J Fluoresc (2009) 19:487–493
DOI 10.1007/s10895-008-0437-8

ORIGINAL PAPER

Spectroscopic Study of 2-(2-pyridyliminomethyl)phenol as a Novel Fluorescent Probe for Superoxide Anion Radicals and Superoxide Dismutase Activity

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Received: 7 July 2008 / Accepted: 27 October 2008 / Published online: 11 November 2008

Abstract A novel spectrofluorometric method, using 2-(2-pyridyliminomethyl)phenol as a fluorescent probe, was developed for the determination of superoxide anion radical ($O_2^{\bullet -}$) and superoxide dismutase activity (SOD). The new fluorescent probe was synthesized and characterized with elemental analysis and IR spectra. It was oxidized by $O_2^{\bullet -}$ to form a less fluorescence product. Based on this reaction, a spectrofluorometric method was proposed and successfully used to determine superoxide anion radicals and SOD activity. The effects of interferences were studied. The reaction was simple, precise and sensitive. It was applied to determine SOD activity in garlic, papaya and spinach successfully.

Keywords 2-(2-Pyridyliminomethyl)phenol · Superoxide anion radical · Superoxide dismutase · Spectrofluorometric method · Fluorescent probe

Introduction

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$) and hydrogen peroxide (H_2O_2), are various forms of activated oxygen. These molecules exacerbate factors in cellular injury and the aging process [1, 2]. Overproduction of ROS and their derivatives occurs in a number of diseases [3]. Superoxide anion radicals are involved in the onset of various diseases such as cancer, cardiovascular diseases, immune system decline and diabetes. So it is important to eliminate $O_2^{\bullet -}$ to prevent diseases and senescence. In recent years, detecting and scavenging of superoxide anion radicals have gained great attentions [4–7]. It is well known that SOD is the scavenger of $O_2^{\bullet -}$ and an important indicator of the amount of $O_2^{\bullet -}$ in organisms. SOD catalyzes $O_2^{\bullet -}$ to O_2 and H_2O_2, a less reactive ROS [8]. So establishing a precise, rapid, and sensitive method to determine SOD activity has important significance. Up to now, the methods commonly used to determine SOD activity are nitro blue tetrazolium method (NBT) [9, 10], chemiluminescence (CL) [11], pyrogallol auto-oxidation assay method [12]. At present, there are few reports on detecting free radicals with Schiff base by spectrofluorometric method, which is superior to the others [6]. So in this study, we synthesized 2-(2-pyridyliminomethyl) phenol as a novel fluorescent probe, it could be oxidized by $O_2^{\bullet -}$ generated in the pyrogallol auto-oxidation system. The product has less fluorescence. Based on this study, a fluorescence quenching method was developed to determine $O_2^{\bullet -}$ indirectly and SOD activity. The fluorescent quenching intensity was linear with SOD activity. The method was simple, precise, and sensitive in detection of SOD activity in garlic, papaya and spinach samples.

Experimental

Apparatus and reagents

All fluorescence measurements were carried out on a RF-5301PC Spectrofluorometer (Shimadzu, Japan), equipped

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with a xenon lamp and 1.0 cm quartz cells. All pH measurements were made with a pH-3CT digital pH meter (Shanghai Da Zhong Device Works, China) with a combined glass–calomel electrode. Elemental analysis was performed on a Vario EL III elementary analytical meter (Elementar, Germany). The IR spectra were recorded on Nicolet 380 IR spectrometer (Thermo, America). Melting point was measured with WRS-1A Digital Melting Point Apparatus (Shanghai Precision Scientific Instruments Corporation, China). ^{1}HNMR were run on Bruker AV-400 (Switzerland). GL-20G-II centrifuge was employed to obtain the SOD extract (Shanghai An ting Scientific Instruments Corporation, China).

Salicylaldehyde was purchased from Aladdin. 2-Aminopyridine was purchased from Shanghai Chemical Reagent Company. All chemicals used were of analytical-reagent grade. Doubly distilled water was used throughout.

Synthesis and characterization of 2-(2-pyridyliminomethyl) phenol

The fluorescent probe was synthesized according to Scheme 1

Two grams (21 mmol) of 2-aminopyridine was dissolved in 10 ml of toluene. After salicylaldehyde was dropwisely added to 2-aminopyridine toluene solution, and the mixture was refluxed for 9 h. Then the solvent was removed under reduced pressure and the yellow crude product was obtained. The crude product was recrystallized from ethanol [13, 14] and dried in vacuum overnight, the yellow crystal was obtained with the yield of 77%. Mp: 67–68 °C. Elemental analysis (%) for $C_{12}H_{10}ON_2$ (calc.): C 70.78 (72.71), H 5.17 (5.09), N 14.54 (14.14), IR (KBr pellet): ν (cm^{-1}) 3456 (O–H), 1609 (C=N), ^{1}HNMR: δ=6.2–8.4(m, 9×CH). The appearance of ν C=N peak demonstrated that the target product (2-(2-pyridyliminomethyl)phenol) was obtained. The synthesis route was shown in Scheme 1.

Fluorescent probe 2.00×10^{-4} M was prepared by dissolving an appropriate amount of 2-(2-pyridyliminomethyl)phenol in ethanol. A stock solution of SOD (0.05 U ml^{-1}) was prepared in water (stored in refrigerator). The following solutions were prepared in doubly distilled water: pyrogallol, 2.00×10^{-4} M; Tris–HCl (pH 8.20, 0.40 M) buffer solution. NaH_2PO_4–Na_2HPO_4 (pH=7.80, 0.05 M) buffer solution.

Experimental procedure

Determination of $O_2^{\bullet-}$

To each of 10-ml test-tubes, add 2.00 ml of pH 8.20 Tris–HCl buffer solution, different volume of 2.00×10^{-4} M pyrogallol, and 1.50 ml of 2.00×10^{-4} M 2-(2-pyridyliminomethyl)phenol. Dilute the mixture to 10 ml with doubly distilled water, shake and stand for 30 min. Measure the fluorescence intensity (F_0) in a 1-cm quartz cell at excitation and emission wavelength of 294 and 355 nm. The fluorescence intensity of the solution without pyrogallol was denoted as F. So a linear plot of ΔF vs pyrogallol concentration was obtained, where $\Delta F=F-F_0$. For a pH 8.20 Tris–HCl buffer solution including 3.00×10^{-5} M 2-(2-pyridyliminomethyl)phenol in the absence and presence of 2.00×10^{-5} M pyrogallol, the excitation spectra from 260 to 340 nm were measured at the emission wavelength of 355 nm with a 3.0 nm slit width. The emission spectra of these solutions from 310 to 460 nm were also monitored at the excitation wavelength of 294 nm.

Determination of scavenging effect of $O_2^{\bullet-}$

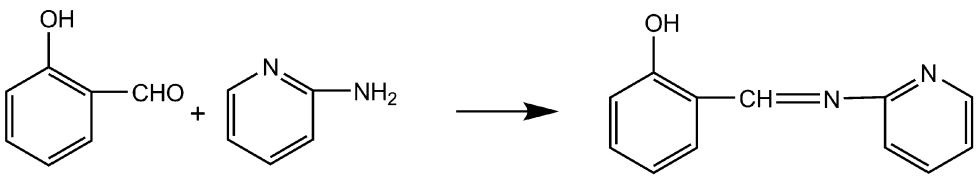
A certain amount of SOD solution was added into the system containing 2-(2-pyridyliminomethyl)phenol, pyrogallol and buffer solution. The fluorescence intensity was detected as F_S. Then the scavenging percentage (P) of $O_2^{\bullet-}$ by SOD was calculated as

$$P(\%)=(F_S-F_0)/(F-F_0)\times 100\% \quad (1)$$

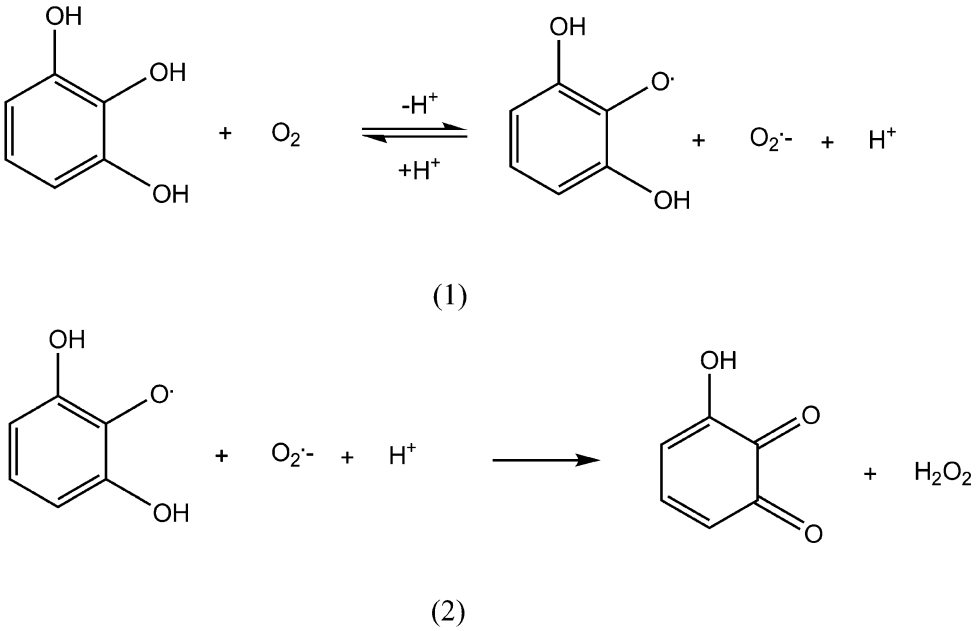
Determination of SOD activity

Extract of SOD in the samples: garlic, papaya and spinach (tegument and root of Garlic were removed), washed, and dried in air; 3.0000 g of each sample and 6.00 ml of phosphate buffer solution (pH 7.80; 0.05 M) were mixed together. Having been frozen for 2 h, they were pounded into a paste and then diluted to 24.00 ml, frozen for 1 h continuously, and centrifuged (12,000 rpm, 4 °C) for 15 min. Supernatants were transferred into three centrifuge tubes, and then cold ethanol (1.50 ml) and $CHCl_3$ (1.50 ml) were added. After being equilibrated, they were centrifuged (12000 rpm, 4 °C) for 5 min [15]. Then the supernatants were incubated in water at 55–60 °C for 20 min and stored in a refrigerator as SOD extract.

Scheme 1 The synthesis of 2-(2-pyridyliminomethyl)phenol



Scheme 2 Steps of pyrogallol auto-oxidation



A certain amount of SOD solution was added into the system containing 2-(2-pyridyliminomethyl)phenol, pyrogallol and buffer solution. The fluorescent quenching intensity was measured at $\lambda_{ex}/_{em}$=294/355 nm against a reagent blank.

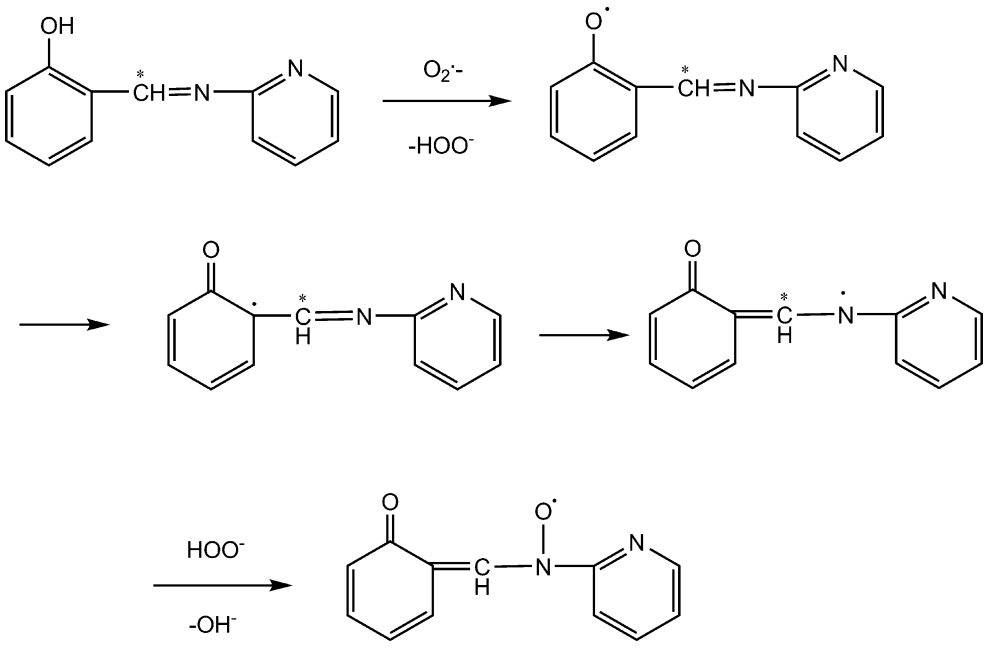
Determination of SOD activity by pyrogallol auto-oxidation

Into a 10 ml test-tube was added 5.00 ml of Tris–HCl (pH 8.20) buffer solution, 0.15 ml of pyrogallol (6.00×10^{-4} M), and 0.20 ml of SOD extract [15]. The mixture was diluted to 10 ml with doubly distilled water. The solution was equilibrated and its absorbance was measured at 320 nm against a reagent blank. The amount of SOD that could restrain the auto-oxidation rate of pyrogallol to 50% $min^{-1}ml^{-1}$ at 25 °C was defined as a SOD activity unit (U ml^{-1}). The calculation formula of SOD activity is as follows:

$$\text{SOD activity}\left(\text{U ml}^{-1}\right) = \left[\left(v_p - v_s\right)/\left(v_p \times 0.5\right)\right] \times V_T n/V_s \quad (2)$$

where v_p is the auto-oxidation rate of pyrogallol, v_s is the auto-oxidation rate of samples, V_T is the total volume of the solution to be determined (ml) and, n is the dilution fold of SOD extract. V_s is the volume of sample solution.

Scheme 3 The oxidation reaction of 2-(2-pyridyliminomethyl)phenol with $O_2^{\bullet-}$



Results and discussion

The mechanism of the reaction of $O_2^{\bullet -}$ with 2-(2-pyridyliminomethyl)phenol was as follows:

– Generation of $O_2^{\bullet -}$

$O_2^{\bullet -}$ was generated by pyrogallol auto-oxidation in Tris–HCl (pH 8.20) buffer solution [16]. It had been proved by UV spectrum [17]. The product of pyrogallol auto-oxidation was 3-hydroxycyclohexa-3, 5-diene-1, 2-dione (Scheme 2). Compared with the reaction of step 2, step 1, which produced $O_2^{\bullet -}$, was more quickly.

– Oxidation by $O_2^{\bullet -}$

2-(2-Pyridyliminomethyl)phenol, which has strong fluorescence, could be oxidized by $O_2^{\bullet -}$ to form a less fluorescent product. In the past, some investigations found that many nitroxyl radicals, such as *N*, *N* -diphenylnitroxyl radical, were stable in aqueous buffered solution [18, 19]. So we deduced the oxidation mechanism as shown in Scheme 3. O–H bond of 2-(2-pyridyliminomethyl)phenol was ruptured in the presence of $O_2^{\bullet -}$ and a quinoid radical and a hydrogen peroxide ion were produced. After free radical rearrangement, the π bond was broken up between the C* and the nitrogen atom, a double bond generated between the C^* and the C atom in benzene ring, and a single electron was assigned to the nitrogen atom. The nitrogen atom was attacked by hydrogen peroxide ion to form a stable nitroxyl radical which was less fluorescent. Thus the fluorescence intensity decreased obviously after the probe reacted with $O_2^{\bullet -}$.

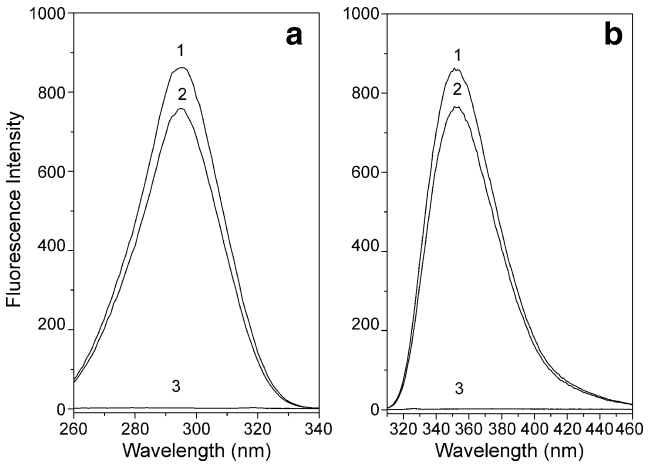


Fig. 1 Excitation and emission spectra of the probe reagent. a. excitation spectra at λ_{em} 355 nm, b. emission spectra at λ_{ex} 294 nm. *1* Buffer solution+2-(2-pyridyliminomethyl)phenol; *2* buffer solution+pyrogallol+2-(2-pyridyliminomethyl)phenol; *3* buffer solution+pyrogallol. Buffer solution, pH 8.20, 0.08 M; pyrogallol, 2.00×10^{-5} M; 2-(2-pyridyliminomethyl)phenol, 3.00×10^{-5} M

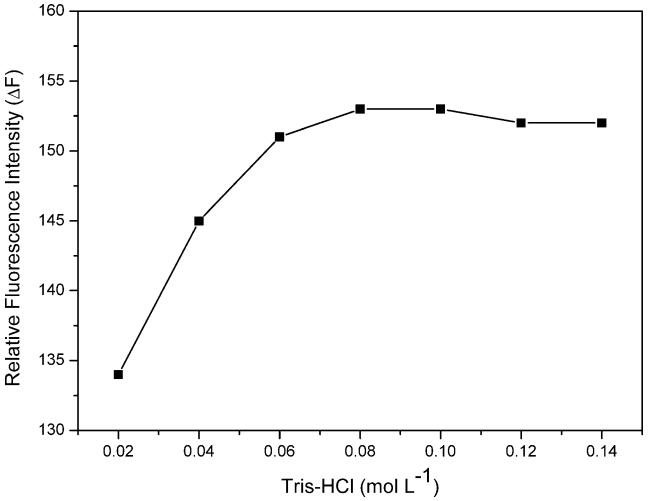


Fig. 2 Effect of buffer solution concentration. 2-(2-pyridyliminomethyl) phenol, 2.00×10^{-5} M; pyrogallol, 4.00×10^{-5} M

Excitation and emission spectra of the probe reagent

It could be found that 2-(2-pyridyliminomethyl)phenol had strong fluorescence in the buffer solution of pH 8.20 and the fluorescence intensity decreased obviously after 2-(2-pyridyliminomethyl)phenol reacted with $O_2^{\bullet -}$ (Fig. 1).

Effect of pH and buffer solution concentration

With the increase of pH value, the rate of pyrogallol auto-oxidation increased. Moreover, the inhibition rate of SOD reduced. However, the inhibition rate of SOD had no significant changes in the pH range 7.70–8.20 [20]. By comprehensive consideration of the influence of every factor, pH 8.20 Tris–HCl was selected.

The relative fluorescence intensity (ΔF) of the system was high and stable when the buffer solution concentration

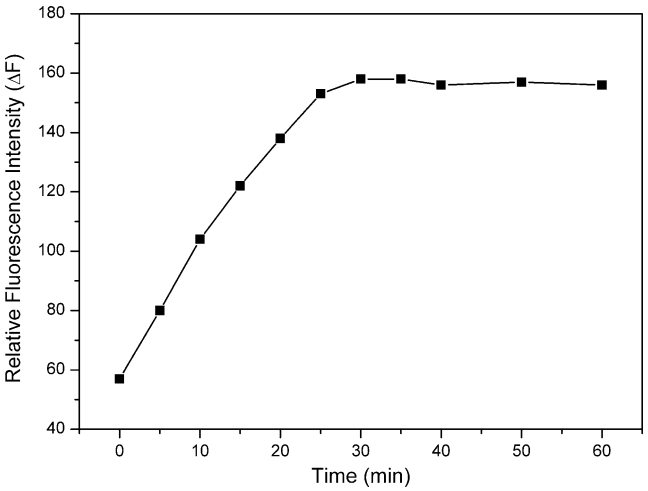


Fig. 3 Effect of reaction time. Buffer solution, 0.08 M; 2-(2-pyridyliminomethyl)phenol, 2.00×10^{-5} M; pyrogallol, 4.00×10^{-5} M

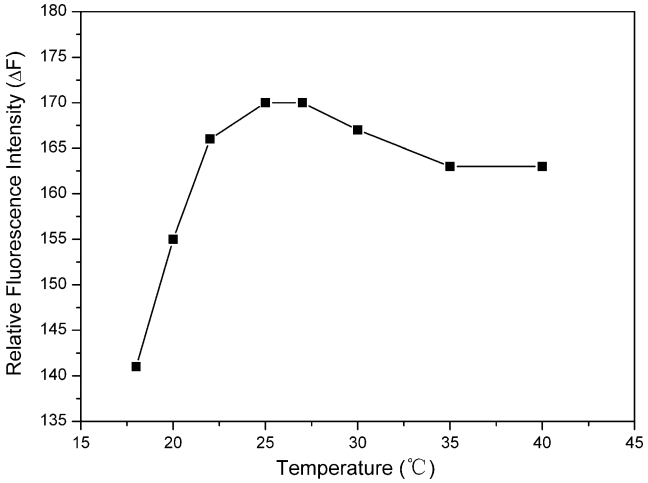


Fig. 4 Effect of temperature. Buffer solution, 0.08 M; 2-(2-pyridyliminomethyl)phenol, 2.00×10^{-5} M; pyrogallol, 4.00×10^{-5} M; t= 30 min

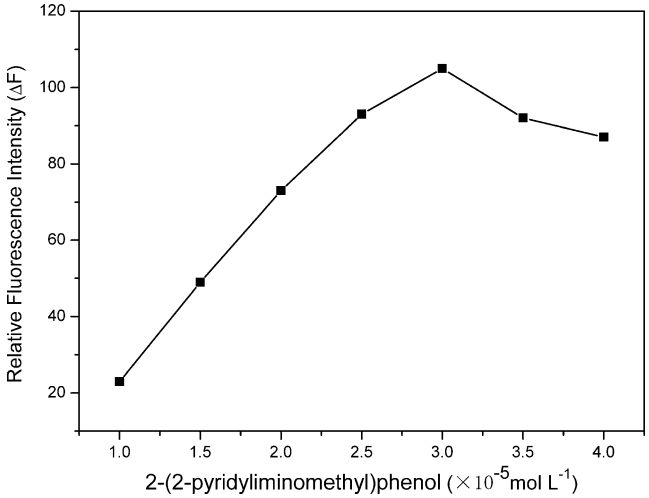


Fig. 6 Effect of the fluorescent probe concentration. Buffer solution, 0.08 M; pyrogallol, 2.00×10^{-5} M; t=30 min; T=25 °C

was from 0.06 to 0.14 M (Fig. 2). Thus 0.08 M buffer solution was chosen throughout the experiment.

Effect of reaction time

Reaction time affected the yield of $O_2^{\bullet-}$. The experimental results showed that the relative fluorescence intensity (ΔF) increased within 0–30 min, remained constant after that (Fig. 3). So the reaction time was selected as 30 min.

Effect of reaction temperature

The relative fluorescence intensity (ΔF) was related with reaction temperature, so the effect of reaction temperature was studied (Fig. 4). Figure 4 showed that 25 °C was the optimal reaction temperature. The reason was that radicals were not stable, the decay rate turned fast with increasing temperature.

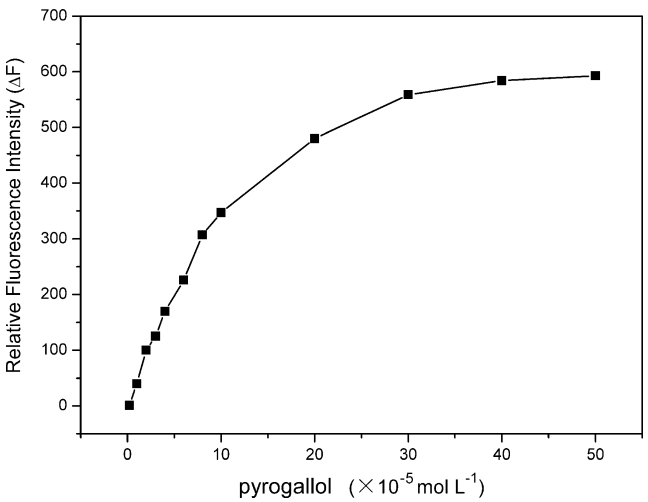


Fig. 5 Effect of pyrogallol concentration. Buffer solution, 0.08 M; 2-(2-pyridyliminomethyl)phenol, 2.00×10^{-5} M; t=30 min; T=25 °C

Effect of pyrogallol concentration

The effect of pyrogallol concentration was tested in the range from 2.00×10^{-6} to 5.00×10^{-4} M. The ΔF increased with pyrogallol concentration increasing up to about 10^{-4} M. There was a linear relationship with pyrogallol concentration in the range from 2.00×10^{-6} to 1.00×10^{-4} M and then the increasing tendency became slow (Fig. 5). Considering the effect of sensitivity, 2.00×10^{-5} M pyrogallol was selected.

Effect of fluorescent probe concentration

Because of the fluorescent probe 2-(2-pyridyliminomethyl) phenol as a trapper of $O_2^{\bullet-}$, its concentration decided directly whether $O_2^{\bullet-}$ in the system was trapped completely, which decided the accuracy of the method. The effect of its concentration on the ΔF was studied under the selected conditions. The ΔF increased with the trapper concentration increasing up to about 3.00×10^{-5} M, above which it decreased (Fig. 6). A suitable concentration of 2-(2-pyridyliminomethyl)phenol was advantageous while super-

Table 1 Effect of addition sequence of reagents

Addition sequence of reagents	ΔF
(1) Buffer+2-(2-pyridyliminomethyl)phenol+pyrogallol	87
(2) Buffer+pyrogallol+2-(2-pyridyliminomethyl)phenol	105
(3) 2-(2-pyridyliminomethyl)phenol+pyrogallol+buffer	90
(4) 2-(2-pyridyliminomethyl)phenol+buffer+pyrogallol	90
(5) Pyrogallol+buffer+2-(2-pyridyliminomethyl)phenol	94
(6) Pyrogallol+2-(2-pyridyliminomethyl)phenol+buffer	94

Table 2 Effect of organic reagents

	Methanol	Ethanol	Isopropanol	Acetonitrile	DMF	Acetone	Water
F_0	826	869	893	898	911	0	886
F	758	809	822	808	857	0	779
ΔF	68	60	71	90	54	0	105

fluous 2-(2-pyridyliminomethyl)phenol could absorb its own fluorescence. Therefore, the concentration of 2-(2-pyridyliminomethyl)phenol was selected as 3.00×10^{-5} M.

Effect of addition sequence of reagents

The addition sequence of reagents was experimented in Table 1. The results demonstrated the second group was the best one.

Effect of organic reagents

Different organic solvents (2.00 ml) were added in the system, and the results were showed in Table 2. It could be seen that ΔF was the highest in the system without any organic reagents. So water was selected as solvent.

The reproducibility of the method and effects of interferences

Under the optimum experimental conditions, ten determinations were measured. The standard deviation of the method was 1.62 and the relative standard deviation was 1.58%, the results indicated that this method had a good reproducibility.

The influences of foreign substances were studied with 2.00×10^{-5} M pyrogallol. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. No interference was encountered from (mole ratio): Na^+, Cl^- (1,500); glucose (1,000); sucrose, K^+ (500); Ca^{2+}, Br^-, NO_3^- (200); SO_4^{2-}, I^- (120); L-phenylalanine (80); uracil (40); DNA (20); Cu^{2+} (20); DL-tyrosine, L-arginine (10); Co^{2+} (8); BSA (7.5); Guanine (6). The interference experiment proved that most components in biological materials had no or little effect on the determination of $O_2^{\bullet-}$, which indicated that the proposed method had a high selectivity. So the method could be used to determine $O_2^{\bullet-}$ and SOD activity in biology systems.

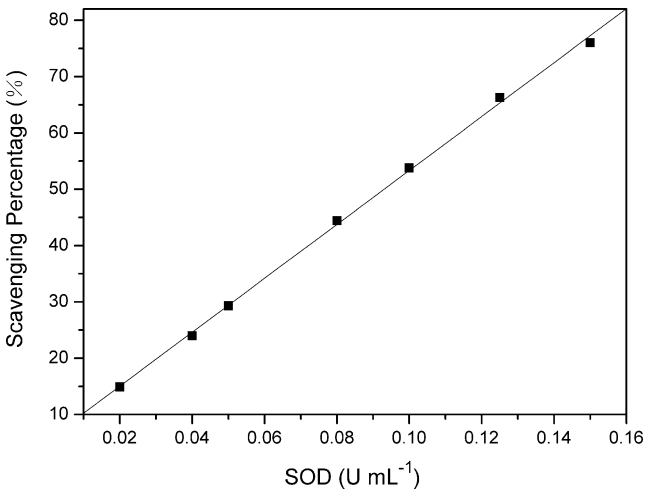


Fig. 7 Relationship between SOD and scavenging percentage

Scavenging effect of SOD on $O_2^{\bullet-}$

SOD is the specific scavenger of $O_2^{\bullet-}$, whose scavenging effect on $O_2^{\bullet-}$ could be used to verify the efficiency of the proposed method. It could be seen from Fig. 7, there was a good linear relationship between scavenging effect and SOD quantity with a correlation coefficient (r) of 0.9994. It demonstrated that the proposed method was effective.

Determination of SOD activity in samples

SOD activity in garlic, papaya and spinach were determined by the proposed method and the classical standard method [15], respectively (Table 3). This indicated that the results obtained by the suggested method were consistent with those obtained from the standard method, and this proposed method had higher precision. Obviously, the method had less interference by other substances presented in the samples.

Conclusions

In the paper, alkaline pyrogallol was adopted as the source of $O_2^{\bullet-}$, a novel fluorescent probe, 2-(2-pyridyliminomethyl)phenol was synthesized and used to determine $O_2^{\bullet-}$ indirectly, the linear calibration range between the

Table 3 Determination of SOD activity in samples (n=6)

Samples	Proposed method X±SD	Standard method X±SD
Garlic	120.25±3.20	119.64±8.12
Papaya	274.67±8.64	272.32±8.94
Spinach	115.32±6.88	113.32±7.32

relative fluorescence intensity (ΔF) and pyrogallol was in the range 2.00×10^{-6}–1.00×10^{-4} M and the detection limit was 1.38×10^{-6} M. Moreover, the spectrofluorometric method was applied to verify the scavenging effect of SOD in which there was a good linear relationship with a correlation coefficient (r) of 0.9994 between ΔF and SOD activity. In addition, SOD activity in garlic, papaya and spinach was successfully determined. The results showed that 2-(2-pyridyliminomethyl)phenol had a good recognition, the reaction was simple, precise and sensitive, which was well suited for the determination of SOD activity.

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