Spectrophotometric Determination of Peroxidase by the Oxidative Decomposition of Copper–Phthalocyanine Complex using Peroxomonosulfate

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Peroxidase (POD) was found to act as an excellent catalyst during the oxidative decomposition reaction of the copper–phthalocyanine complex (Cu-pts) using peroxomonosulfate. POD also decomposed with Cu-pts during the reaction. Therefore, the simultaneous decomposition of indicator and catalyst provides a characteristic L-shape absorbance–time curve. Based on these findings, an indicator system of POD, which has a slight variation in the measured absorbance for time and high precision, was established. This spectrophotometry was able to make determinations over the range of 0.1 to 30 μg/ml of POD. The detection limits (3σ) were 0.02 μg/ml and the relative standard deviation was 3.7% for 0.5 μg/ml of POD (10 determinations). The proposed method has been applied to the determination of human interleukin-8 which was added to human serum using POD as the label in an immunoassay.

Key words spectrophotometric determination; peroxidase; copper–phthalocyanine complex; simultaneous decomposition; L-shape absorbance–time curve; immunoassay

In living tissues, iron–porphyrin complexes such as hemoglobin, myoglobin, catalase and peroxidase (POD), are well-known to play important roles. Among them, POD has been extensively used as a label for immunoassay in clinical analysis. For this purpose, a highly sensitive, high precision, rapid and simple determination of POD has been strongly desired. The common methods used for the determination of POD are catalytic determinations[1—4] based on peroxidase activity. These catalytic determinations are highly sensitive because of the chemical amplification of the enzyme. However, they have some problems with regard to the variation in measured absorbance with respect to time and precision. In addition, the chromogenic reagents have the disadvantages of toxicity and lability.

On the other hand, some highly sensitive determinations have been reported based on the oxidative decomposition of porphyrin compounds as a chemical amplification probe. For example, the catalytic determination of the ruthenium(III) ion which selectively accelerated the decomposition of the porphine ring with potassium bromate, has been reported.[5] Moreover, the determination of cobalt ion has been reported[6] based on the autocatalytic reaction by the oxidative decomposition of the cobalt–phthalocyanine complex. These methods are highly sensitive, but had the limitation of measuring time.

Furthermore, the indicator system for the determination of the iron–porphyrin complexes such as the iron–chlorophyllin complex and hemoglobin has been previously proposed.[7] For example, hemoglobin acts as a catalyst during the oxidative decomposition of copper–phthalocyanine complex (Cu-pts) using peroxomonosulfate and it is decomposed together with Cu-pts during the reaction. Based on this finding, a highly sensitive determination for hemoglobin has been reported. The simultaneous decomposition of an indicator and catalyst provides the characteristic L-shape absorbance–time curve which has a slight variation in absorbance with time and has a high precision. In this paper, the rapid and high precision spectrophotometric determination of POD having an iron–porphyrin complex will be demonstrated, and this method will be applied to an immunoassay using POD as a label.

Experimental

Apparatus All spectrophotometric measurements were made on a Hitachi Model 200-100 double-beam spectrophotometer, equipped with a 1 cm cell or microcell and a thermostated cell holder. A Horiba Model F-8AT pH meter was used for the pH measurements.

Reagents All of the chemicals used were of analytical reagent grade and were used as received. All working solutions were prepared just before use. Distilled water was used in all of the experiments. A standard POD (from horseradish, Wako) solution was prepared by dissolving in distilled water. A Cu-pts (copper-phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt, dye content (*ca.* 85%, Aldrich)) solution was prepared by dissolving in distilled water and was adjusted to 10^{-5} M. A pH buffer solution was prepared by mixing 1 M sulfuric acid (Kanto) and 1 M sodium sulfide (Kanto), diluted and then adjusted to 0.1 M. A potassium peroxomonosulfate (OXONE, $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, Aldrich) was prepared by dissolving in distilled water and adjusting to 1.5×10^{-2} M. A commercial human interleukin-8 (hIL-8) immunoassay kit (Biosource International) was used. Human serum (blood type AB, Whittaker Bioproducts) was used.

Procedure Determination of Peroxidase: A 5 ml sample solution containing POD, 2 ml of 10^{-4} M Cu-pts and 2 ml of sulfuric acid–sodium sulfate pH buffer solution (pH 2.0) was placed in a 10 ml volumetric flask, and the mixture was diluted to the mark with water. Two ml of the mixture was used in a 1 cm light-path cell and the cell was placed in the spectrophotometer. A 1.1 ml of 4.2×10^{-2} M peroxomonosulfate was injected into the mixture in order to start the reaction. The change in absorbance was measured at 613 nm which is the maximum absorption wavelength of Cu-pts. The concentration of POD was determined after 5 min using the calibration curve of ΔA, which is given by $\Delta A=(Asample(0)-Asample(5))-(Ablank(0)-Ablank(5))$, where the absorbance of the blank solution and that of the sample solution containing POD at the reaction times of 0 and 5 min is given by Ablank(0), Ablank(5), Asample(0) and Asample(5), respectively.

Determination of hIL-8 in Human Serum: A commercial hIL-8 immunoassay kit was used. The antigen–antibody reaction was performed according to the procedure in the kit instructions. However, the dilution number and added volume of each reagent was changed as follows. Human serum (50 μl) and 100 μl of biotinylated anti-IL-8 (biotin conjugate) solution were added to the hIL-8 antibody-coated well. The mixture was incubated for 90 min at room temperature. The well was then washed 4 times for B/F separation. A 150 μl solution of streptavidin-POD (50-fold dilution of concentrate) was added and was incubated for 30 min. The well was also washed 4 times for B/F separation. The immobilized POD was determined according to the proposed method: 100 μl of Cu-pts solution as the indicator

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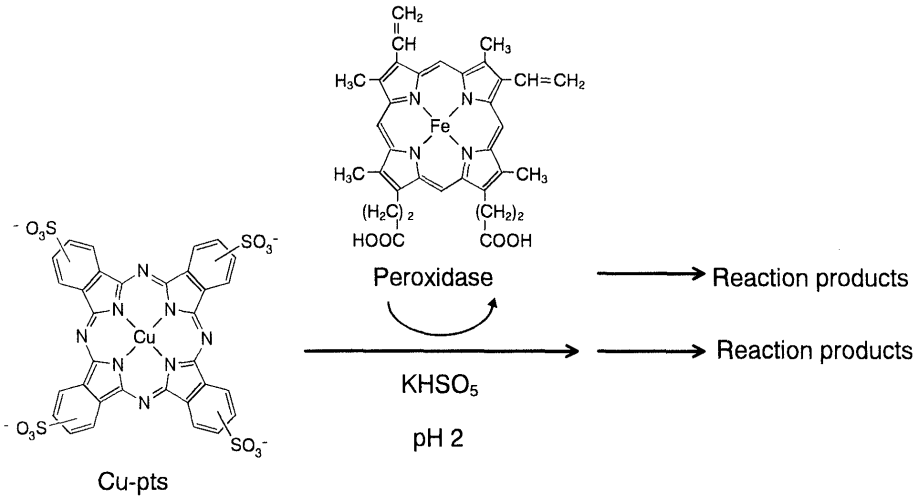

Fig. 1. Scheme for the Simultaneous Oxidative Decomposition Reaction as an Indicator System

and sulfuric acid–sodium sulfate mixed solution as the pH buffer was added and the peroxomonosulfate as the oxidizing reagent was then added in order to start the indicator reaction. After 5 min, the absorbance of the mixture was recorded with a microcell.

Results and Discussion

The scheme for the oxidative decomposition of Cu-pts, which becomes an indicator reaction, is shown in Fig. 1. The Cu-pts was decomposed with peroxomonosulfate. In the presence of POD having an iron–porphyrin complex, the oxidative decomposition of the Cu-pts was accelerated. The decomposition of the Cu-pts depended on the POD concentration added. The absorbance–time curve is shown in Fig. 2A. The slope of the absorbance–time curve of the sample solution becomes gentle and equal to that of the blank solution after 5 min. This means that the uncatalyzed reaction (blank reaction) proceeds after the decomposition of POD as a catalyst. By considering this influence of the blank reaction, a $\Delta A'$–time curve, which was given by $\Delta A' = (\text{Ablank}(0) - \text{Ablank}(t)) - (\text{Asample}(0) - \text{Asample}(t))$, is shown in Fig. 2B, where the absorbance of the blank solution and that of the sample solution were at reaction times of 0 and t, respectively, given by Ablank(0), Ablank(t), Asample(0) and Asample(t). This figure shows the characteristic L-shape absorbance–time curve and the measured absorbance that gives a constant signal for the measuring time.

Optimization of Conditions in the Recommended Procedure pH: The sulfuric acid–sodium sulfate solution was selected as the pH buffer solution because the oxidizing agent is peroxomonosulfate consisting of $KHSO_5$, $KHSO_4$ and K_2SO_4 and an influence of the nature of the salt was observed. The effect of pH was examined over the pH range of 1.5 to 3.0. A change in ΔA was not observed in this pH range. The procedure was also carried out at pH 2.0.

Salt Concentration: The effect of salt concentration (*i.e.*, the concentration of pH buffer solution) was examined by the addition of sodium sulfate. A change in ΔA was not observed over the range of 0.08 to 0.12 M total salt concentration. With the decrease in salt concentration, ΔA was decreased due to the aggregation of the Cu-pts. The salt concentration was adjusted to 0.10 M.

Peroxomonosulfate Concentration: The effect of peroxomonosulfate concentration was tested over the range of

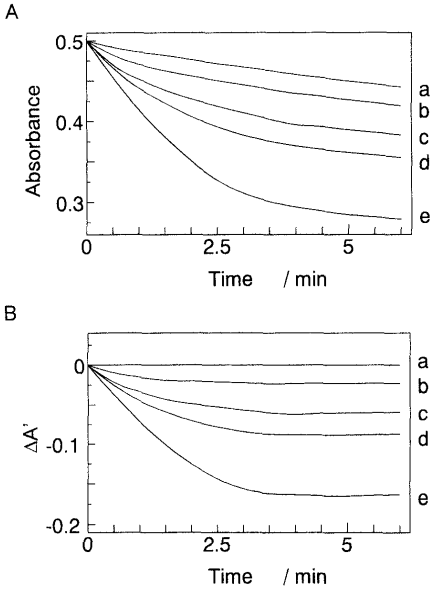

Fig. 2. Absorbance–time Curve (A) and $\Delta A'$–time Curve (B)
[POD]: a, 0 μg/ml; b, 0.2 μg/ml; c, 0.4 μg/ml; d, 0.8 μg/ml; e, 2.0 μg/ml.

0.5×10^{-2} to 2.0×10^{-2} M. With the increase to a peroxomonosulfate concentration of 1.5×10^{-2} M, the decomposition of Cu-pts was accelerated and ΔA was increased. Above 1.5×10^{-2} M, ΔA was decreased because the blank reaction was accelerated and the decomposition of POD was also accelerated. The peroxomonosulfate concentration was adjusted to 1.5×10^{-2} M.

Temperature: The effect of temperature was evaluated over the range of 15 to 40 °C. The absorbance of the Cu-pts decreased with the increase in temperature. The change in ΔA was not observed at room temperature (25 °C). In order to run the antigen–antibody reaction at room temperature, the recommended procedure was carried out at room temperature without controlling the temperature.

Stability of Reagent: The working solutions of indicator, catalyst and oxidizing agent were stable for at least 6 h.

Calibration Graph The proposed method was able to make accurate determinations over the range of 0.1 to 30 μg/ml of POD. The detection limits (3σ) were 0.02 μg/ml, which corresponded to three times the standard deviation of

Table 1. Influence of Foreign Substances[a]

Amount added[b]	Foreign substances	Error[c] %	Foreign substances	Error[c] %
×1000	Na_2SO_4	+1.51	Racemic acid	+3.13
	Na_2CO_3	+3.03	Citric acid	+4.62
	Na_3PO_4	+4.84	Glutamic acid	+3.49
	K_2SO_4	+4.35		
×100	$NaNO_3$	+4.54	Heparin	+3.30
	$Zn(NO_3)_2$	±0	Glycine	−1.45
	$MnSO_4$	−3.13	Histidine	+2.33
	$CuSO_4$	−1.54		
×10	Cysteine	+1.56		
×1	NaCl	+1.61	Albumin	−2.94
	$Mg(NO_3)_2$	+1.61	Globulin	−1.94
×0.1	$CaCl_2$	+3.13	EDTA	−1.54
×0.05	$FeSO_4$	−4.48	Hemoglobin	+4.17
	$Fe(NO_3)_3$	−4.48		
×0.01	$NiCl_2$	−4.48	Bilirubin	+1.52
	$CoCl_2$	−4.48		

a) Under coexistence of 0.5 μg/ml POD. *b*) Ratio of weight: [foreign substances]/[POD]. *c*) The value (%) of error was calculated for ΔA(0.066).

the blank reaction. For 0.5 μg/ml of POD, the relative standard deviation was 3.7% (10 determinations). The apparent molar absorptivity calculated from the 0.5 μg/ml of POD was $5.8\times10^6\,M^{-1}\,cm^{-1}$. This value corresponds to a molecule of POD turnover which decomposes approximately 120 molecules of Cu-pts within 5 min at room temperature.

Influence of Foreign Substances The tolerance amount and the error value (%) are shown in Table 1. Metal ions and organic compounds were also examined. For 0.5 μg/ml of POD, an error of ±5% in ΔA was permitted. Metal ions such as iron, cobalt and nickel were not deleterious up to 0.01—0.05 fold the weight of POD. By conversion to molar ratio, these metal ions were permitted up to 10—100 fold. Since only the POD was left by B/F separation in the antigen–antibody reaction, the removal of foreign substances was not carried out.

Application to a Practical Sample The proposed method was applied to the determination[8] of hIL-8 in human serum using POD as a label. hIL-8 is a chemotactic and activating cytokine for neutrophils, which play an important role in acute inflammatory responses. The results obtained for the determination of hIL-8 in human serum are summarized in Table 2. As an aberrant signal, known amounts (5.0 ng/ml) of hIL-8 were added to the human serum. A 99% recovery of hIL-8 was obtained, and the inactivation of immobilized POD was not observed. The tetramethylbenzidine method, which is an indicator system in the kit, requires 30 min to determine hIL-8. The proposed method requires 5 min and was able to make a rapid determination. Also, the proposed method has a slight variation of measured absorbance for time and the relative standard deviation was 2.5% (5 determinations). In the near future, by immunoassay using POD as a label, the proposed method could be applied for the detection of a drug in biological fluids such as TDM (therapeutic drug monitoring) which requires speed as well as sensitivity.

Table 2. Determination of hIL-8 in Human Serum

Sample	Added ng/ml	Found ng/ml	Recovery %	R.S.D.[a] %
Human serum	0	N.D.	—	—
	5.00	4.95	99	2.5

a) 5 determinations.

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