

Enzymatic nitration of phytophenolics: evidence for peroxynitrite-independent nitration of plant secondary metabolites

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Abstract Peroxynitrite (ONOO[−]), a reactive nitrogen species, is capable of nitrating tyrosine residue of proteins. Here we show *in vitro* evidence that plant phenolic compounds can also be nitrated by an ONOO[−]-independent mechanism. In the presence of NaNO₂, H₂O₂, and horseradish peroxidase (HRP), monophenolic *p*-coumaric acid (*p*-CA, 4-hydroxycinnamic acid) was nitrated to form 4-hydroxy-3-nitrocinnamic acid. The reaction was completely inhibited by KCN, an inhibitor for HRP. The antioxidant ascorbate suppressed *p*-CA nitration and its suppression time depended strongly on ascorbate concentration. We conclude that nitrogen dioxide radical (NO₂[•]), but not ONOO[−], produced by a guaiacol peroxidase is the intermediate for phytophenolic nitration.

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Key words: Horseradish peroxidase; Nitric oxide; Nitrite; Peroxynitrite; Plant phenolics; Reactive nitrogen species

1. Introduction

Nitric oxide (NO) is a free radical that is produced in vertebrates, invertebrates, fungi and bacteria by the activity of NO synthases (NOSs) [1]. It has been shown that plants also produce NO endogenously through enzymatic as well as non-enzymatic mechanisms [2–6]. As NO plays essential roles in animal physiology, the free radical is involved in a wide spectrum of metabolic regulation in plants: pathogen response [3,4], stomatal movements [7,8], respiration [9], and photosynthesis [10]. A plant NOS structurally similar to the mammalian one had been presumed as an enzymatic mechanism for NO production in plants [11,12]. Despite many efforts, however, no plant homologue of such an enzyme has been found to date [13]. Alternatively, there is a growing body of evidence to support that assimilatory nitrate reductase (NR) produces

NO using nitrite (NO₂[−]) as the substrate [5–7,14,15]. Recently, a variant form of the P protein of glycine decarboxylase complex has been found to have an iNOS-like activity [16]. It is becoming apparent that plants produce NO in cells by mechanisms fundamentally different from those of animals and others [13].

NO and its reaction products, referred to as reactive nitrogen species (RNS), exert not only beneficial roles but also harmful effects similar to reactive oxygen species (ROS). Peroxynitrite (ONOO[−]), a reaction product between NO and the ROS superoxide (O₂^{•−}), is the most toxic RNS that potentially oxidizes biomolecules including amino acids, lipids and DNA [17–19]. Thus, ONOO[−] is considered to be a major factor of RNS cytotoxicity [20]. In addition to the activities for oxidative destruction of biomolecules, ONOO[−] is unique because the RNS nitrates tyrosine residues of proteins to form nitrotyrosine [21]. Since the reaction proceeds without any aid of enzyme, the formation of nitrotyrosine had previously been used as an indicator of ONOO[−] production in cells [22]. Recently another mechanism has been found for nitrotyrosine formation that is independent of ONOO[−] production; hemeperoxidases, including horseradish peroxidase (HRP) [23], myeloperoxidase (MPO) [24,25] and lactoperoxidase [26], are reported to be capable of nitrating tyrosine residues through nitrogen dioxide radical (NO₂[•]) formation.

Nitrotyrosine is formed by nitration of the benzene ring of tyrosine. Plant tissues contain abundant aromatic compounds, in particular phytophenolics including flavonoids and hydroxycinnamic acids (HCAs) are ubiquitous in plants. These plant phenolics are known as effective natural antioxidants because they can scavenge a wide range of ROS, e.g. O₂^{•−}, hydrogen peroxide (H₂O₂), hydroxyl radicals (•OH) and singlet oxygen (¹O₂) through direct and indirect mechanisms [27–31]. Recent *in vitro* studies have suggested that these plant phenolics may scavenge RNS, as well as ROS [17,32–34]. In fact, Pannala et al. [35] have shown that HCAs suppress the ONOO[−]-dependent tyrosine nitration and HCAs are nitrated as the result. This is due to a structural similarity between tyrosine and plant HCAs. Thus, it is probable that plant phenolic compounds are nitrated by HRP as similar to tyrosine. However, to date there is no report for peroxidase-dependent nitration of plant phenolics. In this study we show *in vitro* evidence for ONOO[−]-independent nitration of plant phenolics catalyzed by HRP using *p*-coumaric acid (*p*-CA) as a model plant phenolic compound.

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Abbreviations: HRP, horseradish peroxidase; HCA, hydroxycinnamic acid; NO, nitric oxide; NO₂[•], nitrogen dioxide radical; ONOO[−], peroxynitrite; *p*-CA, *p*-coumaric acid; RNS, reactive nitrogen species

2. Materials and methods

The reaction mixture contained 50 mM potassium phosphate (pH 7.4), 100 μ M *p*-CA, 0.5 mM H₂O₂ and 10 mM NaNO₂. The reaction was initiated by the addition of 1 nM HRP and incubated at room temperature. The nitration of phenols was monitored by absorbance increase at 440 nm with a double beam spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). The oxidation of *p*-CA was measured by monitoring absorbance decrease at 286 nm using absorption coefficients of 10.5 mM⁻¹ cm⁻¹.

High-performance liquid chromatography (HPLC) analysis was carried out with a reverse-phase C-18 column (Cadenza CD-C18, 4.6×50 mm; Intakt, Kyoto, Japan). After 60 min incubation, the reaction mixture was applied to a HPLC system using 90% potassium phosphate (50 mM, pH 7.0) containing 10% acetonitrile as the solvent at a flow rate of 1 ml/min. Peaks were monitored absorbance at 440 nm with a photodiode array detector (Waters 996, Waters, Milford, MA, USA).

4-Hydroxy-3-nitrocinnamic acid (nitrated *p*-CA) was synthesized from 4-hydroxy-3-nitrobenzaldehyde and malonic acid by a published procedure [36]. Yellowish needles; EI-MS, *m/z* (rel. int., %): 209 (M⁺, 100), 192 (12), 89 (12), 44 (17), 40 (19), ¹H-NMR (CD₃OD, 270 MHz): 6.46 (1H, d, *J*=16.0), 7.18 (1H, d, *J*=8.8), 7.63 (1H, d, *J*=16.0), 7.89 (1H, dd, *J*=8.8 and *J*=2.2), 8.25 (1H, d, *J*=2.2).

The *p*-CA was purchased from Nacalai Tesque (Kyoto, Japan). HRP and NaNO₂ were obtained from Toyobo (Tokyo, Japan) and Kanto Chemica (Tokyo, Japan), respectively.

3. Results and discussion

3.1. HRP-dependent *p*-CA nitration in the presence of nitrite

Fig. 1 shows absorption spectra of *p*-CA and its reaction products. HRP and H₂O₂ rapidly oxidized *p*-CA with a decrease in absorbance at 286 nm (Fig. 1B). In the presence of 10 mM NaNO₂, *p*-CA was oxidized and a new absorbance increase was observed at 440 nm (Fig. 1B). This absorbance increase depended on concentrations of NaNO₂ and *p*-CA. No *p*-CA oxidation nor the absorbance increase at 440 nm was detected in the absence of HRP or H₂O₂. Addition of KCN, a strong inhibitor for HRP, completely suppressed the

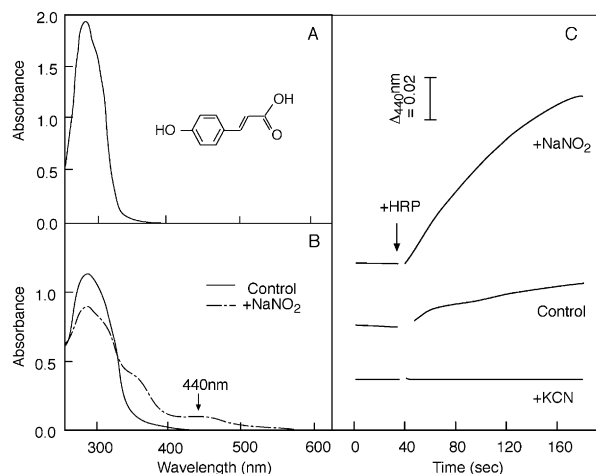


Fig. 1. Absorbance changes in *p*-CA upon nitration catalyzed by the HRP/H₂O₂ system. A: Absorption spectrum of the reduced form of *p*-CA. B: Absorption spectrum of *p*-CA 1 min after the addition of 1 nM HRP in the absence (solid line) or presence (dashed line) of nitrite. The basal reaction mixture contained 50 mM potassium phosphate (pH 7.4), 100 μ M *p*-CA, 0.5 mM H₂O₂ and 10 mM NaNO₂. C: Nitrite-dependent nitration of *p*-CA in the HRP/H₂O₂ system. Upper trace, with nitrite (30 mM); middle trace, without nitrite; lower trace, with nitrite and KCN (5 mM). The reaction was initiated by the addition of HRP at the arrow indicated.

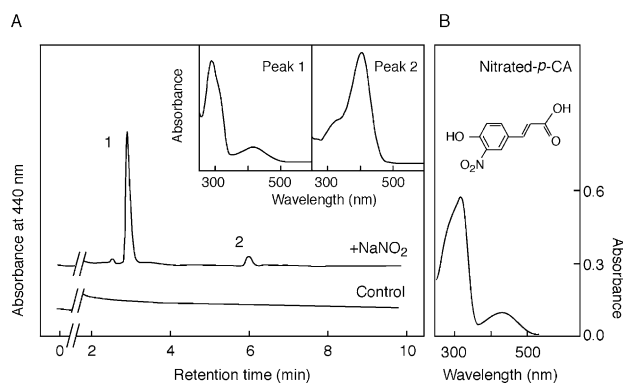


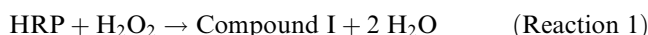
Fig. 2. HPLC analysis for reaction products of HRP-catalyzed *p*-CA nitration. A: HPLC chromatograms of the reaction products. Upper trace, with nitrite (40 mM); lower trace, without nitrite. The inset figures show UV/Vis absorption spectra of the reaction products. The reaction mixture contained 50 mM potassium phosphate (pH 7.4), 25 mM *p*-CA, 40 mM H₂O₂ and 6 μ M HRP. B: UV/Vis absorption spectrum of the synthesized nitrated *p*-CA.

increase in absorbance at 440 nm (Fig. 1C). Pannala et al. [35] have reported the detection of a similar absorbance increase at 430 nm due to production of nitrated *p*-CA by ONOO⁻. Furthermore, van der Vliet et al. [23] have reported that tyrosine, possessing a similar part structure to *p*-CA, is nitrated by a HRP/H₂O₂/nitrite system. To confirm the formation of nitrated *p*-CA, the reaction products were analyzed with a HPLC system by monitoring the absorbance change at 440 nm. No peak was detected in a reaction mixture without NaNO₂. However, in a NaNO₂-containing reaction mixture, new peaks were detected (Fig. 2A). The UV/Vis spectrum of peak 1 showed absorbance maxima at 280 and 440 nm (Fig. 2, inset). The retention time and absorption spectrum of peak 1 showed good agreement with those of synthesized nitrated *p*-CA (Fig. 2B). These results indicate that nitrated *p*-CA was formed from *p*-CA by enzymatic reaction of HRP in the presence of H₂O₂ and NaNO₂.

3.2. Reaction mechanism for plant phenol nitration mediated by HRP

Fig. 3 shows suppression of the *p*-CA nitration by ascorbate, a ubiquitous antioxidant (vitamin C) contained in plant tissues, such as leaves and fruits. In the absence of ascorbate, *p*-CA nitration was immediately observed just after the HRP addition. In contrast, the nitration was initiated 35 s after the addition of HRP when 100 μ M ascorbate was present. This significant time lag of *p*-CA nitration was strongly correlated with the concentration of ascorbate added (Fig. 3, inset). During the suppression of the nitration, *p*-CA oxidation was also suppressed. We previously showed that ascorbate inhibits apparent oxidation of phenolics by reducing short-lived phenoxyl radicals to their parent compounds [37,38]. Consistent with this, *p*-CA nitration did not occur when nitrite was added long (500 s) after initiation of the reaction by HRP in the presence of H₂O₂. These results suggest that *p*-CA radical is required for the HRP-dependent *p*-CA nitration.

Shibata et al. sequence [39] have shown that HRP in conjunction with H₂O₂ oxidizes NO₂⁻ to form nitrogen dioxide radical (NO₂[•]) as in the following reaction:



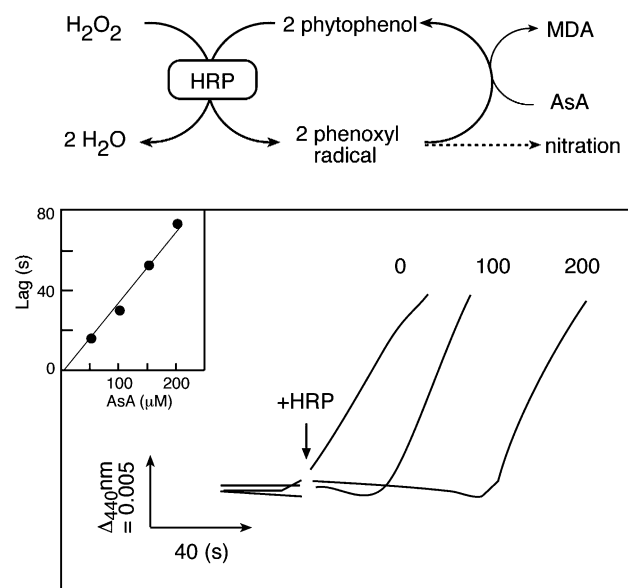
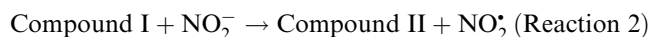
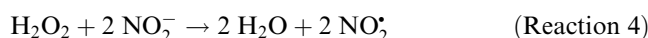


Fig. 3. Retardative effects of ascorbate on HRP-catalyzed *p*-CA nitration in the presence of nitrite. The arrow indicates the addition of HRP. The inset figure shows the relationship between ascorbate concentration and time lag of the *p*-CA nitration. Other experimental conditions were similar to those in Fig. 1. AsA, ascorbate; MDA, monodehydroascorbate.



Reactions 1–3:



It has been reported that NO_2^{\bullet} is capable of nitrating tyrosine in the absence of ONOO^- [21,40,41]. When both nitrite and phenolics are contained in a HRP/ H_2O_2 system reaction medium, phenolics can be oxidized by HRP to form phenoxyl radicals (Phe^{\bullet}) (Reaction 5) [37,38]. NO_2^{\bullet} is also oxidized by HRP to form NO_2^{\bullet} (Reaction 3). Then, phenolics can be nitrated through free radical reaction between Phe^{\bullet} and NO_2^{\bullet} (Reaction 6):



Like ascorbate, other radical scavengers such as glutathione also inhibited *p*-CA nitration by the HRP/ H_2O_2 system. The rates of *p*-CA nitration were decreased to 46.2 and 69.2% of control by 10 and 100 μM glutathione, respectively. Glutathione is known as an effective scavenger for ONOO^- [42,43]. To investigate the involvement of ONOO^- in the HRP-dependent *p*-CA nitration, we tested the effect of O_2 on this nitration. Since ONOO^- production under physiological conditions eventually requires superoxide ($\text{O}_2^{\bullet-}$) generation [44], depletion of O_2 (precursor for $\text{O}_2^{\bullet-}$) should eliminate the nitration of *p*-CA via the suppression of ONOO^- production. However, we did not observe substantial changes in the reaction even under anaerobic conditions (data not shown). Glutathione can react with NO_2^{\bullet} resulting in formation of nitroglutathione [26,45]. Thus, glutathione may compete with Phe^{\bullet}

for NO_2^{\bullet} . These results suggest that ONOO^- is not involved in the mechanism for HRP-dependent *p*-CA nitration.

3.3. Implication for physiological function of HCA nitration in plants

As shown above, *p*-CA can be nitrated by an HRP/ H_2O_2 system in the presence of nitrite, a manner similar to tyrosine nitration. HRP belongs to the guaiacol-type peroxidases (GuPX) which differ from the H_2O_2 scavenging enzyme ascorbate peroxidase. Both HCAs and GuPX are localized in the apoplasts as well as vacuoles of plant cells [46]. Phenoxyl radicals can be produced in the apoplasts as intermediates for lignin biosynthesis. Normally, nitrite is produced through one electron reduction of nitrate by NR in the cytosol. The concentration of nitrite in cells is very low due to its rapid transport into chloroplasts and subsequent reduction to NH_4^+ by nitrite reductase [47]. Recently, a plasma membrane-bound NR, a novel type of NR, has been found in tobacco roots [48], suggesting that nitrite can be produced not only in the cytosol but also in the apoplasts. In this study we used a relatively high concentration of nitrite (such as 10 mM in Fig. 1) to show in vitro *p*-CA nitration. It should be noted that the reaction itself is detectable even at 1 mM nitrite or lower. Thus, the HRP/nitrite-dependent nitration of plant phenolics may occur in the apoplasts rather than the cytosol. Although the data presented here provide in vitro biochemical evidence, the physiological significance of nitro-phenolics formation remains an open question.

Monohydroxy phenolics (monophenolics such as *p*-CA) and polyhydroxy phenolics (polyphenolics such as caffeic acid or chlorogenic acid) display different scavenging mechanisms against RNS [17,32,35]. Monophenolics, which are less efficient antioxidants than polyphenolics [49], can remove ONOO^- (or NO_2^{\bullet}) without nitrite production reaction [35]. In contrast, nitrite can be produced as the result of reductive destruction of ONOO^- (or NO_2^{\bullet}) by antioxidant polyphenolics [32]. Although its toxicity is lower than ONOO^- , nitrite is still a potential cytotoxin for both plants and animals. Recent human atherosclerosis studies have shown that MPO-initiated oxidative damage involves nitrite oxidations [25,52]. Likewise, nitrite exhibits harmful effects on plants [14] such as chlorophyll bleaching [50] and inhibition of enzymatic reactions [51] though molecular mechanisms are largely unknown. We consider it plausible that the nitrating reaction of monophenolics sustains the antioxidant activity of polyphenolics in oxidative stress conditions by suppressing unfavorable increases in cellular nitrite levels, a reservoir function against nitrite and RNS. In this context, a fine combination of anti-oxidative phenolics (polyphenols) and anti-nitrating phenolics (monophenols) would be necessary to suppress both ROS and RNS toxicity that likely occur simultaneously under in vivo situations in both plants and animals.

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