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Spectrofluorimetric determination of both hydrogen peroxide and –O–O–H in polyethylene glycols (PEGs) using 2-hydroxy-1-naphthaldehyde thiosemicarbazon (HNT) as the substrate for horseradish peroxidase (HRP)

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Abstract

2-Hydroxy-1-naphthaldehyde thiosemicarbazon (HNT) had been synthesized and used as a new kind of substrate for horseradish peroxidase (HRP) in spectrofluorimetric determination of hydrogen peroxide (H_2O_2). The oxidation reaction of HNT with H_2O_2 under the catalysis of HRP was studied in detail. The possible reaction mechanism was discussed. Under optimum experimental conditions, the oxidized product of HNT had excitation and emission maxima at 260 and 450 nm, respectively. The linear range of this method was 1.30×10^{-9}–1.25×10^{-5} mol l^{-1} with a detection limit of 3.89×10^{-10} mol l^{-1}. The effect of interferences, surfactants and organic solvents on the determination of H_2O_2 had been investigated. A study to prove the existence of –O–O–H in PEGs was carried out. The proposed method was successfully applied to the determination of –O–O–H in polyethylene glycols.

Keywords: Hydrogen peroxide determination; HNT; HRP; Catalytic spectrofluorimetry; Polyethylene glycols

1. Introduction

In recent years, various methods for hydrogen peroxide (H_2O_2) determination have been proposed, including the use of enzymatic assays, which have been widely used in analytical biochemistry because of their rapidity and high selectivity. The horseradish peroxidase (HRP)-catalyzed reaction is one of the most widely used enzymatic reactions in bioanalytical chemistry [1–4].

The characteristics of the enzyme were systematically studied with H_2O_2 as oxidizing agent and various substances as fluorogenic substrates. Based on these catalytic reactions, various highly sensitive spectrofluorimetric methods for determination of H_2O_2 have been developed [5–14]. For example, homovanillic acid, hydroxyphenylacetic acid, tyrosine and tyramine have been used as the fluorescent substrates of HRP in H_2O_2 determination by Guilbault et al. [5,6]. Zaitsu et al. [7] found

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that a series of *p*-hydroxy-phenyl group compounds can be oxidized to fluorescent dimers by H_2O_2 under the catalysis of HRP. Ci et al. [9–13] had systematically studied the reactive characteristics of a series of substrates such as tyrosine and chavicol. They researched the reaction mechanism and proposed the best structure of substrate. Based on fluorescence quenching of scopoletin in the reaction with H_2O_2 catalyzed by HRP, the fluorimetric HRP-scopoletin method was chosen for H_2O_2 determination by Holm et al. [14].

2-Hydroxy-1-naphthaldehyde thiosemicarbazone (HNT) was used to manganese determination in coffee by Perez-Bendito et al. in 1984 [15], they found that HNT can be oxidized to form fluorescent by H_2O_2 under the catalysis of Mn^{2+}. Based on the reaction, 7.5–48 ng of Mn^{2+} was spectrofluorimetricly determined.

In our studies, we found that the oxidization of HNT with H_2O_2 could be catalyzed by HRP, the product of HRP-catalyzed reaction was same as that of Mn^{2+}-catalyzed reaction. Therefore, in this paper, HNT have been synthesized and used as a new kind of substrate for HRP in spectrofluorimetric determination of H_2O_2 with high sensitivity. The effects of surfactants and organic solvents were studied. The possible mechanism of the reaction was discussed.

Polyethylene glycols (PEGs) is a kind of widely used polymer in medicine manufacture, whose molecular formula is expressed as CH_3–(CH_2–CH_2–O–)$_n$–OH. Because of its special linear molecular structure, the hydroxyl group (–OH) in PEGs can be easily activated to form hydroperoxide (ROOH). Moreover, ether chains in PEGs having low degree of polymerization can be oxidized to form hydroperoxyl group (–O–O–H) in the air. The higher the content of –O–O–H in PEGs, the lower the degree of polymerization and pureness of PEGs. Therefore, the pureness and degree of polymerization of PEGs can be detected by determining the –O–O–H content in PEGs. In this work, a study to prove the existence of –O–O–H in PEGs was carried out. The proposed method was applied to the determination of –O–O–H in polyethylene glycols with satisfactory results.

2. Experimental

2.1. Apparatus

All fluorescence measurements were carried out on a Perkin–Elmer (Norwalk, CT, USA) LS-5 spectrofluorimeter, equipped with a xenon lamp, 1.0 cm quartz cells and a Perkin–Elmer model 561 recorder. All pH measurements were made with a pHS-3C digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. The elemental analysis was carried out on a Yanaco (Japan) model MF-3 elemental analyzer.

2.2. Reagents

All chemicals used were of analytical-reagent or higher grade. De-ionized water was used throughout. A 10 μg ml^{-1} stock solution of HRP was prepared by dissolving an appropriate amount of HRP (Shanghai Dongfeng Boichemical Technology, China) in distilled deionized water. H_2O_2 solution was prepared by dilution of a 30% solution with distilled deionized water (standardized by titration with potassium permanganate). The boric acid–sodium hydroxide (H_3BO_3–NaOH) buffer (0.2 mol l^{-1}, pH 11.5) was used. HNT solution (8.0×10^{-4} mol l^{-1}) was prepared by dissolving an appropriate amount of the reagent (synthesized following the method in the literature [16], mp 245 °C, anal. found (calc.): C, 58.82; (58.75), H, 4.58;(4.52)) in dimethylformamide (DMF). All polyethylene glycols solutions were prepared to 10% solutions with distilled deionized water.

2.3. Determination of H_2O_2

In a 10 ml color comparison tube were placed 2.0 ml of buffer solution (pH 11.5), 0.4 ml of 8.0×10^{-4} mol l^{-1} HNT solution, 0.5 ml of 10 μg ml^{-1} HRP solution and different amount of H_2O_2 standard solutions. After dilution to volume with deionized water, the mixture was equilibrated at room temperature for 10 min, then the fluorescence intensity of the solution was measured at 450 nm with excitation at 260 nm against a reagent blank. A standard addition method was used to

determine amount of –O–O–H in polyethylene glycols.

3. Results and discussion

3.1. Excitation and emission spectra

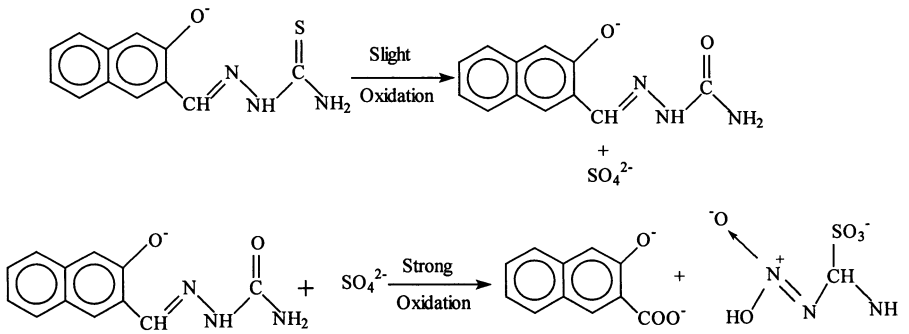
In order to determine the optimum working wavelength, the spectral characteristics of the oxidized product of HNT by H_2O_2 were studied at various pH values. The corrected excitation and emission spectra (Fig. 1) showed that the wavelengths of maximum excitation and emission of the oxidized product of HNT at pH 11.5 were 260 and 450 nm, respectively.

The concentrations of HNT, HRP and H_2O_2 were 3.2×10^{-5} mol l^{-1}, 0.5 μg ml^{-1} and 3.8×10^{-6} mol l^{-1}, respectively, in 0.2 mol l^{-1} H_3BO_3–NaOH buffer (pH 11.5). Peaks: 1 and 1′, HNT; 2 and 2′, oxidized product of HNT.

3.2. Preliminary discussion of reaction mechanism

The reaction mechanism of H_2O_2 with 2-hydroxy-1-phenylaldehyde thiosemicarbazone (HBTS) have been studied by Morene et al. [17]. They considered that the C=N bond of HBTS was broken off to form a cyclic fluorescent product in the oxidation reaction with H_2O_2.

In the experiments, we studied the spectral characteristics of HNT and its oxidized product by H_2O_2. The results (Fig. 1) showed that the fluorescence response ($\lambda_{ex}/\lambda_{em}$) of HNT and its oxidized product were 410/475 and 260/450 nm, respectively. So we inferred that the C=N bond of HNT was broken off in the HRP-catalyzed reaction with H_2O_2 as oxidizing agent, just as that of HBTS having similar structure to HNT. The



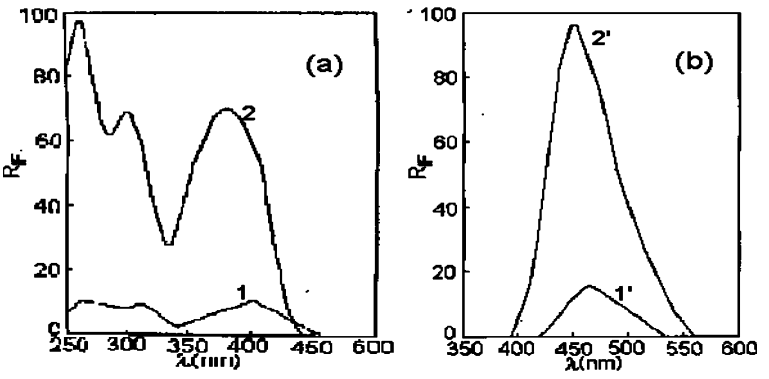
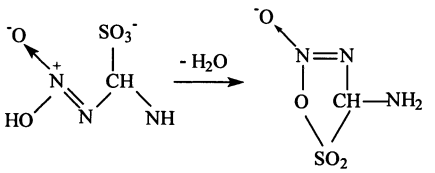


Fig. 1. Excitation (a) and emission (b) spectra of HNT and its oxidized product by H_2O_2.

possible mechanism of the reaction was as follows:



3.3. Optimization of experimental variables

The pH of the medium had an important effect on the fluorescence intensity of the reaction system. The experimental results showed that the optimum pH range was between 11.0 and 12.0. Therefore, a pH of 11.5 was fixed with the use of H_3BO_3–NaOH buffer.

As the volume of the buffer added (from 1.0 to 3.0 ml) had little effect on the fluorescence intensity, 2.0 ml was used in subsequent experiments.

The influence of HNT and HRP concentrations were studied. The results showed that the optimum concentrations of HNT and HRP were 3.2×10^{-5} mol l^{-1} and 0.5 μg ml^{-1}, respectively.

The effect of reaction time was discussed. The results showed that the oxidation reaction was completed within 7–10 min at room temperature; the fluorescence intensity reached a maximum 10 min after the regents had been added and remained constant for at least 1 h. Hence, after all the oxidation reactions were carried out for 10 min, the subsequent fluorescence measurements were made at room temperature within 1 h.

3.4. Effect of interferences

A systematic study of the interferences by foreign substances in determination of H_2O_2 (3.8×10^{-6} mol l^{-1}) was carried out. The result in Table 1 showed that the anions had little effect on the fluorescence intensity, but most of the cations had quenching effect on reaction system.

3.5. Analytical characteristics

Under the optimum experimental conditions, there was a linear relationship between the fluorescence intensity and H_2O_2 concentration in the range of 1.30×10^{-9}–1.25×10^{-5} mol l^{-1} with a correlation coefficient of 0.9983. The regression equation was $R_{IF} = 2.16 \times 10^7$ C (mol l^{-1}) − 0.169. The R.S.D. was 2.3% obtained from a series of 12 standards, each containing 3.8×10^{-6} mol l^{-1} H_2O_2. The standard deviation of the fluorescence measurements was 0.0028 obtained from a series of 11 blank solutions. The limits of detection ($k = 3$) and of determination ($k = 10$) of the method were established according to the IUPAC definitions ($c_1 = kS_0/s$, where c_1 is the limit of detection, S_0 is the standard error of blank determination, s is the slope of the standard curve and k the constant related to the confidence interval) [18] and the values found were 3.89×10^{-10} and 1.30×10^{-9} mol l^{-1}, respectively. In Table 2, characteristics of the method are compared with those of similar published fluorescence methods for H_2O_2 determination, which showed that the proposed method offered higher sensitivity than other methods.

Table 1
Effect of interferences on the determination of H_2O_2 (3.8×10^{-6} mol l^{-1})

Interferent	Additions (μg)	Relative error (%)
SO_4^{2-}	500	−7
Ca^{2+}	0.2	−5
I^-	100	−6
Fe^{3+}	3	−6
F^-	12	−5
V_{B6}	15	+6
V_c	5	+5
V_{B2}	1	+5
Phenylalanine	3	+5
Tyrosine	2	+4
NO_3^-	500	0
Zn^{2+}	1	−3
Mn^{2+}	1	+4
Cu^{2+}	2	0
Cl^-	1000	−2
Tryptophane	10	+5
CO_3^{2-}	200	−3
V_{B1}	4	−2
Sucrose	1000	+5
Glucose	900	+5

3.6. Effect of surfactants

A study of the effect of surfactants on reaction system was carried out. The results are given in

Table 2
Comparison of the characteristics of the proposed method with those published previously

Substrate	λ_{ex} (nm)	λ_{em} (nm)	Sensitivity (nmol l^{-1})	Reference
Homovanillic acid	315	425	0.01	[5]
p-Hydroxyphenylacetic acid	320	400	20	[8]
Tyrosine	316	412	169	[11]
p-Hydroxyphenylpropionic acid	325	430	0.71	[7]
Chavico	312	410	0.527	[12]
HNT	260	450	0.389	This work

Table 3
Effect of surfactants on the determination of H_2O_2 (3.8×10^{-6} mol l^{-1})

Surfactant[a]	Concentration (%)	R_{IF}	F (%)
–	–	97.8	0
Tween-80	0.1	24.0	−75.7
CTMAB	0.1	77.4	−20.9
PVA	0.5	45.8	−53.2
Triton X-100	0.1	51.4	−47.4
SDS	0.1	44.6	−54.4
β-CD	2.0×10^{-3}	58.8	−39.9

[a] CTMAB, bromocetyltrimethyl amine; SDS, sodium dodecyl sulfate; PVA, polyvinyl alcohol; β-CD, β-cyclodextrin.

Table 3. At first, the effective factor (F) was defined as follows: $F = [I_{RF} - (I_{RF})_0]/(I_{RF})_0$, where $(I_{RF})_0$ is the relative fluorescence intensity of the reaction system when surfactant had not been added. When $F > 0$, the surfactant has sensibilization effect on the system. When $F < 0$, that means the surfactant has an restraint effect. Therefore, according to the values of F in Table 3, we found that almost all of the surfactants added have restraint effect on the system. The possible reason is that HNT and its oxidized product can not be solubilized in or on the micelle of surfactants because they are insoluble in water [19].

3.7. Influence of organic solvents

The influence of organic solvents was discussed. After adding organic solvents to the reaction system, the reaction regents and products were solubilized in organic solvent, which could strengthen the fluorescence intensity of system. At the same time, HRP could be denatured in organic solvents so that part of HRP had been activeless [20], which caused the fluorescence quenching of the system. As a result, all of the absolute values of F were not very large, which indicated that the general effect of organic solvents on the fluorescence intensity of system was not very noticeable.

3.8. Existence of –O–O–H in PEGs

In order to prove the existence of –O–O–H in PEGs, a series of study was carried out as follows.

3.8.1. Oxidizing property of PEGs

Tyrosine (Tyr) could be oxidized to fluorescent dimer by H_2O_2 under the catalysis of HRP. Under

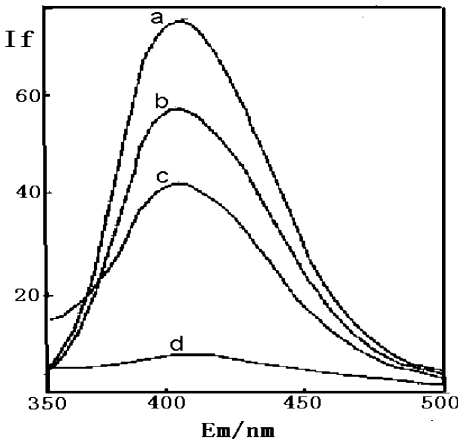


Fig. 2. Emission spectra of Tyr after oxidation by H_2O_2 (a) and PEGs (b), catalyzed by HRP. Tyr, 0.04 mg ml^{-1}; HRP, 0.1 μg ml^{-1}; H_2O_2, 3×10^{-4} mol l^{-1}; $PEGs_{400}$, 15%; $PEGs_{600}$, 15%. (a) HRP+Tyr+H_2O_2; (b) HRP+Tyr+$PEGs_{400}$; (c) HRP+Tyr+$PEGs_{600}$; (d) regent blank.

the same experimental conditions, –O–O–H in PEGs also oxidized Tyr to fluorescence dimer. The emission spectra of Tyr after oxidation by H_2O_2 and by –O–O–H in PEGs were given in Fig. 2(a) and (b), respectively. The result in Fig. 2 showed that the oxidized product of Tyr by H_2O_2 had a maximum emission at 408 nm, whose emission spectra was similar to that of the oxidized product by –O–O–H in PEGs, which indicated that the oxidized product by H_2O_2 was same as that by –O–O–H in PEGs. So we inferred that the mechanism of the catalytic oxidation of Tyr by H_2O_2 was same as that by –O–O–H. Thus, –O–O–H in PEGs had oxidizing property.

3.8.2. Reaction of KI with PEGs

H_2O_2 could oxidize KI to I_2, which had two absorption peaks at 288 and 353 nm (Fig. 3(a)). Under the same experimental conditions, after KI was treated with $PEGs_{400}$, the position of the two peaks of I_2 did not shift (Fig. 3(b)), which indicated that the reaction product and mechanism of KI with H_2O_2 was same as those of KI with PEGs.

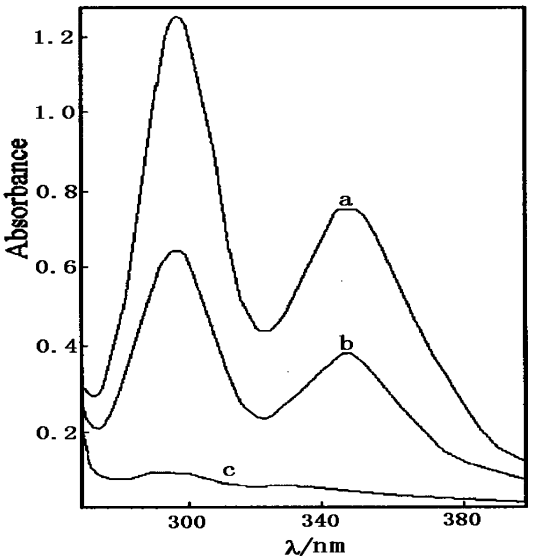


Fig. 3. Absorbance spectra of I_2 generated by the reaction of KI with H_2O_2 (a) and PEGs (b). $PEGs_{400}$, 15%; KI, 1.125×10^{-3} mol l^{-1}; H_2O_2, 3×10^{-4}%. (a) KI+H_2O_2 (b) KI+$PEGs_{400}$ (c) regent blank.

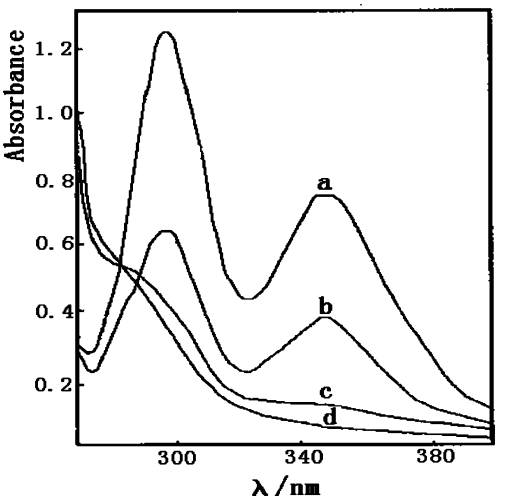


Fig. 4. Absorbance spectra of I_2 generated by the reaction of KI with PEGs, in which –O–O–H was destroyed. $PEGs_{400}$, 15%; KI, 1.125×10^{-3} mol l^{-1}; heating time: (a) 0 min; (b) 10 min; (c) 20 min; (d) 40 min.

The solution of $PEGs_{400}$ was deaerated with pure nitrogen when it was heated to boiling for 40 min in order to decompose the –O–O–H in PEGs. After cooling to room temperature, the solution was treated with KI, then the absorbance spectrum was detected. The results (Fig. 4) showed that the absorption peaks of I_2 disappeared after –O–O–H in PEGs was destroyed.

Liquid PEGs having low degree of polymerization (degree of polymerization < 400) can easily be oxidized to form –O–O–H in the air, but solid PEGs (degree of polymerization > 600) can not be easily to. So the solid PEGs was solved in water, after passing pure oxygen through the solution for different times, the solution was reacted with KI. The result showed that the height of peaks of I_2 increased with the increasing of oxygen supplied to the solution, which indicated that the content of –O–O–H in PEGs was increased after pure oxygen was supplied.

3.8.3. Thin-layer chromatographic spectrum analysis of PEGs

The silicic acid H chromatographic plate was used. The development agent was the mixture solution of acetoacetate, acetic acid and water (70:16:15). The saturated solution of KI was used

Table 4
Determination of –O–O–H in polyethylene glycols ($P = 0.95$, $n = 6$)

Sample	H_2O_2 added (μmol l^{-1})	Mean found (μmol l^{-1})	Recovery value (μmol l^{-1})	Mean recovery (%)
$PEGs_{400}$	0	3.71 ± 0.01		
	2.00	5.80 ± 0.03	2.09 ± 0.02	105.0
	4.00	7.65 ± 0.04	3.94 ± 0.03	99.0
$PEGs_{600}$	0	2.59 ± 0.01		
	2.00	4.55 ± 0.03	1.96 ± 0.02	98.0
	4.00	6.66 ± 0.02	4.07 ± 0.01	102.0
$PEGs_{800}$	0	1.76 ± 0.01		
	2.00	3.70 ± 0.02	1.94 ± 0.01	97.0
	4.00	5.84 ± 0.03	4.08 ± 0.02	102.0

as color-developing agent. The result showed that there was only one color-developing point ($R_f = 0.312$), which indicated that there was no impurity having oxidizing property except for –O–O–H in PEGs.

3.9. Sample analysis

In the experiment, we found that –O–O–H in PEGs having different degree of polymerization can oxidize HNT under the catalysis of HRP. The oxidized product of HNT by PEGs was same as that by H_2O_2. The working solutions of $PEGs_{400}$, $PEGs_{600}$, $PEGs_{800}$ were prepared by diluting to 500, 200, 250 times of 10% stock solutions of $PEGs_{400}$, $PEGs_{600}$, $PEGs_{800}$, respectively. To 1.00 ml of the working solution of PEGs were added various amounts of standard H_2O_2 solutions, then the –O–O–H amount of PEGs was determined spectrofluorimetrically by the proposed method for the determination of H_2O_2. The standard additions method [21] was used in the sample analysis procedure. From Table 4, it can be seen that the results obtained were satisfactory.

4. Conclusions

In this work, HNT has been successfully used as the new kind of substrate for HRP in highly sensitive spectroflourimetric determination of H_2O_2. Compared with other substrates used in the published methods [5–14], HNT offers higher sensitivity. (Table 2) Moreover, a study to prove the existence of –O–O–H in PEGs was carried out in this work. The proposed method was applied to the determination of –O–O–H in polyethylene glycols with satisfactory results.

Acknowledgements

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