}$) in sodium phosphate buffer (100 mM, pH 7.5) were added to the wells of black polystyrene microtiter plates. After incubating for 12 h at 4°C, unbound antibody was removed by washing three times with sodium phosphate buffer (10 mM, pH 7.5) including 0.5% Tween-20 and NaCl (150 mM). Then 300 μl of sodium phosphate buffer (10 mM, pH 7.5) including 5% bovine serum albumin, 0.5% Tween-20 and NaCl (150 mM) was put into the well. After incubating for 2 h at 25°C, the solution was removed by decantation.

CLEIA of Mouse IgG

One hundred microliter aliquots of Mouse IgG in sodium phosphate buffer (10 mM, pH 7.5) including 5% bovine serum albumin, 0.5% Tween-20 and NaCl (150 mM) were added to the antibodies-coating wells of the above plates. After incubating for 30 min at 25°C, unbound Mouse IgG was removed by washing three times with sodium phosphate buffer (10 mM, pH 7.5) including 0.5% Tween-20 and NaCl (150 mM). The commercially available goat anti-Mouse IgG antibody labeled with β -Gal was diluted 1000-fold with sodium phosphate buffer (10 mM, pH 7.5) including 5% bovine serum albumin, 0.5% Tween-20 and NaCl (150 mM) and then 100 μl of aliquots were added to each well. After incubating for 30 min at 25°C, the wells were washed

Fig. 2 Effect of β -cyclodextrins on the detection of β -Gal. Black columns indicate the light intensity in the absence of β -Gal. Dots columns indicate the light intensity in the presence of β -Gal (173 fmol/assay). Error bars denote $\pm$ SD ($n=3$). The concentration of β -cyclodextrins was 10 mM.

three times with sodium phosphate buffer (10 mM, pH 7.5) including 0.5% Tween-20 and NaCl (150 mM). The substrate solution for β -Gal was prepared as follows: β -Gal-MCLA dimethylsulfoxide solution (100 μM) was diluted 10-fold with sodium phosphate buffer (200 mM, pH 8.0) including 1 mM MgCl_2 . One hundred microliters of the solution was added to the wells. After incubating for 20 min at 40°C, the plate was put into a microplate reader (Luminous CT-9000D, Dia-latron Co. Ltd.). The light intensity of each well was measured for 10 s.

Results and Discussion

Effect of β -cyclodextrins on the chemiluminescent detection of β -Gal

First of all, β -Gal activity in the presence of β -cyclodextrins was investigated. The activity was measured by spectrophotometry using 2-nitrophenyl- β -D-galactopyranoside as a substrate in Tris-HCl buffer (100 mM, pH 8.0). Three kinds of β -cyclodextrins: β -cyclodextrin (β -CD), 2,6-di-*O*-methyl- β -cyclodextrin (2,6- β -CD), and 2,3,6-tri-*O*-methyl- β -cyclodextrin (2,3,6- β -CD) were examined. The activity was not changed and was almost independent of the kinds and the concentrations of β -cyclodextrins in the range from 0.1 to 10 mM. These results prove that the activity of β -Gal was not inhibited by the addition of β -cyclodextrins.

Next, the effect of β -cyclodextrins on the light intensity of chemiluminescent detection of β -Gal was investigated. In the absence or presence of β -Gal (173 fmol/assay), the light intensities were measured on three kinds of β -cyclodextrins. The results were shown in Fig. 2. In all cases, the intensities in the presence of β -Gal were enhanced compared with the case of no β -cyclodextrins,

while in the absence of β -Gal, no appreciable intensities were observed. Especially, in the case of 2,6- β -CD, the light intensity was 6 times higher than that of no β -cyclodextrins. The enhancement order was 2,6- β -CD, 2,3,6- β -CD, and β -CD. The quantum yields of MCLA in the presence of 2,6- β -CD, 2,3,6- β -CD and β -CD were reported to be 1.0, 0.48 and 0.34%, respectively, in the phosphate buffer (pH 7.0).⁸ This enhancement order was compatible with that reported for the quantum yields of MCLA by autoxidation. From these results, we presumed that the addition of β -CDs increased the quantum yields of the MCLA which was produced from the reaction of β -Gal-MCLA with β -Gal. We proved that 2,6- β -CD was the most effective enhancer on the chemiluminescent detection of β -Gal by using β -Gal-MCLA.

Effect of 2,6- β -CD on the CLEIA for Mouse IgG

Mouse IgG was chosen as an antigen of the CLEIA by using β -Gal and β -Gal-MCLA. As antibodies, goat anti-Mouse IgG antibody and β -Gal labeled antibody were used. The sandwich immunoassay using these antibodies was performed by using a microtiter plate. The light from the reaction of β -Gal with β -Gal-MCLA was detected by the microplate reader. In the absence of 2,6- β -CD, Mouse IgG was detected in the range from 4 to 125 fmol/assay.

The enhancement effect of 2,6- β -CD on the detection limit of Mouse IgG was investigated. The result is shown in Fig. 3. It seems that the difference of light intensity in Figs. 2 and 3 was caused by the sensitivity of the detectors. In the absence of 2,6- β -CD, the light intensity slightly increased with the quantity of Mouse IgG in the range from 0 to 8 fmol/assay. By using 2,6- β -CD, the light intensity and the slope were increased in comparison with those of no addition. The detection limit was increased from 4 to 2 fmol/assay. It was proved that 2,6- β -CD is a good enhancer on the CLEIA by using β -Gal and β -Gal-MCLA.

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Fig. 3 Effect of 2,6- β -CD on the CLEIA for Mouse IgG. (●), in the presence of 2,6- β -CD; (■), in the absence of 2,6- β -CD. Error bars denote $\pm$ SD ($n=16$).

References

1. M. Maeda and A. Tsuji, in "Kagaku Hakkou Immunoassay (Chemiluminescent Immunoassay, in Japanese)", ed. A. Tsuji and T. Kanno, p. 3, Life Science, Tokyo, 1992.
2. A. Tsuji, M. Maeda and H. Arakawa, *Anal. Sci.*, **5**, 497 (1989).
3. L. J. Kricka, *Clin. Chem.*, **37**, 1472 (1991).
4. A. Mayer and S. Neuenhofer, *Angew. Chem. Int. Ed. Engl.*, **33**, 1044 (1994).
5. I. Bronstein, B. Edwards and J. C. Voyta, *J. Biolumin. Chemilumin.*, **4**, 99 (1989).
6. H. Arakawa, M. Maeda and A. Tsuji, *Anal. Biochem.*, **199**, 238 (1991).
7. M. Mitani, S. Sakaki, Y. Koinuma, Y. Toya and M. Kosugi, *Anal. Sci.*, **10**, 813 (1994).
8. Y. Toya, *Nippon Nogeikagaku Kaishi*, **66**, 742 (1992).
9. Y. Toya, to be published.
10. K. Tanaka and E. Ishikawa, *Anal. Lett.*, **19**, 433 (1986).

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