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A novel method for determination of peroxynitrite based on hemoglobin catalyzed reaction

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Abstract

A novel spectrofluorimetric method for the determination of peroxynitrite is proposed. The method is based on a mimetic enzyme catalyzed reaction with hemoglobin as the catalyst and L-tyrosine as the substrate. A new fluorescent substance is produced that might probably be the coupled dimmer of tyrosine, which, instead of nitryl-tyrosine, is likely to be a new marking substance of $ONOO^-$ injury in vivo. Kinetics of the reaction is studied and the possible reaction mechanism is also recommended. The proposed method is simple and highly sensitive with a detection limit of 5.00×10^{-8} mol L^{-1} of peroxynitrite. A liner calibration graph is obtained over the peroxynitrite concentration range 5.60×10^{-7} to 2.10×10^{-5} mol L^{-1}, with a correlation coefficient of 0.9983. Interferences from some amino acids and metal ions normally seen in biological samples, and also some anions structurally similar to $ONOO^-$ are studied.

Keywords: Peroxynitrite; Enzymatic method; Hemoglobin; L-Tyrosine; Kinetics

1. Introduction

Reactive oxygen species (ROS) have been implicated as an important causative factor in cell damage, including apoptosis and necrosis. Their proposed actions comprise lipid peroxidation, DNA damage, the mitochondrial respiratory chain destruction and protein modifications [1–3]. Recent experiments have fastened on the importance of peroxynitrite, the reaction product of the two reactive species nitric oxide and superoxide [4]. Cells injury aroused by peroxynitrite is considered to contribute to the pathogenesis of a series of diseases, including inflammatory processes, ischemia-reperfusion, septic shock and neurodegenerative processes [5,6]. Evaluation of the potentially injuring mechanism of peroxynitrite has proved difficult due to its short life span, low concentration in vivo [7,8]. In recent years, various approaches have been tried to detect peroxynitrite [9–13], such as fluorescent probes method (usually use dihydrodichlorofluorescein (DCFH) and dihydrorhodamine-123 (DHR-123) as the probes), which is considered to be ideal and has been widely employed to monitor peroxynitrite in various biological systems [14,15]. Generally, the above methods are based on the oxidation of reduced, non-fluorescent forms of fluorescent dyes such as fluorescein and rhodamine by peroxynitrite to produce the parent dye molecule, resulting in a dramatic increase in fluorescence response. However, though these assays are commonly used, there are some controversy in the literatures concerning the mechanism of peroxynitrite-mediated oxidation of fluorescent indicators [16–18], and the synthesis of these probe molecules is rather difficult and inconvenient [7]. Additionally, the use of organic dyes is very likely to result in environmental pollutants and usually should be avoided.

Since peroxynitrite always originates and functions in vivo, it is significant to seek for some methods for the determination of peroxynitrite that fit to living biological system. On basis of our experiences in researching ROS utilizing enzymatic reactions [19–22], we consider this kind of method might also be an interesting and qualified approach for peroxynitrite that meets the above demand. In the present tentative work, a peroxidase catalyzed redox reaction between

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peroxynitrite and another reducer is studied and applied in determination. Hemoglobin (Hb) is used as the mimetic enzyme for substituting horseradish peroxidase (HRP). There are two main reasons for doing so. Firstly, just like in our previous work, it is for the purpose of the mildness of reaction conditions and the minimization of cost. Hb is the respiring albumen in blood cells of the amniotes, due to the high similarity of its porphyrin (Fe) structure to that of HRP, Hb is capable of being used as a peroxidase substitute in many catalytic oxidation reactions. Its peroxidatic characteristics and enzymatic kinetics have been studied systematically in our laboratory during last few years [22–25]. Secondly and more weightily, since Hb is a kind of nature protein in human body it is much likely to coexist with peroxynitrite in cell or organism.

Tyrosine is chosen as the reduced substrate in this work. As we all have known, based on the nitration of tyrosine with peroxynitrite, 3-nitro-tyrosine has normally been acted as the detecting target for the determination of peroxynitrite with spectrophotometry [26,27]. It is also considered as the marking substance of $ONOO^-$ injury in vivo. Whereas in this work, with Hb catalyzing, L-tyrosine is directly oxidized by peroxynitrite to produce a fluorescent product other than 3-nitro-tyrosine. Thus a new fluorometric method is built up for peroxynitrite determination. The kinetics and possible mechanism of the enzymatic reaction is also discussed.

The other notable and important point of the work is that since all partners of the reaction system are seen in living organism and such reaction is very likely to occur in vivo, it is reasonably presumed that the enzymatic reaction may be another pathway in which the biologic macromolecules are injured by $ONOO^-$ and the fluorescent product, the molecular structure of which is still in further research, may be a new marking substance of $ONOO^-$ injury. And we believe the work might give a fillip to peroxidase-based researches on peroxynitrite in future.

2. Experimental

2.1. Reagents and apparatus

L-Tyrosine (Tianyuan Biotechnological Co., Wuhan, China), a stock solution (1.00×10^{-3} mol L^{-1}) is prepared by dissolving 0.0090 g of L-tyrosine in 50.00 mL water (containing 1.00 mL 0.10 mol L^{-1} HCl); hemoglobin (Lizhu Reagent Co., Shanghai, China), a stock solution (1.00×10^{-4} mol L^{-1}) is prepared by dissolving 0.3400 g Hb in 50.00 mL water; Superoxide dismutase (SOD, 3000 U mL^{-1}) is purchased from Sigma; A 0.10 mol L^{-1} dimethyl sulfoxide (DMSO) solution is prepared; 0.20 mol L^{-1} Tris–HCl buffer solutions with varying pH values are prepared. Distilled, de-ionized water is used throughout. All the solutions are stored in a refrigerator (4 °C).

For fluorometric measurements, a Perkin-Elmer LS55 spectrofluorometer equipped with 1 cm quartz cell is used. The excitation and emission wavelength slits are respectively set at 15.0 and 5.0 nm. An UVIKON-941 (Kontron Inc.) spectrophotometer is used for absorbance measurements. Kinetic experiments are performed on an SX18MV-R stopped-flow analyzer (Applied Photophysics, UK). The experimental temperature, both for the quantitative determination and kinetic researches, is controlled with a Shimadzu TB-85 thermostat bath. For pH measurement, a TOA Electronics Model PHS-3C precision pH meter (Shanghai, China) is used.

2.2. Preparation of peroxynitrite standard solution

The standard peroxynitrite is prepared according to methods reported previously [28], to simply describe the procedure, 25.00 mL solution containing 1.00 g sodium nitrite of analytical reagent purity is added with 25.00 mL of distilled water containing 1.50 mL of 35% hydrogen peroxide and 0.40 mL of 96% sulphuric acid. The resulting mixture is promptly poured into 50 mL of aqueous solution containing 2.50 g sodium hydroxide. The excess H_2O_2 is removed by 0.40 g MnO_2. The product is diluted by 0.30 mol L^{-1} NaOH and filtrated to eliminate the excess MnO_2 and placed in a thermostat. The product is determined with UV–vis spectrophotometry (λ_{max} = 302.0 nm) and the concentration of peroxynitrite is found to be approximately 4.00–7.00 $\times 10^{-4}$ mol L^{-1}. Aliquots of peroxynitrite are monitored spectrophotometrically to accurately determine the concentration before each experiment.

2.3. Preparation of NO donor standard solution

About 50.00 mL of reduced GSH (2.00×10^{-2} mol L^{-1}) is prepared in citric acid buffer (1.00×10^{-3} mol L^{-1}, pH = 2.00). About 50.00 mL of KNO_2 (2.00×10^{-2} mol L^{-1}) is prepared in NaCl solution (9.0%). The above two solutions are mixed and the pH is adjusted to 2.00. A yellow solution of NO donor is obtained and stored in a refrigerator (4 °C). The concentration of this NO donor solution is determined accurately with UV–vis spectrophotometry (λ_{max} = 338.0 nm).

2.4. Methods for determination of peroxynitrite

In a set of 10 mL-colorimetric tubes, 0.20 mL of L-tyrosine (1.00×10^{-3} mol L^{-1}), 5.00 mL of Tris–HCl buffer solution (pH = 8.50), a series of different amount of $ONOO^-$ and 20.00 μL of hemoglobin (1.00×10^{-4} mol L^{-1}) are added in order, and then the reaction solution is diluted to the mark with the buffer solution. The reaction solution is kept at room temperature for 10 min. The fluorescence intensity of the solution is recorded at 410.0 nm with the excitation wavelength of 320.0 nm.

2.5. Detection of peroxynitrite in adriamycin-treated yeast cells

When yeast culture enters the stationary phase, cells are harvested by centrifugation for 3 min at 5000 rpm and subsequently washed twice with 0.20 mol L^{-1} Tris–HCl (pH = 8.5). The same amounts of cells are suspended in the same volume of buffer in closed screw-cap cuvettes. A taken group with adriamycin (100 μg mL^{-1}) added and a control group are prepared synchronously. Both groups are then incubated for 20 h with shaking at 28 °C. Cells are harvested by centrifugation and washed twice with 0.20 mol L^{-1} Tris–HCl (pH = 8.5), transferred to a glass homogenizer and homogenized completely on ice. The homogenate is centrifuged for 10 min at 12000 rpm under 4 °C. The suspension is taken for analysis. DMSO (0.10 mol L^{-1}), a specific scavenger for $^{\bullet}OH$ and SOD (3 U mL^{-1}), a specific scavenger for $O_2^{\bullet -}$, are introduced to the cell extraction to eliminate the possible interference of $^{\bullet}OH$ and $O_2^{\bullet -}$. Under the optimal experimental conditions, the samples including the taken group and control group are detected fluorimetrically. For standard additions and recovery experiments, yeast cells treated with various concentration of adriamycin are taken as samples.

2.6. Procedures for dynamic experiments

Stopped-flow fluorescence analysis is performed to acquire kinetic parameters. One stream containing various amount of L-tyrosine, buffer and Hb is mixed with the other stream containing $ONOO^-$ and buffer in the flow cell with a 20 μL cubage. The slit width of excitation and emission wavelength are both set at 2.5 nm and the dead time is set to 1 ms. The fluorescence at 320.0/410.0 nm (excitation/emission) is recorded under a constant temperature within the initial 10 s after sampling, and the initial reaction rate (represented as dFI/d*t*) under different concentrations of L-tyrosine are calculated from such kinetic curves (FI–*t*). Further, an initial rate versus tyrosine concentration curve is plotted so that the Michaelis–Menten constant K_m and V_{max} are obtained.

3. Results and discussion

3.1. Spectra characteristics of the system and possible structure of the product

The substrate, L-tyrosine has its inherent fluorescence with the maximum excitation and emission wavelength located at 225.0 and 305.0 nm, respectively. After being oxidized by $ONOO^-$ with Hb catalyzing, the fluorescence of L-tyrosine decreased greatly or disappeared completely, meanwhile a new fluorescent substance appeared with the maximum emission wavelength located at 410.0 nm. The fluorescence spectra of both the substrate and the product are shown in Fig. 1. It is seen the oxidization of the substrate is quite complete

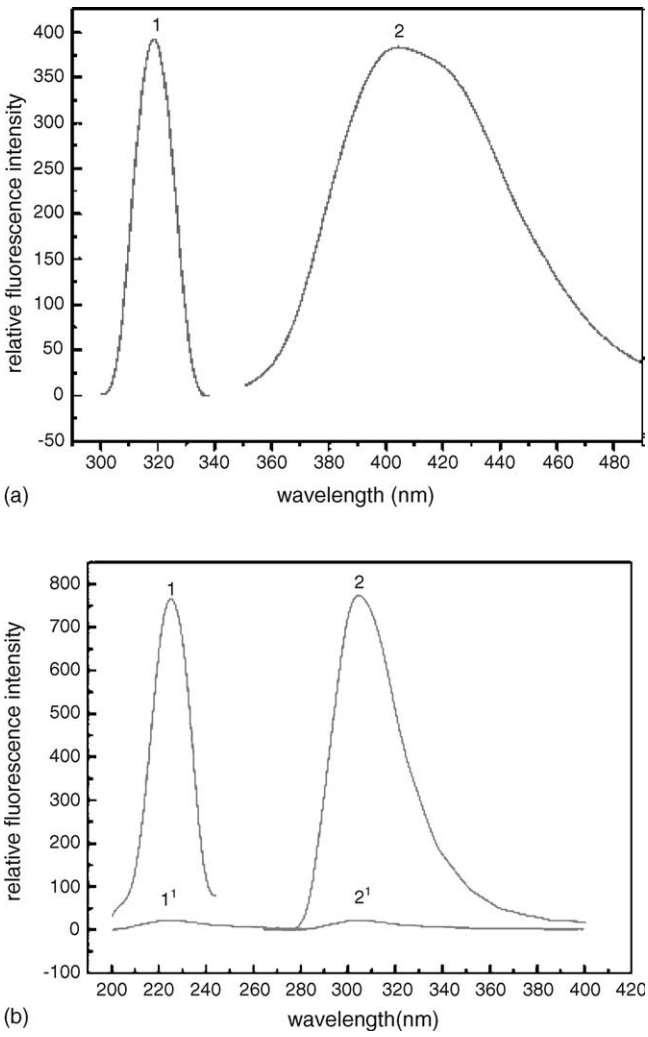

Fig. 1. (a) (1) The excitation spectra of the product, (2) the emission spectra of the product; (b) (1) the excitation spectrum of L-tyrosine, (2) the emission spectrum of L-tyrosine, (1′) the excitation spectrum of L-tyrosine after adding $ONOO^-$, (2′) the emission spectrum of L-tyrosine after adding $ONOO^-$.

and the spectral signal of the product is fully free from interference from that of the substrate. The fluorescence of the product has two excitation peaks, which locates at 250.0 and 320.0 nm, respectively. Consider that strong violet light may probably result in the decomposing of the compounds in the reaction system, the relatively long wavelength, 320.0 nm is chosen as the excitation wavelength in the following experiments.

In those works concerning $ONOO^-$ injury, it is well established that the tyrosine residue is nitrified to generate 3-nitrotyrosine that has no intrinsic fluorescence but a maximum absorbance around 428.0 nm [29,30], which is detected spectrophotometrically. Whereas in our experiments, the product is a strong fluorescent substance with no absorbance around 428.0 nm.Thus it is safely excluded the production of 3-nitro-tyrosine in this reaction. As has been proved in literatures, peroxidase-catalyzed oxidation of substrates having similar molecular structure to that of tyrosine, i.e., *p*-cresol,

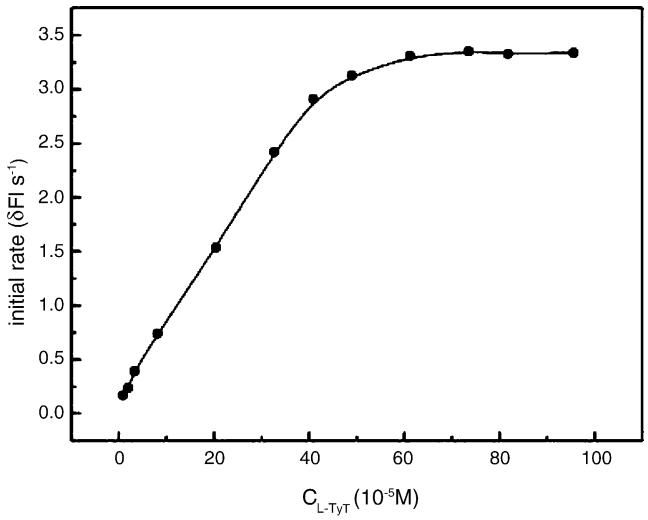

Fig. 2. Relationship between the reaction rate and reaction time under different concentrations of L-tyrosine. Concentration: $ONOO^-$, 2.40×10^{-5} mol L^{-1}; Hb, 2.00×10^{-6} mol L^{-1}; Tris–HCl buffer solution, pH 8.50, 22 °C.

would always yield polymers of the substrate [31,32]. And an early research reported that L-tyrosine was directly oxidized by H_2O_2 with manganese-tetrasulfonatophthalocyanine catalyzing to produce coupled dimer [33]. In this work, the formation of the dimer of tyrosine is further verified with NO donors–inhibitors of dimer [34]. With introducing the standard solution of NO donor to the reaction mixture before peroxynitrite, the fluorescence signal disappears, which indicates that the NO donor restrains the formation of the dimeric product.

3.2. Kinetics and possible mechanism of the reaction

With dynamic experimental approach, the kinetics of this catalytic reaction is studied. By recording the FI–*t* (relative fluorescence intensity versus time) curves under different concentrations of tyrosine, the relationship between the initial reaction rate (represented as dFI/d*t*) and the substrate concentration is thus obtained, which is shown in Fig. 2. It is discovered that the initial rate is proportional to the concentration of tyrosine at comparatively lower concentration level, which accords with the kinetic property of first-order reactions; with the concentration of tyrosine increasing, the reaction turns to be the so-called mixed order reaction. And finally, when the concentration of tyrosine becomes even higher, i.e., up to 4.90×10^{-4} mol L^{-1} in this system, the initial rate does not increase any longer, in other words, the reaction has turned into a zero-order reaction due to the fact that Hb, the enzyme, has already been saturated by tyrosine, the substrate. All the experimental results are corresponding to the Michaelis–Menten's law, which describes enzyme-based kinetic behaviors, indicating the oxidation of tyrosine by peroxynitrite in the presence of Hb is a quite typical enzymatic reaction. According to Fig. 2, the values of Michaelis–Menten constant K_m and V_{max} are acquired as 2.27 mmol L^{-1} and 3.34 ΔFI s^{-1}, respectively.

Studies have shown that the oxidation of various substrates by peroxynitrite ($ONOO^-$/ONOOH) can take place via different pathways [7]: (i) peroxynitrite can directly oxidize the substrates; (ii) peroxynitrite may decompose into highly reactive species ($^\bullet OH$, $^\bullet NO_2$), which subsequently oxidizes the substrate or hydroxylates and nitrates aromatic compounds. In the present work, the possible pathway of the oxidation of tyrosine by peroxynitrite is studied. DMSO (0.10 mol L^{-1}), a specific scavenger for $^\bullet OH$, is introduced to the reaction mixture before the addition of peroxynitrite, and the signal of the system is found to be almost unchanged, indicating that $^\bullet OH$ does not contribute to the fluorescence increase of the system. Similarly, a specific scavenger for $O_2^{\bullet -}$, SOD (3 U mL^{-1}), is added to the reaction system, and the fluorescence of the system also shows no changes compared with that in the absence of SOD, proving that $O_2^{\bullet -}$ does not mediate the generation of the fluorescent product. The above results suggest that the oxidation of tyrosine must have raised from peroxynitrite itself, but not its decomposed reactive species. Combined the above results of kinetic studies with other reported opinions concerning the mechanism of the reaction between peroxynitrite and tyrosine [35–37], the reaction pathway is proposed as follows. Firstly, hemoglobin is oxidized by peroxynitrite to form methemoglobin, which will promote the isomerization of peroxynitrite. Subsequently, in the presence of the carbonate radical anion – the product of peroxynitrite isomerization, tyrosyl radicals are generated. Meanwhile, methemoglobin is quickly back-reduced to Hb. Finally, the dimeric product is formed through the polymerization of tyrosyl radicals.

3.3. Optimization of determination conditions

As is well known, in an acid circumstance, the majority of peroxynitrite will present as its conjugate acid, ONOOH, which is rather unstable and easy to decompose. Thus the determination is performed under alkaline conditions controlled with buffer solutions. Having studied several kinds of buffer solutions with similar pH range, i.e., Tris–HCl, NH_3–NH_4Cl, Na_2HPO_4–KH_2PO_4 and so on. Tris–HCl is preferred due to the highest sensitivity. Then the pH dependence of the system is investigated over the range from pH 7.20 to 9.10 using Tris–HCl buffer solution, which is shown in Fig. 3. It can be seen the detection signal (FI) of the system increases with the pH value up to 8.40, and there exists a platform on the curve within the pH range of 8.40–8.80. Hence the Tris–HCl buffer with pH 8.50 is chosen in the subsequent experiments. It is reported that peroxynitrite has a pK_a value of 6.80, that is to say, in a slightly alkaline environment (pH 8.50), about 85% of peroxynitrite will present in the form of $ONOO^-$ [27], indicating that it is mainly $ONOO^-$ who oxidized tyrosine to yield a highly fluorescent product in this system, which is consistent with the oxidizing nature of $ONOO^-$.

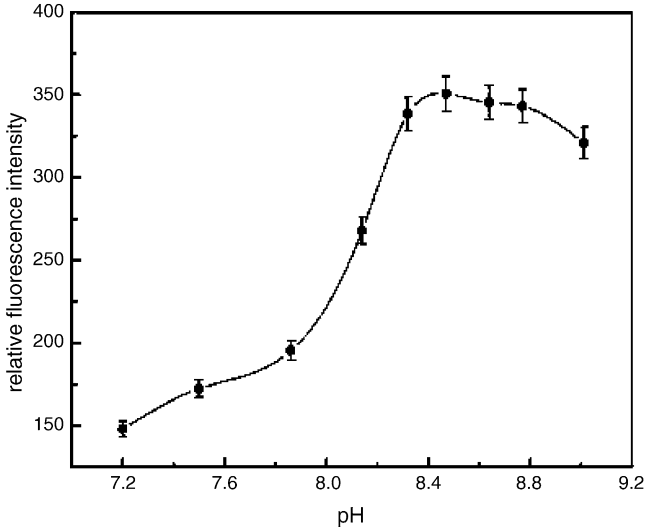

Fig. 3. The influence of pH on the system. Concentration: L-tyrosine, 2.00×10^{-5} mol L^{-1}; $ONOO^-$, 1.00×10^{-5} mol L^{-1}; Hb, 1.00×10^{-7} mol L^{-1}; Tris–HCl buffer solution.

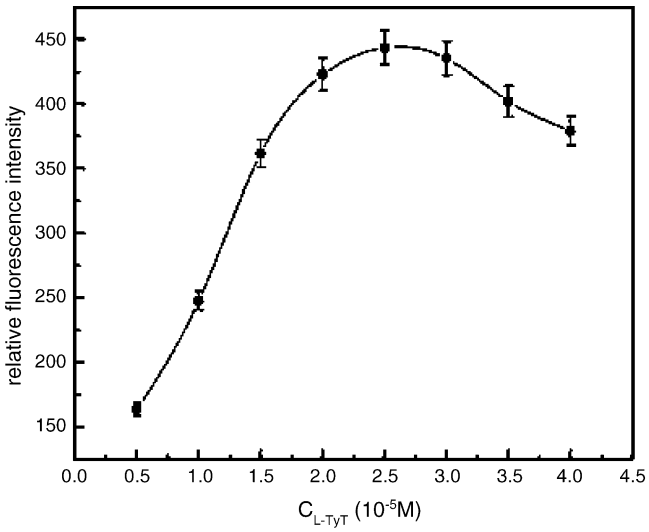

Fig. 5. The influence of the concentration of L-tyrosine on the system. Concentration: $ONOO^-$, 2.40×10^{-5} mol L^{-1}; Hb, 1.00×10^{-7} mol L^{-1}; Tris–HCl buffer solution, pH 8.50.

As has been pointed out above, the Hb catalyzed reaction between L-tyrosine and $ONOO^-$ is thoroughly different from that without Hb catalyzing. Like most works concerning enzymatic reactions, the concentration of the catalyst is always an important factor influencing the performance of the method and is essential to decide its appropriate amount. In our experiment, there is nearly no fluorescent product generated in the absence of Hb, and the fluorescence intensity increases with Hb concentration increasing till 2.00×10^{-7} mol L^{-1}, as is shown in Fig. 4. With Hb concentration higher than 2.00×10^{-7} mol L^{-1} the fluorescence signal decrease instead, which is often seen in enzymatic reactions. Besides, the reproducibility of the signal becomes poor with even higher Hb. Considering the sensitivity as well as the reproducibility, 2.00×10^{-7} mol L^{-1} of hemoglobin is recommended in the following determination.

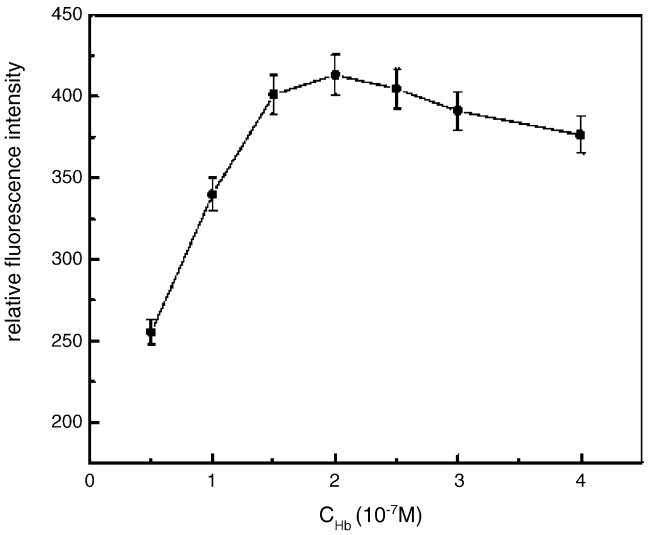

Fig. 4. The influence of the concentration of Hb on the system. Concentration: L-tyrosine, 2.00×10^{-5} mol L^{-1}; $ONOO^-$, 2.40×10^{-5} mol L^{-1}; Tris–HCl buffer solution, pH 8.50.

The concentration of substrate in enzymatic reactions is always a very important factor for the system. Too low concentration of substrate will unavoidably cause signal loss because of the incompleteness of reactions. Meanwhile, improperly high concentration of substrate will be extremely detrimental to the catalyst and also contribute to the blank. For the designed reaction completed and the signal fully obtained, less consumption of substrate is certainly better. The influence of L-tyrosine concentration on the fluorescence intensity of the system is also studied in this work. It is shown in Fig. 5 that the detection signal (FI) reaches to the maximum at about 2.00×10^{-5} mol L^{-1} of L-tyrosine, which is then used during the following experiments.

The temperature dependence of the reaction rate is one of the characteristics of enzymatic reactions and the experimental temperature is always precisely controlled. The reaction rate in the range of 4–50 °C was obtained, which is shown in Fig. 6 as a typical bell-mouthed curve. The optimal temperature of this Hb catalyzed reaction is located at 22 °C.

3.4. Analytical performances of the method

The calibration curve for peroxynitrite obtained from the above mentioned determination procedure is linear ranging from 5.60×10^{-7} to 2.10×10^{-5} mol L^{-1} with a correlation coefficient 0.9983, which is shown in Fig. 7. The detection limit, calculated according to the three S_b/m criterion, in which 'm' is the slope of the range of the linearity used and 'S_b' the standard deviation ($n = 9$) of the blank solution, is found to be 5.00×10^{-8} mol L^{-1}. The relative standard deviation (R.S.D.) at a 5.60×10^{-6} mol L^{-1} peroxynitrite is 3.80% ($n = 7$), and is 4.30% ($n = 8$) at 8×10^{-7} mol L^{-1}

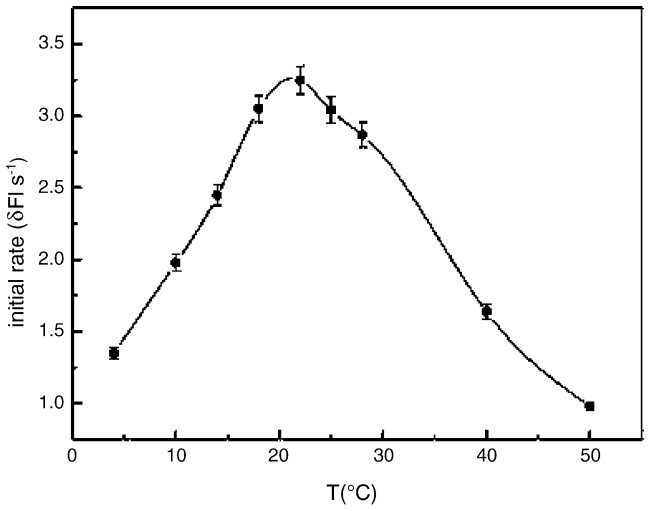

Fig. 6. The influence of temperature on the reaction rate. Concentration: L-tyrosine, 5.00×10^{-4} mol L^{-1}; $ONOO^-$, 2.40×10^{-5} mol L^{-1}; Hb, 1.00×10^{-7} mol L^{-1}; Tris–HCl buffer solution, pH 8.50.

peroxynitrite, which shows a good reproducibility of this method.

In order to demonstrate the accuracy of the new method, standard control experiments are carried out with spectrophotometry, the standard quantitative method for peroxynitrite based on its absorbance at 302.0 nm, as the comparison. A series of peroxynitrite solution with varying concentrations are parallel determined by both spectrophotometry and the proposed enzymatic fluorometric method. The results obtained from both methods, as shown in Table 1, reveal a good correlation ($r = 0.998$) and no statistical difference between the two means using a paired *t*-test. It is seen that the new method is comparatively precise.

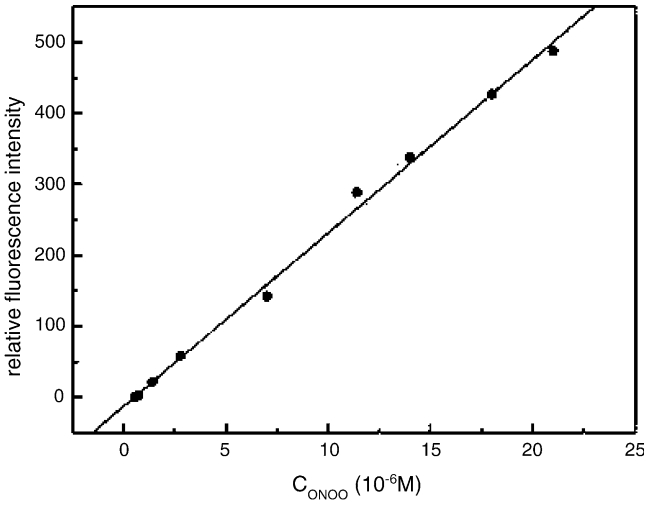

Fig. 7. The calibration curve in the range of 5.60×10^{-7} to 2.10×10^{-5} mol L^{-1} $ONOO^-$. Concentration: L-tyrosine, 2.00×10^{-5} mol L^{-1}; Hb, 1.00×10^{-7} mol L^{-1}; Tris–HCl buffer solution, pH 8.50.

Table 1
Comparison on determination results (mol L^{-1}) between this method and standard spectrophotometry

Sample no.	Standard method	This method	Relative error (%)
1	1.57×10^{-5}	1.59×10^{-5}	−1.3
2	1.25×10^{-5}	1.19×10^{-5}	5.0
3	9.19×10^{-6}	9.28×10^{-6}	−0.9
4	5.36×10^{-6}	5.30×10^{-6}	1.1
5	6.35×10^{-7}	6.63×10^{-7}	−4.0

r: correlation coefficient between the two methods (0.998).

3.5. Interference studies

Interferences from some amino acids, metal ions normally seen in biological samples, as well as anions structurally similar to $ONOO^-$ are considered. At the same time, since a little amount of H_2O_2 is possibly remained in the prepared $ONOO^-$ solution [28], and it is also capable of oxidizing the substrate as a kind of ROS, it is obligatory to investigate the disturbance of H_2O_2 on the system. With the $ONOO^-$ concentration fixed at 2.40×10^{-6} mol L^{-1}, those foreign substances under study are added into the system with different concentrations. Their influences on the determination are presented as tolerance, which means the added intruder has lead to a change of the relative fluorescence intensity with less than 5%. Results in Table 2 show a fairly satisfactory selectivity of the method. Especially, the tolerance of H_2O_2 is quite high, which may be explained in that the reactivity of peroxynitrite is 2000 times greater than H_2O_2 [38], it is prior to react with the reduced substrate. But the method allows rather low amount of Mn^{2+} and Fe^{3+}, which might have come from the disturbance of them on the catalytic center of Hb, the iron contained heme.

3.6. Analysis of biological samples

It is well known that $ONOO^-$ is generated in adriamycin-induced cell injury. Results of fluorimetric detection of yeast cell samples are shown in Fig. 8. It clearly exhibits the char-

Table 2
Tolerance of the method to some foreign substances

Foreign substances	Tolerance
NO_2^-	1000
NO_3^-	1000
H_2O_2	100
Glucose	1000
Albumin	400
Glycine	200
Glutamine	100
Phenylalanine	100
Tryptophan	500
Cu^{2+}	200
Mn^{2+}	8
Ca^{2+}	600
Na^+	1000
K^+	1000
Fe^{3+}	10

Table 3
Standard additions and recovery data of yeast samples[a]

Sample no.	$ONOO^-$ in samples ($\times 10^{-6}$ mol L^{-1})	$ONOO^-$ added ($\times 10^{-6}$ mol L^{-1})	$ONOO^-$ found ($\times 10^{-6}$ mol L^{-1})	Recovery (%)
1	2.47	0.65	2.92	93.6
		2.34	4.68	97.3
2	2.01	0.65	2.34	88.0
		2.04	3.92	96.8
3	1.40	0.65	1.79	87.3
		1.17	2.29	89.1

[a] The final adriamycin concentration for samples 1–3 are 83, 50 and 17 μg mL^{-1}, respectively.

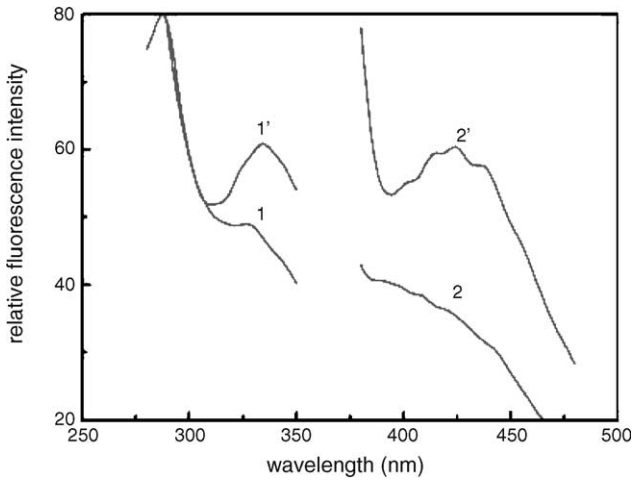

Fig. 8. The fluorescence spectra of biological samples: (1,1′) the excitation spectra of the yeast homogenate with and without adriamycin treatment; (2,2′) the emission spectra of the yeast homogenate with and without adriamycin treatment. Adriamycin: 100 μg mL^{-1}.

acteristic excitation and emission spectra in the taken group, i.e., the homogenate of yeast cells treated with adriamycin, whereas no such signal is detected in the control group. However, because of the rather strong background in the samples, quantitative results are not obtained yet. With addition of external peroxynitrite standard solutions to cells treated with adriamycin, the recovery data are obtained and shown in Table 3, which are average values of three repeated determinations.

4. Conclusion

A new method for determination of peroxynitrite, an attention-getting ROS, is built up with satisfactory sensitivity and selectivity. It is based on the oxidation of L-tyrosine by peroxynitrite in the presence of Hb. Biological sample is analyzed, and peroxynitrite in adriamycin-treated yeast cells is detected with the proposed method. The enzymatic essence of the redox reaction is revealed by kinetic studies, and Michaelis–Menten constant K_m and V_{max} are acquired. Possible pathway of the reaction and the structure of the product are recommended. Peroxynitrite is thought to be able to directly oxidize L-tyrosine under the catalysis of Hb, which produces a dimeric substance with strong intrinsic fluorescence emitted at 410.0 nm. The new product obviously differs from 3-nitro-tyrosine, the recognized product in non-enzymatic reactions, which is traditionally thought of as the marking substance of peroxynitrite injury of cells. Since Hb is much likely to coexist with peroxynitrite within living body, the enzymatic reaction might probably be another pathway of such injury to cells or organism, especially proteins with tyrosine residual.

Acknowledgements

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References

[1] R.P. Patel, D. Moellering, M.U. Joanne, Free Radical Biol. Med. 28 (2000) 1780.
[2] C. Laura, B.A. Freeman, Nutrition 17 (2001) 161.
[3] P. Sovitj, M.R. Gerald, J. Chem. Soc., Perkin Trans. 998 (1998) 1507.
[4] D.U. Victor, H. Wiseman, B. Halliwell, FEBS Lett. 369 (1995) 131.
[5] C.M. Boulanger, J. Mol. Cell. Cardiol. 31 (1999) 39.
[6] C. Szabo, Brain Res. Bull. 41 (1996) 133.
[7] X.F. Yang, X.Q. Guo, Y.B. Zhao, Talanta 57 (2002) 883.
[8] H. Possel, H. Nosck, A. Wolfgang, FEBS Lett. 416 (1997) 175.
[9] J. Liang, R.-X. Cai, Z.X. Lin, J. Wang, Chem. J. Internet 5 (2003) 42.
[10] D. Kang, A.G. Vlessidis, N.P. Evmiridis, Talanta 59 (2003) 55.
[11] R.A. Wheatley, M. Saryahmeto¢glu, B. Çakýcý, Analyst 125 (2000) 1902.
[12] S.B. Digerness, K.D. Harris, J.W. Kirklin, F. Urthaler, L. Vierera, J.S. Beckman, Free Radical Biol. Med. 27 (1999) 1386.
[13] T. Hayashi, K. Yamada, T. Esaki, H. Kano, Atherosclerosis 147 (1999) 349.
[14] N.W. Kooy, J.A. Royall, H. Ischiropoulos, J.S. Beckman, Free Radical Biol. Med. 16 (1994) 149.
[15] H. Possel, H. Noack, W. Augustin, G. Keilhoff, G. Wolf, FEBS Lett. 416 (1997) 175.
[16] D. Jourd'heuil, F.L. Jourd'heuil, P.S. Kutchukian, R.A. Musah, D.A. Wink, M.B. Grisham, J. Biol. Chem. 276 (2001) 28799.
[17] N.W. Kooy, J.A. Royall, H. Ischiropoulos, Free Radical Res. 27 (1997) 245.

[18] J. Glebska, W. Koppenol, Free Radical Biol. Med. 35 (2003) 676.
[19] Z.-H. Liu, R.-X. Cai, L.Y. Mao, H.P. Huang, W.H. Ma, Analyst 124 (1999) 173.
[20] H. Yuan, R.-X. Cai, Z.T. Pan, Anal. Lett. 36 (2003) 278.
[21] Z.-H. Liu, Q.L. Wang, L.Y. Mao, R.-X. Cai, Anal. Chim. Acta 413 (2000) 167.
[22] K. Zhang, L.Y. Mao, R.-X. Cai, Talanta 51 (2000) 179.
[23] Y.H. Chen, R.-X. Cai, Spectrochim. Acta A 59 (2003) 3357.
[24] K. Zhang, R.-X. Cai, D.H. Chen, L.Y. Mao, Anal. Chim. Acta 413 (2000) 109.
[25] R.Y. Wang, Z.-H. Liu, R.-X. Cai, X.Y. Li, Anal. Sci. 18 (2002) 977.
[26] A.R. Butler, T.J. Rutherford, D.M. Short, J.H. Ridd, Chem. Commun. 7 (1997) 669.
[27] J.S. Beckman, W.H. Koppenol, Am. J. Physiol. 271 (1996) 1424.
[28] Dr. Henry, M. Papee, Dr. Gianna, L. Petriconi, Nature 204 (1964) 142.
[29] H.P. Claudia, K. Sailesh, E.G. Shuker David, Nitric Oxide: Biol. Chem. 2 (1998) 324.
[30] L.F. Liao, Y.Y. He, Ch.X. Liu, Y.L. Yuan, G.R. Li, Prog. Biochem. Biophys. 24 (1997) 450.
[31] G.G. Guillbault, E. Hockney, Anal. Chem. 40 (1968) 1256.
[32] Z.-H. Liu, R.-X. Cai, L.Y. Mao, H.P. Huang, W.H. Ma, Analyst 124 (1999) 173.
[33] Ch.Q. Zhu, D.H. Li, H. Zheng, Q.Zh. Zhu, J.G. Xu, J. Xiamen Univ. (Nat. Sci. Ed.) 40 (2001) 68.
[34] P. Ferdinandy, R. Schulz, Antioxid. Redox Signal 3 (2001) 165.
[35] S. Herold, K. Shivashankar, Biochemistry 42 (2003) 14036.
[36] X.C. Célio, et al., Arch. Biochem. Biophys. 377 (2000) 146.
[37] D. Pietraforte, et al., Biochemistry 40 (2001) 15300.
[38] G.T. Vatassery, Biochem. Pharmacol. 52 (1996) 579.