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Study of 2-(2-Pyridyl)benzothiazoline as a Novel Fluorescent Probe for the Identification of Superoxide Anion Radicals and the Determination of Superoxide Dismutase Activity in Scallion Genus Foods

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This paper presents a novel spectrofluorometric method using the novel fluorescent probe 2-(2-pyridyl)benzothiazoline for the determination of superoxide dismutase (SOD) activity. The fluorescent probe was synthesized in house and fully characterized by elemental analysis and by IR and ^{1}H NMR spectra. It could specially identify and trap superoxide anion radicals ($O_2^{\bullet-}$), and then was oxidized by $O_2^{\bullet-}$ to form a strong fluorescence product. On the basis of this reaction, the spectrofluorometric method was proposed and successfully used to determine SOD activity. The proposed method has a better selectivity in the determination of reactive oxygen species, because the probe can be oxidized to afford a highly fluorescent product only by $O_2^{\bullet-}$ excluding hydrogen peroxide and hydroxyl radical. As a kind of simple, rapid, precise, and sensitive technique, it could avoid the errors caused by detection time and was applied to the measurement of SOD activity in scallion genus foods with satisfactory results.

KEYWORDS: Superoxide anion radicals ($O_2^{\bullet-}$); 2-(2-pyridyl)benzothiazoline (H. Py. Bzt); fluorescent probe; superoxide dismutase (SOD)

INTRODUCTION

Various free radicals are generated in the course of biological metabolism, such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($HO^\bullet$), and $ROO^\bullet$, which are three typical ones (*1*). Free radicals, especially $O_2^{\bullet-}$, have been considered extensively in myocardial ischemia and reperfusion injury in the past several years (*2*). Excessive $O_2^{\bullet-}$, which is a toxic reactive oxygen, does great harm to photosynthetic pigment and membrane and further damages the photosynthetic systems. Moreover, $O_2^{\bullet-}$ can be diverted into other more toxic radicals such as $HO^\bullet$, H_2O_2, and $^1O_2$ (*3*), which can damage photosynthetic membranes and many biological molecules. In addition, $O_2^{\bullet-}$ can cause coronary, arteriosclerosis, and tumor diseases (*4*−*6*). For these reasons, eliminating $O_2^{\bullet-}$ from organisms is of great importance to prevent diseases and senescence.

It is well-known that superoxide dismutase (SOD) is the scavenger of $O_2^{\bullet-}$ and an important indicator of the amount of $O_2^{\bullet-}$ in organisms. Therefore, establishing a precise, rapid, and sensitive method to determine SOD activity is of great applied value. At present, the common methods of determining SOD activity are autoxidation of pyrogallol (*7*) and hypoxanthine−xanthine oxidase (HX-XO)−cytochrome reduction (*8*), both of which are spectrophotometric methods. In recent years, some new methods such as chemiluminescence (CL) (*9*), electron spin resonance (ESR) (*10*, *11*), high-performance liquid chromatography (HPLC) (*12*), polarographic oxygen electrode (*13*), and immunity (*14*) have been developed. However, all of them have their disadvantages; for example, the instruments needed in ESR are too expensive, the operation in HPLC is complicated, the selectivity of CL is poor, and the sensitivity of spectrophotometry is low. Compared with the methods mentioned above, spectrofluorometry is simpler, more sensitive, and more selective. However, there are only a few papers on detecting SOD activity with spectrofluorometry so far. The other aspect well-known is that the selectivity of reagents for reactive oxygen species (ROS) is important but difficult, because there are chain reactions of ROS in organisms, which result in the existence of many kinds of ROS. Commonly, $O_2^{\bullet-}$, $HO^\bullet$, and H_2O_2 are the three main coexisting substances.

In this work, a novel fluorescent probe, namely, 2-(2-pyridyl)-benzothiazoline (H. Py. Bzt) synthesized in house, was applied to the identification of $O_2^{\bullet-}$ and the determination of SOD activity by spectrofluorometry. In the experiment, we found that only $O_2^{\bullet-}$ could oxidize H. Py. Bzt to form a strong fluorescence product, but H_2O_2 could not do so. Moreover, the same amount of hydroxyl radicals ($HO^\bullet$) also had no influence on the determination of $O_2^{\bullet-}$, because it could not make the fluorescence intensity of the reaction system change at the measuring wavelengths. Therefore, the probe has a better selectivity. In addition, the reaction completed instantly, so the reaction time had no influence on the determination, which avoided the error

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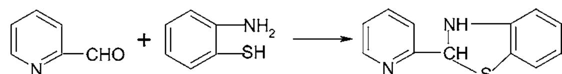
Scheme 1. Synthesis of 2-(2-Pyridyl)benzothiazoline



caused by time. A comparison between the proposed method and the established method showed that the former had a satisfactory accuracy and good precision. The method was applied to determine SOD activity in garlic, scallion, and onion successfully.

MATERIALS AND METHODS

Apparatus. The fluorescence spectra and intensity were measured on a Cary Eclipse spectrofluorometer with a xenon lamp and 1.0 cm quartz cells (Varian). All pH measurements were made with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, China) with a combined glass−calomel electrode. The IR spectra were recorded on a PE-983 IR spectrometer (KBr disks cm^{-1}, Perkin-Elmer, Norwalk, CT). Elemental analysis was performed on a PE-240 CHN elementary analytical meter (Perkin-Elmer). The ^1H NMR spectra were recorded on an FX-90Q nuclear magnetic resonance spectrometer (DMSO as solvent, JEOL). The absorbance was recorded on a UV-265 spectrophotometer (Shimadzu).

Reagents. × Fluorescent probe (1.00×10^{-4} mol L^{-1}, synthesized in house) was prepared by dissolving an appropriate amount of 2-(2-pyridyl)benzothiazoline in ethanol. The $Na_2S_2O_4$ solution (6.80×10^{-3} mol L^{-1}) was prepared by dissolving an appropriate amount of $Na_2S_2O_4$ in 0.10 mol L^{-1} NaOH. A stock solution of SOD (1.00×10^{-2} g L^{-1}) was prepared in water (stored in a refrigerator). A solution of H_2O_2 (3.40×10^{-4} mol L^{-1}) was standardized by titration with potassium permanganate. The following solutions were prepared in doubly distilled water: pyrogallol, 3.00×10^{-3} mol L^{-1}; $Na_2B_2O_7$−NaOH (pH 11.60, 0.10 mol L^{-1}) buffer solution, and Tris-HCl (pH 8.20, 0.10 mol L^{-1}) buffer solution. 2-Pyridinaldehyde and *o*-aminobenzenothiol were purchased from Fluka. All chemicals used were of analytical reagent grade, and doubly distilled water was used throughout.

Synthesis and Properties of H. Py. Bzt. 2-(2-Pyridyl)benzothiazoline was prepared by condensation of the appropriate 2-pyridinaldehyde (0.030 mol) with *o*-aminobenzenothiol in a 1:1 molar ratio in benzene (*15*). The synthesis reaction is shown in **Scheme 1**. The mixed solution was heated under reflux for ∼2−3 h. After the solvent was removed under reduced pressure, the yellow crude solid was obtained. Then it was recrystallized from benzene and dried in a vacuum. Melting point: 81−81.5 °C; ^1H NMR (90 MHz, DMSO-d_6, 25 °C, TMS) δ 6.3 (s, 1H, C−H), 4.3 (s, 1H, N−H), 7.2−8.5 (4H, pyridine-H), 6.6−7.2 (4H, benzol-H); IR (KBr pellet) ν (cm^{-1}) 3250 (N−H), 3105 (C−H), 1595 (C═C). Elemental analysis (%) calcd for $C_{12}H_{10}N_2S$ (found): C, 67.24 (67.31); H, 4.70 (4.81); N 13.70 (13.60). The data were in agreement with the values reported in refs *15* and *16*.

Experimental Procedure. *Determination of $O_2^{\bullet-}$ and Scavenging Effect of $O_2^{\bullet-}$ by Standard SOD.* Into a 10-mL graduated color comparison tube were added 0.50 mL of $Na_2S_2O_4$ (6.80×10^{-3} mol L^{-1}), 1.50 mL of $Na_2B_4O_7$−NaOH buffer solution (pH 11.60, 0.10 mol L^{-1}), and 3.00 mL of H. Py. Bzt in turn. Then it was diluted to volume with doubly distilled water and equilibrated. The fluorescence intensity was measured at $\lambda_{ex/em} = 377/528$ nm against a reagent blank. The excitation and emission slits were both set to 5 nm.

A certain amount of standard SOD solution (0.20, 0.40, 0.60, 0.80, and 1.00 mL, respectively) was added additionally. The fluorescence intensity with SOD and $Na_2S_2O_4$ was recorded as F_s, only with $Na_2S_2O_4$ was F, without SOD or $Na_2S_2O_4$ was F_0. Then the scavenging percentage (P) of SOD to superoxide anion radicals was calculated by $P = (F - F_s)/(F - F_0) \times 100\%$.

Determination of the Activity of SOD Extracted from Samples. In actual sample analysis, 0.20 mL of SOD extracted from scallion genus foods was added additionally and the reagent blank was carried out at the same time. The SOD amount with the P at 50% was assumed as a unit, and then the calculation formula of SOD activity was as follows: SOD activity (U mL^{-1}) $= PV_Tn/(50\% V_M)$, where V_T (mL) is the total volume of the solution to be determined, V_M is the volume of the SOD extract, and n is the dilution fold of the SOD extract (V_T/V_M). Then the SOD activity unit could be converted into units per gram according to the SOD content in the samples.

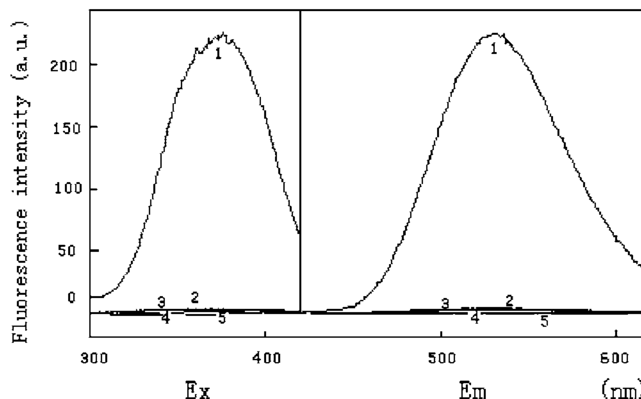


Figure 1. Excitation and emission spectra: 1, $Na_2S_2O_4$ + buffer + H. Py. Bzt; 2, buffer + H. Py. Bzt; 3, H_2O_2 + buffer + H. Py. Bzt; 4, HO• [obtained from H_2O_2 (3.40×10^{-4} mol L^{-1}) + Co^{2+}] + H. Py. Bzt; 5, buffer + $Na_2S_2O_4$ (3.40×10^{-4} mol L^{-1}), H. Py. Bzt (3.00×10^{-5} mol L^{-1}), buffer solution (pH 11.60, $Na_2B_4O_7$–NaOH), H_2O_2 (0.003%), Co^{2+} (5.00×10^{-5} mol L^{-1}).

Extract of SOD in the Samples. Garlic, scallion, and onion tegument and root were removed and dried in air; 1.0000 g of each sample and 2.00 mL of phosphate buffer solution (pH 7.80, 0.050 mol L^{-1}) were mixed together. Having been frozen for 2 h, they were pounded into a paste and then diluted to 8.00 mL, frozen for 1 h continuously, and centrifuged (4000 rpm) for 15 min (*17*). After that, 5.40 mL of supernatants was transferred into three centrifuge tubes, and then cold ethanol (0.50 mL) and $CHCl_3$ (0.50 mL) were added. After being equilibrated, they were centrifuged for 5 min. The supernatant was the SOD extract and stored in a refrigerator.

Determination of SOD Activity by Classic Autoxidation of Pyrogallol. Into a 10-mL color comparison tube were added 5.00 mL of Tris-HCl (pH 8.20) buffer solution, 0.30 mL of pyrogallol (3.00×10^{-3} mol L^{-1}), and 0.20 mL of SOD extract in turn. The mixture was diluted to volume with doubly distilled water. The solution was equilibrated, and its absorbance was measured at 320 nm against a reagent blank (*7*). The amount of SOD that could restrain the autoxidation rate of pyrogallol to 50% min^{-1} mL^{-1} at 25 °C was defined a SOD activity unit (units mL^{-1}). The calculation formula of SOD activity is as follows: SOD activity (units mL^{-1}) $= [(v_p - v_s)/(v_p \times 0.5)] \times V_Tn/v_s$, where v_p is the autoxidation rate of pyrogallol, v_s is the autoxidation rate of samples, V_T (mL) is the total volume of the solution to be determined, and n is the dilution fold of the SOD extract. Then the SOD activity unit could be converted into units per gram according to the SOD content in the samples.

RESULTS AND DISCUSSION

Mechanism of the Determination. In this study, $O_2^{\bullet-}$ was produced by the reaction $S_2O_4^{2-} + O_2 + 4HO^- = 2SO_3^{2-} + 2H_2O + O_2^{\bullet-}$ (*18*), and the reaction equilibrium constant (K) and conditional reaction equilibrium (K') were 7.6×10^{18} and 7.6×10^8, respectively, which were calculated according to the standard and conditional redox potentials of SO_3^{2-}−$S_2O_4^{2-}$ and O_2−$O_2^{\bullet-}$ (*19*). Therefore, the reaction could take place spontaneously and completely in the alkaline solution saturated by O_2, and the concentration of $O_2^{\bullet-}$ could remain stable for at least 1.5 h in 0.10 mol L^{-1} of NaOH.

Because H. Py. Bzt could react with $O_2^{\bullet-}$ to form a strong fluorescence product, the amount of $O_2^{\bullet-}$ was determined indirectly by measuring the changes of fluorescence intensity. According to the proposed method, the excitation and emission spectra were recorded (**Figure 1**) and the fluorescence intensity was measured at $\lambda_{ex/em} = 377/528$ nm. It could be found that

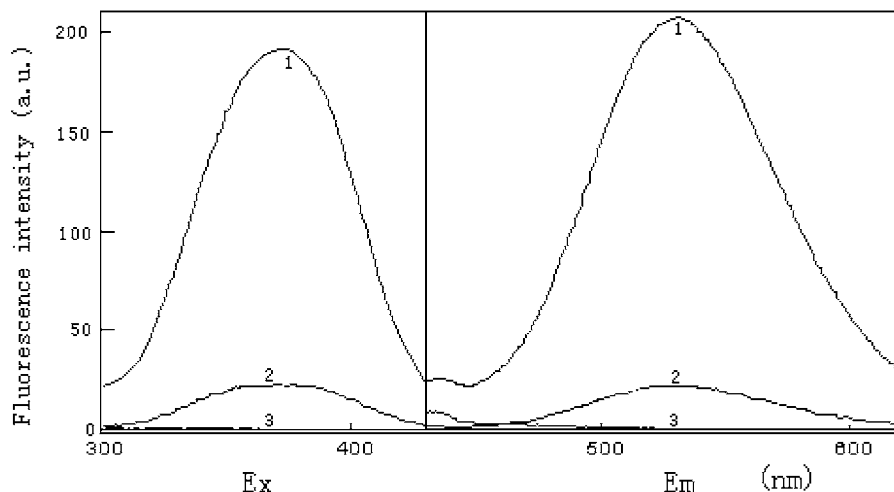
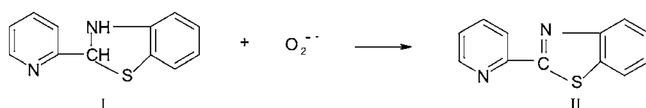


Figure 2. Autoxidation of pyrogallol: 1, pyrogallol + buffer + H. Py. Bzt; 2, buffer + H. Py. Bzt; 3, pyrogallol + buffer pyrogallol (3.40×10^{-4} mol L^{-1}), H. Py. Bzt (3.00×10^{-5} mol L^{-1}), buffer solution (pH 8.20, Tris-HCl).

Scheme 2



H. Py. Bzt itself had no fluorescence and the fluorescence intensity increased obviously after the oxidation reaction took place. However, H_2O_2 could not oxidize the probe [**Figure 1**(**3**)], and the same amount of hydroxyl radical ($HO^{\bullet}$) could not make its fluorescence intensity change at the measuring wavelengths [**Figure 1**(**4**)]. Although a 10-fold amount of $HO^{\bullet}$ can make the fluorescence intensity increase, in real samples $HO^{\bullet}$ is derived from $O_2^{\bullet -}$ and its life is only 10^{-4} s with a very low concentration of 10^{-8} mol L^{-1} (*20*), so the amount of $HO^{\bullet}$ existing in biology cannot reach the amount of $O_2^{\bullet -}$. In addition, the reaction of H. Py. Bzt trapping $O_2^{\bullet -}$ was sensitive and rapid. Therefore, $HO^{\bullet}$ could not interfere with the determination of $O_2^{\bullet -}$ in real samples. The probe could identify $O_2^{\bullet -}$ especially, and the proposed method has a better selectivity in the determination of ROS.

To investigate the reaction mechanism, the oxidization product was synthesized; 0.050 g of H. Py. Bzt was dissolved in 10 mL of ethanol, 2.00 g of $Na_2S_2O_4$ was dissolved in 10 mL of NaOH (0.10 mol L^{-1}), they were mixed together, and 20 mL of buffer solution was added. Then the solvent was removed, and the residue was extracted by dichloroethane. The crude product was obtained as a green solid. The pure brown product was obtained by recrystallization in ethanol: melting point, 131−132 °C; ^{1}H NMR (90 MHz, DMSO-d_6, 25 °C, TMS) δ 7.6−8.6 (4H, pyridine-H), 7.3−7.6 (4H, benzol-H); IR (KBr pellet) ν (cm^{-1}) 1637 (C=N), 3105 (C−H), 1595 (C=C). Elemental analysis (%) calcd for $C_{12}H_8N_2S$ (found): C, 67.90 (67.92); H, 3.75 (3.77); N, 13.26 (13.21). As shown in ^{1}H NMR spectra, peaks corresponding to N−H (4.3) and C−H (6.3) of H. Py. Bzt disappeared and the other signals moved to a lower field, which indicated that the product had a larger conjugated system. This also could explain the fluorescence intensity increase. In the IR spectrum, N−H (3250 cm^{-1}) and C−H (3105 cm^{-1}) in H. Py. Bzt disappeared and the C=N absorption band at 1673 cm^{-1} appeared. All spectral data were consistent with structure II in **Scheme 2**. The structure was further confirmed by elemental analysis. Therefore, the mechanism could be presumed as given in **Scheme 2**. $O_2^{\bullet -}$ oxidized the probe by deleting hydrogen to yield the compound 2-(2-pyridyl)benzothiazol (**Scheme 2**, II), which had better rigidity and a larger conjugated system (free electron pairs on S also conjugating with phenyl ring). Due to its larger conjugated system, the fluorescence intensity increased obviously after the probe reacted with $O_2^{\bullet -}$.

To confirm that it was $O_2^{\bullet -}$ obtained in alkaline $Na_2S_2O_4$ that made the fluorescence increase, several related compounds containing sulfur were tested. It was found that neither Na_2SO_4, Na_2SO_3, nor Na_2S could make the fluorescence intensity increase. Neutral and alkaline solutions of $Na_2S_2O_4$ reacting with H. Py. Bzt were also tested, and the results showed that the fluorescence intensity did not change in the neutral water solution, but it increased obviously in the alkaline solution. This could be taken as evidence that only $O_2^{\bullet -}$ obtained in alkaline $Na_2S_2O_4$ solution could oxidize the probe and make the fluorescence intensity increase. To validate the conclusion further, pyrogallol was applied as the source of $O_2^{\bullet -}$ to react with the probe. The results were the same as those with alkaline $Na_2S_2O_4$ (**Figure 2**), and the fluorescence intensity increased with longer time due to the slow autoxidation rate of pyrogallol. This demonstrated that there existed $O_2^{\bullet -}$ in alkaline $Na_2S_2O_4$ solution, which also could be confirmed from the experiment of SOD scavenging $O_2^{\bullet -}$. Compared with autoxidation of pyrogallol as the source of $O_2^{\bullet -}$, alkaline $Na_2S_2O_4$ had a rapid rate, so it was unnecessary to consider the influence of time on determination. This not only avoided experimental error but also improved determination speed.

Optimization of Experimental Variables. *Effect of pH and Amount of Buffer Solution.* As shown in **Figure 3a**, the experiment showed that the optimal pH for producing $O_2^{\bullet -}$ was in the range of 11.00−11.80. In comparison, $Na_2B_4O_7$−NaOH was more sensitive within NH_4Ac−NaOH, NH_4Cl−NH_3·H_2O, and Tris-HCl buffer solution systems. Therefore, $Na_2B_4O_7$−NaOH buffer solution was selected. The relative fluorescence intensity (ΔF) of the system was high and stable when the amount of buffer solution was from 1.00 to 2.00 mL (**Figure 3b**). Thus, 1.50 mL of $Na_2B_4O_7$−NaOH (0.10 mol L^{-1}) buffer solution (pH 11.60) was chosen throughout the experiment.

Effect of Reaction Time. Different reaction times were tested. The results showed that the ΔF did not change with different lay-side time (**Figure 4**). This indicated that the reaction was rapid; it could react completely in an instant due to its rapid reaction rate, so time had no influence on the determination

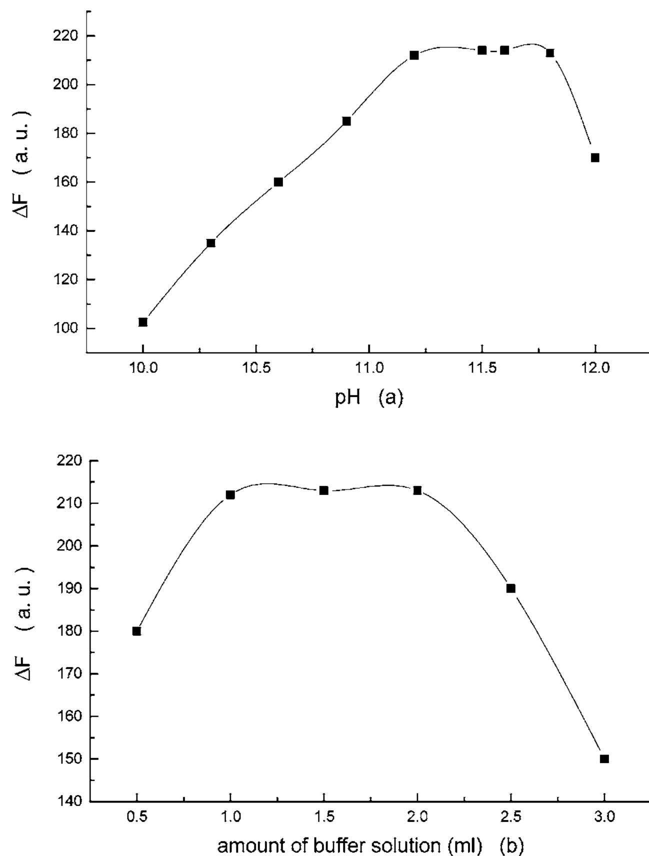


Figure 3. Effect of pH (**a**) and amount of buffer solution (**b**).

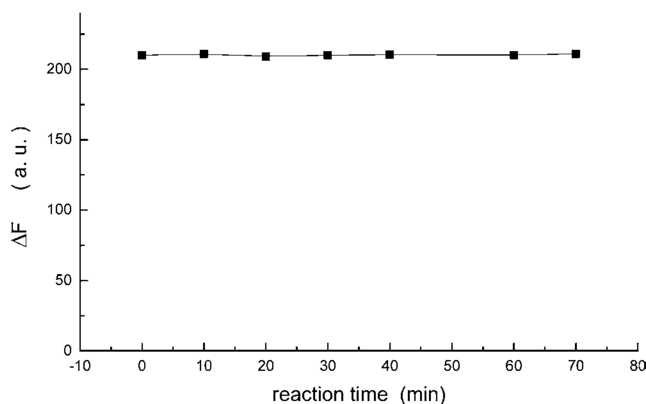


Figure 4. Effect of reaction time.

and did not need to be considered. This not only improved the determination rate but also minimized the errors caused by detection time.

Effect of Concentration of $Na_2S_2O_4$. The concentration of $Na_2S_2O_4$ determined the yield of $O_2^{\bullet-}$. The experimental results showed that ΔF had a linear relationship with $Na_2S_2O_4$ in the range from 0.00 to 3.20×10^{-4} mol L^{-1}, and then remained high and stable when the concentration of $Na_2S_2O_4$ was between 3.20×10^{-4} and 3.60×10^{-4} mol L^{-1} (**Figure 5**). Therefore, 3.40×10^{-4} mol L^{-1} of $Na_2S_2O_4$ was selected throughout the experiment.

Effect of the Concentration of Fluorescent Probe. Because H. Py. Bzt was the trapper of $O_2^{\bullet-}$, its concentration directly determined whether $O_2^{\bullet-}$ was trapped completely, which decided the precision and sensitivity of the method. ΔF increased with the increase of the trapper concentration and then remained stable when it was between 2.50×10^{-5} and 3.50×10^{-5} mol L^{-1}. Therefore, 3.0×10^{-5} mol L^{-1} of the probe was selected.

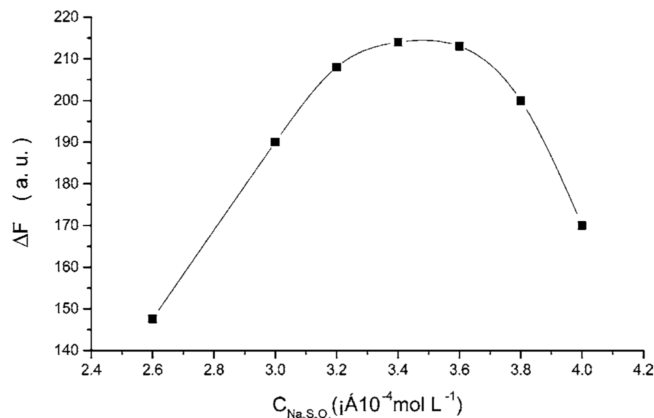


Figure 5. Effect of $Na_2S_2O_4$.

Table 1. Effect of Addition Order of Reagents

addition sequence of reagent	ΔF (au)
(1) buffer + H. Py. Bzt + $Na_2S_2O_4$	150
(2) buffer + $Na_2S_2O_4$ + H. Py. Bzt	230
(3) H. Py. Bzt + $Na_2S_2O_4$ + buffer	163
(4) H. Py. Bzt + buffer + $Na_2S_2O_4$	171
(5) $Na_2S_2O_4$ + H. Py. Bzt + buffer	89
(6) $Na_2S_2O_4$ + buffer + H. Py. Bzt	268

Table 2. Effect of Organic Solvents

au	methanol	ethanol	2-propanol	acetonitrile	DMF	acetone	water
F	40	33	43	39	61	42	13
F_0	138	137	133	194	175	149	214
ΔF	98	134	90	155	114	107	201

Effect of Addition Sequence of Reagents. The addition sequence of reagents is detailed in **Table 1**. The results indicated the sixth one was the best. The reason was that $Na_2S_2O_4$ in alkaline solution could yield more $O_2^{\bullet-}$.

Effect of Organic Solvents. Two milliliters of different organic solvents was added additionally, and then the fluorescence intensity was measured according to the experimental method above. The results are shown in **Table 2**. It can be seen that ΔF of the system without any organic solvent was highest. Therefore, water but not organic solvent was selected to dilute to volume.

Reproducibility of the Method. According to the experimental method, 11 determinations were made every other day. The standard deviation was 1.25, and the relative standard deviation of the methods was 0.46%, which showed that the reproducibility of the proposed method was very good.

Effect of Interferences. Thirty-one interferences (common components in organisms) were studied individually to investigate their effects on the determination of $O_2^{\bullet-}$ obtained from 3.4×10^{-4} mol L^{-1} of $Na_2S_2O_4$ by the procedure. An error of ±5% in the relative fluorescence intensity was considered to be tolerable. No interference was encountered from (tolerable ration in moles) Na^+, Cl^- (over 1500); glucose, lactin (1000); sucrose, K^+, Mg^{2+} (500); Cu^{2+}, Ca^{2+}, Br^-, NO_3^- (200), lactose, SO_4^{2-}, I^- (120); Zn^{2+} (100); L-phenylalanine, Co^{2-}, Pb^{2+} (80); HAS, thymine, cytosine (40); adenine, guanine (30); thiourea, DNA (20); RNA, BSA (15); tryptophan, DL-tyrosine (10); and Fe^{3+}, Al^{3+} (2). The experimental results showed that there was no interference for most biological components.

Scavenging Effect of $O_2^{\bullet-}$ by Standard SOD. SOD is the specific scavenger of $O_2^{\bullet-}$; its scavenging effect on $O_2^{\bullet-}$ could

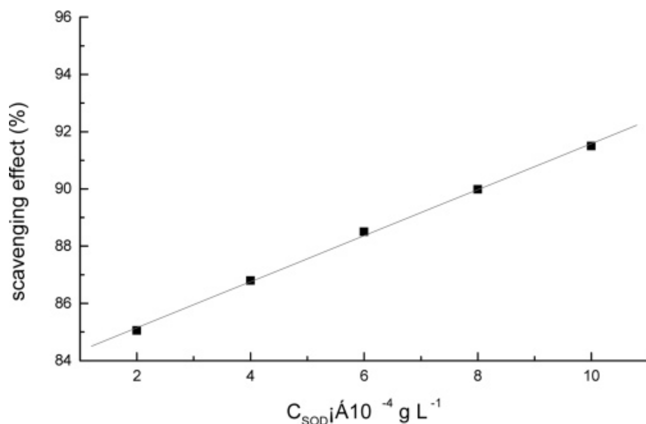


Figure 6. Relationship between SOD and scavenging percentage.

Table 3. Determination of SOD Activity in Samples

		$\bar{x}$ ± SD (units g^{-1})	
sample	no.	proposed method	standard method (*7*)
garlic	7	184.2 ± 5.2	182.1 ± 5.6
scallion	7	70.5 ± 4.5	72.2 ± 5.2
onion	7	50.3 ± 4.8	51.2 ± 6.0

be used to verify the efficiency of the proposed method. The experimental results showed that there was a good linear correlation ($R = 0.9995$) between scavenging percent and SOD quantity (**Figure 6**). The calibration equation was $\Delta F = 82.02 + 0.995C$ (C is in 10^{-4} g L^{-1}). Therefore, the proposed method was effective.

Determination of SOD Activity in Samples. On the basis of the proposed experimental method, SOD activity in garlic, scallion, and onion was determined successfully. The results obtained were compared with the autoxidation of pyrogallol method (**Table 3**). The former results were similar to the latter ones, which proved that the proposed method was efficient and useful. Compared with the classical method, the proposed method had a higher sensitivity and more rapid detection rate, because the autoxidation rate of pyrogallol was determined, then the detection time had to be controlled strictly in the classical method, and each rate determination needed at least 25 min, whereas the proposed method needed to measure only the fluorescence intensity, which could be recorded at any time after mixing without considering time, and each determination was completed in 1 min. The autoxidation of pyrogallol method had a close relationship with the detection time, which made the determination discommodious and caused bigger errors, and metal ions had more interference on the classical method.

ABBREVIATIONS USED

$O_2^{\bullet -}$, superoxide anion radicals; H. Py. Bzt, 2-(2-pyridyl)-benzothiazoline; SOD, superoxide dismutase; $HO^{\bullet}$, hydroxyl radicals.

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