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Original Article

An easy and cost-effective colorimetric assay of hydrogen peroxide based on iodide-catalyzed oxidation of 3,3,5,5-tetramethylbenzidine

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Abstract: Objective: Hydrogen peroxide (H_2O_2) is a key reactive oxygen species in industrial production and biological processes. Detection of H_2O_2 in an easy and cost-efficient way still remains challenging. Methods: Here we first reported a novel catalytic reaction system in which iodide could catalyze the oxidation reaction of a colorless peroxidase substrate 3,3,5,5-tetramethylbenzidine into a yellow product by H_2O_2. Since the noticeable color change can be distinguished by naked eye or spectrophotometric measurement, it can be utilized to establish a colorimetric detection method for H_2O_2. Results: The results of our study showed that using the iodide-mediated catalytic reaction strategy, a quantitative and selective assay of H_2O_2 in solution was successfully achieved with an incubation at 55°C for 10 min in the presence of 0.1 M H_2SO_4. It was found that a concentration of H_2O_2 as low as 0.5 μM could be discriminated by naked eye and a concentration as low as 0.2 μM could be detected using spectroscopic analysis. In addition, this testing approach could also be applied effectively to detect iodide with a detection limit of 0.25 μM. Conclusion: Our study demonstrated a simple, rapid, low-cost, sensitive and selective method for detection of both H_2O_2 and iodide, which can be used for the analysis of various H_2O_2-related substances.

Keywords: Hydrogen peroxide, iodide, catalytic oxidation, 3,3,5,5-tetramethylbenzidine, colorimetric detection

Introduction

Hydrogen peroxide (H_2O_2) has a strong oxidizing property and is widely used in various fields, including organic synthesis, food production, paper bleaching, as well as pharmaceutical, clinical and environmental analysis [1]. In addition, as one of the major reactive oxygen species in living organisms, H_2O_2 also plays a significant role in many biological processes such as cell signaling [2]. At present, a variety of methods, including fluorimetric, chemiluminescent, high performance liquid phase chromatography (HPLC)-based, and electrochemical assays have been developed for detecting H_2O_2 [3-9]. Although these techniques have been used in the H_2O_2-related studies, they still have some disadvantages. For example, the fluorimetric and chemiluminescent assays require fluorescent compounds or chemiluminescent nanoparticles (NPs) and the preparation processes are quite complicated [3, 4]; the instruments required in HPLC assays are expensive, which can limit the application of this method; the electrochemical technique has unstable electrode modification and troublesome washing steps, despite the fact that it has intrinsic sensitivity, high selectivity and low cost [7-9]. Thus, there is a great demand for an easy, inexpensive, selective and sensitive method to detect H_2O_2.

With advantages of simplicity, easy operation and no need of any costly or advanced instrument, colorimetric assay is of particular interest in chemical and biological analytical research fields [10]. Based on different mechanisms, including the catalyst- or enzyme-mediated oxidation-reduction reaction of H_2O_2 and variation in the absorption spectra of substrates after direct reaction with H_2O_2, colorimetric methods for detecting H_2O_2 have been rationally established [11, 12]. In particular, kinetic catalytic colorimetric assays have unique properties of fast response, high efficiency and high selectivity, thus getting much researchers' attention [13, 14]. Several novel enzyme mimic-mediated catalytic colorimetric reaction systems have also been reported and used for H_2O_2 analysis, which involve positively-charged gold NPs, hollow Mn ferrite nanostructures, $CoFe_2O_4$ ferrite nanocubes, and prussian blue NPs [15-17]. Considering the catalytic effect of enzyme mimics on the oxidation-reduction reaction of H_2O_2, these approaches may serve as candidates for an easy and rapid colorimetric method to determine H_2O_2. However, they still exhibit some shortcomings such as troublesome preparation process (complicated NP synthesis and purification procedure) and high detection limit.

Interestingly, we recently discovered for the first time that iodide could catalyze the oxidation of 3,3,5,5-tetramethylbenzidine (TMB) by H_2O_2. In this reaction system, iodide is a commercially available and low-cost reagent, while TMB is a commonly used substrate in peroxidase-based detection system due to its soluble oxidized product with high absorption coefficients for color discrimination by naked eye and spectrophotometric quantification [18-21]. Therefore, with the aim of developing a simple, rapid and cost-efficient test method for H_2O_2, we investigated the iodide-mediated catalytic reaction of TMB and H_2O_2 for the first time and optimized a series of influence factors, including pH, temperature and time. A sensitive and selective colorimetric assay of H_2O_2 has thus been proposed based on the iodide-catalyzed oxidation of TMB in this study. In addition, we have also found that this reaction system can be applicable for detecting iodide.

Materials and methods

Chemicals and materials

TMB was purchased from Sigma-Aldrich (St. Louis, MO). Potassium iodide (KI) was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Unless otherwise noted, all reagent-grade chemicals were used as received without further purification. Deionized water was prepared by the Milli-Q ultrapure water system (18.2 MΩ·cm^{-1}, Millipore System Inc.).

Instruments

A multifunctional microplate reader (Infinite M1000, TECAN Austria GmbH) was used to record the absorption spectra from 300 nm-600 nm and the absorbance intensity at 450 nm of the reaction product at room temperature [22]. The photographs were taken with an Olympus C-370 digital camera.

Investigation of iodide-catalyzed TMB-H_2O_2 reaction

Feasibility of iodide-catalyzed TMB-H_2O_2 reaction was first investigated, during which 125 μL of 0.4 mM KI and H_2O (control) were mixed with 25 μL of 1 mM H_2O_2, 50 μL of 1.7 μM TMB and 50 μL of 5 M H_2SO_4, respectively. Next, all of the mixed solutions were incubated in a water bath at 45°C for 20 min and then cooled to room temperature. Photographs were taken immediately and 150 μL of the resulting solutions were added into a 96-well plate respectively. The absorption spectra of the oxidation product of TMB from 300 nm-600 nm were recorded.

Effect of the iodide concentration on the TMB-H_2O_2 reaction was analyzed as follows: 125 μL of 3 μM, 10 μM, 20 μM, 100 μM of KI were mixed respectively with 50 μL of 1.7 μM TMB, 50 μL of 0.5 M H_2SO_4 and 25 μL of 1 mM H_2O_2. Next, all of the mixed solutions were incubated in a water bath at 55°C for 10 min and then cooled to room temperature. Afterwards, 150 μL of the resulting solutions were added into a 96-well plate and the absorption spectra from 300 nm-600 nm were recorded. The specificity of the TMB-H_2O_2 reaction system for I^- was measured by the method similar to the one described above. In this method, I^- (10 μM) and other ions (100 μM) were compared, 150 μL of the resulting solutions were added into a 96-well plate and the absorption spectra at 450 nm were recorded. The experiments were repeated for three times.

Optimization of reaction conditions

To study the effects of different factors on the catalytic activity of iodide in the TMB-H_2O_2 reaction system, the pH, temperature and reaction time were investigated for the catalytic reaction. First, 50 μL of 5 M H_2SO_4, 0.5 M H_2SO_4, 0.05 M H_2SO_4, H_2O and 0.5 M NaOH were respectively mixed with 50 μL of 1.7 μM TMB, 125 μL of 1 mM H_2O_2 and 125 μL of 0.4 mM KI. Next, all of the mixed solutions were incubated in a water bath at 45°C for 20 min before

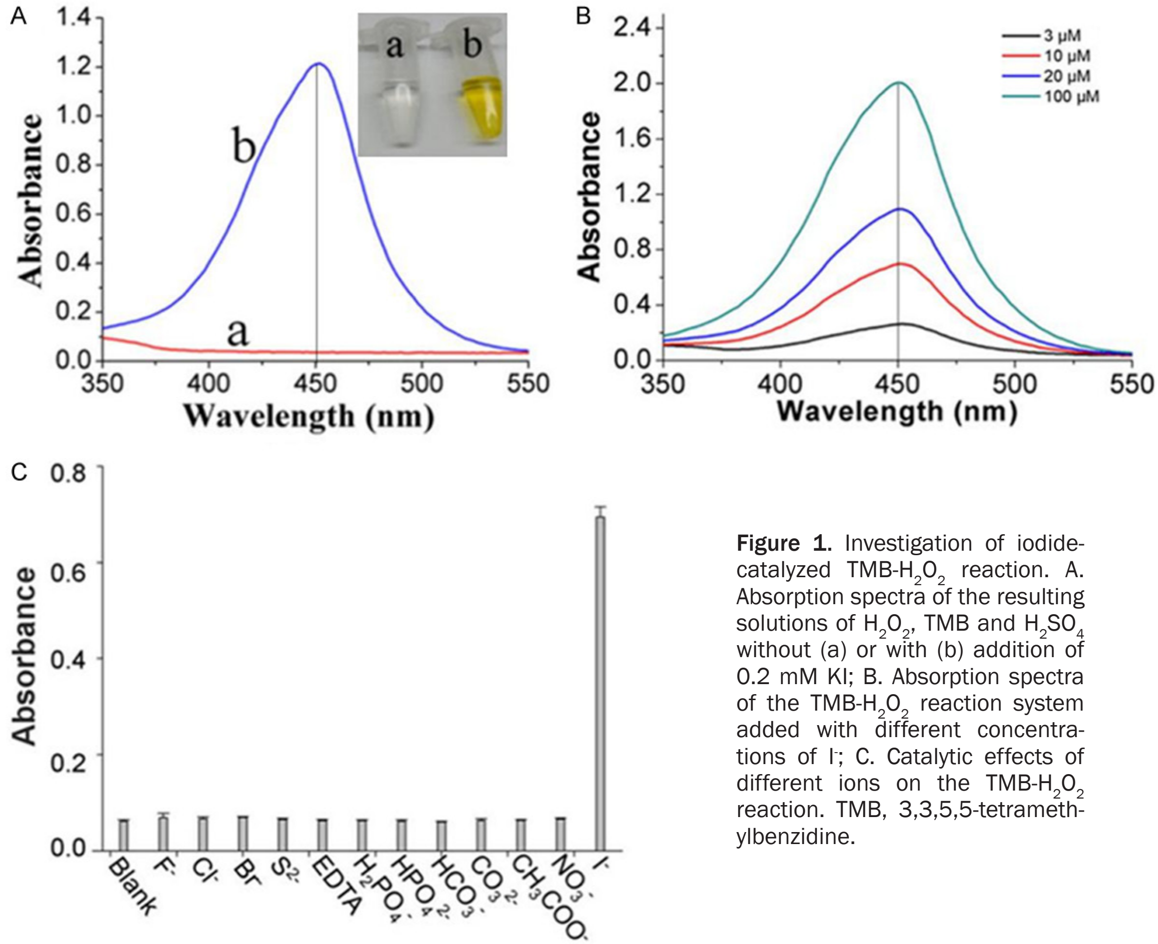

Figure 1. Investigation of iodide-catalyzed TMB-H_2O_2 reaction. A. Absorption spectra of the resulting solutions of H_2O_2, TMB and H_2SO_4 without (a) or with (b) addition of 0.2 mM KI; B. Absorption spectra of the TMB-H_2O_2 reaction system added with different concentrations of I^-; C. Catalytic effects of different ions on the TMB-H_2O_2 reaction. TMB, 3,3,5,5-tetramethylbenzidine.

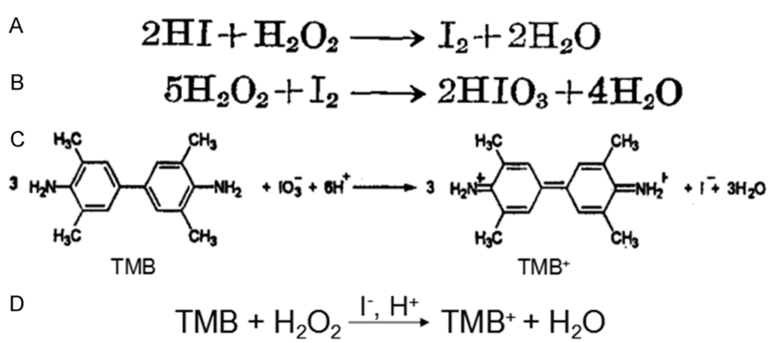

Figure 2. Possible chemical reaction mechanism of TMB-H_2O_2 reaction. A, B: I^- was oxidized to IO_3^- in the presence of H_2O_2 and H^+; C: IO_3^- was reduced to I^- while TMB was oxidized to a colored product; D: Possible chemical equation of TMB-H_2O_2 reaction. TMB, 3,3,5,5-tetramethylbenzidine.

cooled to room temperature. Afterwards, 150 μL of the resulting solutions were added to a 96-well plate and the optical density of each well at 450 nm was measured. Reaction temperature and time were investigated under the optimal condition of pH. The experiments were repeated for three times.

Detection of H_2O_2 using the iodide-catalyzed TMB-H_2O_2 reaction system

A typical colorimetric analysis for H_2O_2 detection was conducted as follows: 125 μL of 0 (H_2O), 0.5, 1, 2, 3, 4, 5, 20, 50, 500, 1,000 μM of H_2O_2 were added with 50 μL of 1.7 μM TMB, 50 μL of 0.5 M H_2SO_4 and 25 μL of 4 mM KI respectively. Next, all of the mixed solutions were incubated in a water bath at 55°C for 10 min and then cooled to room temperature. Photographs were taken immediately and 150 μL of the resulting solution was added into a 96-well plate, followed by the recording of the absorption spectra from 300 nm-600 nm and 450 nm. The experiments were repeated for three times.

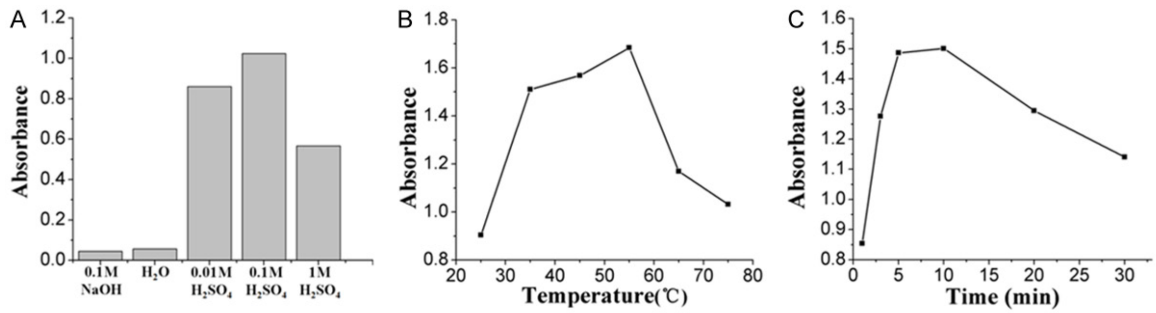

Figure 3. Effects of different conditions on the iodide-catalyzed TMB-H_2O_2 reaction. A: pH; B: Reaction temperature; C: Reaction time. TMB, 3,3,5,5-tetramethylbenzidine.

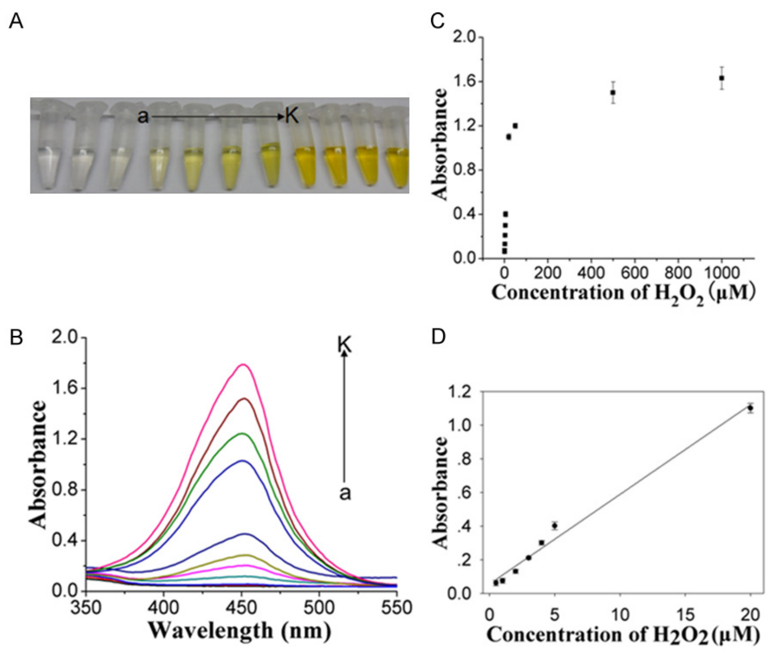

Figure 4. Detection of H_2O_2 with different concentrations based on the iodide-catalyzed oxidation of TMB. A: Photographs; B: Absorption spectra; C: The relationship between the absorbance at 450 nm and the concentration of H_2O_2; D: The linear response at low concentrations of H_2O_2 (from a to k: 0, 0.5, 1, 2, 3, 4, 5, 20, 50, 500 and 1,000 μM). TMB, 3,3,5,5-tetramethylbenzidine.

Detection of iodide using the iodide-catalyzed TMB-H_2O_2 reaction system

Detection of iodide was conducted as follows: 125 μL of 0 (H_2O), 1, 3, 4, 5, 10, 20, 40, 100, 200, 1,000 μM KI were mixed with 50 μL of 1.7 μM TMB, 50 μL of 0.5 M H_2SO_4 and 25 μL of 1 mM H_2O_2 respectively. Next, all of the mixed solutions were incubated in a water bath at 55°C for 10 min before cooled to room temperature. Afterwards, absorbance intensity at 450 nm of the resulting solution was recorded. The experiments were repeated for three times.

Results

Investigation of the iodide-catalyzed TMB-H_2O_2 reaction

In order to validate the catalytic activity of iodide in the TMB-H_2O_2 reaction, absorption spectra of mixed solutions containing TMB, H_2O_2 and H_2SO_4 added with or without I^- were measured and the corresponding photographs were taken. As shown in **Figure 1A**, it was found that the solution added without I^- exhibited no evident adsorption peak ranging from 300 to 600 nm, while with the addition of I^-, a noticeable peak centered at 450 nm appeared, which could be attributed to the oxidation of TMB producing a colored chemical TMB^+ that could be distinguished by naked eye. **Figure 1** shows the color change of the corresponding samples, and the result was in accord with the spectra variation mentioned above, demonstrating the ability of I^- for catalytic oxidation of TMB in the presence of H_2O_2. Absorption spectra of mixed solutions containing TMB, H_2O_2 and H_2SO_4 added with different concentrations of I^- were measured. As shown in **Figure 1B**, the absorbance intensity gradually changed from low to high with increasing I^- concentration, indicating the catalytic oxidization rate of TMB in the TMB-H_2O_2 reaction system was dependent on iodide concentration. Furthermore, the specificity of the TMB-H_2O_2 reaction system for I^- was measured. As displayed in **Figure 1C**, evident absorbance

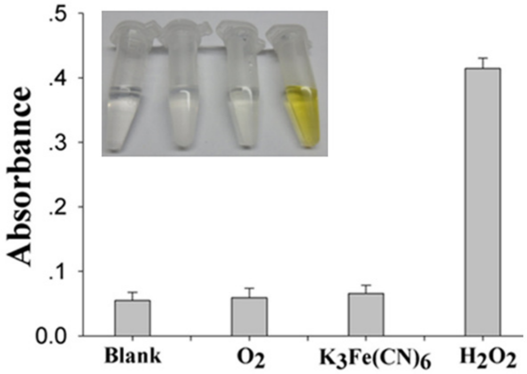

Figure 5. Selectivity of the iodide-catalyzed TMB-H_2O_2 reaction system for H_2O_2 detection. Comparison among 10 μM H_2O_2, 100 μM $K_3Fe(CN)_6$ and dissolved O_2 were conducted (inset: the corresponding photos showing the color change). TMB, 3,3,5,5-tetramethylbenzidine.

at 450 nm was observed in the presence of I^- compared with other ions, suggesting the dependent specificity of the catalytic system for I^-. We speculated that I^- was first oxidized to IO^-_3 in the presence of H_2O_2 and H^+ (**Figure 2A** and **2B**) and IO^-_3 was then reduced to I^-, while TMB was oxidized to a colored product (**Figure 2C**) [23]. Possible chemical equations are presented in **Figure 2D**. As demonstrated above, iodide can obviously accelerate the reaction rate for catalytic oxidization of TMB and works as a catalyst in the TMB-H_2O_2 reaction system.

Optimization of reaction conditions

The results of our study showed that factors such as pH, reaction temperature and reaction time all had impacts on the catalytic activity of iodide in the TMB-H_2O_2 reaction system.

When we studied the effect of pH on the iodide-catalyzed TMB-H_2O_2 reaction, we recorded the relationship between A_{450} (the absorption intensity of the oxidization product of TMB at 450 nm) and different pH conditions. As shown in **Figure 3A**, the result showed that the catalytic oxidation rate of TMB by H_2O_2 in the presence of iodide was much higher in acidic solution than in neutral and basic solution. In acidic solution, the reaction rate enhanced with increasing H_2SO_4 concentration up to 0.1 M and then decreased at higher concentrations. The reason for this may be due to the formation of IO^-_3, which can promote TMB to be oxidized to a colored product, depending on the co-existence of H_2O_2 and H^+. Therefore, a concentration of 0.1 M H_2SO_4 was chosen as the optimal acidic concentration for the colorimetric assay in the subsequent experiments.

Similarly, in order to obtain the optimal reaction temperature for this colorimetric assay, different temperatures ranging from 25°C to 75°C were explored and the relationship between A_{450} and temperature was examined. As shown in **Figure 3B**, it was found that A_{450} first gradually increased with the rise of temperature up to 55°C and then decreased at higher temperature. The decrease in A_{450} at high temperature might be caused by the inhibition of the reaction or the destruction of the TMB oxidation product. Thus, 55°C was chosen as the optimal reaction temperature for the colorimetric assay in the whole experiment.

Also, the reaction time was optimized as displayed in **Figure 3C**. We found that an increase in reaction time from 1 to 10 min could lead to an increase in the absorption intensity. However, no further elevation of the absorption intensity was observed afterwards and the absorbance even dropped a little during a longer reaction time (>10 min), showing the completion of the reaction between TMB and H_2O_2 in the presence of iodide. Therefore, after the optimization for this catalytic reaction, 0.1 M H_2SO_4, 55°C and 10 min were used as the parameters for the subsequent experiments.

Detection of H_2O_2 based on the iodide-catalyzed oxidation of TMB

In order to investigate the feasibility of the iodide-mediated TMB-H_2O_2 reaction system for H_2O_2 detection, absorption spectra of mixed solutions containing TMB, KI and H_2SO_4 added with different concentrations of H_2O_2 were recorded and the corresponding photographs were taken. **Figure 4A** shows that the solution color changed from colorless to yellow as the concentration of H_2O_2 increased and a concentration of 0.5 μM H_2O_2 could even be observed by the naked eye. **Figure 4B** shows the corresponding absorbance spectrum change, which aligns with the result in **Figure 4A**. As shown in **Figure 4C**, it was found that the absorbance intensity at 450 nm increased with an increasing H_2O_2 concentration from 0.5 μM to 1 mM, implying a broad response range using this catalytic reaction system. There was a good linear correlation between absorbance intensity

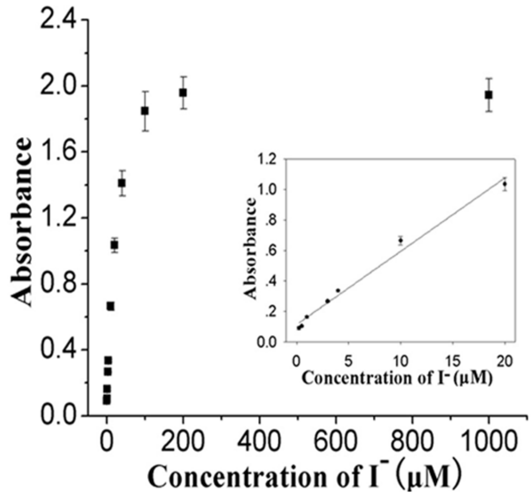

Figure 6. The relationship between the absorbance at 450 nm and the concentration of I^-. Solutions of 0, 1, 3, 4, 5, 10, 20, 40, 100, 200, 1,000 μM I^- were added to the TMB-H_2O_2 reaction respectively, absorbance of resulting solutions at 450 nm was shown above (inset: the linear response at low concentrations of I^-). TMB, 3,3,5,5-tetramethylbenzidine.

and H_2O_2 concentration in the range of 0.5-20 μM (R^2=0.987). The detection limit was 0.2 μM (**Figure 4D**), which was lower than the limit reported in other colorimetric methods [15-17]. Our study results demonstrated that this colorimetric assay could be applied for quantitative and sensitive detection of H_2O_2.

In order to test the specificity of the colorimetric method for analyzing H_2O_2, we also used $K_3Fe(CN)_6$ (100 μM) and sufficient amount of dissolved O_2 to replace H_2O_2 (10 μM) for comparisons. **Figure 5** shows the selectivity of this system toward H_2O_2. A yellow color solution was obtained in the presence of H_2O_2, however, no evident color change was observed for other interferent chemicals including $K_3Fe(CN)_6$ and dissolved O_2, although the concentration of $K_3Fe(CN)_6$ was 10-fold that of H_2O_2 and the dissolved O_2 was saturated. **Figure 5** shows the corresponding absorbance intensity at 450 nm for all of these samples and the results were consistent with the phenomenon detected above. Thus, the colorimetric method developed here showed high selectivity toward H_2O_2 detection. In summary, the method we developed here for the colorimetric detection of H_2O_2 based on the iodide-catalyzed TMB-H_2O_2 reaction. was easy, rapid, cost-efficient, sensitive and selective. The reagents used in this reaction system were commercially available and low-cost. Furthermore, the reaction was simple, rapid and effective, and the oxidized product could be distinguished both by naked eye and spectrophotometric quantification. Therefore, this method has a great potential in the analysis of H_2O_2-related substances.

In addition, considering that the TMB-H_2O_2 reaction rate was iodide concentration-dependent, we further utilized this system to detect iodide, a substance that is of special interest due to its confirmed essential roles in neurological activities and thyroid gland functions [24]. As displayed in **Figure 6**, it was revealed that the absorbance intensity at 450 nm increased with increasing I^- concentration from 0.25 μM to 1 mM, indicating a broad response range. Furthermore, there was a good linear correlation between absorbance intensity and I^- concentration in the range of 0.25-20 μM (R^2=0.986) (**Figure 6**). The detection limit was 0.25 μM, which was lower than the limit reported in the colorimetric iodide recognition method using citrate-stabilized core/shell Cu@Au NPs (6 μM) [25].

Moreover, in order to ensure that the amounts of I^- or H_2O_2 added were sufficient, the concentration for I^- was 0.4 mM in the detection of H_2O_2 and the concentration for H_2O_2 was 0.1 mM in the detection of I^-. As shown in **Figure 4**, 0.4 mM of I^-, which was 2.85 times the concentration (0.14 mM) used in the tests for optimizing reaction conditions, was enough for detecting 1 mM H_2O_2. The concentration of H_2O_2 (0.1 mM) was chosen to be used here based on the findings in **Figure 4C**. When the concentration of H_2O_2 reached 100 mM, the absorbance value was almost saturated; furthermore, the detection limit would be too high and the sensitivity would be low if excessive H_2O_2 was used.

Discussion

In this study, we discovered that iodide could catalyze the oxidation of peroxidase substrate TMB by H_2O_2 to present a yellow color in an aqueous solution, which provided a key basis for a novel, facile, rapid, cost-efficient, sensitive and selective colorimetric assay for H_2O_2 detection. Through condition optimization, it was found that the iodide-catalyzed TMB-H_2O_2 reaction could present a relatively high efficiency in the presence of 0.1 M H_2SO_4 after incubation at 55°C for 10 min. Under the optimal condition, H_2O_2 in solution could be quantitatively and selectively determined by both naked eye and spectroscopy measure with low detection limits of 0.5 μM and 0.2 μM, respectively. Similarly, the catalytic colorimetric reaction system was also utilized for the measurement of iodide with excellent sensitivity and high selectivity. Considering H_2O_2 is a by-product in many enzyme catalytic reactions, such as glucose oxidase, cholesterol oxidase and oxalate oxidase, the iodide-mediated catalytic oxidation reaction of TMB might be applicable in detecting various H_2O_2-related substances as mentioned above [26-28].

However, there are still some limitations to our study. For example, there have been only few reports on the hydrogen peroxide and iodide detection. We optimized the reaction conditions based on relevant literatures, followed by the analysis of hydrogen peroxide and iodide. However, in the future, we will consult some computational chemistry and statistical experts regarding the calculation of the optimal reaction conditions via fitting curve for further verification.

In conclusion, iodide can catalyze the oxidation of TMB by H_2O_2 to a yellow product. The optimal condition for the reaction is a treatment with 0.1 M H_2SO_4 after incubation at 55°C for 10 min. This method is simple, rapid, low-cost, sensitive and selective, which can be applicable to the analysis of various H_2O_2-related substances.

Disclosure of conflict of interest

None.

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