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Monitoring the reaction of hemoglobin with hydrogen peroxide by capillary electrophoresis-chemiluminescence detection

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Abstract

The reaction of hemoglobin (Hb) with hydrogen peroxide (H_2O_2) leads to fluorescent product and heme degradation. We applied capillary electrophoresis-chemiluminescence (CE-CL) detection to monitor the course of Hb reacting with H_2O_2. Hb and released free iron ion (Fe^{3+}) were detected based on their enhancement effects on CL of the luminol-H_2O_2 system. In this study, we discovered an intermediate of this reaction which intensely enhances the luminol-H_2O_2 CL system. The ratio of max CL signals of Fe^{3+}, Hb and this intermediate is circa 1:10:60.
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Keywords: Capillary electrophoresis; Chemiluminescence; Hemoglobin; Hydrogen peroxide; Heme degradation

1. Introduction

Proteins are the most versatile macromolecules in living systems and serve crucial functions in essentially all biological processes. The separation and analysis of protein is important in the area of clinical therapy, pharmaceutics, food detection, and bioscience and bring an increasing interest to many scientists. Capillary electrophoresis (CE) has become an important analysis method and technique to proteins owing to high separation efficiency, low sample needed and relatively short analysis time [1,2]. Ultraviolet absorbance (UV) detection and laser-induced fluorence (LIF) are the most frequently used methods coupling to CE-based analysis of protein in laboratories [3]. Electrochemical detection and mass spectrometry [4] are also reported to be used in the CE-based detection of proteins. Chemiluminescence (CL) detection is characterized by simple optical systems requiring no light sources, avoiding the effects of stray light, and thus providing low background with high sensitivity [5–8].

Hemoglobin (Hb) is the major interplasmic protein of red blood cell (RBC) with the relative molecular weight (M_r) of 64.5 kDa and the primary function of oxygen and carbon dioxide transport in the body [9]. Hydrogen peroxide (H_2O_2) is a reactive oxygen species involved in the propagation of cellular injury during various pathophysiological conditions [10]. The concentration of H_2O_2 in normal human plasma is 4–5 μM [11] and the toxicity of H_2O_2 is enhanced in the presence of Hb [12–14]. In the red blood cell, Most of the H_2O_2 is eliminated by catalase and glutathione peroxidase, resulting in a steady state concentration of H_2O_2 of approximately 2×10^{-10} M [15]. So the reactions between Hb and H_2O_2 have been extensively characterized [16–19].

The CL burst [20] during the reaction of Hb with H_2O_2 was reported by Lissi et al. in 1994. Hammouda and Fakeir [16] reported in 1995 the formation of fluorescence (fluorescent products) when H_2O_2 was added to RBCs. Nagababu and Rifkind [17–19] further suggested that the fluorescence generated by hydrogen peroxide in the red cell originate from the heme peroxidation.

The enhancement effects of Hb on the CL of luminol-H_2O_2 system has long been studied [21] and applied for Hb's detection [22,23]. Tsukagoshi et al. [24] developed a method for direct separation and detection of hemoglobin with a luminol-hydrogen peroxide-Cu(II)-catalyzed chemiluminescence reaction in a capillary electrophoresis-chemiluminescence detection system. Ju [25] studied the CL from the reaction of hemoglobin with hydrogen peroxide in an aqueous carbonate solution. Yumiko et al. [26] studied the CL by hemoglobin in the reaction with H_2O_2 and hydrogen donors. Teselkin et al. [27] measured the activity of blood plasma antioxidant by the hemoglobin hydrogen peroxide luminol system. Zhang and co-workers [28] developed a CL

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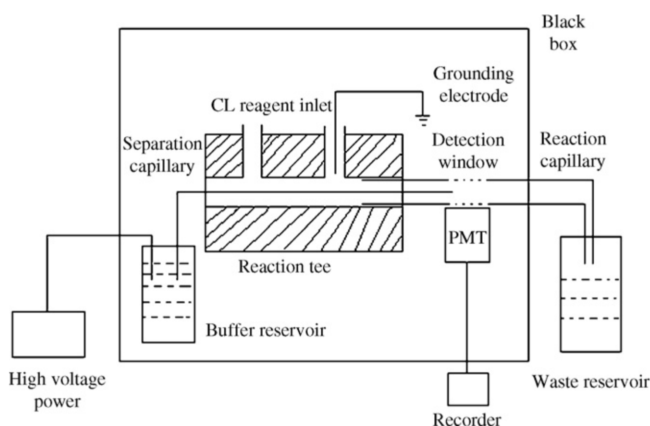


Fig. 1. Schematic diagram of the post-column CL detector for CE.

flow-through sensor for hydrogen peroxide by immobilization of hemoglobin. However, application of this effect for monitoring Hb reacting with other chemicals was rarely reported. It is a novel application to detect iron ion release of hemoglobin reacting with H_2O_2 using CE-CL device. In this study, we applied CE-CL detection to monitor the reaction of Hb with H_2O_2. Hb and the free iron ion (Fe^{3+}) were detected based on their enhancement effects on the CL of luminol-H_2O_2 system. During the reaction course, we discovered an intermediate product of heme degradation which intensely enhances the CL of luminol-H_2O_2 system. The ratio of max CL signals of Fe^{3+}, Hb and this intermediate is circa 1:10:60.

2. Experimental

2.1. Apparatus

The CE-CL apparatus was self-assembled in the laboratory [29] (Fig. 1). A 0–30 kV power supply (Peking University) provided the applied high voltage. Separation capillary of 50 μm i.d. × 375 μm o.d. was from Yongnian Optical Fiber Factory (Hebei, China). A 5 cm coating section of one end of the separation capillary was burned and then etched with hydrofluoric acid for 1.5 h to about 200 μm o.d. (before etch the tip of capillary was sealed by wax to avoid the inner wall being etched). The hydrofluoric acid treated end of the separation capillary was then inserted into a reaction capillary of 530 μm i.d. These two capillaries were held in a plexiglass four-way joint. The post-column reagents were delivered by gravity through a reagent capillary of 320 μm i.d. The outlet of the reagent capillary was also led to the four-way joint. Plexiglass nuts and polyimide ferrules were used to fix the above mentioned three capillaries inside the four-way joint. The grounding electrode was also put into the joint to complete the CE electrical circuit. The outlet of the reaction capillary was 2 cm lower than the other end to make the solution flow out of the reaction capillary more easily and quickly. A 1 cm detection window was formed on the reaction capillary by burning off the polyimide coating. In order to collect the most intensive CL signal, the detection window was situated just in front of the photon-counting photomultiplier tube (PMT R928, Hamamatsu Photonics). The distance between the reaction capillary detection window and PMT was 3 mm. The high voltage of PMT was set at −750 V. The CL emission was collected with a type HX-2 signal magnifier (Institute of Chemistry, Chinese Academy of Sciences, Beijing, China) and then recorded using a computer with TL9000 software. The whole CL detection system was held in a large light-tight box to exclude stray light.

2.2. Reagents and solutions

All chemicals used were of analytical grade. Hb (hemoglobin-A_0, Hb), luminol and carbonate anhydrase (CAII) were purchased from Sigma and hemin was purchased from the Sino-American Biotechnology Co. Ltd. (Wuhan, China). All solutions were prepared with the 18.2 MΩ cm^{-1} water purified with a Water PRO PS system (Labconco, Kansas City, KS). Phosphate buffer solutions (PBS) (50 mM) were prepared by dissolving appropriate amount of NaH_2PO_4 into water. The pH of phosphate buffer solutions was adjusted to 7.4 with 1 M NaOH. All Hb solutions were prepared with PBS (pH 7.4), and then stored in refrigerator. All solutions were filtered through a 0.22 μm membrane prior to use.

2.3. Procedure

The reaction of Hb and H_2O_2 was carried out under 4 °C in PBS (50 mM pH 7.4). The solution of 20 μM Hb in 50 mM PBS (pH 7.4) was placed in a cuvette (10 mL), and 5 μL of H_2O_2(1 M) was placed in the cuvette to produce a final H_2O_2 of 0.5 mM. 10 μL aliquot from mixture on the time course of the reaction of Hb with H_2O_2 was mixed in PBS (10 mM pH 7.4) to 1 mL. The final dilution was analyzed with CE-CL. Similar experiments were performed at the same concentration of Hb and different concentration of H_2O_2 (1.0 mM, 2.0 mM, 5 mM).

The similar experiment is performed on the erythrocyte lysate. 50 μL blood samples from a healthy man were diluted to 2 mL with physiological saline solution (ingredients (mM): NaCl 140; KCl 2.8; $CaCl_2$ 5; $MgCl_2$ 1; HEPES 25, adjust to pH 7.4 with NaOH). The sample was spun in a centrifuge at 2000 rpm for 5 min. Repeated above process; removed the plasma, serum, and white blood cell [30]. Then the cellular membrane lysed by sonication for 5 min, diluted to 2 mL with 50 mM PBS (pH 7.4) and stored in refrigerator. 100 μL stored RBC Hb sample was diluted with 50 mM PBS (pH 7.4), and 5 μL of H_2O_2 (1 M) was added to produce a final H_2O_2 of 0.5 mM (the final volume of solution is 10 mL). 10 μL aliquot from mixture on the time course of the reaction of the lysate with H_2O_2 was mixed in PBS (10 mM pH 7.4) to 1 mL. The final dilution was analyzed with CE-CL.

2.4. Treatment of capillary

In this study, the new capillaries were rinsed sequentially with 2 M NaOH–CH_3OH (i.e. 2 g NaOH dissolved in 25 ml (4:1) methanol/water solution), 1 M NaOH, 1 M HCl and water for 30 min, and then were equilibrated with the running buffer solution for 30 min. The separation capillary was filled with the running buffer while the four-way joint and reaction cap-

illary were filled with the post-column reagent. After each run, the separation capillary was treated with the running buffer for 5 min.

3. Results and discussion

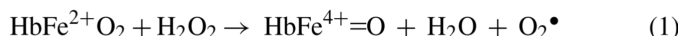
3.1. Separation conditions

Hb is a biological macromolecular and its isoelectric point is 6.7. In CE, the adsorption of the capillary significantly affected the separation efficiencies of proteins. The covalent coating and the dynamic modification of capillaries were normally used to eliminate adsorption of proteins [31–33]. But the sensitivity, the peak shape, and the migration time of the protein were unsatisfactory. In fact, the use of the high pH buffer was one of the widely used methods for suppressing the adsorption of proteins [34], we studied the effects of pH on migration times and signal/noise ratios ranging from 7.0 to 10.0. The reproducibility of the protein peak increased due to the adsorption of hemoglobin decreased when the pH value of buffer increased. The migration time decreased with the increase of pH value. To assure the stability of the electrophoresis baseline and Hb, we chose pH 10 and 10 mM to be the pH value and running buffer concentration, respectively. Samples were injected electrokinetically at 10 kV for 5 s. Separations were carried out at an applied voltage of 16 kV.

3.2. Intermediate product in the reaction

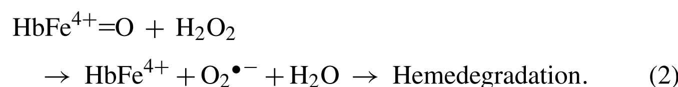
When the reaction time is very short, only Hb peak is observed on the electropherograms (Fig. 2a). When the reaction time is long enough, a sharp and intense peak appear after the Hb peak (Fig. 2b). The change of electropherograms illustrates that a substance which intensely enhance the CL of luminol-H_2O_2 system is produced in the reaction. The peak M is first observed when the reaction time is over 30 min, a sharp peak M appears after the Hb peak (Fig. 2b) in the electropherograms.

According to reports, the reaction of Hb with H_2O_2 leads to fluorescent product [16–19], this product may intensely enhance the CL of the luminol-H_2O_2 system [20,35]. The peak M increases as the reaction going on (Figs. 2 and 3) and becomes relatively steady and reaches highest when the reaction proceeds for 3–4 h (Fig. 3). The peak M becomes unstable when the reaction carries on and absolutely disappeared when the react time is over 6 h. Recent mechanistic studies showed that both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms of Hb react with H_2O_2 to form the oxyferryl species ($HbFe^{4+}$).



$$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O + O_2^{\bullet} \quad (1)$$

The formation of the Fe^{4+} form of Hb results in the formation of heme degradation products [19].



$$HbFe^{4+}{=}O + H_2O_2 \rightarrow HbFe^{4+} + O_2^{\bullet -} + H_2O \rightarrow \text{Hemedegradation}. \quad (2)$$

The migration time of M shifts from 10.7 min to 11.9 min, may be due to the configuration change of the intermediate. The primary oxidation reactions involve the formation of ferryl Hb and are attributed to the accessibility of the heme pocket. The secondary oxidation reactions involve the formation of hombic heme (distorted geometry) and fluorescent degradation products [19].

Our study also shows that the Hb sample (dissolved in 50 mM PBS (pH 7.4)), even without the addition of H_2O_2, was detected

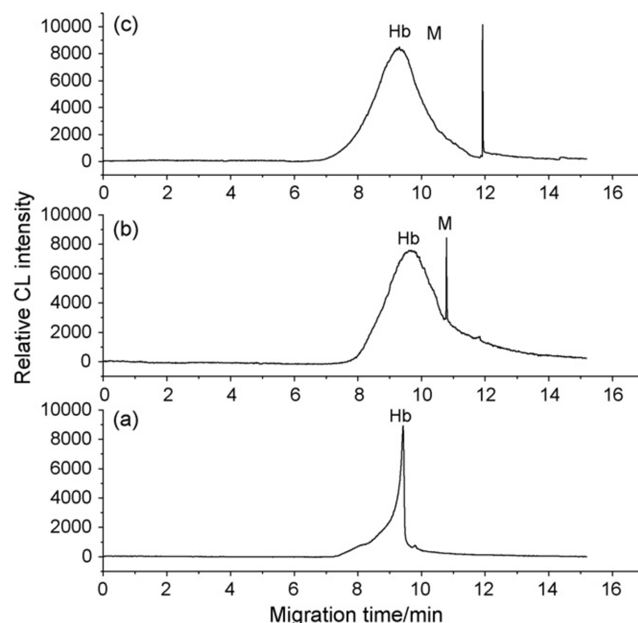


Fig. 2. Changes of electropherograms on the time course of the reaction of Hb and H_2O_2. The assay sample contains Hb (2×10^{-7} M) dissolved in 10 mM phosphate buffer. The sample was dilution of 10 μL aliquot from mixture of Hb react with H_2O_2 at 4 °C to 1 mL after (a) 5 min; (b) 30 min; (c) 45 min. Running buffer: 10 mM phosphate buffer pH 10; post-column reagent: 30 mM H_2O_2, 0.5 mM luminol, pH 11.5. Peak M stands for intermediate of the reaction.

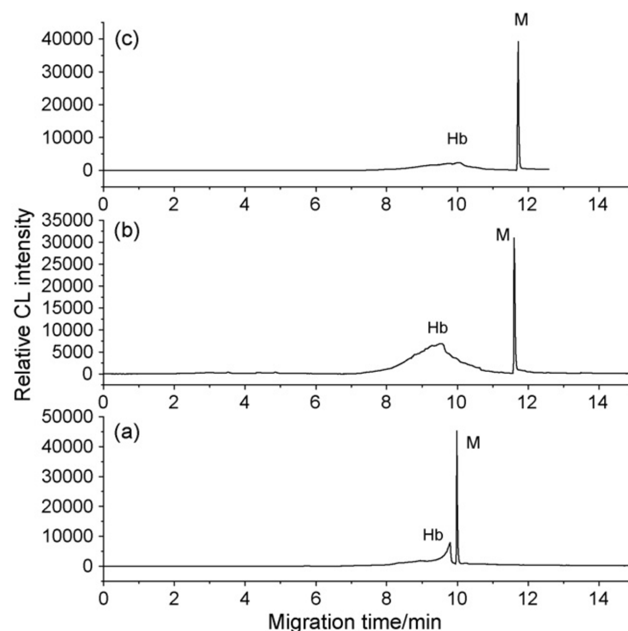


Fig. 3. Peak M reached a relative steady height after Hb reacted with H_2O_2 for (a) 2 h; (b) 3 h; (c) 4 h. Peak M is the intermediate.

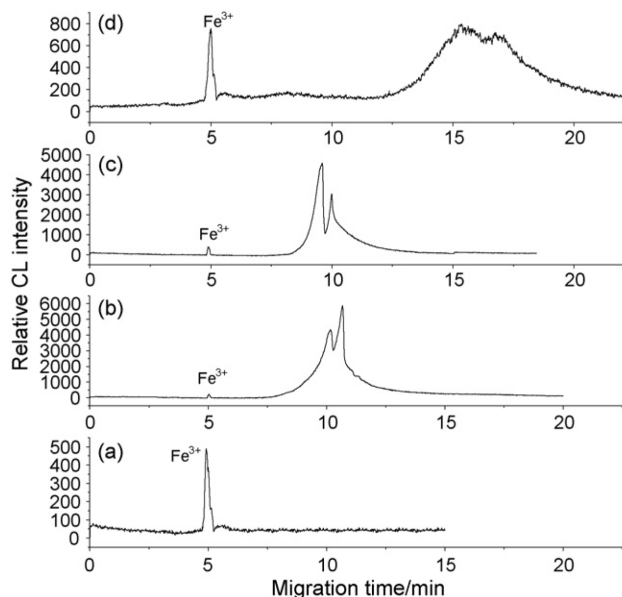


Fig. 4. Fe^{3+} peak appeared after peak M disappeared. Electropherograms for the reaction of Hb and H_2O_2 after (b) 6 h; (c) 8 h; (d) 12 h with Fe^{3+} peak increasing. (a) is the electropherograms of standard Fe^{3+} (5×10^{-7} mol L^{-1}).

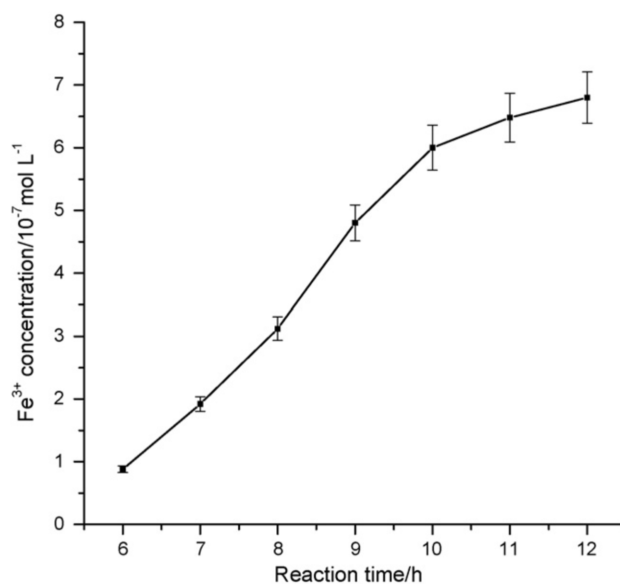


Fig. 5. Fe^{3+} concentration in sample changes as reaction proceeds on. The same conditions as Fig. 2.

with the intermediate peak appeared after stored under room temperature for 1 week. It is possibility the result of the autoxidation of oxhemoglobin [36].

3.3. Fe^{3+} release in the reaction

As reported, the reaction of Hb and H_2O_2 leads to fluorescent product and results in Fe release. In our study, the Fe^{3+} peak appears in front of the Hb peak in the electropherograms while the peak M disappears (Fig. 4b–d). The Fe^{3+} is identified by standard Fe^{3+} sample (Fig. 4a). The Fe^{3+} peak is sharp and symmetrical due to the fast kinetics of Fe^{3+} catalyzing the luminol's CL reaction. The concentration of Fe^{3+} increases as the reaction proceeds on (Fig. 5).

The Hb peak broadens due to the joule effect and the adsorption of protein after long run of the apparatus. In Fig. 4, the Hb peak bifurcates due to the oxidation of part Hb to metHb. The experiment also indicates that the lower the H_2O_2 is, the longer time needed for the Fe^{3+} peak's appearance. In consistent, the higher concentration of H_2O_2 caused the quicker appearance and increase of Fe^{3+} peak except that when the H_2O_2 concentration is over 5 mmol L^{-1}, no peak M is observed during the reaction course. Other oxidants, i.e. sodium chlorate and bromate instead of H_2O_2 in the similar experiment showed the heme degradation of Hb but no intermediate peak was observed.

3.4. The reaction of H_2O_2 with lysate of RBC

The main component of RBC lysate is Hb (97%). However, the lysate of RBC brings on different electropherograms from the purchased Hb. The intermediate peak appears at a latter time and exhibits a relatively lower height compared to the Hb peak (16:10) (Fig. 6). It is reported that in the red blood cell, most of the H_2O_2 is eliminated by catalase and glutathione peroxidase. It is possible that the catalase, glutathione peroxidase, other enzymes or biochemicals which coexist in the RBC with Hb restrain the intermediate production [22].

3.5. Analysis results

Based on the enhancement effect of Hb to the CL of luminol-H_2O_2 system, the LOD (S/N = 3) of Hb in CE-CL detection is 1.0×10^{-9} mol L^{-1} (25 amol) in the optimized conditions (running buffer: 10 mM phosphate buffer pH 10; post-column reagent: 30 mM H_2O_2, 0.5 mM luminol, pH 11.5). The LOD of Fe^{3+} is 2.3×10^{-8} mol L^{-1} in optimized conditions and 4.4×10^{-8} mol L^{-1} in chosen conditions. In this study, the ratio

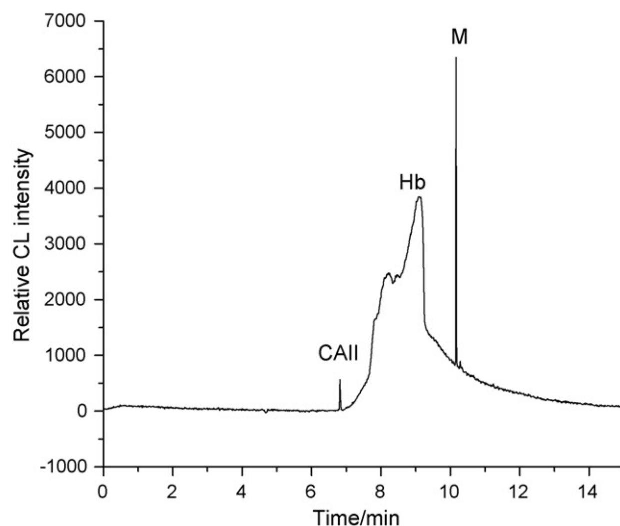


Fig. 6. Electropherograms for the reaction of H_2O_2 and lysate of human red blood cell. The assay sample contained Hb from human red blood cell (1:500 diluted from stock solution) and reacted with H_2O_2 for 6 h at 4 °C. Peak CAII is carbonate anhydraseII. M is the intermediate.

of max CL signals of Fe^{3+}, Hb and the intermediate is circa 1:10:60. The intermediate peak becomes relatively steady when the reaction proceeds for 3–4 h and the optimal reaction time is 3.5 h. The signal of M in standard sample is about 6 times to Hb's. The signal of M in lysate RBC is about 1.6 times to Hb's.

3.6. Conclusion

The combination of high separation efficiency of CE and ultra-sensitivity of CL detection leads to powerful technique in analytical science. The reaction of Hb with H_2O_2 leads to fluorescent products and results in heme release. In this study, we found that the intermediate product of this reaction could intensely enhance the CL of the luminol-H_2O_2 system. We applied CE-CL detection to monitor the course of Hb reacting with H_2O_2. Hb and the release iron ion (Fe^{3+}) were detected at the same time and an intermediate which intensely enhances the luminol-H_2O_2 CL system was found in the reaction course. The ratio of max CL signals of Fe^{3+}, Hb and the intermediate is circa 1:10:60 and a more sensitive detection method of hemoglobin may be established with the intermediate.

Acknowledgements

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