Inactivation and Heme Degradation of Horseradish Peroxidase Induced by Discharge Plasma[a]

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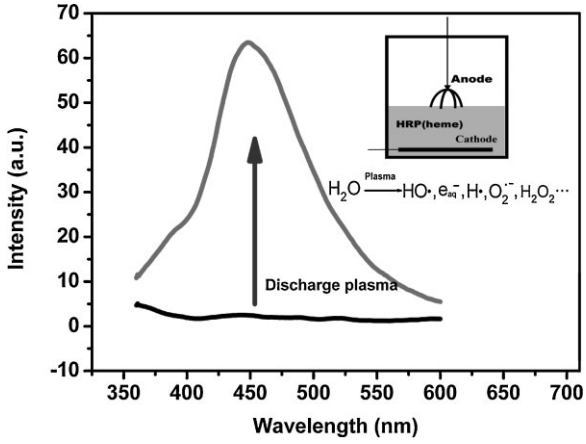
The effects of non-thermal plasma at the argon gas–solution interface on horseradish peroxidase (HRP), a classical heme-containing protein, were assessed quantitatively. Evidence from spectroscopic approaches showed that enzymatic activity together with the contents of heme and iron in HRP decreased dramatically and concomitantly upon plasma treatment. The plasma induced both long- and short-lived reactive species which inactivated HRP in the solution. In particular, hydrogen peroxide broke the heme prosthetic group into fluorescent products, mainly dipyrrols and their derivatives, while other factors such as hydroxyl radicals and UV radiation generated during plasma discharge accelerated this heme protein degradation process.



1. Introduction

Because of its advantages in eliciting chemical reactions and flexible operation, atmospheric-pressure non-thermal plasma has attracted more and more attention in the field of biology and biomedicine, such as sterilization,[1–6] healing of wounds,[7,8] blood coagulation,[9,10] cancer therapy,[11–14] and so on.

But even though many papers addressing plasma-induced biological effects have been published, fundamental understanding of the interaction between plasma and biomolecules has remained insufficient. To gain an in-depth insight, it is not only necessary to study the bio-effects in the plasma treated cells and tissues, but also required to scrutinize the interaction of plasma with the involved biomolecules directly. Up to now, there are also some researchers have endeavored to study the effects and mechanisms of plasma on biomolecules.[15–21] Li et al. have confirmed that it is the reactive species rather than heat, UV radiation, and intense electric field that induce the break of plasmid DNA during plasma treatment.[15] Yan et al. have reported the alteration of plasmid DNA topology from super-coiled form to open circular and linearized form induced by plasma plume.[16] The effects of plasma on some model proteins have also been investigated by some researchers. Julák et al. found that the infectivity of prion was significantly decreased by the negative corona discharge, and studied the mechanisms for the prion inactivation which are deemed due to the acidification of samples together with the denaturation and/or formation of insoluable high

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molecular weight complex.[17] For different proteins, the plasma-induced effects should be different. For example, the enzymatic activity of lysozyme was decreased upon plasma treatment,[18] but the activity of lipase increased within one minute plasma treatment.[19] The reason for the contrary phenomenon are due to the chemical modification and change of protein structure induced by generated reactive species.[18,19] Except for the conditions as mentioned above in which the plasma was created in contact with liquid water, biomolecules in dry conditions such as proteins deposited on stainless-steel can also be degraded by plasma treatment.[20,21] Generally, the reactive oxygen species (ROS) play an important role in these processes.

Herein, the damage of model protein, horseradish peroxidase (HRP), induced by non-thermal discharge plasma at the gas–solution interface in argon (Ar) atmosphere, which is a continuation of our previous work on amino acids, bases, and peptides,[22–26] is reported. HRP, which contain iron (III) protoporphyrin IX (heme) as the prosthetic group, can catalyze the oxidation of a wide variety of organic and inorganic substrates at the expense of hydrogen peroxide (H_2O_2) or hydroperoxide (ROOH). By virtue of its extraordinary enzymatic activity, it has been extensively applied in several fields, such as chemical synthesis,[27,28] environmental biocatalysis,[29,30] analytical diagnosis,[31,32] and so on. Upon plasma treatment, HRP inactivation, fluorescent heme degradation product formation, and iron release were observed and analyzed quantitatively. The factors responsible for heme degradation were determined and the mechanism was discussed. The information obtained from this work is helpful for understanding the inactivation mechanism of other heme-containing proteins under plasma treatment.

2. Experimental Section

2.1. Chemicals

Horseradish peroxidase (HRP, Rz > 3.0), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfunic acid) (ABTS) (>98%, in diammonium salt form), and hematin were obtained from Sangon Biotech (Shanghai) Co., Ltd. and used without further purification. Unless otherwise indicated, all HRP solutions were obtained by dissolving an appropriate quantity of lyophilized HRP powder in 10 mM phosphate buffer (PBS) solution, pH 7.2. Its concentration was determined spectrophotometrically using the molar excitation coefficient of 102 $mM^{-1} cm^{-1}$ at 403 nm.[33] Protoporphyrin IX (PPIX) was purchased from Sigma–Aldrich. All other chemicals including H_2O_2 (30% by volume), buffer substances (analytical reagent grade), and ammonium formate (analytical reagent grade) were obtained from Sinopharm Chemical Reagent Co., Ltd. The concentration of H_2O_2 applied in experiments was determined with spectrophotometric absorbance at 240 nm (molar excitation coefficient = 43.6 $M^{-1} cm^{-1}$).[34]

2.2. Discharge Apparatus

The experimental setup for the generation of non-thermal discharge plasma at the gas–solution interface has been described in detail previously.[22–26,35] Certain volume of HRP solution was contained in the columniform plexiglas reactor. The needle-like anode made of stainless steel was placed 3–5 mm above the solution surface, and the plate-like cathode, also made of stainless steel, was submerged in solution. Both electrodes were connected to a DC power supply. Argon gas was introduced into the reactor before discharge in order to remove air. Discharge plasma was generated between the tip of anode and solution surface. When the discharge was steady the current was about 20 mA and the voltage was about 1 200 V (±10%). Certain volume of treated HRP samples was drawn from the reactor periodically for analysis.

2.3. Sample Analysis

2.3.1. Measurement of Enzyme Activity

The peroxidase activity of HRP was measured by determining the rate of H_2O_2-mediated oxidation of the used substrate ABTS spectrophotometrically at 414 nm.[36] 2.7 mL ABTS solution (9.1 mM) in 0.1 M PBS, pH 5.5, were placed in a quartz cuvette with 1 cm optical path, and then 0.3 mL 100 mM H_2O_2 were added and mixed sufficiently. The reaction was initiated by addition of 10 μL HRP sample after dilution. The changes of absorbance at 414 nm were followed for 3 min.

2.3.2. Spectroscopic Analysis

Steady state UV–Vis absorption spectra of native and treated HRP sample were obtained by a UV–Vis spectrometer (SHIMADZH UV-2550) at ambient temperature. The fluorescence spectra determinations were conducted on a fluorescence spectrometer (VARIAN Cary Eclipse). Both absorbance and fluorescence scans were performed with quartz cuvettes with 1 mm optical path. Unless otherwise indicated, all spectra measurements were conducted after the plasma-treated HRP samples had been laid overnight in order to wait for all oxidized HRP intermediates had been converted to the resting state.

2.3.3. Determination of Free Iron Released from HRP

Free iron released from HRP due to heme destruction was measured colorimetrically using ferrozine following the published method[37] and the reference cited therein. To 100 μL of treated HRP sample 100 μL of ascorbic acid solution (2 mM) was added. After incubation at room temperature for 5 min, 50 μL of ammonium acetate (16%) and 50 μL of ferrozine (16 mM) was added to the mixture and mixed sufficiently. Then the absorbance at 562 nm of the reaction mixture was measured after incubation at room temperature for 5 min. A standard curve was constructed by using ammonium Fe(III) sulfate.

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2.3.4. Determination of H_2O_2 Concentration Produced in PBS Buffer after Discharge Plasma Treatment

The concentration of H_2O_2 in plasma-treated PBS buffer was determined spectrophotometrically at 410 nm, after mixing with titanium sulfate in acidic condition.[38]

3. Results and Discussion

3.1. Inactivation, Heme Destruction, and Iron Release by Plasma Treatment

Figure 1 shows the effect of non-thermal discharge plasma at the Ar gas–solution interface on the enzymatic activity, iron (Fe) content, and heme content (represented by the residual Soret absorbance at 403 nm, denoted as A_{403}) of HRP. Fe content in HRP was determined by subtracting the Fe concentration measured colorimetrically in solution with primary HRP concentration determined spectrophotometrically. Steady decreases of enzymatic activity, Fe and heme content were observed with increase of discharge time. The declining degree of enzymatic activity was almost identical to the decrease of A_{403}, indicating that the enzyme inactivation induced by discharge plasma can be probed as heme destruction, which involves deprivation of heme iron.

3.2. Fluorescent Product Formation Originating from Heme Destruction

One fluorescent band was identified after treatment of HRP solution by discharge plasma, which exhibited the maximum emission wavelength at 450 nm with an excitation wavelength at 330 nm (Figure 2). The inset shows the dependence of fluorescence intensity at 450 nm on discharge time.

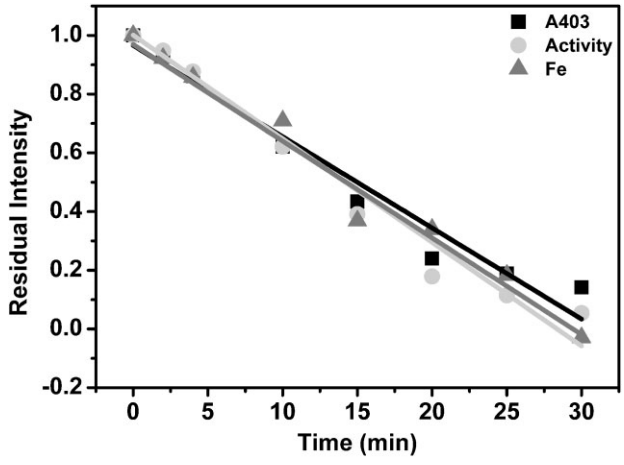

Figure 1. Dependence of peroxidase activity, Fe and heme content of HRP on discharge time. The initial HRP concentration was 2×10^{-5} M and the volume was 25 mL.

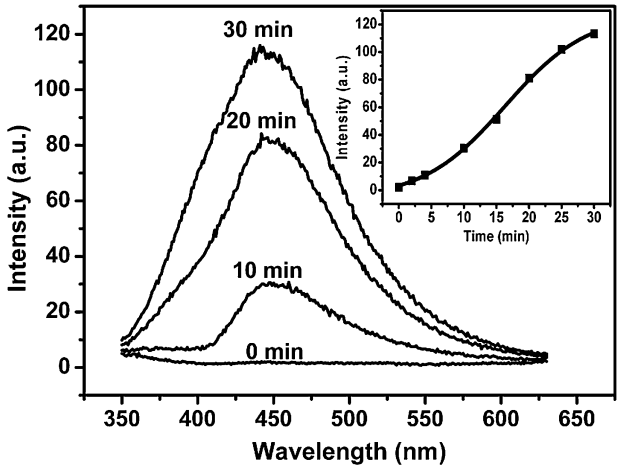

Figure 2. Fluorescence spectra of HRP with different discharge time. Inset: Dependence of the fluorescence intensity at 450 nm on discharge time. The initial HRP concentration was 2×10^{-5} M and the volume was 25 mL. Excitation wavelength was 330 nm and both excitation and emission slit were 10 nm.

In order to ascertain the origin of the fluorescent product, several control experiments were conducted. Apo-HRP obtained by removing heme with methyl ethyl ketone in acidic condition at 4 °C[39,40] were also treated by discharge plasma under the same conditions applied for HRP. The sample shows much weaker fluorescence intensity at 450 nm compared with the HRP sample after plasma treatment (Figure S1 in Supporting Information). The intenser background signal from apo-HRP is due to residual ketone. Additionally, the solution of hematin (iron-protoporphyrin complex with similar structure to the heme in HRP) after discharge plasma treatment also displays similar fluorescent band at 450 nm, although its intensity is much weaker compared with that of HRP (Figure S2 in Supporting Information). All these results unambiguously indicate that the fluorescent product formed in HRP solution by discharge plasma treatment originated from heme destruction. In the present study, therefore, fluorescence intensity at 450 nm was used as a measurable index to investigate the mechanism of heme degradation and inactivation of HRP induced by discharge plasma.

3.3. Contribution of H_2O_2 to the Fluorescent Heme Degradation Product Formation

The dependence of the fluorescent heme degradation product formation on H_2O_2 during plasma treatment can be confirmed by adding H_2O_2 to HRP solution directly. Certain volume of HRP solution (4×10^{-5} M) was mixed with the same volume of H_2O_2 with different concentrations. The ratio for H_2O_2 to HRP concentration was from 0

to 1 000, which is similar to the condition under plasma treatment for 0–30 min. The fluorescence spectra of the mixture were detected after incubating at room temperature overnight (Figure 3) and the same characteristic fluorescence peak as with that of HRP sample after discharge plasma treatment was observed. With the increase of $[H_2O_2]$/[HRP] ratio the fluorescence intensity of the mixture increases linearly (the inset of Figure 3). All these results indicate that the fluorescent heme degradation product formation in HRP depends on the concentration of H_2O_2. Indeed, as high as millimole H_2O_2 was generated in PBS buffer by plasma treatment and its concentration increased exponentially with discharge time (Figure S3 in Supporting Information). Therefore, the fluorescent heme degradation product formed in HRP solution by plasma treatment originated from the reaction of HRP with plasma-generated H_2O_2.

3.4. Comparison of the Fluorescent Heme Degradation Product Formation in HRP by Plasma Treatment with That by External H_2O_2 Solely

As mentioned above, the loss of HRP enzymatic activity by discharge plasma was concomitant with the heme destruction, and H_2O_2 could lead to the formation of the fluorescent heme degradation product directly. In order to quantify the role of H_2O_2 in the fluorescent heme degradation product formation by plasma treatment, the curve of the fluorescence intensity at 450 nm in HRP solution induced by discharge plasma (the inset in Figure 2) was compared with that induced by H_2O_2 (the inset in Figure 3) and the result is shown in Figure 4. The axis of the inset in Figure 2 was converted to H_2O_2 concentration in

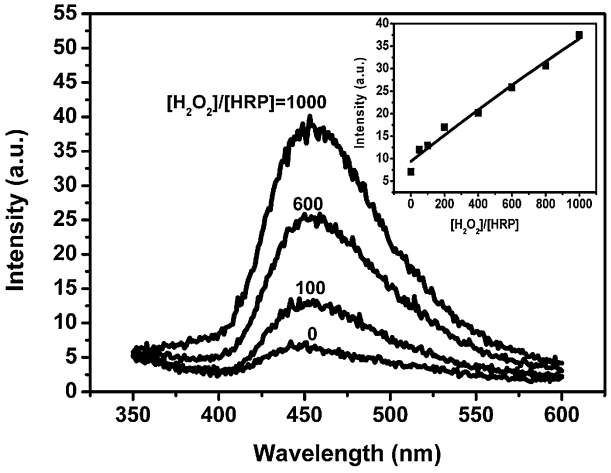

Figure 3. Fluorescence spectra of HRP and H_2O_2 mixture with different $[H_2O_2]$/[HRP] ratios. Inset: Dependence of the fluorescence intensity at 450 nm on $[H_2O_2]$/[HRP]. The final HRP concentration was 2.0×10^{-5} M. Excitation wavelength was 330 nm and both excitation and emission slit were 10 nm.

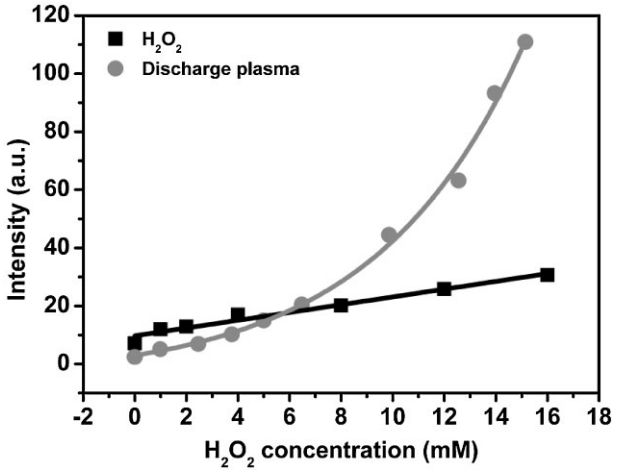

Figure 4. Comparison of the fluorescence intensity at 450 nm in HRP by discharge plasma with that by H_2O_2.

Figure 4 based on Figure S3 in Supporting Information. Within the same H_2O_2 concentrations, the fluorescence intensity in HRP by discharge plasma was intenser than that induced by H_2O_2 solely, implying that other factors besides H_2O_2 during plasma treatment must also play an important role in the formation of the fluorescent heme degradation product.

3.5. Effect of Hydroxyl Radical on the Fluorescent Heme Degradation Product Formation by Plasma Treatment

It is known that hydroxyl radicals play an important role in plasma-induced chemical processes due to its strong oxidation capability.[41,42] To investigate the possible role of hydroxyl radicals on the fluorescent heme degradation product formation, formate which can scavenge hydroxyl radicals was added to HRP solution prior to plasma treatment. The effect of formate on the fluorescence intensity in HRP solution by plasma treatment is presented in Figure 5. Obviously, inhibitory effect on the fluorescence intensity was observed with the addition of formate. This result confirms that hydroxyl radicals indeed contribute to the fluorescent heme degradation product formation in HRP during plasma treatment.

3.6. Protection of Heme against Degradation by Protein Conformation/Function

The native structure/function of HRP on the fluorescent heme degradation product formation was also investigated. Partially disordered HRP by high temperature at 70 °C for 15 min was mixed with certain amounts of H_2O_2 and then the fluorescence intensity at 450 nm of the mixture

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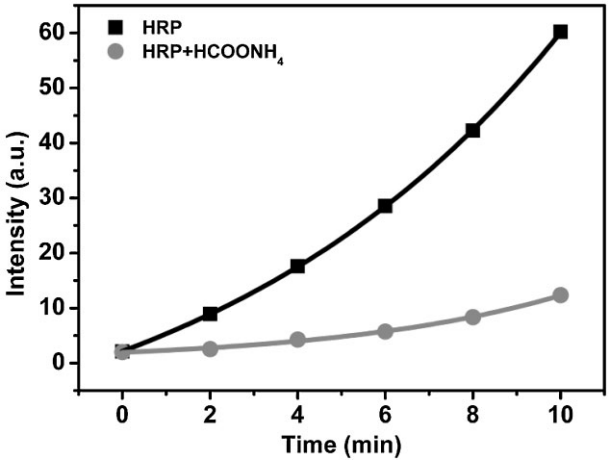

Figure 5. Effect of formate on the fluorescence intensity at 450 nm of HRP induced by plasma treatment. In both experiments the initial HRP concentration was 1.25×10^{-5} M and the volume was 10 mL. The formate concentration was 0.5 M. Excitation wavelength was 330 nm and both excitation and emission slit were 10 nm.

was detected after incubating at room temperature overnight (Figure 6). It is clearly seen that in the presence of same amount of H_2O_2 the fluorescence intensity of the partially disordered HRP is intenser than that of native one. In other words, heme in disordered or inactivated HRP is more readily to be degraded by H_2O_2 to form fluorescent product than that in native state. Therefore, native structure/function of HRP indeed can protect its heme against degradation by H_2O_2 during discharge plasma treatment. In addition, the inhibitory role of protein structure/function in heme degradation can also be confirmed from the experiment conducted with hematin. The fluorescence intensity of hematin solution with different $[H_2O_2]$/[Hematin] ratios was detected and the results were compared with that of HRP under the same conditions (Figure S4 in Supporting Information). Stronger fluorescence intensity was observed in hematin than in native HRP, implying that the native structure/function of HRP can really protect its heme against degradation by H_2O_2 during plasma treatment.

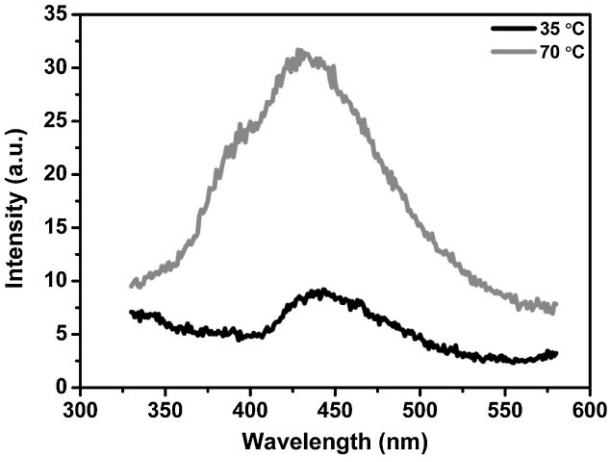

Figure 6. Fluorescence spectra of the mixture of H_2O_2 with native/partially disordered HRP. The partially disordered HRP was obtained by high temperature at 70 °C for 15 min. In both experiments the HRP concentration was 1.25×10^{-5} M and $[H_2O_2]$/[HRP] was 600. Excitation wavelength was 330 nm and both excitation and emission slit were 10 nm.

3.7. Influence of Plasma-generated UV Radiation on the Fluorescent Heme Degradation Product Formation

It is known that intense UV light is emitted from discharge plasma.[43] The influence of plasma-generated UV radiation on the fluorescent heme degradation product formation was thus also studied. Certain volumes of HRP mixed with H_2O_2 with different $[H_2O_2]$/[HRP] ratios were enclosed in a quartz cuvette with 1 mm optical path and placed into discharge container for accepting the plasma-generated UV radiation. Fluorescence spectra of the mixture after UV radiation was measured immediately and the results are shown in Figure 7. UV radiation distinctly enhanced the fluorescence intensity of HRP and H_2O_2 mixture. However, no effect of UV in the absence of H_2O_2 on the fluorescence intensity was observed. Therefore, it is concluded that the UV light accelerated the heme destruction mediated by H_2O_2 during discharge plasma treatment.

3.8. Mechanisms

The mechanism for the reactive species formation in solution by discharge plasma at the argon gas–solution interface has been summarized previously.[35] Briefly, water molecules in solution are ionized and activated by collision with bombarding H_2O^+ ions under the acceleration of cathode drop near the solution surface, and consequently, highly reactive radicals and molecular species including hydroxyl radical, hydrogen radical, aqueous electron, hydrogen peroxide, and so on are produced in solution.[44,45] Additionally, intense UV radiation is also generated by discharge plasma.[43] The damage of the solute in solution is due to the combined action of the radicals, non-radical oxidants, UV radiation, and so on.

As shown in Figure 1, the declining curve of enzymatic activity of HRP induced by discharge plasma is accordant with that of Soret absorbance, implying that heme destruction can be utilized as a probe to detect the inactivation of HRP by discharge plasma treatment.

The heme destruction can also be evaluated by measuring the fluorescence intensity of the heme degradation

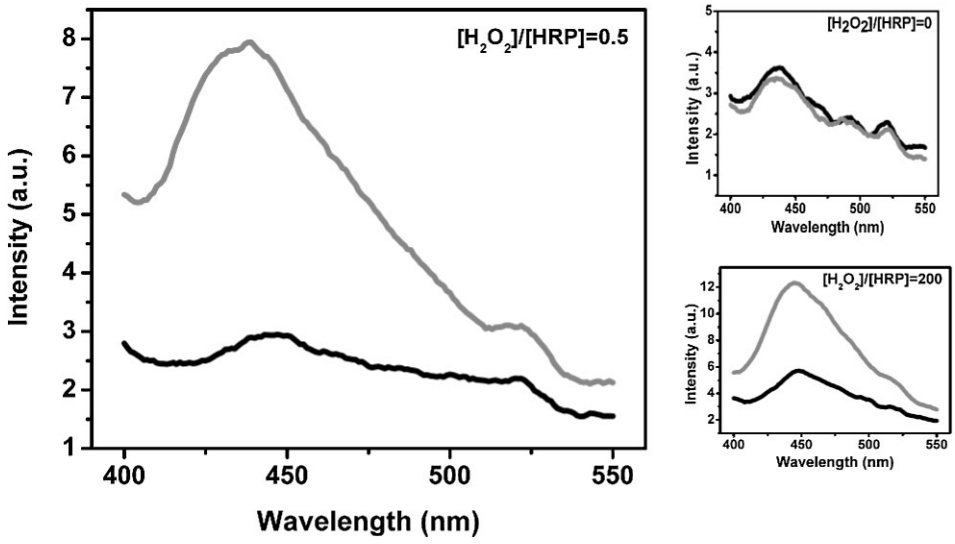

Figure 7. Fluorescence spectra of HRP and H_2O_2 mixture with (gray line, 10 min) and without (black line) the UV radiation generated during plasma treatment. The final HRP concentration was 1.25×10^{-5} M. The spectra were with smoothing. Excitation wavelength was 330 nm and both excitation and emission slit were 10 nm.

product. The phenomenon for the fluorescent heme degradation product formation with the maximum emission wavelength at 450 nm have been investigated and interpreted clearly by other researchers previously. Protoporphyrin or heme (iron-protoporphyrin) can be degraded by superoxide radicals generated by potassium superoxide in aprotic solvent systems or by xanthine oxidase/xanthine system to form the fluorescent product with maximum emission wavelength at about 450 nm.[46,47] The fluorescent heme degradation product is due to the action of superoxide radicals on the tetrapyrrols of the protoporphyrin or heme.[46,47] As for the classical heme-containing protein Fe(II) hemoglobin (Hb(II)), similar fluorescent heme degradation products can also be generated due to the action of superoxide radicals generated in the heme pocket from the reaction of Hb(II) with H_2O_2 on heme.[47–50]

Based on the information from plasma chemistry, superoxide radicals can also be produced from ionization of water by discharge plasma.[51] However, the contribution of superoxide radicals from this source to the fluorescent heme degradation product formation is neglectable because inhibitory effect on the fluorescence intensity was observed (Figure S3 in Supporting Information) with the addition of formate which can convert primary free radicals to superoxide in the presence of oxygen.[52,53] In addition, PPIX solution after plasma treatment under the same conditions showed no fluorescent band at 450 nm (Figure S2 in Supporting Information), further confirming this conclusion. The reason may be the short lifetime of superoxide radicals in solution,[48] after it is released from the discharge zone, it cannot reach the heme site inside the protein moiety before it is quenched. In other words, the folded structure of HRP can protect its heme against the attack by the superoxide radicals generated by discharge plasma to form fluorescent heme degradation product.

However, the superoxide radicals produced in the catalytic cycle of HRP in the absence of reducing substrate, as shown in Scheme 1[54] which has been studied extensively,[55–59] may contribute to the fluorescent heme degradation product formation in HRP solution during plasma treatment. In the early stage of plasma treatment, HRP was in native state and it reacted with plasma-generated H_2O_2. Superoxide radicals were generated in the heme pocket in the reactions of H_2O_2 with compound 1 and 3, which can attack the tetrapyrrols and further induce the formation of fluorescent heme degradation product. In order to verify whether this catalytic cycle (Scheme 1) is involved in the fluorescent heme degradation product formation, the reducing substrate ascorbic acid (Vc)[60] which can inhibit the catalytic reaction was added to HRP solution prior to H_2O_2 addition. Indeed, it was found that the formation of the fluorescent product was significantly inhibited (Figure S5 in Supporting Information).

Except for peroxidase activity, HRP also possesses catalase-like activity which is a major protective mechanism against inactivation by H_2O_2 especially at pH values greater than approximate 6.5.[55,61] Because of this protection mechanism the fluorescent heme degradation product formation within the catalytic cycle is ineffective and the efficiency is lower than hematin (Figure S4 in Supporting Information), and the latter is much lower

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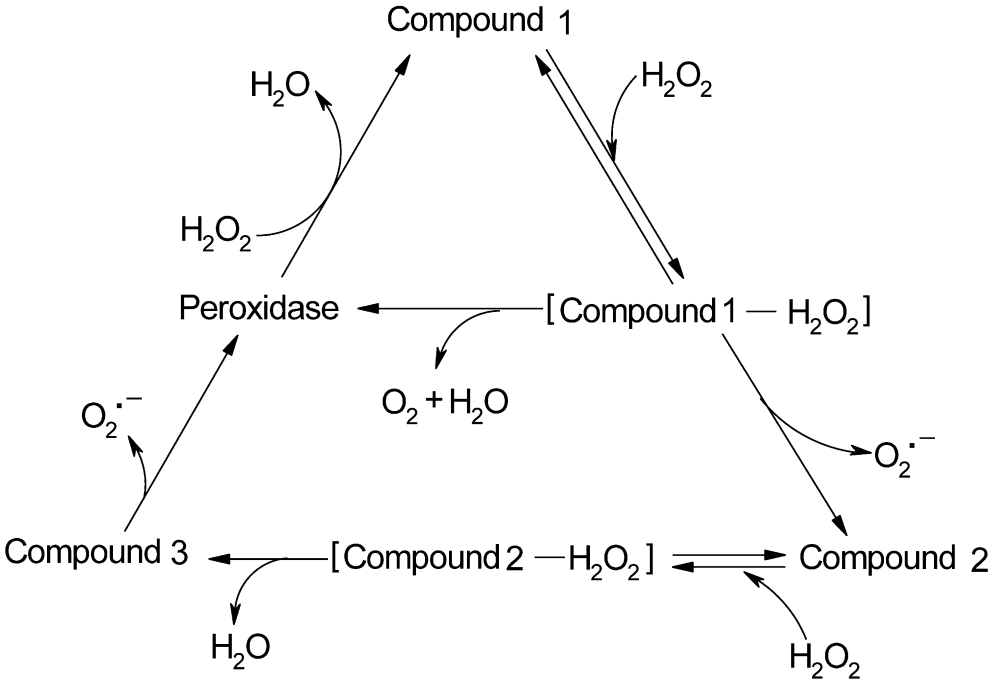

Scheme 1. Reaction pathway of peroxidase with H_2O_2 in the absence of reducing substrate.

than Hb(II) because Hb(II) is more readily to react with H_2O_2 to form superoxide radicals.[48] As confirmed in Figure 4, the fluorescence intensity in HRP by discharge plasma was much intenser than that induced by H_2O_2 solely within the same H_2O_2 concentrations. Therefore, besides H_2O_2 there must be some other factors that contribute to the fluorescent heme degradation product formation in HRP during plasma treatment.

Although hydroxyl radicals are not the direct reason for the fluorescent heme degradation product formation (Figure S6 in Supporting Information), they anyway play an important role in accelerating the formation of the fluorescent product during plasma treatment (Figure 5). As noted in "Results" section that the native structure of HRP has the ability to protect its heme against degradation by H_2O_2 to form fluorescent product, and so the factors which can destroy the native structure of HRP can accelerate the processes of heme degradation during plasma treatment. The HRP samples with different periods of discharge time have been separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S7 in Supporting Information). With the increase of discharge time the native HRP amount decreased and the amount of smaller peptide increased substantially, implying the peptide moiety was really destroyed and consequently the structure/function of HRP was damaged. HRP with destroyed structure will lose its peroxidase and catalase-like activity, so it will react with H_2O_2 in the way similar to hematin,[48] leading to more fluorescent heme degradation product as compared to native HRP (Figure S4 in Supporting Information).

$$\text{Heme} - \text{Fe(III)} + H_2O_2 \xrightarrow{\text{Heme Fe}} \text{(II)} + O_2^{\bullet -} + 2H^+$$

The weaker fluorescence intensity of hematin by plasma treatment (Figure S2 in Supporting Information) was probably due to the attack of primary free radicals on the produced fluorescent product because in hematin the fluorescent product is exposed to plasma-generated free radicals directly, while in HRP it is in the heme pocket protected by the protein moiety. Direct attack of H_2O_2 on tetrapyrrols cannot induce the formation of this fluorescent product because it was not formed in PPIX solution in the presence of excessive H_2O_2, as illustrated in ref.[47] and Figure S4 in Supporting Information.

Another reason for the inhibitory effect of formate on the fluorescence intensity was lower H_2O_2 concentration due to hydroxyl radical scavenging because the later is an important source to H_2O_2 formation during plasma treatment. The H_2O_2 concentration in PBS with 0.5 M formate after discharge plasma treatment was also measured and the result is shown in Figure S3 in Supporting Information, which shows lower H_2O_2 concentration upon addition of formate.

Intense UV radiation was generated during discharge plasma and its effect on the fluorescent heme degradation product formation could not be neglected because of the photosensitivity of heme-containing protein.[62] Heme

destruction induced by H_2O_2 represented by the fluorescence intensity at 450 nm was remarkably accelerated by the UV radiation generated during discharge plasma treatment (Figure 7). The accelerating effect resulted from the synergistic action of UV light and H_2O_2, and when the light was used to irradiate free HRP solution the effect was not observed. The possible mechanism for the accelerating effect of UV radiation on heme destruction is that it accelerates the reaction of HRP with H_2O_2 as shown in Scheme 1,[62] and so more superoxide radicals are produced in the heme pocket. Certain volume of HRP III obtained by mixing HRP solution with H_2O_2 was enclosed in a quartz cuvette and placed into discharge container for testing the influence of plasma-generated UV radiation on the conversion between native HRP and its compound. It is clear that UV radiation really accelerated the conversion of HRP III to native form (Figure S8 in Supporting Information).

4. Conclusion

The inactivation and heme destruction of HRP induced by non-thermal discharge plasma at the argon gas–solution interface was investigated. The degree of HRP inactivation was accordant with the prosthetic group heme destruction which gave rise to fluorescence with maximum emission at 450 nm. Therefore, monitoring the intensity change for this fluorescence band could lead to the quantitative assessment of the effect of discharge plasma on HRP. The plasma elicited reactive species, among which H_2O_2 degraded the heme into fluorescent products, while other factors such as hydroxyl radicals could destroy the structure of HRP and thus accelerate the degradation process. Additionally, the UV radiation generated during plasma treatment also played a role in acceleration of the heme degradation and HRP inactivation.

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