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Fluorometric Determination of Hydrogen Peroxide Based on Formation of Amprochrome

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A fluorometric determination of H_2O_2 based on the formation of amprochrome was developed. Amprochrome is an oxidation product of amprolium, an agent used for treating coccidiosis of chickens, and has a strong fluorescence. Though potassium ferricyanide has been used for oxidizing amprolium, H_2O_2 is also considered to be useful for the reaction. The analytical procedure was as follows. To a sample solution containing a suitable quantity of H_2O_2 , 1 ml of amprolium (500 $\mu\text{g}/\text{ml}$), NaOH (10 %) and peroxidase solution (20 units/ml) were added. The mixture was shaken for 45 min at room temperature and amprochrome produced was extracted with 10 ml of isoBuOH after saturating the solution with sodium sulfate. Relative fluorescence intensity of the separated isoBuOH layer was measured (excitation wavelength 400 nm and emission wavelength 460 nm) against that of 10^{-6} M quinine sulfate in 0.1 N H_2SO_4 . The calibration plot was linear up to H_2O_2 0.2 $\mu\text{g}/\text{tube}$. On the basis of the proposed method, remaining H_2O_2 in Japanese noodles (udon) could be determined with a recovery of 82.4–108 %.

Keywords—hydrogen peroxide; fluorometric determination; amprochrome; peroxidase; food

Introduction

In the previous paper,¹⁾ we investigated the fluorometric determination of H_2O_2 with thiamine. However, thiamine is contained in foods, and this may influence the analysis. Thus, we searched for another chemical not contained in foods and having a structure expected to show fluorescence, and we selected amprolium (1-[(4-amino-2-*n*-propyl-5-pyrimidinyl) methyl]-methylpyridinium chloride). Amprolium is an effective agent for the control of coccidiosis and is converted to a fluorescent substance, namely amprochrome, by a reaction analogous to the oxidative production of thiochrome from thiamine (Chart 1). That is, amprochrome is formed through dehydration and double bond formation in conjugation with

the pyrimidine ring. Though potassium ferricyanide has generally been used for the production of amprochrome,²⁾ H_2O_2 is also considered to be suitable based on the results of the present examination. The present fluorometric method should be applicable for H_2O_2 assay in foods.

Experimental

Samples—The following four kinds of foods purchased from a local market in Kanazawa were used in the present examination; Japanese noodles (udon), small bait (shirasuboshi), fish paste (chikuwa) and herring roe (kazunoko).

Reagents—Amprolium standard was obtained from Dainippon Chemical Ind. Ltd., Osaka. All the other reagents used were as described in the previous paper.¹⁾

Chart 1. Principles of Fluorometric Determination of H_2O_2

Apparatus—The same apparatus was used as described in the previous paper.¹⁾

Procedure—The procedure is summarized in Chart 2.

Preparation of Sample Solution: A sample was cut into pieces (0.1–1.0 g) and taken into a centrifuge tube. One gram of potassium bromate, 1 drop of silicone and 10 ml of MeOH were added to the tube. Then the mixture was homogenized in an ice bath, centrifuged (3000 rpm, 5 min) and filtered through Toyo filter paper No. 5C. These procedures were repeated, and the filtrates were mixed and made up to 25ml with MeOH.

Development of Fluorescent Substance: Sample solutions 1 ml each were taken into three test tubes, A, B and C. Then 4 ml of water was added to A, 3 ml of water and 1 ml of catalase solution (0.2 %) to B, and 3 ml of water and 1 ml of H_2O_2 standard solution (0.2 $\mu\text{g}/\text{ml}$) to C, and the three test tubes were allowed to stand for ten minutes. During this time, hydrogen peroxide, if it exists in the sample solution of B, is decomposed by catalase. Next, 1 ml of amprolium solution (500 $\mu\text{g}/\text{ml}$), NaOH solution (10 %) and peroxidase solution (20 units/ml) were added to the three test tubes. After shaking for 45 min at room temperature, each reaction mixture (A, B and C) was salted out with Na_2SO_4 and amprochrome formed was extracted with 10 ml of isoBuOH.

Reading of Fluorescence: About 2 ml of the isoBuOH extract was transferred to a cuvette and the relative fluorescence intensity was measured

against 10^{-6} M quinine sulfate in 0.1 N H_2SO_4 . The wavelengths for excitation and emission were adjusted to 400 nm and 460 nm, respectively.

Calculation: Hydrogen peroxide content in the food sample was calculated by means of the following formula;

$$\text{H}_2\text{O}_2 (\mu\text{g}/\text{g}) = 0.2 \times \frac{a-b}{c-a} \times 25 \times \frac{1}{\text{sample weight (g)}}$$

where a, b and c are the fluorescence readings for A, B and C, respectively.

Results and Discussion

Influence of MeOH Concentration on Fluorescence Intensity

The sample solution was prepared with MeOH in this procedure for the purpose of preventing H_2O_2 decomposition. Since the MeOH concentration in a reaction mixture may influence the peroxidase activity, the extractability of amprochrome with isoBuOH and the measurement of fluorescence, we investigated the optimum MeOH concentration. As shown in Fig. 1, MeOH tended to increase the fluorescence intensity somewhat, probably because of its solvent effect on amprochrome. However, the fluorescence intensity became variable when the MeOH concentration exceeded 20 %, and so 1 ml of sample solution was used in the standard procedure (equivalent to 12.5 % MeOH concentration in a reaction mixture of 8 ml).

Chart 2. Procedure for Fluorometric Determination of H₂O₂ with Amprolium

Effect of Amount of Amprolium on Fluorescence Intensity

As shown in Fig. 2, the fluorescence of a standard sample containing 0.1 μg of H₂O₂ increased with increasing amount of amprolium added. The fluorescence of the reagent blank also increased, which may be attributable to oxygen in the air or in the reaction mixture. Removal of the oxygen is under investigation. The amount of amprolium to be added was set at 500 μg per tube, since the ratio of

fluorescence intensity of the standard sample to the reagent blank was almost constant above 300 μg of amprolium.

Effect of NaOH Concentration on Fluorescence Intensity

Amprolium is converted to a fluorescent substance in alkaline solution. We examined the optimum NaOH concentration (Fig. 3).

As 1–2% NaOH concentration was most suitable for the formation of amprochrome, 1 ml of 10% NaOH was added to the standard

Fig. 1. Effect of MeOH Concentration on Fluorescence Intensity

—○—: H₂O₂ 0.1 µg/tube, —●—: reagent blank.

Fig. 2. Effect of Amount of Amprolium

—○—: H₂O₂ 0.1 µg/tube, —●—: reagent blank.

reaction mixture, giving an NaOH concentration of 1.25 %.

Effect of Peroxidase on Fluorescence Intensity

Twenty units of peroxidase was used in this procedure since the fluorescence intensity of amprochrome reached a maximum above 10 units of peroxidase.

Effect of Reaction Temperature on Fluorescence Intensity

Fluorescence intensity increased with increasing reaction temperature for both the stan-

Fig. 3. Effect of NaOH Concentration on Fluorescence Intensity

—○—: H₂O₂ 0.1 µg/tube, —●—: reagent blank.

Fig. 4. Effect of Reaction Temperature

—○—: H₂O₂ 0.1 µg/tube, —●—: reagent blank.

dard sample and the reagent blank, as shown in Fig. 4. The reaction was carried out at room temperature, since the ratio of the fluorescence intensity of the standard sample to that of the reagent blank was optimum in the temperature range of 15–37°C.

Relationship between Reaction Time and Amprochrome Formation

The amount of amprochrome formed reached the maximum 45 min after the beginning

TABLE I. Effects of Salting-out Agents on Extractability

Amount of isoBuOH (ml)	Extraction time	Extractability (%) salting-out agents		
		—	Na ₂ SO ₄	NaCl
5	1	85.2	95.5	94.7
10	1	88.0	96.1	96.8
5	2	93.5	97.2	97.5

Fig. 5. Calibration Plots

—◇—: herring roe (kazunoko), —□—: fish paste (chikuwa), —○—: Japanese noodles (udon), —△—: small bait (shirasuboshi), —●—: MeOH.

of the reaction and was stable for at least 120 min.

Effect of Salting-Out Agents and Solvents on Extractability of Amprochrome

The extractability of amprochrome with isoBuOH was improved by the use of salting-out agents, Na₂SO₄ and NaCl, as shown in Table I. Further, the extractability of amprochrome with isoBuOH was the same as that with *n*-amyl alcohol; both solvents have been generally used for amprochrome extraction. Thus, the reaction mixture was salted-out with Na₂SO₄ and then extracted with 10 ml of isoBuOH.

Calibration Plots

The calibration plots were prepared with four sample solutions containing added H₂O₂

TABLE II. Tolerance of This System to Various Materials

Materials	Tolerance limit for the material in the detn. of 0.1 μg H ₂ O ₂ /tube
KBrO ₃	>40 mg/tube
Silicone	> 2 mg/tube
NaCl	> 2 mg/tube
CaCO ₃	> 2 mg/tube
Glutamic acid	> 2 mg/tube
Glycine	> 2 mg/tube
Saccharose	> 2 mg/tube
Sorbic acid	> 2 mg/tube
Sodium polyphosphate	200 μg/tube
Oleic acid	20 μg/tube
Ammonium persulfate	2 μg/tube
Ascorbic acid	0.5 μg/tube
BHA	0.5 μg/tube
Thiamine hydrochloride	0.4 μg/tube

in the range of 0–0.2 μg. All the calibration plots were linear, as shown in Fig. 5. The maximum wavelengths of all the reaction products were observed with excitation at 400 nm and emission at 460 nm (the same as the wavelengths of amprochrome produced by the oxidation with potassium ferricyanide). The slopes of the plots differed from sample to sample, even among the same kind of food, though generally animal food samples gave gentler slopes than vegetable ones. The spectrum of the blank (no added H₂O₂) of each sample had similar maximum wavelengths, which indicated that added amprolium was converted to amprochrome through the agency of dissolved oxygen in the medium, oxidizing substances in samples, and so on.

Tolerance of This System to Various Materials

Tolerance limits of the assay system for various food components, food additives and reagents used (KBrO₃ and silicone) were examined. Recovery tests were carried out on the standard solution containing 0.1 μg H₂O₂ in the presence of test materials, and the results are shown in Table II. Each tolerance

TABLE III. Recovery of H_2O_2 from Various Foods

Samples	N	Added H_2O_2 $\mu\text{g/g}$	Found H_2O_2 $\mu\text{g/g}$	Recovery % M. $\pm$ S.D.	C.V. %
Japanese noodles (udon)	4	0	N.D.	-	-
	4	5	4.12	82.4 ± 15.9	22.0
	3	10	8.78	87.8 ± 8.80	10.0
	4	25	27.0	108 ± 6.86	6.35
	4	50	52.5	105 ± 7.13	6.79
Small bait (shirasuboshi)	4	0	N.D.	-	-
	4	5	N.D.	-	-
	4	10	4.76	47.6 ± 18.6	39.1
	4	25	18.9	75.5 ± 12.1	16.0
	4	50	39.1	78.2 ± 11.5	14.7
Fish paste (chikuwa)	4	0	N.D.	-	-
	3	5	N.D.	-	-
	4	10	8.38	83.8 ± 24.0	28.6
	4	25	22.1	88.4 ± 5.30	6.00
	4	50	52.0	104 ± 3.70	3.56
Herring roe (kazunoko)	4	0	N.D.	-	-
	4	5	N.D.	-	-
	4	10	N.D.	-	-
	4	25	22.8	91.7 ± 21.9	24.0
	4	50	50.0	100 ± 12.0	12.0

limit exceeded the maximum amount expected to be present in 1 ml of sample solution, indicating that the interference due to these materials is minor.

Recoveries of H_2O_2 from Various Foods

For practical application, recovery tests of H_2O_2 were carried out on food samples to which H_2O_2 had been added at the concentration of 0, 5, 10, 25 or 50 $\mu\text{g/g}$. Hydrogen peroxide was not detected in any sample to which no H_2O_2 had been added. In Japanese noodles, recovery of 82.4–108% and coefficients of variation of 10.0–22.0% below H_2O_2 10 $\mu\text{g/g}$ and 6.35–6.79% above H_2O_2 25 $\mu\text{g/g}$ were obtained, indicating that the proposed method is adequate for H_2O_2 assay in Japanese noodles provided that the H_2O_2 content is rather high. On the other hand, recoveries

from animal food samples were lower, especially when the concentration of H_2O_2 added was below 10 $\mu\text{g/g}$, and the coefficients of variation were comparatively large. Though the recoveries, namely 0–78.2% for small bait, 0–104% for fish paste and 0–100% for herring roe, were more satisfactory than the results reported in the previous paper,¹⁾ it is clear that enzymes, oxidizing substances, reducing substances and others in animal food samples still influenced this assay. Removal of oxygen is currently being investigated in an attempt to improve the procedure.

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