The Determination of a Small Amount of Biological Constituent by the Use of Chemiluminescence. V. An Iron-dyestuff Complex as a Catalyst

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Synopsis. The catalytic activity of the iron(III)-α,β,γ,δ-tetraphenylporphinetrisulfonic acid complex for the chemiluminescence reaction between luminol and H_2O_2 has been found to decrease in the presence of protein. On the basis of this phenomenon, 2×10^{-5}—2×10^{-3} g dm^{-3} bovine serum albumin was determined with the detection limit of 4 ng, lower than that in the previous paper.

It was previously reported[1] by the present authors that the catalytic activity of Cu(II) for the chemiluminescence(CL) reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) and hydrogen peroxide(H_2O_2) decreased in the presence of protein, and that the sensitive determination of protein could be carried out on the basis of this phenomenon. In order to make the method of determining protein more sensitive, the use of Co(III) instead of Cu(II) as a catalyst was attempted.[2] The results were slightly improved, but they not very satisfactory, because there was not enough bond formation between Co(III) and a protein molecule.

On the other hand, various dyestuff combination methods have been reported for the determination of a small amount of protein. The dyestuff molecule used for this method generally has several aromatic rings and sulfonic acid groups, which have a high affinity against the residues of lysine, arginine, and histidine, and the amino terminal in a protein molecule.[3] The dyestuff combination method[3,4] could be carried out only by mixing a dyestuff solution with a sample solution and by measuring the absorbance of the mixed soluion. With the object of establishing a highly sensitive method of determining protein by means of the CL method, a metal-complex compounds which had a structure similar to that of a dyestuff in the dyestuff-combination method and which had a catalytic activity for the CL reaction was used in the present study.

Several iron(III) porphine complexes have previously been reported[5] by the present authors to show a high catalytic activity for the CL reaction. Accordingly, the iron(III) α,β,γ,δ-tetraphenylporphinetrisulfonic acid complex([Fe(tpps)]), which contains aromatic rings and sulfonic acid groups in the molecule, was used for the determination of a small amount of protein by means of the CL method. This method was about 50 times as sensitive as the previous method, using the Cu(II) catalyst for the determination bovine serum albumin(BSA) as a model protein.

Experimental

The [Fe(tpps)] was prepared in accordance with Nomura's method.[6] The α,β,γ,δ-tetraphenylporphinetrisulfonic acid was purchased from Dojin Laboratories as its disulfonic acid salt. The absorption spectrum of the solution of the synthesized [Fe(tpps)] was in agreement with that reported by Nomura *et al.* The concentration of the [Fe(tpps)] stock solution was determined by means of an absorbance measurement at 395 nm.

A sample solution containing protein was diluted with a 0.01 mol dm^{-3} phosphate buffer solution(pH 7.2), and to the mixture a definite amount of [Fe(tpps)] stock solution was added to yield 5.7×10^{-9} mol dm^{-3}. The catalytic activity of the [Fe(tpps)] in the mixed solution was measured by mean of the flow-injection-analysis apparatus reported in a previous paper.[5] The ratio of the CL intensity of a protein sample solution to that of a 5.7×10^{-9} mol dm^{-3} [Fe(tpps)] solution was plotted against the amount of protein, and this graph was used as a calibration curve of the protein.

Results and Discussion

Though iron(III) porphine complexes had been reported by the previous authors to show a high catalytic activity for the CL reaction between luminol and H_2O_2, the catalytic activity of [Fe(tpps)] still remained unknown. Therefore, the catalytic activity of [Fe(tpps)] was measured; the results are shown in Fig. 1. [Fe(tpps)] was about 3 times as active as the previous iron(III) porphine complex for the CL reaction. Therefore, the effect of the protein on the catalytic activity of [Fe(tpps)] was examined. Human

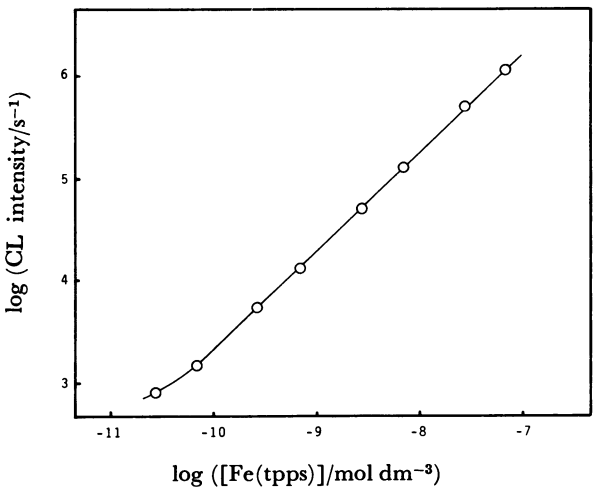

Fig. 1. Calibration curve of [Fe(tpps)].

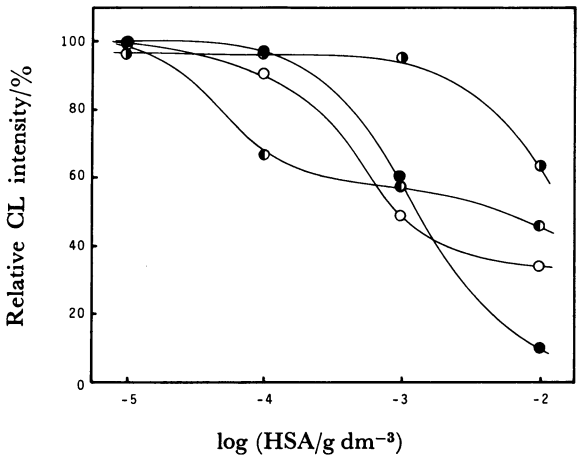

Fig. 2. Effect of pH value on the catalytic activity of [Fe(tpps)].
◐: pH 2.5, ○: pH 7.2, ●: pH 9.1, and ◑: pH 12.1.

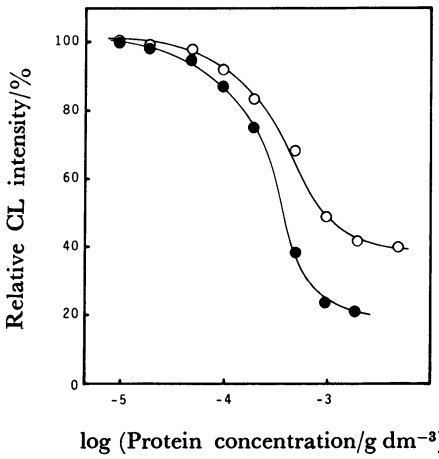

Fig. 3. Calibration curve of HSA and BSA.
○: HSA and ●: BSA.

serum albumin(HSA) was dissolved in a buffer solution and diluted with the same buffer solution as the preceding one, and then to it the [Fe(tpps)] stock solution was added so as to make it 5.7×10^{-9} mol dm^{-3}; this showed a CL intensity of about 10^5 cps, corresponding to the full scale of a photon counter in the absence of any protein. The mixed solution was left standing for 1 h at room temperature, and its catalytic activity was measured. This experiment was carried out at various pH values(Fig. 2). The catalytic acivity of [Fe(tpps)] for the CL reaction was found to decrease in the presence of protein. The change in the catalytic activity in the presence of protein was also found to decrease with an increase in the pH value. However, the CL intensity of a sample solution of less than pH 5 greatly decreased in the absence of protein; moreover, its CL intensity was not so reproducible. Hence, the solution containing [Fe(tpps)] and protein was adjusted to pH 7.2, and its CL intensity was measured.

The change in the catalytic activity of [Fe(tpps)] caused by HSA, BSA, human serum γ-globulin-(HγG), and bovine serum γ-globulin(BγG) was examined. HSA and BSA lowered the catalytic activity, while even 1.0×10^{-3} g dm^{-3} HγG and BγG did not affect the catalytic activiy. It is well known that Methyl Orange, Amido Black 10B, Coomassie Brilliant Blue, and so on, which are used for the dyestuff-combination method, do not react with a protein such as globulin.[3,4] Judging from the effect of the pH on the reaction between [Fe(tpps)] and protein, and the difference in reactivity with various proteins, it may be supposed that the [Fe(tpps)] binds to protein as in the above-mentioned dyestuffs.

The relationship between the reaction time of [Fe(tpps)] with protein and the catalytic activity of [Fe(tpps)] was examined. The CL intensities measured at room temperature at about 1.5 min and 1 h after mixing protein with [Fe(tpps)] were almost the same. Therefore, the CL intensity was measured immediately after a sample solution was mixed with [Fe(tpps)].

Table 1. Coefficient of variation for the determination of protein

HSA concentration/gdm^{-3}	CV*/%	BSA concentration/gdm^{-3}	CV*/%
0	0.54	0	0.54
2.0×10^{-5}	0.69	2.0×10^{-5}	0.79
2.0×10^{-4}	0.74	2.0×10^{-4}	0.44
2.0×10^{-3}	1.20	2.0×10^{-3}	0.73

* Coefficient of variation.

The calibration curves of HSA and BSA obtained by the present method are shown in Fig. 3, while the coefficient of variation(CV) for the determination of protein is shown in Table 1. The detection limits of HSA and BSA were 10 ng and 4 ng respectively.

In comparison with the previous method using the Cu(II) catalyst, the present method was characterized as follows: 1) about 50 times more sensitive, 2) a rapid reaction between catalyst and protein, 3) no necessity to heat the sample solution, and 4) no response for γ-globulin.

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