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Determination of the Hydroperoxil Group Level in Polyethylene Glycols Using a Novel Mimic Enzyme Fe(III)-Mn(II)(HNAPTS)$_2$

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Two kinds of Schiff base metal complexes of 2-hydroxy-1-naphthaldehyde-2-amino-5-phenylthiazole (HANPTS), Mn(II)(HNAPTS)$_2$ and Fe(III)(HNAPTS)$_2$, were synthesized and used to mimic the active group of horseradish peroxidase (HRP). The catalytic characteristics of the mimic enzymes in the oxidation reaction of ascorbic acid (AsA) with the OOH group in polyethylene glycols (PEGs) have been studied by a spectrophotometric method. Fe(III) has remarkable coordinated catalysis to Mn(II)(HNAPTS)$_2$; as a result, the catalytic ability of Fe(III)-Mn(II)(HNAPTS)$_2$ is 75% of that of HRP. The possible mechanism of the reaction was discussed. The linear relationship between $\Delta A^{265}{}_{AsA}$ and OOH group concentrations was in the range of 1.5×10^{-6} to 9.0×10^{-4} mol/L. The proposed method was successfully applied to the determination of the OOH group level in different molecular weight PEGs.

KEYWORDS: Mimic enzyme; spectrophotometry; determination of the OOH group level in polyethylene glycols; Fe(III)-Mn(II)(HNAPTS)$_2$

1. INTRODUCTION

Enzymes such as HRP have been widely used in analytical biochemistry because of their considerable advantages of rapidity and high selectivity in catalytic reactions (*1*), but many enzymes are expensive and their solutions quite unstable. In recent years, studies on the mimic enzyme have become an important branch of enzyme analysis (*2*, *3*).

Polyethylene glycols [PEGs, $CH_3(CH_2CH_2O)_nOH$] are widely used in protein medicine, cosmetics, etc. (*4*), especially as a cleanser and antioxidant in food because of their good water solubility and low level of volatility and because they are flavorless and nontoxic. In agriculture chemistry, PEGs are used as plant growth regulators and as an inhibitory coating for keeping fruits and vegetables fresh. Under normal conditions, PEGs should present no redox reaction, but the hydroxyl group (OH) in it can be easily oxidized to form an OOH group which is similar to methyl peroxide and ethyl peroxide. On the other hand, the OOH group can be reduced to •OH by the catalysis of metal ions in the human body. Some diseases such as hypertension, coronary disease, and senile diseases are due to the abundance of •OH which accelerates the senility of the body. So the analysis of OOH in PEGs used in food has become an important issue. According to this, the reaction system that includes OOH as an oxidizer and horseradish peroxidase (HRP) as a catalyst has been proposed (*5*). But HRP cannot be stored for a long time as common chemical reagents and its solutions are quite unstable. Furthermore, the sensitivity of the substrate that is catalyzed by HRP is low. As common ligands, Schiff bases are easy to synthesize and dissolve and are widely used in metal ion analysis (*6*); to this point, we know their metal complexes are seldom used in mimic enzymes.

In this paper, we synthesize two kinds of Schiff base metal complexes, Mn(II)(HNAPTS)$_2$ and Fe(III)(HNAPTS)$_2$. They were used as catalysts to determine the hydroperoxil group (OOH) levels in polyethylene glycols (PEGs) by a spectrophotometric method based on the oxidation reaction of ascorbic acid (AsA) with the OOH group in PEGs. It was found that they both have catalytic abilities similar to that of horseradish peroxidase (HRP). Also, Fe(III) has remarkable coordinated catalysis to Mn(II)(HNAPTS)$_2$; as a result, Fe(III)-Mn(II)(HNAPTS)$_2$ has a novel catalytic ability equal to 75% of that of HRP.

AsA was oxidized by H_2O_2 to form CO_2, L-threonic acid, and its γ-lactone in the reaction system of AsA and H_2O_2 (*7*). The absorbance of AsA at 265 nm ($A^{265}{}_{AsA}$) decreased as the reaction proceeded because of the conversion of AsA, and there was no absorbance of the products at 265 nm. A linear relationship between $\Delta A^{265}{}_{AsA}$ and ΔC_{AsA} developed. The molar ratio of AsA to H_2O_2 was 1:1 in the reaction, so there was also a linear relationship between $\Delta A^{265}{}_{AsA}$ and $\Delta C_{H_2O_2}$. When the reaction was complete with a excess of AsA, we obtained a linear relationship between $\Delta A^{265}{}_{AsA}$ and $\Delta C_{H_2O_2}$.

In this paper, we used an H_2O_2 solution as a standard solution to obtain a linear relationship between $\Delta A^{265}{}_{AsA}$ and $\Delta C_{H_2O_2}$, which was assumed to be the same with the OOH group in

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PEGs, because H_2O_2 and the OOH group had similar oxidizing properties and equal molar relations in the oxidization reaction of AsA. The proposed method was applied successfully to determine the OOH group level in different molecular weight PEGs.

2. EXPERIMENTAL PROCEDURES

2.1. Apparatus. All fluorescence measurements were carried out on a LS-5 spectrofluorimeter (Perkin-Elmer Co.) equipped with a xenon lamp, 1.0 cm quartz cells, and a Perkin-Elmer model 561 recorder. All absorbance measurements were obtained on a UV-265 spectrophotometer equipped with 1.0 cm quartz cells (Shimadzu). All pH measurements were made with a pHS-3C digital pH-meter (Shanghai Leici Device Works, Shanghai, China) with a combined glass−calomel electrode.

2.2. Reagents. All chemicals were analytical reagent grade or better. Deionized water was used for the preparation of all solutions.

The Fe^{3+} standard solution (5.0×10^{-6} mol/L), a KI solution (2.25×10^{-2} mol/L), a tyrosine solution (0.80 mg/mL), and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (0.20 mol/L) were used. An ascorbic acid (AsA) stock solution (2.0×10^{-2} mol/L) was prepared weekly and kept in a refrigerator, and the working solution (2.0×10^{-4} mol/L) was prepared daily by diluting the stock solution. A hydrogen peroxide (H_2O_2) solution (w/w, 0.003%) was prepared by diluting the 30% (w/w) H_2O_2 solution with deionized water (standardized by titration with potassium permanganate).

PEG_{400}, PEG_{600}, and PEG_{800} were all in solution in water (w/w, 50%). HRP (10 μg/mL) was purchased from Sino-American Biotechnology Co. A silic acid-H thin-layer chromatography board was purchased from Qingdao Haiyang Local Chemical Plant.

Fe(III)$(HNAPTS)_2$ and Mn(II)$(HNAPTS)_2$ were synthesized by the following method.

2.2.1. Synthesis of HNAPTS. 2-Hydroxy-1-naphthaldehyde (0.69 g, 0.004 mol) was added to 10.0 mL of alcohol (95%) in a 100 mL three-neck flask. 2-Amino-5-phenylthiazole (0.71 g, 0.004 mol) dissolved in 15.0 mL of alcohol was dropped slowly into the flask with stirring at room temperature. The mixture was refluxed for 2 h at 80 °C in a water bath; then the excess alcohol was removed, and the mixture was cooled and filtered. The rough product was recrystallized twice with alcohol (95%). Orange needles (40% yield) were obtained: mp 175−176 °C; IR ν 2800−3150 (O−H···N), 1625 (C═N), 1320 (C−N), 1225 cm^{-1} (Ar−O). Anal. Found (calcd) for $C_{20}H_{14}N_2OS$: C, 72.81 (72.73); H, 4.30 (4.24); N, 8.73 (8.60).

2.2.2. Synthesis of Mn(II)(HNAPTS)$_2$. A $Mn(Ac)_2$ solution (0.35 g, 0.002 mol) of alcohol (95%) was dropped slowly into 1.32 g (0.004 mol) of HNAPTS dissolved in alcohol with stirring and heating. The mixture was refluxed at 80 °C in a water bath for 4 h, filtered, washed sequentially with alcohol and water several times, and vacuum-dried. A brown powder (61% yield) was obtained: mp >330 °C; IR ν 3050 (Ar−H), 1620 (C═N), 1370 (C−N), 1305 cm^{-1} (Ar−O). Anal. Found (calcd): C, 67.09 (67.03); H, 4.11 (3.93); N, 7.85 (7.82); Mn, 7.80 (7.66) (determined by titration with EDTA).

Fe(III)$(HNAPTS)_2$ was prepared in the same way: mp >330 °C; IR ν 3051 (Ar−H), 1623 (C═N), 1371 (C−N), 1307 cm^{-1} (Ar−O). Anal. Found (calcd): C, 67.01 (66.94); H, 3.97 (3.93); N, 7.84 (7.81); Fe, 7.82 (7.78) (determined by titration with EDTA). The stoichiometries of the two complexes were studied via a molar ratio and continuous variation method. The two methods both showed that the compositions of the two complexes [Fe(III)HNAPTS and Mn(II)-HNAPTS] are in a 1:2 ratio. The proposed structures of Mn(II)-$(HNAPTS)_2$ (a) and Fe(III)$(HNAPTS)_2$ (b) are shown in **Chart 1**.

2.3. Determination of the Oxidizing Property of the OOH Group in PEGs Compared with That of H_2O_2. HRP could catalyze the oxidant reaction between tyrosine and H_2O_2, producing dimmer fluorescence of tyrosine with excitation and emission at 320 and 408 nm, respectively. Under the same experimental conditions, the oxidant reaction between tyrosine and the OOH group also could be catalyzed by HRP, producing dimmer fluorescence of tyrosine at the same peak place (**Figure 1**). In addition, all kinds of PEGs can function with a KI solution because of the presence of the OOH group, producing I_2

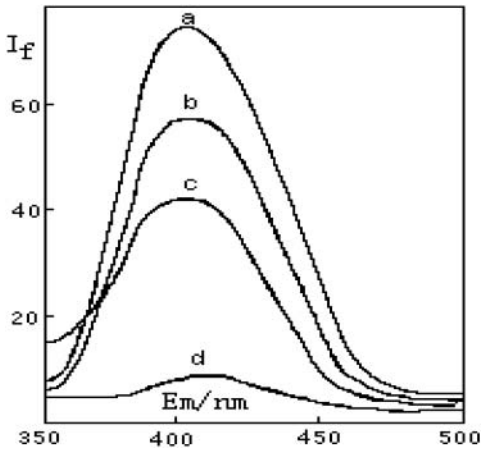


Figure 1. Emission spectra of Tyr after oxidation by H_2O_2 and PEGs: (a) HRP, Tyr, and H_2O_2, (b) HRP, Tyr, and PEG_{400}, (c) HRP, Tyr, and PEG_{600}, and (d) blank solution (HRP and Tyr). [HRP] = 0.1 μg/mL. $[H_2O_2] = 3 \times 10^{-4}$%. [Tyr] = 0.8 mg/mL. $[PEG_{400}]$ = 15%. $[PEG_{600}]$ = 15%.

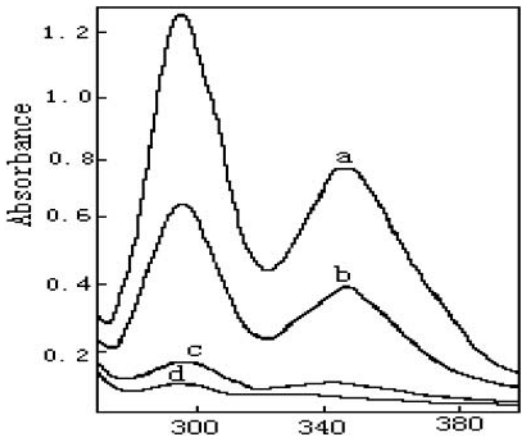
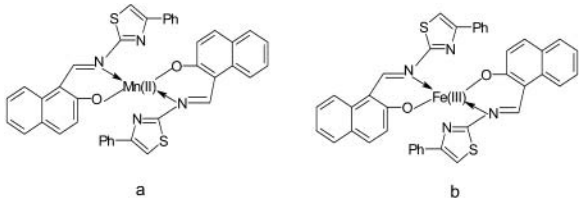


Figure 2. Absorbance spectra of KI after oxidation by H_2O_2 and PEGs: (a) KI and H_2O_2, (b) KI and PEG_{400}, (c) KI and PEG_{600}, and (d) blank solution. $[PEG_{400}]$ = 15%. $[PEG_{600}]$ = 15%. [KI] = 1.125×10^{-3} mol/L. $[H_2O_2] = 3 \times 10^{-4}$%.

Chart 1. Structures of Mn(II)$(HNAPTS)_2$ (a) and Fe(III)$(HNAPTS)_2$ (b)



a b

absorption peaks at 288 and 353 nm. This absorption peak was the same as that for H_2O_2 and KI (**Figure 2**). On the basis of these results, we proposed that the OOH group in PEGs has an oxidizing property similar to that of H_2O_2.

2.4. Procedure. In a 10 mL colorimetric tube, 2.0 mL of Tris-HCl buffer (pH 7.40), 0.20 mL of the Fe^{3+} (5.0×10^{-5} mol/L) standard solution, 0.20 mL of a Mn(II)$(HNAPTS)_2$ (5.0×10^{-4} mol/L) solution, an appropriate amount of 50% (w/w) PEGs, and 0.60 mL of AsA (2.0×10^{-4} mol/L) were added sequentially. The mixture was diluted to the mark with deionized water. After being shaken thoroughly, the solution was put into a 1.0 cm quartz cell, and the absorbance was measured on a spectrophotometer at 265 nm. The initial absorbance (A_i) of AsA at 265 nm was recorded. The absorbance was then recorded at various time intervals for the kinetic calculation. The final absorbance (A_f) of AsA was recorded after 10 min. The absorbance difference was defined by the relation $\Delta A^{265}_{AsA} = A_i - A_f$.

Table 1. Comparison of the Catalytic Activities of Fe(III)$(HNAPTS)_2$, Mn(II)$(HNAPTS)_2$, Fe(III)-Mn(II)$(HNAPTS)_2$, and HRP

	Fe(III)$(HNAPTS)_2$	Mn(II)$(HNAPTS)_2$	Fe(III)-Mn(II)$(HNAPTS)_2$	HRP
C_o (mol/L)	1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}
V_{max}(mol L^{-1} s^{-1})	1.02×10^{-9}	6.68×10^{-9}	2.48×10^{-8}	2.96×10^{-8}
K_{cat} ($\times 10^6$ μmol/s)	1.67×10^{-3}	7.33×10^{-3}	2.60×10^{-2}	3.47×10^{-2}
relative activity (%)	5	21	75	100

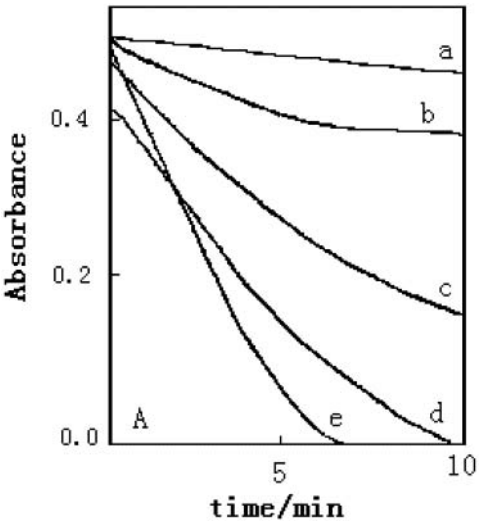


Figure 3. Effect of different catalysts on the ΔA^{265}_{AsA} of the reaction of AsA with H_2O_2: (a) AsA, H_2O_2, and Fe(III)$(HNAPTS)_2$, (b) AsA, H_2O_2, and Mn(II)$(HNAPTS)_2$, (c) AsA, H_2O_2, Mn(II)$(HNAPTS)_2$, and Fe^{3+}, (d) Fe^{3+}, Mn(II)$(HNAPTS)_2$, AsA, and H_2O_2, and (e) HRP, AsA, and H_2O_2. [HRP] = 0.1 μg/mL. [AsA] = 1.2×10^{-5} mol/L. [H_2O_2] = 3×10^{-4}%. Tris-HCl at pH 7.40. [Fe(III)$(HNAPTS)_2$] = 1.0×10^{-6} mol/L. [Mn(II)$(HNAPTS)_2$] = 1.0×10^{-6} mol/L. [Fe^{3+}] = 1.0×10^{-7} mol/L.

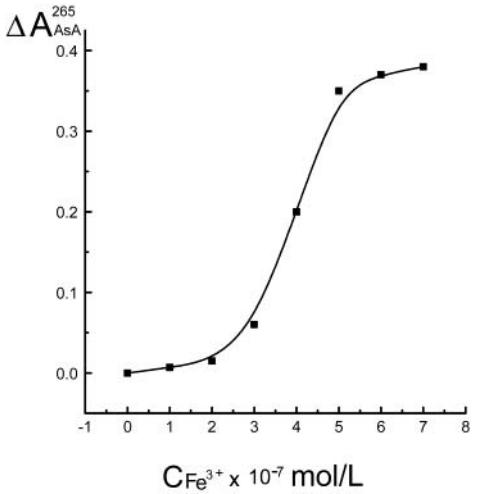


Figure 4. Effect of Fe^{3+} concentration on the ΔA^{265}_{AsA} of the reaction of AsA with H_2O_2. [AsA] = 1.2×10^{-5} mol/L. [H_2O_2] = 3×10^{-4}%. [Mn(II)$(HNAPTS)_2$] = 1.0×10^{-6} mol/L. [Fe^{3+}] = 1.0×10^{-7} mol/L. Tris-HCl at pH 7.40.

3. RESULTS AND DISCUSSION

3.1. Catalytic Mechanism of Fe(III) with Mn(II)(HNAPTS)$_2$. Both Mn(II)$(HNAPTS)_2$ and Fe(III)$(HNAPTS)_2$ could catalyze the reaction between the OOH group and AsA, but their catalytic abilities were unapparent when they were used individually (**Figure 3a,b**). When Fe^{3+} was present, the catalytic ability of Mn(II)$(HNAPTS)_2$ could improve remarkably if the optimal sequence of reagent added was chosen to be Fe^{3+}, Mn(II)$(HNAPTS)_2$, AsA, and H_2O_2 (**Figure 3c,d**). It was suggested that Fe^{3+} has remarkable coordinated catalysis to Mn(II)$(HNAPTS)_2$ (**Figure 4**), but the detailed mechanism of this catalytic reaction is not clear to us at present.

3.2. Comparison of the Catalytic Activities of Fe(III)(HNAPTS)$_2$, Mn(II)(HNAPTS)$_2$, Fe(III)-Mn(II)(HNAPTS)$_2$, and HRP. The kinetics of the enzymatic reaction have been studied with the Michaelis equation. The maximum rate V_{max} was obtained from Lineweaver−Burk plots (*8*) ($1/v$ vs [S], **Table 1**). The transformation constant K_{cat} was obtained from the relation $K_{cat} = \Delta C_{AsA} \times 10^6/t \times C_o$, where C_o is the initial concentration of the enzyme and K_{cat} represents the catalytic activities (*9*). The results showed that the catalytic activity of the mimic enzyme Fe(III)-Mn(II)$(HNAPTS)_2$ is similar to that of HRP.

3.3. Optimization of Experimental Variables. The experimental variables were optimized by applying the univariate method. The optimum pH of the system was 7.40. The optimum concentrations of Mn(II)$(HNAPTS)_2$, AsA, and Fe^{3+} were 1.0×10^{-5}, 1.2×10^{-5}, and 1.0×10^{-7} mol/L, respectively.

3.4. Interference of Inorganic Ions and Organic Compounds. To examine the possible interference, 20 kinds of inorganic ions and organic compounds were studied. When 3.0 mL of 50% (w/w) PEG_{400} was added to a 10 mL colorimetric column and the tolerable error was less than ±5%, the permitted concentrations of various constituents were 200× for Ca^{2+}, Mg^{2+}, Be^{2+}, Mn^{2+}, Cl^-, SO_4^{2-}, NO_3^-, and F^-, 100× for Cu^{2+}, Ni^{2+}, Zn^{2+}, alcohol, glycol, and glycerol, 50× for V^{5+} and Mo^{6+}, 15× for Al^{3+} and Ga^{3+}, and 10× for Co^{2+}.

Table 2. Determination of the OOH Group Content in Different Molecular Weight PEGs ($P = 0.95$)

sample	H_2O_2 added ($\times 10^{-4}$ mol/L)	found[a] ($\times 10^{-4}$ mol/L)	recovery (%)
PEG_{400}	0	1.85 ± 0.02	
	1.00	2.85 ± 0.03	101
	2.00	3.81 ± 0.02	98
PEG_{600}	0	1.30 ± 0.01	
	1.00	2.29 ± 0.04	99
	2.00	3.34 ± 0.02	102
PEG_{800}	0	0.90 ± 0.01	
	1.00	1.88 ± 0.02	98
	2.00	2.94 ± 0.02	102

[a] Each sample was analyzed seven times.

3.5. Analytical Characteristics. Because the OOH group and H_2O_2 have equal molar ratios and similar oxidizing properties in the reaction system, we used an H_2O_2 solution as the standard solution instead of the OOH group to obtain a linear relationship between ΔA^{265}_{AsA} and H_2O_2 concentrations in the range of 1.5×10^{-6} to 9.0×10^{-4} mol/L. The linear regression equation was $\Delta A^{265}_{AsA} = 1.98 \times 10^3 C_{H_2O_2}$ (moles per liter) − 0.030 with a correlation coefficient (r) of 0.9978, which was assumed to be the same as that with the OOH group in PEGs. The RSD was 0.20%, which was obtained from a series of nine standards each containing 1.0×10^{-5} mol/L H_2O_2. The standard deviation was 0.000297, wihch was obtained from a series of 13 blank solutions. The limits of detection ($k = 3$) and of determination

($k = 10$) of the method were established according to the IUPAC definitions ($C_1 = KS_o/S$, where C_1 is the limit of detection, S_o the slope of the standard curve, and K the constant related to the confidence interval) (*10*), and the values that were found were 4.50×10^{-7} and 1.50×10^{-6} mol/L, respectively.

3.6. Application to PEGs. The samples of PEG_{400}, PEG_{600}, and PEG_{800} were diluted to 100×, 40×, and 50×, respectively. Then the OOH group content was determined, and the results, including the recovery experiment values, are shown in **Table 2**.

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