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Determination of tyrosine by sodium fluorescein-enhanced ABEI–H_2O_2–horseradish peroxidase chemiluminescence

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Abstract

In this study, N-(4-aminobutyl)-N-ethylisoluminol (ABEI) was used as an energy donor, while sodium fluorescein was used as an enhancer and energy acceptor, which resulted in it producing resonance energy transfer and greatly increasing the strength of chemiluminiscence (CL). When horseradish peroxidase (HRP) is added, hydrogen peroxide (H_2O_2) will quickly separate into hydroxyl radicals (·OH) and superoxide ions ($O_2·^-$). If tyrosine (Tyr) is present in the system, the hydroxyl group on the benzene ring of Tyr robs ·OH and $O_2·^-$ in the CL system, thereby reducing the intensity of CL. Based on this phenomenon, a luminescence system of ABEI and sodium fluorescein system was established to detect Tyr for the first time. This method has an ultra-low detection limit and a wide linear range, and is cheap and easy to operate. Under various optimal conditions, the linear range is from 3.0×10^{-8} to 3.0×10^{-5} mol/L, and the limit of detection is 2.4×10^{-8} mol/L. It has been successfully used in the detection of dairy products with satisfactory results.

Keywords: *ABEI*, Chemiluminescence, Sodium fluorescein, Tyrosine

Introduction

Chemiluminiscence (CL) refers to the light emitted during chemical reactions (usually in the visible and near-infrared regions). CL method has been widely used in the determination of a variety of samples because of its high sensitivity, simple setup, short measurement time, and free from the interference of background scattered light. So far, it has become a broad and practical analytical method in many fields such as molecular biology, clinical chemistry, environmental science, and food analysis (Wang et al. 2020). Luminol is the most important luminescent substance in the luminescence system, and the application of luminol is very common (He and Cui 2012; Dong et al. 2017); therefore, this article will focus on the CL system of its derivatives.

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This article is mainly based on CL resonance energy transfer (CRET) principle, which mainly refers to the non-radiative energy transfer phenomenon of CL donors by fluorescent acceptors. Compared with fluorescence resonance energy transfer (Cai et al. 2019), CRET is produced by the oxidation of the light-emitting substrate without an excitation light source (Zhao et al. 2010; Li et al. 2009). The sample studied has no autofluorescence, so it can improve the corresponding sensitivity. There have been a few reports on CRET research so far today (Zhou and Yoon 2012; Zhao et al. 2009; Huang and Ren 2010; Liu et al. 2019; Guo et al. 2007; Zhang et al. 2014; Yang et al. 2015; Ohtomo et al. 2012; Yi et al. 2018; Zhou and Yoon 2012).

Tyrosine (Tyr) is a semi-essential amino acid that constitutes protein and plays an important role in maintaining the nitrogen balance of the human body. Secondly, Tyr is the precursor of dopamine, neurotransmitter, and thyroxine in the central nervous system of mammals, played an important role in regulating hormones in the body. When the concentration of Tyr is high, it may lead to increased sister chromosome exchange. When the concentration is low, mood disorders such as Parkinson's disease and depression will generally occur. Therefore, the rapid and accurate determination of Tyr content plays an important role in pharmacology (He et al. 2019). The main methods for determining Tyr are high-performance liquid chromatography (Li et al., 2015, b), gas chromatography-mass spectrometry (Zhou et al. 2019), CL, and enzymatic methods (Liu et al. 2016).

In this study, a new method for detecting Tyr was established, which can maintain a high intensity and stable CL and greatly shorten the reaction time. In this experiment, N-(4-aminobutyl)-N-ethylisoluminol (ABEI) was used as an energy donor. Sodium fluorescein is used as an enhancer and CRET energy acceptor, which can significantly enhance the intensity of the CL system. In this method, Tyr can snatch reactive oxygen radicals such as hydroxyl radicals (·OH) and superoxide ions ($O_2·^-$) produced by hydrogen peroxide (H_2O_2) after being oxidized under weakly alkaline conditions, thereby reducing the CL intensity and showing a wider linearity and a lower detection limit. On this basis, a CL method for determining Tyr content in yogurt, milk, and goat milk was developed.

Experimental

Reagents and chemicals

Trichloroacetic acid (TCA) and horseradish peroxidase (HRP) were purchased from Macleans Biochemical Technology Co. Ltd. (Shanghai, China); ABEI, thiourea, and ascorbic acid (AA) were supplied by Aladdin Chemical Reagent Co. Ltd. (Shanghai, China); sodium fluorescein was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan); and Tyr was from Bailingwei Technology Co. Ltd. (Beijing, China). Sodium hydroxide, 2,2,6,6-tetramethylpiperidinooxy (TEMPO), histidine, superoxide dismutase (SOD), and H_2O_2 (30%, v/v) were obtained from Beijing Chemical Works (Beijing, China). All of the reagents used in the experiment were of analytical grade and were not further purified. Ultrapure water was used throughout the current work.

ABEI (0.022 g) was dissolved in a Britton-Robinson (BR) buffer (2 mL, 0.04 mol/L) to prepare a 4.0 mmol/L ABEI stock solution and stored in the refrigerator for at least 3 days. The H_2O_2 working solution was daily prepared by dilution with deionized water.

Apparatus

For sample processing, a desktop high-speed centrifuge TG16G (Chengdu Yike Instrument Co. Ltd.) was used. The CL spectra were captured by RFL-1 ultra-weak CL detector (Xi'an Remai Analytical Instrument Co.). The CL spectra of the relationship between the wavelength and the intensity were captured by closing the excitation slit on the F-2700 spectrofluorometer (Hitachi, Japan). The absorption spectra were recorded with a UV-3100 UV-VISNIR spectrophotometer (Shimadzu, Japan).

Procedure of CL measurement

First, 20 μL ABEI (4.0 mmol/L), 60 μL H_2O_2 (0.5 mmol/L), 60μL Tyr (0, 3×10^{-8}, 4×10^{-8}, 1×10^{-7}, 1×10^{-6}, 2×10^{-6}, 3×10^{-6}, 5×10^{-6}, 7.5×10^{-6}, 1×10^{-5}, 1.5×10^{-5}, 2×10^{-5}, 3×10^{-5}mol/L), and 1 mL sodium fluorescein (1.5 mmol/L) solution were mixed and were placed at room temperature for 10 min, to make the reaction more fully. Then, the above solution was transferred to the glass tube in the dark room sample chamber. Finally, within 2 s, 50 μL of HRP (3.5 mg/mL) was injected into the glass tube with a static syringe, and the CL dynamic curve was recorded.

Results and discussion

Enhanced effect of sodium fluorescein

Sodium fluorescein can enhance the ABEI–H_2O_2 reaction through the energy transfer process. As shown in Fig. 1a after adding ABEI alone (black line), there is a weak CL peak at 425 nm, and when sodium fluorescein is added, the strong emission of sodium fluorescein at a long wavelength of 535 nm is indicated (blue line). Sodium fluorescein absorbs part of the energy in the excited state ABEI and re-emits it at long wavelengths. However, when only sodium fluorescein is added, there is no change in intensity (red line). The effect of sodium fluorescein concentration on energy transfer is observed in this figure. The enhancement, which can be attributed to the increased overlap between CL and acceptor absorption, modified the ABEI CL maximum from 440 to 410 nm.

We also verified the effect of HRP on the ABEI-H_2O_2 CL reaction as shown in Fig. 1b. First, the entire experimental conditions were carried out under the action of sodium fluorescein. When ABEI and HRP are added to the system, there is no change in the CL intensity (curve a). When ABEI and H_2O_2 were added to it, it was found that the system produced a weak CL strength (curves b). But when HRP was added to it, the weak CL strength was significantly enhanced (curves d), further indicating that the catalytic activity of HRP mainly acts on H_2O_2. However, when Tyr was added to it, the CL intensity was significantly reduced (curve c), so based on this phenomenon, a new method was established to detect Tyr.

Optimization of reaction conditions

In order to establish a sensitive method for the determination of Tyr, according to the principle of the controlled variable method, the reaction conditions of the ABEI–H_2O_2–HRP-sodium fluorescein system were optimized accordingly. Since BR buffer has the widest pH

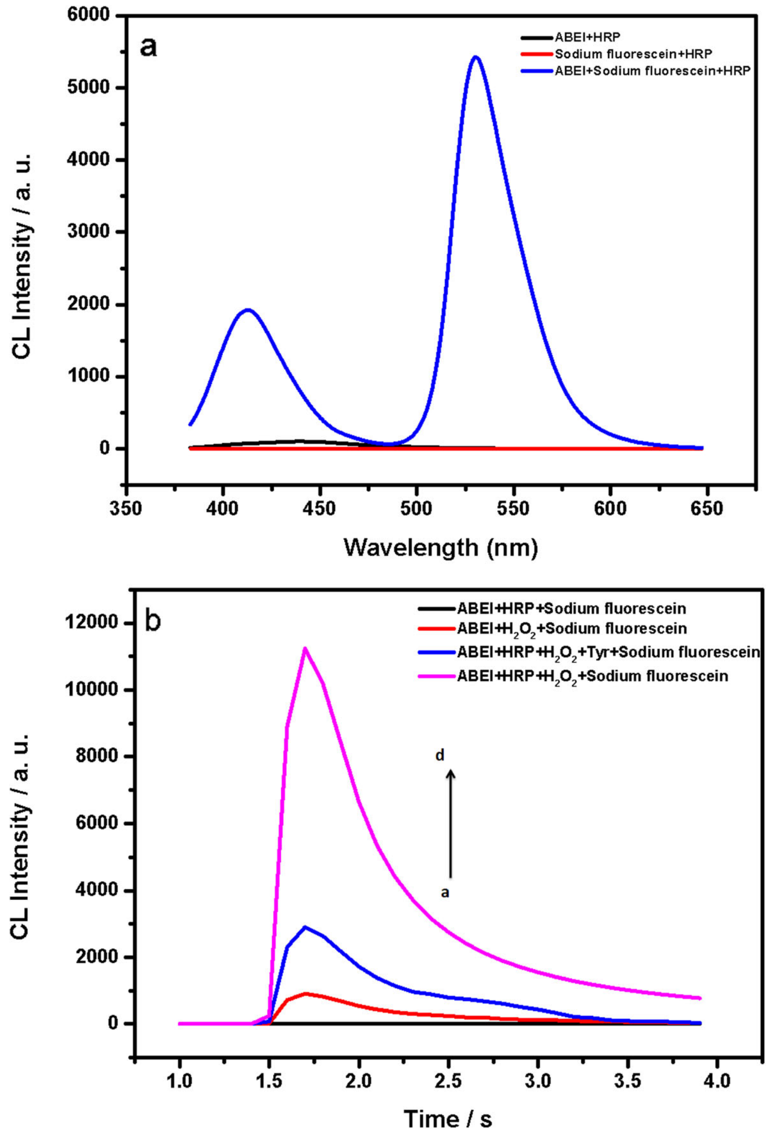

Fig. 1 **a** CL kinetic curves of sodium fluorescein-HRP (red curve) with H_2O_2, ABEI-HRP-sodium fluorescein (blue curve) with H_2O_2, and HRP-ABEI (black curve) with H_2O_2; **b** ABEI+HRP+sodium fluorescein (curve a), ABEI+H_2O_2+sodium fluorescein (curve b), ABEI+HRP+H_2O_2+Tyr+sodium fluorescein (curve c), and ABEI+HRP+H_2O_2+sodium fluorescein (curve d) CL kinetic curve of reaction

range, we use BR buffer as the solution to optimize pH. As shown in Fig. 2a, the CL intensity gradually increased from pH 4.0 to 7.8, and then dropped sharply after 7.8. Therefore, in this experiment, pH 7.8 is the optimal condition for this reaction.

Then, the influence of different buffer solutions on the system was studied. Different buffer solutions (BR buffer solution, Tris-HCl buffer solution, HEPES buffer solution, and PBS buffer solution) were configured with a concentration of 0.018 mmol/L and pH=7.8. As shown in Fig. 2b, the CL intensity of the system was the strongest in the BR buffer solution, so we chose the BR buffer solution (pH=7.8; 0.018 mmol/L) as the buffer solution.

Since the concentration of sodium fluorescein also has a greater impact on the intensity of CL, the change of CL intensity at different concentrations was studied. As shown in Fig. 2c, when other conditions remain unchanged, only with the increase of the concentration of sodium fluorescein, the CL intensity gradually increases. When it is 0.701 mmol/L, the CL intensity reaches the maximum, so 0.701 mmol/L is used as the optimal concentration of sodium fluorescein.

Figure 2d shows the effect of ABEI concentration on CL response. As the concentration of ABEI gradually increases, you will find that CL gradually increases when the concentration is in the range of 4.7×10^{-3} to 3.7×10^{-2} mmol/L, reaches its highest point at a concentration of

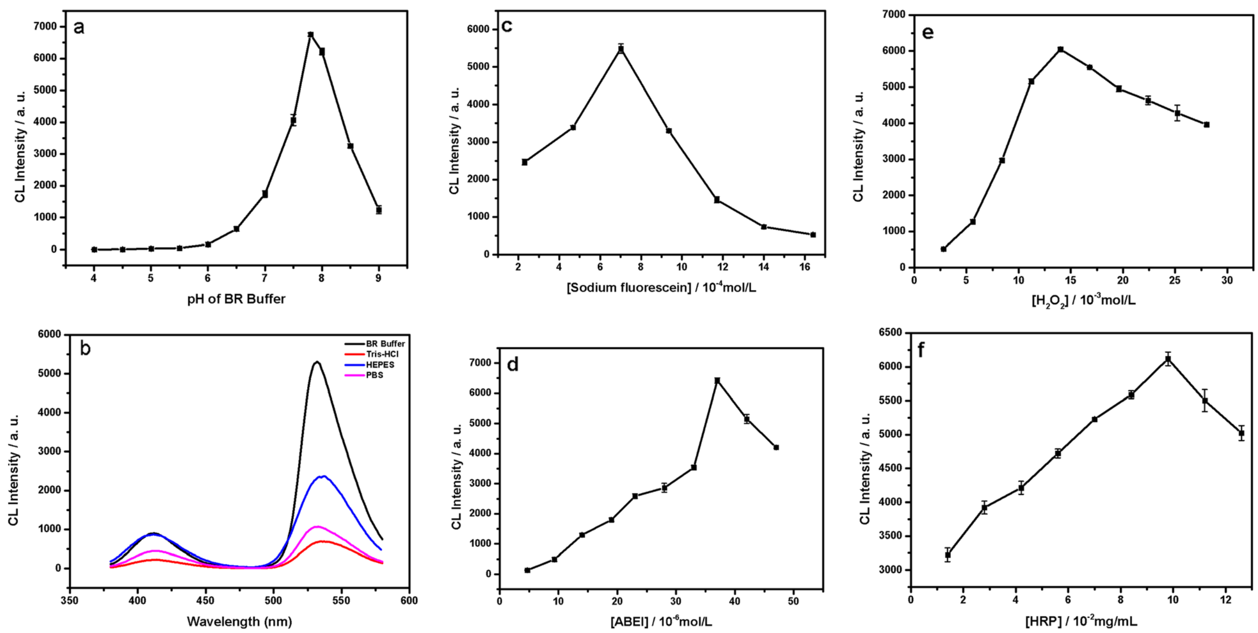

Fig. 2 **a** Effect of BR buffer pH on CL emission. Experimental conditions: c(ABEI)=0.037 mmol/L, c(sodium fluorescein)=0.701 mmol/L, c(HRP)=0.098 mg/mL, c(H_2O_2)=0.014 mol/L. **b** CL kinetic curves of the sodium fluorescein-ABEI–H_2O_2–HRP system at different buffered solution: BR buffer (black curve), PBS buffer (pink curve), Tris-HCl buffer (red curve), HEPES buffer (blue curve). Experimental conditions: c(ABEI)=0.037 mmol/L, c(sodium fluorescein)=0.701 mmol/L, c(HRP)= 0.098 mg/mL, c(H_2O_2)=0.014 mol/L. **c** Effect of sodium fluorescein concentration on CL emission. Experimental conditions: c(BR)=0.018 mmol/L at pH 7.8, c(ABEI)=0.037 mmol/L, c(HRP)=0.098 mg/mL, c(H_2O_2)=0.014 mol/L. **d** Effect of ABEI concentration on CL emission. Experimental conditions: c(BR)=0.018 mmol/L at pH 7.8, c(sodium fluorescein)=0.701 mmol/L, c(HRP)=0.098 mg/ml, c(H_2O_2)=0.014 mol/L. **e** Effect of H_2O_2 concentration on CL emission. c(BR)=0.018 mmol/L at pH 7.8, c(ABEI)=0.037 mmol/L, c(Sodium fluorescein)=0.701 mmol/L, c(HRP)=0.098 mg/mL. **f** Effect of HRP concentration on CL emission. Experimental conditions: c(BR)=0.018 mmol/L at pH 7.8, c(ABEI)=0.037 mmol/L, c(sodium fluorescein)=0.701 mmol/L, c(H_2O_2)=0.014 mol/L

3.7×10^{-2} mmol/L, and then gradually decreases. Therefore, we finally chose the best concentration of ABEI to be 3.7×10^{-2} mmol/L. In addition, we also checked the influence of H_2O_2 concentration. When the concentration of H_2O_2 is in the range of 2.8×10^{-3} to 1.4×10^{-2} mol/L, the CL intensity increases rapidly. When the concentration continues to increase, the intensity will gradually decrease, as shown in Fig. 2e, so we choose 1.4×10^{-2} mol/L as the optimal concentration of H_2O_2. Finally, we optimized HRP accordingly. As shown in Fig. 2f, as the concentration of HRP increases, the intensity of CL gradually increases. Finally, we choose 0.098 mg/mL as its optimal concentration.

In summary, we finally determined that the reaction conditions are in the BR buffer solution with pH=7.8, sodium fluorescein concentration is 0.701 mmol/L, ABEI concentration is 0.037 mmol/L, H_2O_2 concentration is 0.014 mol/L, and HRP concentration is 0.098 mg/mL as the optimal reaction conditions of the system.

Possible mechanism of the CL system

As an oxidoreductase, HRP itself can catalyze the decomposition of H_2O_2, and then react with luminol to generate luminol free radicals, which enhance the strength of CL. ABEI is an analog of luminol, which can follow a similar mechanism to trigger ABEI.

This section roughly speculates the corresponding reaction mechanism (Fig. S1). First, HRP is oxidized by H_2O_2 to generate ·OH and $O_2\cdot^-$ and the first form of HRP. After that, the oxidized HRP continues to interact with ABEI anions (AH^-) generating ABEI free radicals ($A\cdot^-$). Sodium fluorescein is a CL enhancer, and it can produce two effects to increase in the CL intensity of the system. One is sodium fluorescein can increase the production of ABEI free radicals more effectively and the other is CL resonance energy transfer between ABEI and sodium fluorescein. When we add sodium fluorescein, sodium fluorescein free radicals ($SF^{\cdot -}$) are generated, and the

Table 1 Effects of various free-radical scavengers on the ABEI–H_2O_2–HRP-sodium fluorescein CL system

Quenchers	Intermediates	Concentration	Percent inhibition, %
TEMPO	·OH, $^1O_2$	1 mmol/L	63.8
Ascorbic acid	·OH, $O_2\cdot^-$	1 mmol/L	98.7
Thiourea	·OH	1 mmol/L	53.7
Histidine	$^1O_2$	1 mmol/L	12.3
SOD	$O_2\cdot^-$	0.1 μg/mL	90.2

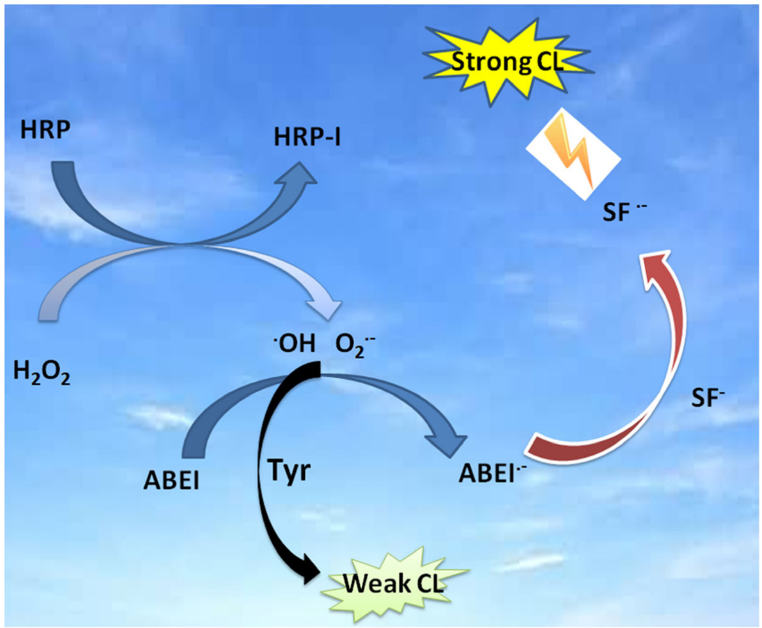

Fig. 3 The reaction process of the whole system

color of solution changes from bright green to orange (Fig. S2). The newly generated sodium fluorescein free radicals can accelerate the production of ABEI free radicals from ABEI-negative ions. When there is no energy transfer in the system, the ABEI free radicals are first oxidized by H_2O_2 to generate ABEI endoperoxide, thereby producing 4-((4-aminobutyl)(ethyl)amino) phthalate formate, and the emission wavelength is 440 nm (Yi et al. 2018; Freeman et al. 2011). When sodium fluorescein anion is present in the solution, the excited state ABEI inner peroxide can react with it to produce the emission wavelength at 535 nm, which is consistent with the

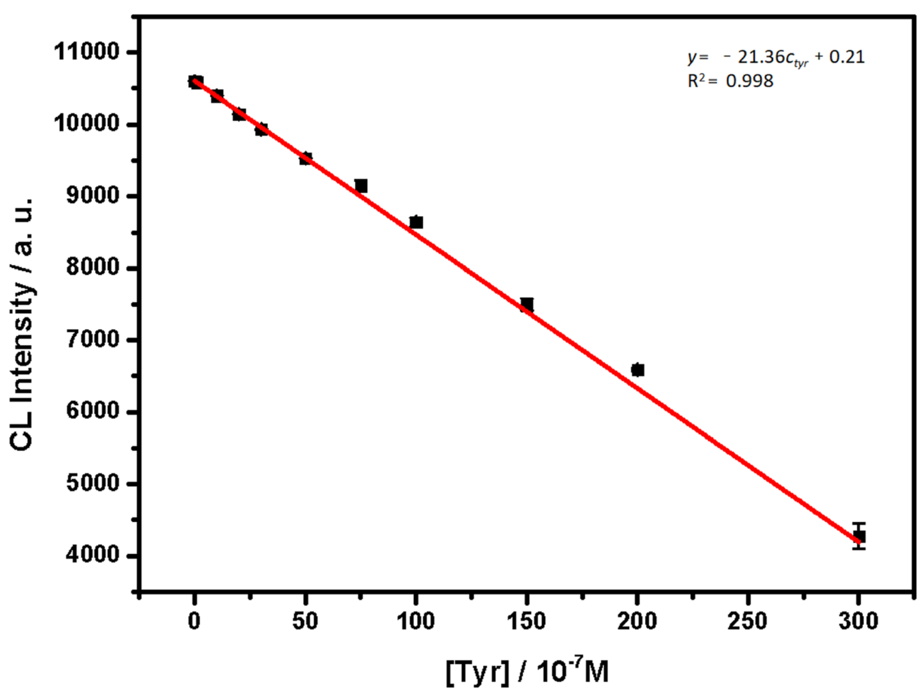

Fig. 4 Calibration curve of Tyr. Experimental conditions: c (BR) = 0.0018 mmol/L at pH 7.8, c (ABEI) = 0.037 mmol/L, c (sodium fluorescein)= 0.701 mmol/L, c (H_2O_2) = 0.014 mol/L, c (HRP) = 0.098 mg/mL

emission wavelength of the fluorescence spectrum of sodium fluorescein (Liu et al. 2015) (Fig. S3).

We also verified the role of different reactive oxygen radicals in the system. According to previous reports, TEMPO and AA are very effective free radical scavengers (Dai et al. 2008). When 1.0 mmol/L AA and TEMPO are added, it can effectively inhibit 63.8% and 98.7% of the CL signal, which proves that the active oxygen radicals generated by H_2O_2 in the whole process play an important role in the oxidation process (Wang et al. 2019). Similarly, several specific quenchers were added to the reaction, including thiourea, histidine, and SOD, which are quenchers of ·OH, singlet oxygen ($^1O_2$), and $O_2\cdot^-$, respectively. After adding a certain concentration of thiourea, histidine, and SOD, the CL signal was inhibited by about 53.7%, 12.3%, and 90.2%, respectively (Table 1). Therefore, we believe that ·OH and $O_2\cdot^-$ play an important role in this system (Liu et al. 2019). The reaction process is shown in Fig. 3. HRP first catalyzes H_2O_2 to produce ·OH and $O_2\cdot^-$, and ABEI in the system further reacts with those radicals to form ABEI radical ($A^{\cdot-}$), which produces strong CL intensity under the action of sodium fluorescein. Sodium fluorescein anion (SFH^-) itself becomes sodium fluorescein anion free

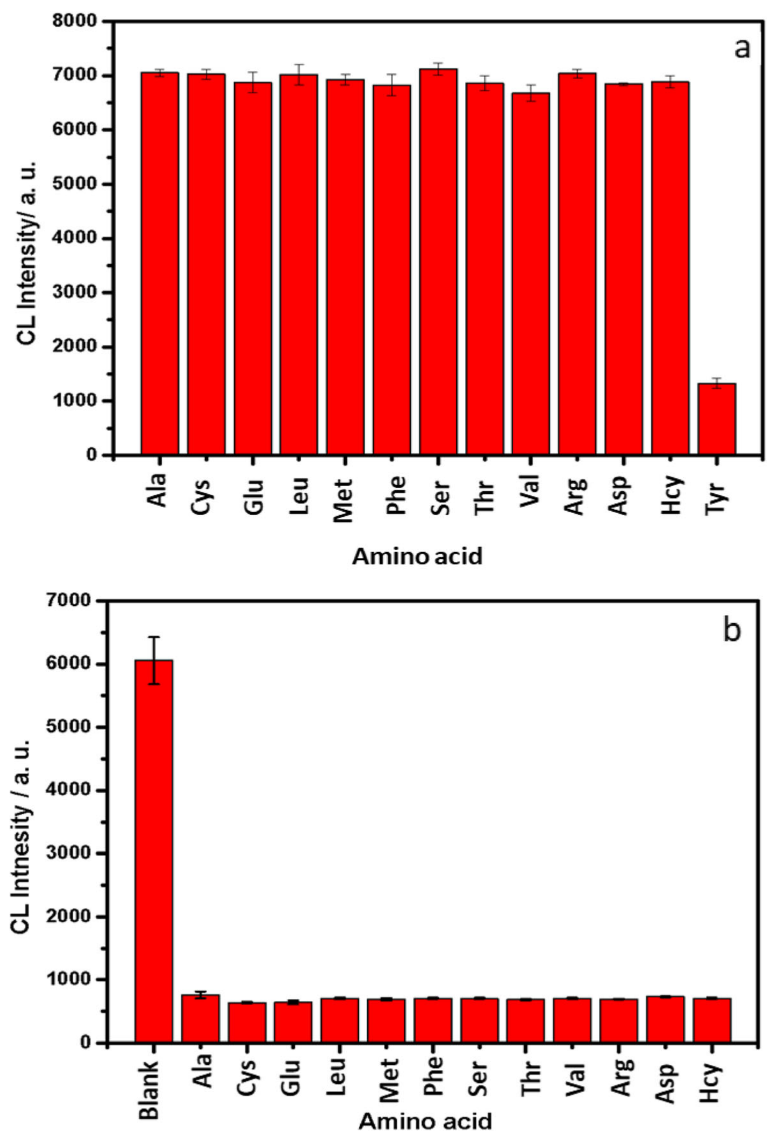

Fig. 5 Selectivity of the CL system for detecting Tyr (**a**) and anti-interference of Tyr (**b**) Experimental conditions: c(amino acids)=0.03 mmol/L, c(Tyr)=0.001 mmol/L, c(BR)=0.018 mmol/L at pH 7.8, c(sodium fluorescein)=0.701 mmol/L, c(ABEI)=0.037 mmol/L, c(H_2O_2)=0.014 mol/L, c(HRP)=0.098 mg/mL

Table 2 Results of the determination of Tyr in milk and its product samples

Samples	Added (μmol/L)	Found (μmol/L)	Recovery (%)
Powdered milk	0	2.46±0.0600	—
	1.5	4.01±0.101	103.2
	3.0	5.42±0.160	98.3
Goat milk powder	0	3.21±0.120	—
	1.5	4.68±0.070	98.0
	3.0	6.19±0.110	99.3
Yogurt	0	1.32±0.130	—
	1.0	2.28±0.090	96.0
	2.0	3.36±0.110	102.0
Fresh milk	0	2.52±0.080	—
	1.0	3.49±0.120	97.0
	2.0	4.57±0.070	102.0

radical ($SF^{\cdot -}$). But when Tyr exists, Tyr can snatch active oxygen free radicals such as ·OH, and $O_2 \cdot^-$ produced by H_2O_2 to produce weak CL strength (Pang et al. 2018).

Sensitive detection of tyrosine

Under the optimal experimental conditions described previously, the relationship between different Tyr concentrations and CL intensity was studied. As shown in Fig. 4, in the concentration range of 3.0×10^{-8} to 3.0×10^{-5} mol/L, CL shows a linear correlation, the detection limit is 2.4×10^{-8} mol/L, the linear equation is y= $-21.36c_{tyr}$+0.21, the relative standard deviation (RSD) of the system is 0.45%, and the corresponding evaluation coefficient is 0.998. In general, this method has good linearity, high accuracy, good sensitivity, and sufficient accuracy for testing Tyr. This result was better than most published literature (Table 2).

Selectivity

In order to test the selectivity of this method, under the same conditions, 0.03 mmol/L Ala, Cys, Glu, Leu, Met, Phe, Ser, Thr, Val, Arg, Asp, and Hcy and 0.001 mmol/L Tyr were added to the ABEI–H_2O_2–HRP-sodium fluorescein CL system. As shown in Fig. 5a, compared with other amino acids, only the addition of Tyr will reduce the strength of CL, and other semi-essential amino acids will not cause changes in CL. Also, in the anti-interference ability, the difference between the concentrations of Tyr and other interfering substances was 30 times. We would find that the addition of Tyr and other interfering substances to the system at the same time does not have a corresponding effect on it (Fig. 5b). The results show that the system has an effect on Tyr. It has good selectivity and anti-interference ability and can be applied to the detection of Tyr.

Inspection of tyrosine in milk samples

According to the previous literature (Yola et al. 2015; Pang et al. 2018), the Tyr in the milk power samples or yogurt sample needed to be extracted and diluted. First, 1 g milk power sample was dissolved with 25 ml of deionized water. Then, 2 mL of the above solution, yogurt sample, or fresh milk sample was mixed with 4 ml of trichloroacetic acid in a mixer for 20 s and centrifuged at 6000 rpm for 14 min, and the supernatant was transferred to another centrifuge tube. The above operation was repeated twice, and the final collected supernatant was filtered with a 0.45 micron filter. The filtrate can be used directly as a sample solution. To evaluate the practicability and dependability of the assay, the method of adding standard recovery experiment was used, and the results are shown in Table 3. The recovery of Tyr in samples ranged from 96.0 to 103.2%, which show that this method can be satisfactorily used for the determination of Tyr in goat milk powder, milk powder, yogurt, and fresh milk.

Conclusions

In summary, this article prepared an ABEI–H_2O_2–HRP CL system enhanced by sodium fluorescein for the detection of Tyr. Based on Tyr inhibitory effect on the CL system, a new method for detecting Tyr content was

Table 3 Comparison of different methods for detecting Tyr

Method	Linear range(mol/L)	Detection limit(mol/L)	References
Spectroscopy	1.0×10^{-7}–1.0×10^{-5}	1.0×10^{-7}	Satheeshkumar and Yang (2015)
Fluorescence	1.66×10^{-6}–1.1×10^{-4}	0.52×10^{-6}	Zhu and Xu (2010)
Fluorescence	1.0×10^{-6}–1.6×10^{-4}	0.5×10^{-6}	Li et al. (2016)
Chemiluminescence	5.5×10^{-6}–5.5×10^{-5}	0.275×10^{-6}	Sanfeliu Alonso et al. (2002)
Chemiluminescence	-	1.37×10^{-8}	Li et al. (2015, b)
Electrochemistry	1.0×10^{-5}–1.6×10^{-4}	2.3×10^{-6}	Fan et al. (2011)
Electrochemistry	8.0×10^{-7}-6.0×10^{-5}	7.0×10^{-8}	Baig and Kawde (2015)
HPLC	5.0×10^{-6}–7.5×10^{-4}	5.0×10^{-6}	Roman and Pavla (2009)
Chemiluminescence	3.0×10^{-8}–3.0×10^{-5}	2.4×10^{-8}	This work

proposed. The method has high sensitivity, wide linear range, low detection limit, and high accuracy, and is suitable for the detection of Tyr content in dairy products. Under the best experimental conditions, the detection range of Tyr is 3.0×10^{-8} to 3.0×10^{-5} mol/L, and the limit of detection (LOD) is 2.4×10^{-8} mol/L. The discussion on the detection mechanism believes that Tyr snatches ·OH and $O_2\cdot^-$ radicals and reduces its CL intensity. This system not only provides a new method for Tyr detection, but also needs a further development of its potential practical application.

Abbreviations
ABEI: N-(4-aminobutyl)-N-ethylisoluminol; Tyr: Tyrosine; CRET: Chemiluminescence resonance energy transfer; CL: Chemiluminescence; BR: Britton-Robinson; AA: Ascorbic acid; TEMPO: 2,2,6,6-tetramethylpiperidinooxy; SOD: Superoxide dismutase; TCA: Trichloroacetic acid; HRP: Horseradish peroxidase; Ala: Alanine; Lys: Cysteine; Glu: Glutamic; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Ser: Serine; Thr: Threonine; Val: Valine; Arg: Arginine; Asp: Aspartic; Hcy: Homocysteine; OH: Hydroxyl radicals; $O_2\cdot^-$: Superoxide ions; $^1O_2$: Singlet oxygen

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s40543-021-00272-8.

Additional file 1: Fig. S1. The mechanism of Sodium fluorescein enhanced reaction. $SF^{\cdot-}$=Sodium fluorescein free radical, AH^-=ABEI anion, $A^{\cdot-}$=ABEI radical, SFH^- = Sodium fluorescein anion. **Fig. S2.** The color of solution before and after chemiluminescence. **Fig. S3.** Fluorescence spectroscopy of sodium fluorescein (Excitation wavelength is 440nm).

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Authors' contributions
Fei Qiang and Shan Hongyan modified the format of the article, Feng Guodong and Xun Yanfu sorted out the data, Li Ming and Fan Qian conducted corresponding experiments, and Dong Bin finally wrote and summarized the text. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets of this manuscript are available upon request.

Declarations

Competing interests
The authors declare that they have no competing interest.

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