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Degradation of chlorophyll by nitrogen dioxide generated from nitrite by the peroxidase reaction

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Abstract

Nitrite, but not nitrate, added to a mixture containing horseradish peroxidase (HRP) and H_2O_2, bleached chlorophyll (Chl). The optimum pH for Chl bleaching was 4.0. Ascorbate, *p*-hydroxyphenyl acetate (HPAA), glycyltyrosine (Gly-Tyr) and amines such as morpholine and diethylamine inhibited Chl bleaching. The reaction products from HPAA and Gly-Tyr showed an absorption peak at 438 nm in an alkaline solution. The laser-Raman spectrum of the product from HPAA showed a band at 1336 cm^{-1} identical to that of authentic 2-nitrotyrosine. These results indicated the formation of nitrogen dioxide, the one-electron oxidation product of nitrite, which caused Chl bleaching in the nitrite/H_2O_2/HRP system. Nitrite caused neither inhibition of the ascorbate peroxidase reaction nor Chl bleaching with the H_2O_2/ascorbate peroxidase system.

Keywords: Chlorophyll bleaching; Hydrogen peroxide; Nitrite; Nitrogen dioxide; Peroxidase

1. Introduction

Peroxidase (EC 1.11.1.7) isozymes appear to be present throughout the plant kingdom and to play important roles in many physiological processes, including the oxidation of indoleacetic acid, biosynthesis of ethylene and lignification of cell wall materials [1,2]. The enzymatic reactions of peroxidase are well established, namely that of the peroxidatic cycle, involving H_2O_2 and a large array of electron donors. The reaction sequence is found to be as follows:

$$\text{Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} + H_2O$$

$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + AH^{\cdot}$$

$$\text{Compound II} + AH_2 \xrightarrow{k_3} \text{Peroxidase} + AH^{\cdot}$$

AH_2 is an electron donor and gives the primary radical product expressed as $AH^{\cdot}$. Many organic and inorganic compounds are known to be the substrate of the peroxidase reaction [2]. We found that 4-thiouridine, a thiol-containing nucleoside, was oxidized to the 4-thiouridine dimer via the formation of thiyl radicals in the H_2O_2/HRP mixture [3]. On the other hand, Chance [4] pointed out that nitrite (nitrous acid was the active species) was the electron donor to Compounds I and II of HRP; $k_2 = 2 \cdot 10^7$ s^{-1} M^{-1} and $k_3 = 2.4 \cdot 10^5$ s^{-1} M^{-1}, at pH 5.4 and 26° C. Dunford and Stillman [2] also reported the reduction of Compound I to II with nitrite. The nature and the fate of the species of the $AH^{\cdot}$ derived from nitrite, however, remains to be elucidated.

Nitrite is a normal intermediate in the sequential reduction of nitrate to ammonia prior to synthesis of amino acids within plant cells. Substantial amounts of nitrite (10–40 nmol/cm^2 of leaf) were detected in normally grown spinach and kidney bean leaves [5]. Plants incorporate nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$), which are very reactive radicals and thus important air pollutants. NO_2 gas entering a leaf dissolves in the extracellular water of the substromatal cavity to form both HNO_2 and HNO_3,

Abbreviations: APx, ascorbate peroxidase; Chl, chlorophyll; Gly-Tyr, glycyltyrosine; HPAA, *p*-hydroxyphenylacetic acid; HRP, horseradish peroxidase; NO_2-Tyr, 2-nitrotyrosine.

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whose dissociated forms, nitrite and nitrate, distribute to cytoplasm as well as chloroplasts [6]. Short-term exposure to NO_2 gas does not usually inhibit growth, but at high concentrations visible leaf injury occurs. Shimazaki et al. [5] examined the visual injuries in related to the accumulation of nitrite in spinach and kidney bean leaves after fumigation with NO_2 gas. Leaves fumigated with a relatively high concentration of NO_2 (8 ppm, v/v) for 3 h in the dark accumulated nitrite and showed severe necrotic symptoms (visible injuries). In the light, however, this injury and accumulation of nitrite did not occur. The authors have suggested that nitrite is the toxic species generated by fumigation with NO_2 gas, because the reduction of nitrite by light to form ammonia in chloroplasts is dependent upon six electrons arriving via ferredoxin from the photosynthetic electron transport chain spanning thylakoids. Wellburn [6] has also indicated that the most phytotoxic effects of NO and NO_2 arise from the accumulation of nitrite and the toxic effects of nitrite are due to the acidification in plants, although stresses due to cellular acidification are not fully evaluated. On the other hand, there is a clue to the elucidation of the mechanism of nitrite-dependent phytotoxicity. When combinations of either NO or NO_2 gas (50–150 nl l^{-1} each) were fumigated along with 50 nl l^{-1} O_3, extensive and significant visible injury occurred in pea seedlings [7]. These authors have suggested that peroxidative events may occur inside plant cells which can ultimately cause macroscopic leaf necrosis, and have concluded that oxides of nitrogen enhance O_3-mediated injury. O_3 incorporated into plant cells has been reported to cause an oxidative stress by its degradation into superoxide, H_2O_2 and hydroxyl radicals [8].

In the present study, we have detected the generation of a reactive species which bleaches Chl in the nitrite-dependent H_2O_2/HRP reaction, and have identified it as NO_2 by examining the nitrations of HPAA and Gly-Tyr. We have found also that ascorbate peroxidase (APx), a H_2O_2-scavenging enzyme localized in chloroplasts and non-photosynthetic tissues of higher plants and in algae [1], can not utilize nitrite as an electron donor. From these results, the possibility of cellular damage caused by nitrite accumulation, in relation to the generation of NO_2 from the nitrite-dependent HRP reaction, is discussed.

2. Materials and methods

A crude HRP preparation was obtained from Wako Pure Chemical Industries, Osaka, Japan, then purified by CM-cellulose chromatography [9]. The *RZ* value (A_{403nm}/A_{275nm}) for the purified HRP was approximately 3.0. The HRP concentration was calculated with a millimolar absorbance coefficient of 100 at 403 nm. A solution of sodium nitrite dissolved in prechilled water was prepared daily and kept in ice before use. Chl *a* and Chl *b* were purified from spinach leaves according to the methods reported [10], and then dissolved in cold ethanol. Thylakoid membranes solubilized with 0.2% (v/v) Triton X-100 were prepared from isolated intact spinach chloroplasts and stored at −30° C in a 50% (v/v) glycerol solution before use.

The standard reaction mixture for the nitrite-dependent HRP reaction contained 50 mM potassium phosphate buffer (pH 6.0), 5 μM HRP, 2 mM $NaNO_2$ and 1 mM H_2O_2 in a total volume of 1 ml. Chl bleaching was followed by monitoring absorbance changes at 500–700 nm in the standard reaction mixture containing Chl *a*, *b* or thylakoid membranes, which gave an initial absorbance of about 1.0 at 670 nm. The APx reaction was followed by monitoring the decrease in A_{290nm} in a mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM H_2O_2, 0.5 mM ascorbate and 5 μM cytosolic isoform of APx [11]. PD-10 column for gel-filtration was a product of Pharmacia. HPAA, Gly-Tyr and NO_2-Tyr were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were reagent grade. Spectrophotometric analysis was performed using a Beckman DU-640 spectrophotometer. Raman spectra were obtained with a JASCO R-800 resonance Raman spectrophotometer at room temperature using the excitation line at 488 nm generated by a Spectra Physics model-164 argon ion laser. Spectra were determined at pH 10, where the 438 nm absorption band of NO_2-Tyr was most intense and favored resonant Raman enhancement of the NO_2-Tyr derivative [12].

3. Results

Chance [4], and Dunford and Stillman [2] showed that nitrite was the electron donor of Compound II by analyzing the kinetic parameters. We examined spectral changes revealing the peroxidatic cycle involving H_2O_2 and nitrite. The ferriperoxidase spectrum, showing the familiar maxima at 403, 495 and 640 nm, did not change upon the addition of nitrite alone, indicating no coordination of nitrite to HRP, nor oxidation of ferri-HRP. The addition of H_2O_2 brought about a spectrum having maxima at 418, 527 and 555 nm and a shoulder between 610 and 620 nm: the change implying the formation of HRP Compound II [13]. These results indicated clearly that nitrite worked as a one-electron donor of the HRP reaction, forming a free radical as an intermediate.

Chl in the Triton-solubilized thylakoid membranes was bleached by the NO_2^-/H_2O_2/HRP system. NO_2^- or H_2O_2 alone or NO_2^- plus H_2O_2 did not cause bleaching. Changes in the absorption spectra (Fig. 1A) were recorded every 2 min after the start of the reaction. The amounts of Chl *a* and *b* were estimated in acetone extracts during the course of the reaction (Fig. 1B). Bleaching of Chl *a* proceeded with an initial rapid linear phase within about 10 min from the addition of H_2O_2 to start the reaction, after which bleaching continued for another 10 min, but more slowly,

Table 1
Effect of additives upon Chl bleaching induced by the nitrite-dependent HRP reaction

Additives	Chl *a* bleaching	
	nmol/min	% of control
Control	5.42	100.0
$-NaNO_2$	0.22	4.1
$-H_2O_2$	0.44	8.1
−HRP	0.25	4.6
Mannitol (2.5 mM)	5.28	97.4
Ascorbic acid (1.0 mM)	0.23	4.2
HPAA (1.0 mM)	2.05	37.8
Gly-Tyr (1.0 mM)	0.88	16.2
Morpholine (15 mM)	2.24	41.3
Piperidine (15 mM)	2.13	39.3
Dimethylamine (15 mM)	1.67	30.8
Triethylamine (15 mM)	2.28	42.1

Bleaching of Chl *a* was examined in the standard reaction mixture. The initial rate of bleaching was compared. The additives were introduced before the addition of H_2O_2 to start the reaction.

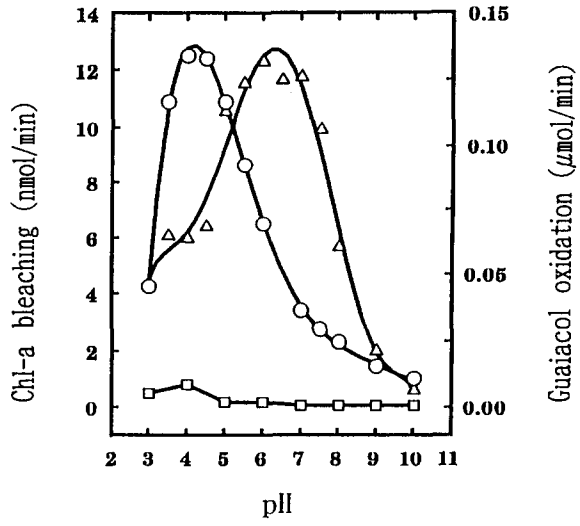

Fig. 2. Effect of pH on bleaching of Chl *a* by the nitrite-dependent HRP reaction. The pH was adjusted with 2 M NaOH to the indicated pH of the solution containing each 20 mM citric acid, KH_2PO_4, boric acid and diethylbarbituric acid. The Chl bleaching reaction was performed in the mixture containing 9 μM HRP, 2 mM $NaNO_2$, 1 mM H_2O_2 and Chl *a* at the indicated pH. Guaiacol oxidation was assayed in the mixture contained 10 nM HRP, 1 mM H_2O_2 and 5 mM guaiacol at the indicated pH. ○, Bleaching of Chl *a*; □, bleaching of Chl *a* without H_2O_2; △, oxidation of guaiacol.

and then appeared to be finished, because the Chl *a* level gradually attained an almost constant level. About 50% of the Chl *a* was bleached in the course of the reaction. Further addition of H_2O_2 or NO_2^- once the reaction had ceased bleached more Chl *a* (data not shown). In contrast, further addition of HRP did not bleach more Chl *a*, indicating no inactivation of HRP during the course of the reaction. Chl *b* in thylakoids appeared to be stable in the nitrite-dependent HRP reaction. Isolated Chl *b* in ethanol, added to the mixture was indeed bleached with a rate of 1.8–2.1 nmol/min (data not shown), whereas Chl *a* in ethanol was bleached at 5.4 nmol/min (Table 1). These results imply that Chl *a* is more sensitive than Chl *b* to the radicals derived from nitrite by the HRP reaction. Nitrate added to the mixture in place of nitrite did not cause bleaching of Chl *a* and *b*, suggesting that nitrate could not work as the electron donor for HRP Compounds I and II.

The dependency of the initial rate of bleaching of Chl *a*

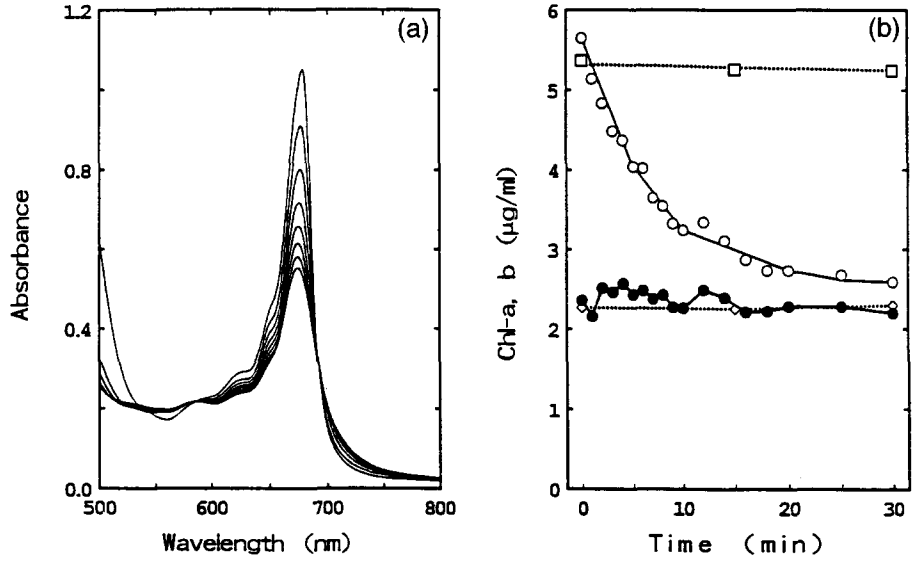

Fig. 1. (a) Chl bleaching by the nitrite-dependent HRP reaction. Thylakoid membranes solubilized by Triton X-100 were added to the HRP standard reaction mixture. Spectra were recorded before the start of the reaction and then every 2 min after the addition of H_2O_2. (b) Amounts of bleached Chl *a* and *b* during the course of the $NO_2^-/HRP/H_2O_2$ reaction. Before and after the start of reaction, to the aliquots of the reaction mixture, acetone was added to make 80% (v/v). After centrifugation of the solution, amounts of Chl *a* and *b* were determined spectrophotometrically. Chl *a* (○) or *b* (●) in the $NO_2^-/HRP/H_2O_2$ mixture. Chl *a* (□) or *b* (◇) in the HRP-H_2O_2 mixture containing 2 mM nitrate but without nitrite.

on pH in the nitrite-dependent HRP reaction is shown in Fig. 2. The oxidation of guaiacol, which is a convenient substrate of HRP, assayed without nitrite, is also indicated. The optimum pH for guaiacol oxidation was 6.8, in contrast to 4.0 for the bleaching of Chl *a*. As the pK_a of nitrite is reported to be 3.3 [6], the protonated nitrite (HNO_2) may be the active form of the electron donor as reported by Chance [4]. The decreased rates for bleaching at pH lower than 4.0 may imply not only changes in charges and/or the inactivation of HRP, but also a decrease of the reacting species responsible for Chl bleaching. A slight amount of Chl *a* was bleached without H_2O_2 at a pH lower than 5.0 (Fig. 2), suggesting that nitrite, HNO_2 or a trace amount of the dismutation products of HNO_2 such as NO, N_2O_3 and NO_2 generated non-enzymatically under acidic conditions [14,15], might bleach Chl.

As shown in Table 1, isolated Chl *a* was bleached with an initial rate of 5.42 nmol/min in the standard reaction mixture. Without nitrite or H_2O_2, very small amount of Chl *a* was lost, suggesting the non-enzymatic production of a trace amount of reactive species as mentioned above. No effect on the rate was found upon the addition of mannitol, a scavenger for hydroxyl radical, indicating no participation of this radical in the bleaching. Ascorbate protected the bleaching almost completely. HPAA, Gly-Tyr and four kinds of amine partially inhibited Chl *a* bleaching. Ascorbate is known to work as the electron donor for HRP [16], and it is easily oxidized by NO_2, thus the inhibitor of the NO_2-induced nitration of Gly-Tyr [17], Phenyl moieties of Gly-Tyr and HPAA are nitrated by NO_2 as well as NO_2^+ [17]. Amine compounds such as triethanolamine [18], morpholine, piperidine and triethylamine are known to react with NO_2 to yield nitrosamine [19]. The results on the inhibition of Chl *a* bleaching by NO_2-reactive compounds suggested that the radical species generated in the HRP reaction might be NO_2, a kind of free radical [20] that acted on Chl, resulting in bleaching.

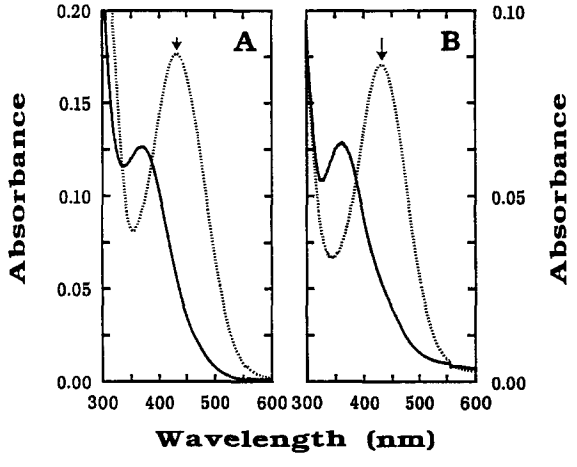

Fig. 3. Absorption spectra of the reaction products of HPAA (A) and Gly-Tyr(B) incubated in the NO_2^-/HRP/H_2O_2 mixture. HPAA or Gly-Tyr at 1 mM each was incubated in the standard reaction mixture at 25° C. After 1 h, the mixture was applied to a PD-10 column to separate the product from HRP. Solid lines and dashed lines are the spectra of the products in 50 mM potassium phosphate (pH 6.0) (absorbance maximum, 356 nm) and 50 mM sodium bicarbonate (pH 10.0) (absorbance maximum, 438 nm, indicated by arrows), respectively.

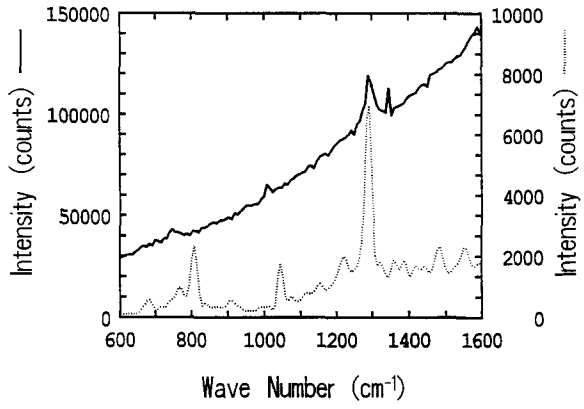

Fig. 4. Laser Raman spectra of the reaction product of HPAA with the NO_2^-/HRP/H_2O_2 system. Spectra of 0.5 mM authentic NO_2-Tyr and of the product in the 50 mM sodium bicarbonate (pH 10) were recorded with the following parameters; laser excitation line, 4880 Å; laser output power, 300 mW; slit height, 10 mm; resolution, 5 cm^{-1}; scan rate, 1 $wavenumber^{-1} \cdot s^{-1}$. Solid line, the product from HPAA; dashed line, NO_2-Tyr.

To confirm the formation of NO_2 in the nitrite-dependent HRP reaction, HPAA or Gly-Tyr was added to the standard reaction mixture. After separation of the products from HRP by gel-filtration using a PD-10 column, the spectra of the reaction products of HPAA (Fig. 3A) and Gly-Tyr (Fig. 3B) were recorded. They were highly pH-dependent; absorption peaks were at 356 nm in acidic solution and at 438 nm in alkaline buffer (pH 10.0). The pH-dependent spectral shift between 356 and 438 nm was identical to those of the tetranitromethane-modified superoxide dismutase [12] and nitrated phenolics [17]. The Raman spectrum of the reaction product of HPAA modified in the nitrite-dependent HRP reaction is shown in Fig. 4, which shows also the spectrum of authentic NO_2-Tyr. The intense band of NO_2-Tyr at 1366 cm^{-1}, which was assigned to the NO_2 symmetric stretching band [12], was detected also in the modified HPAA, indicating the formation of nitro-HPAA. From these results, shown in Table 1 and Figs. 3 and 4, we conclude that the reacting species derived from nitrite via the one-electron oxidation by the HRP reaction is NO_2, not NO_2^+, the two-electron oxidation product of nitrite.

On the other hand, nitrite added to the reaction mixture (0.5 mM ascorbate, 1 mM H_2O_2, 5 μM APx and 50 mM potassium phosphate (pH 7.0)) had no effect on the oxidation of ascorbate by APx, indicating that nitrite did not compete with ascorbate as the substrate of APx and did not inhibit the APx reaction. Moreover, Chl *a* was not bleached by the NO_2^-/H_2O_2/APx system. These results showed that nitrite would not be the electron donor for APx and

that monodehydroascorbate generated by the APx reaction could not bleach Chl.

4. Discussion

The HRP-type peroxidases use a wide range of electron donors [2]. We have shown here that NO_2^- is the one-electron donor of HRP and that NO_2 generated from nitrous acid by the HRP reaction bleaches Chl. Free radicals derived from some phenolics such as 2,4-dichlorophenol, resorcinol, *p*-hydroxybenzoic acid, HPAA and so forth [21–23] in H_2O_2/HRP system have been shown to bleach Chl. In the present HRP reaction mixture, in which nitrite was the electron donor, HPAA and Gly-Tyr inhibited the bleaching of Chl *a* (Table 1) and the phenolic moieties were nitrated (Figs. 3 and 4). The free-radical mechanism of nitration of phenolics (PheOH) by NO_2 is confirmed and the related reactions are also presented [17].

$$NO_2 + PheO^-(PheOH) \rightarrow NO_2^- + PheO^{\cdot}(+H) \quad (1)$$

$$PheO^{\cdot} + NO_2 \rightarrow PheO - NO_2 \quad (2)$$

$$2PheO^{\cdot} \rightarrow \text{biphenolic compound} \quad (3)$$

$$2NO_2 \leftrightarrow N_2O_4 \quad (4)$$

$$N_2O_4 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+ \quad (5)$$

In the absence of NO_2, the phenoxyl radical, $PheO^{\cdot}$, generated by the HRP reaction acts on Chl to bleach or dimerizes to produce the *o,o'*-biphenolic species (3), depending on the reaction constants and upon the concentrations present. While the reaction (1) is comparatively slow at acidic pH [15], in the co-presence of nitrite and phenolics the $PheO^{\cdot}$ derived from H_2O_2/HRP system will react with NO_2, the reaction product of NO_2^-/H_2O_2/HRP system, to form the nitrated phenolic compound, PheO-NO_2 (2). The nitration would inhibit the reaction of Chl with NO_2, resulting in the inhibition of Chl bleaching. The spontaneous disproportionation of NO_2 (reactions 4 and 5) occurs efficiently in acidic solution [14,15]. This may be one reason for the decreased rates of Chl bleaching at pH < 4.0 (Fig. 2).

NO_2 gas dissolves in aqueous media of plant cells such as the extracellular fluid and cytoplasm to form both HNO_3 and HNO_2, which are strong acids [6]: HNO_3, $pK_a = 1.4$; HNO_2, $pK_a = 3.3$. Therefore, fumigation of oxides of nitrogen and uptake of NO and NO_2 gases from a polluted atmosphere or acid rain containing SO_2, NO and NO_2 result in the accumulation of HNO_2 and the cellular acidification. Generation of NO_2 from HNO_2 in the HRP system by analyzing Chl bleaching occurred significantly at pH 3 to 6 (Fig. 2). Recently Floris et al. [24] have reported that HRP reacts with peroxynitrous acid (ONOOH) to form Compounds I and II. Although peroxynitrite ($ONOO^-$) with a half-life of 1 s under physiological conditions, is the reaction product of superoxide with NO [12], and its pK_a is 6.8, we have reported the production of peroxynitrous acid by a reaction of H_2O_2 with nitrite under acidic conditions (< pH 5.0) [25]. At pH 6.0, no production of the acid was observed, but a substantial amount of Chl bleaching occurred at this pH (Fig. 2). In the NO_2^-/H_2O_2/HRP system, the involvement of peroxynitrous acid in the formation of Compounds I and II would be excluded above pH 6.0.

Plants exposed to high concentrations of NO_2 gas usually take on a characteristic water-soaked appearance before necrosis takes place [6]. The one-electron oxidation/reduction potential of NO_2 in (neutral) aqueous solution, $E(NO_2/NO_2^-) = 0.9$ V, is comparable with the values for some halide and pseudohalide radical anions; therefore, NO_2 will exhibit a high degree of reactivity with molecular constituents of living cells [17]. NO_2 initiates the formation of the disulfide anion radical from thiolated cysteine residues [17], the autoxidation of unsaturated fatty acids [20], and nitration of tyrosine residues in peptides and proteins [17]. Nitration of the functionally linked tyrosine brings about conformational changes [26] of the protein as well as inactivation of enzymatic activities [27,28]. Furthermore, nitration interferes with phosphorylation to the tyrosine involved in the regulation by the phosphorylation and dephosphorylation cycle of some proteins [29]. These phenomena may be the initial molecular events caused by NO_2 generated by the NO_2^-/H_2O_2/HRP reaction in NO_2-fumigated plants. The cellular plasmolysis, one of physiological events observed after fumigation by NO_2 gas, is concluded to be a consequence of the lipid breakdown that is related to the peroxidation of unsaturated fatty acids in membranes [6].

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