EPR detection of phytophenoxyl radicals stabilized by zinc ions: evidence for the redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system

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Abstract Chlorogenic acid (CGA; 3-*o*-caffeoylquinic acid), a phenylpropanoid metabolite of plants, was oxidized by H_2O_2 in the presence of horseradish peroxidase. The primary and secondary oxidized products both were free radicals which gave EPR multiline signals at $g = 2.0044$ and 2.0042 in the presence of zinc as a spin stabilizing agent. The EPR kinetics showed that ascorbate functioned as a cooperative reductant by regenerating CGA from its corresponding radicals. These results provide evidence to support the idea that the ascorbate-phenolic redox couple in conjunction with guaiacol peroxidase is an efficient H_2O_2 scavenging mechanism in higher plants.

Key words: Antioxidant; Chlorogenic acid; Hydrogen peroxide; Peroxidase; Phenylpropanoid; Electron paramagnetic resonance

1. Introduction

Ascorbate (vitamin C) is abundant throughout plant tissues such as fruits and leaves [1]. There is increasing evidence to indicate that ascorbate plays an important role in scavenging reactive oxygen intermediates (ROIs) in both animals [2] and plants [3]. In addition to this non-enzymatic antioxidative role, ascorbate is required in plants as an electron donor for a unique ascorbate peroxidase (APX) localized in both the chloroplast and cytoplasm [4]. APX is highly specific for the removal of H_2O_2 and is thus pivotal in defense against oxidative damage induced by environmental stress [5]. However, a considerable amount of ascorbate is present in other plant organelles such as the vacuole [1,6], where there is guaiacol peroxidase (GuPX) but no APX [7]. The vacuole is also the major storage compartment of phenolics [6], which can act as electron donors in the GuPX reaction [7]. Although GuPX has been classified as a 'metabolic' peroxidase whose oxidation products have diverse physiological functions in plants [4], the phenolic/GuPX system may also participate in the H_2O_2 scavenging mechanism to complement the ascorbate/APX system in other metabolic compartments [8,9]. This aspect has been recently emphasized by Mehlhorn et al. [10], Takahama and Oniki [11] and Yamasaki et al. [12,13].

In contrast to phenolic substrates, ascorbate is a poor electron donor to GuPX [4,7,11,13]. In spite of this, ascorbate is rapidly oxidized by GuPX in the presence of phenolics [9,10,14]. Takahama et al. have recently suggested that this synergistic effect is due to an indirect electron donation to GuPX via reduction of phenoxyl radicals by ascorbate [9,11]. However, unlike radical species generated from phenolics containing a single aromatic hydroxyl group [15,16], semiquinone radicals derived from *o*-dihydroxy phenolics are extremely unstable and rapidly convert to unknown compounds at physiological pHs [11]. Because of the difficulties in detecting these radicals even in vitro, until now there has been no direct evidence to confirm the proposals of Takahama et al.

To clarify the interaction between ascorbate and plant phenolics in the GuPX reaction, here we demonstrate the spin stabilization approach for detecting phenoxyl radicals. This technique enables short-lived *o*-semiquinone radicals to be detected in high concentrations required for measurements with a static EPR system [17]. Chlorogenic acid (CGA, an ester of caffeic acid and quinic acid), which is naturally abundant in many plant species [18], was used as a model *o*-dihydroxy phenolic compound. CGA is one of many stress-induced phenylpropanoid metabolites [19] and it may function as an in vivo electron donor to GuPX contained in the vacuoles in a manner similar to flavonoids [11]. Also, the simple structure of CGA, compared with flavonoids, facilitates the analysis of the reaction mechanism. In this paper we present direct EPR evidence for the phenolic-mediated redox coupling between ascorbate and peroxidase in the H_2O_2 scavenging reaction.

2. Materials and methods

The oxidation of CGA was monitored as a decrease in absorbance at 320 nm using a single-beam spectrophotometer (Pharmacia Ultrospec III, USA) modified with a constant stirring device maintained at 25°C.

EPR spectra were measured with a Bruker ESP 300E spectrometer (X band, 9.5 GHz) at room temperature (approx. 15°C). The operating conditions were as follows: microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 0.4 gauss, gain 4×10^5, time constant 328 ms. The kinetics of MDA and CGA radicals were monitored at $g = 2.0049$ (second peak of MDA hyperfine signal) and 2.0064 (first peak of CGA hyperfine signal), respectively. The signal overlap was minimal at those fields. A computer simulation of the hyperfine coupling constants was carried out using the WIN-

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Abbreviations: AsA, ascorbic acid; APX, ascorbate peroxidase; CGA, chlorogenic acid; DHA, dehydroascorbic acid; EPR, electron paramagnetic resonance; GuPX, guaiacol peroxidase; HRP, horseradish peroxidase; MDA, monodehydroascorbic acid; ROIs, reactive oxygen intermediates

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EPR software package (Bruker, USA). Kinetic simulations were carried out with Mathematica software (Wolfram Research, USA).

Horseradish peroxidase (HRP) was used as a model GuPX for the H_2O_2-dependent oxidation of CGA because this peroxidase may be localized in vacuoles [11]. The HRP concentration was determined with a molar absorption coefficient of 2.02×10^5 M^{-1} cm^{-1} [20]. Ascorbate (L-ascorbate), CGA and horseradish peroxidase (type II) were purchased from Sigma (USA).

3. Results

Fig. 1 shows the effect of ascorbate on the CGA oxidation in the H_2O_2-HRP system. Despite being a poor electron donor to HRP [13], ascorbate was rapidly oxidized by the H_2O_2-HRP system when CGA was also present (data not shown). As a result, ascorbate prevented the apparent oxidation of CGA under these conditions (Fig. 1). The lag period for CGA oxidation was directly proportional to the initial ascorbate concentration (Fig. 1, inset). Thus, ascorbate presumably acts as a secondary reductant in the CGA peroxidase reaction but not as a primary electron donor to the peroxidase, consistent with the recent suggestions of Takahama and Oniki [11].

We explored the possibility that CGA can form a one-electron redox cycle between ascorbate and the peroxidase in the CGA/ascorbate/H_2O_2 peroxidase reaction as represented in Fig. 2A. The differential equations that describe the rate law for each reaction can be written as follows.

$$d[CGA]/dt = -(k_1[HRP])[CGA][H_2O_2] + k_2[Asc][CGA^\bullet] \quad (1)$$

$$d[CGA^\bullet]/dt = (k_1[HRP])[CGA][H_2O_2] - k_2[Asc][CGA^\bullet] \quad (2)$$

$$d[Asc]/dt = -k_2[Asc][CGA^\bullet] + k_3[MDA]^2 \quad (3)$$

$$d[MDA]/dt = k_2[Asc][CGA^\bullet] - 2k_3[MDA]^2 \quad (4)$$

where CGA, CGA$^\bullet$, Asc, MDA and HRP are chlorogenic acid and its radical, ascorbate and its radical and horseradish peroxidase. Since HRP is a catalyst, its concentration does not change during the course of the reaction. The rate constant of the CGA radical reduction by ascorbate (k_2) was estimated to be 15 000 M^{-1} s^{-1} from analysis of a kinetic model when k_1 and k_3 are assumed to be 80 000 and 4500 M^{-1} s^{-1}, respectively. This value was in good agreement with a recent study of the interaction between ascorbate and flavonoid radicals [21]. Fig. 2B shows the simulated kinetics of MDA and CGA radical concentrations during the reaction when initial reactant concentrations are 10 mM CGA, 5 mM H_2O_2, 2 mM ascorbate and 3.6 nM HRP.

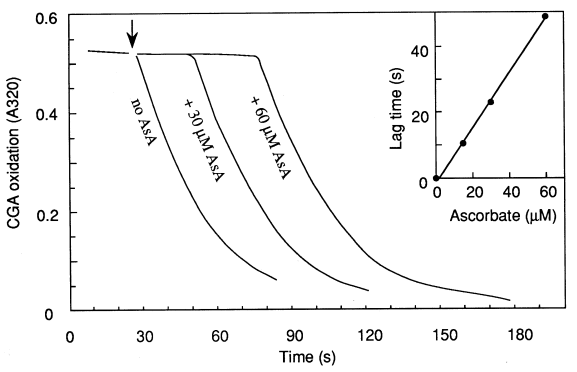

Fig. 1. Retardative effects of ascorbate on CGA oxidation in the HRP system. The reaction medium contained 50 mM Na phosphate (pH 6.0), 30 μM chlorogenic acid, 20 mU/ml HRP. The reaction was initiated by addition of H_2O_2 (200 μM) as indicated by the arrow. Ascorbate was present in the reaction medium before adding H_2O_2 at concentrations indicated beside each trace. The inset shows the ascorbate-dependent prolongation of a lag time of the chlorogenic acid oxidation.

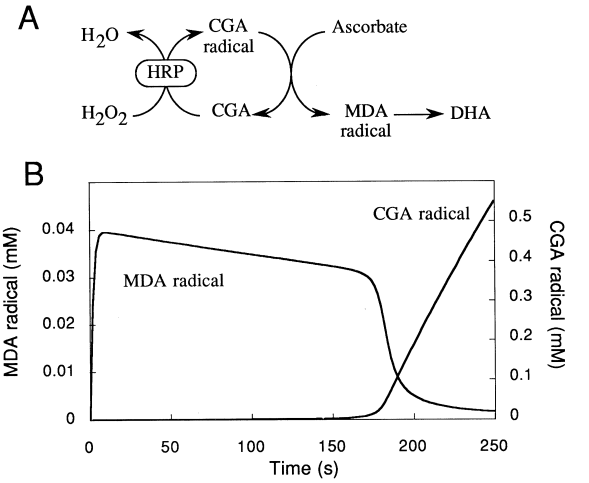

Fig. 2. Kinetics simulation of MDA and CGA radicals in the redox coupled system. A: Scheme of the redox coupling between chlorogenic acid and ascorbate in the H_2O_2-peroxidase system. B: Simulated kinetics of the changes in concentration of both radicals. HRP catalyzes the direct oxidation of CGA to generate the primary CGA radical. Ascorbate acts as an secondary electron donor in this system by reducing the CGA radical back to the parent compound. In the absence of regenerating systems, the MDA radical decays by second-order disproportionation to DHA and ascorbate.

This simulation clearly predicts a rapid increase in the concentration of CGA radical coinciding with the disappearance of MDA radical from the reaction medium. Therefore, the CGA radical should be directly detected after the MDA radical has been exhausted in the redox coupled reaction. However, previous attempts to detect the EPR signal from CGA radicals under static conditions have failed [11], presumably due to the short lifetime of the CGA radical, as has been observed for other *o*-semiquinone radicals [17]. Spin stabilizing agents such as divalent metals have been shown to decrease the rate of second-order recombination of *o*-semiquinone radicals by a factor of 10^4 [17]. We applied zinc as a spin stabilizing agent to detect CGA radicals in the coupled ascorbate/phenolic peroxidase system.

Fig. 3 demonstrates that ascorbate is preferentially oxidized in the peroxidase reaction by acting as a secondary electron donor for the CGA radical. In the fully coupled system, the only EPR signal to be observed in the first few minutes of the reaction was a quasi-stable two-line spectrum at $g = 2.0056$ (Fig. 3A) which was ascribed to the ascorbate radical MDA [22]. The intensity of this signal in the presence of CGA was more than three-fold greater than in its absence (not shown). The MDA signal decayed over several minutes and was followed by a short-lived transition signal (Fig. 3B), which eventually gave rise to an EPR multiline signal at $g = 2.0044$ (Fig. 3C). In the absence of ascorbate, the EPR spectrum shown in Fig. 3C was generated immediately upon addition of H_2O_2 to the peroxidase system. The simulated spectrum (Fig. 4) revealed hyperfine coupling to four inequivalent protons

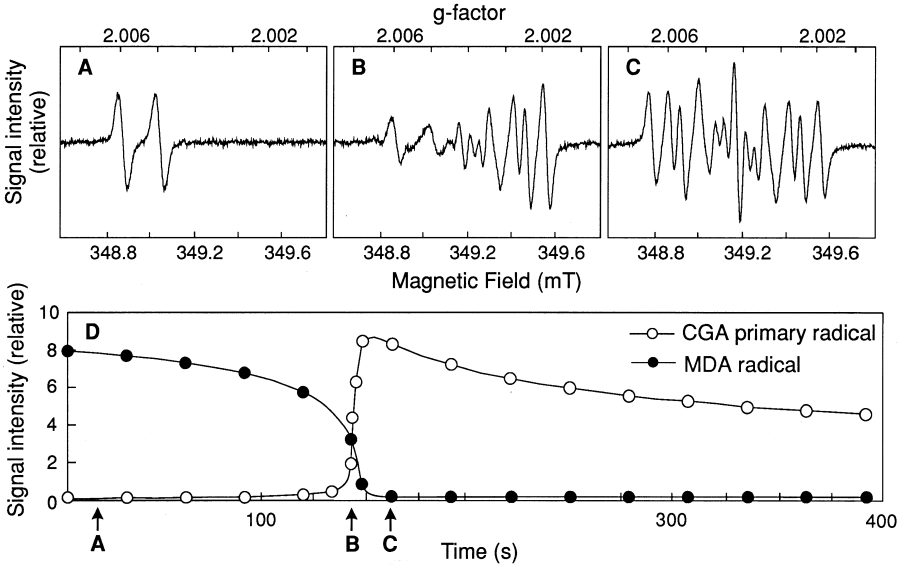

Fig. 3. Transition of radicals formed during the peroxidase reaction. A–C: Typical EPR spectra in the presence of zinc as a spin stabilization agent. A: MDA; B: intermediate; C: primary CGA radical signals. The kinetics of MDA radical (●) and primary CGA radical (○) are shown beneath the EPR spectra (D). The recording time of each spectrum is indicated by the arrows. The reaction medium contained 50 mM MES-KOH (pH 5.5), 200 mM $ZnSO_4$, 10 mM CGA, 50 mU/ml HRP, 5 mM H_2O_2 and 3 mM ascorbate.

($a_3^H = 0.86$G, $a_6^H = 1.38$G, $a_5^H = 2.54$G, $a_\beta^H = 2.98$G), confirming that the spectrum arises from an *o*-semiquinone zinc-CGA radical complex.

The kinetics of the CGA radical were measured from the changes in the intensity of the low-field EPR peak at $g = 2.00647$. This signal showed an abrupt increase which coincided precisely with the disappearance of the MDA radical signal (Fig. 3D). A slow decay of the CGA signal occurred subsequently, the kinetics of which were dominated by a first-order component with a half-time of approximately 178 s, suggesting the involvement of an intramolecular reaction, such as cyclization of the molecule, rather than dismutation in the decay mechanism. An asymmetric EPR signal with 13 peaks centered at $g = 2.0042$ increased in intensity in conjunction with the slow disappearance of the primary CGA radical signal (not shown). This spectrum arose from a mixture of primary and secondary radicals. Computational subtraction using the magnetic parameters ascribed to the primary radical allowed the identification of a nine-line secondary CGA radical spectrum (Fig. 4). Simulation of this spectrum indicated hyperfine coupling with three equivalent (0.54G) and two inequivalent protons (1.34G and 1.17G), suggesting greater delocalization of the radical character in this species as compared to the primary radical. The secondary radical was stable for more than 30 min under both aerobic and anaerobic conditions (not shown), consistent with a low reactivity towards oxygen as well as *o*-semiquinone radicals [23]. The color of reaction medium was blue green when the secondary radical dominated the EPR spectrum.

4. Discussion

4.1. Ascorbate/phenolics/GuPX system as an H_2O_2 scavenging mechanism of vascular plants

The results presented in this study clearly indicate that CGA acts as a one-electron redox mediator between ascorbate and GuPX in conjunction with H_2O_2. Although a novel isozyme of class III peroxidase which is specific for ascorbate rather than guaiacol has been recently found in tea leaves [24], GuPXs do not preferentially use ascorbate as an electron donor in comparison with phenolics. In plants the vacuole and apoplast are the major compartments containing phenolics and GuPX [6,9] and ascorbate can be present at mM order in the vacuoles [25]. If the concentrations of phenolics such as CGA or flavonols in these compartments are also present at mM concentrations [26], the ascorbate/phenolics/GuPX system can efficiently reduce H_2O_2 without any accumulation of oxidized phenolic products (e.g. Figs. 1 and 3) as discussed by Takahama and Oniki [11]. Recently, we have proposed that the vacuoles and apoplasts can function as sinks of H_2O_2 in plant cells, which allow the 'delocalized detoxification' mechanism against H_2O_2 produced in other

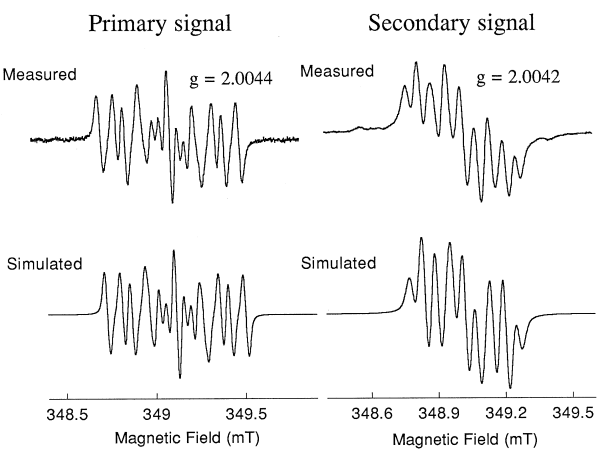

Fig. 4. EPR spectra of the primary and secondary CGA radicals produced by the H_2O_2-peroxidase reaction. Simulated EPR spectra were drawn with the hyperfine coupling constants reported in the text. The spectrum of secondary CGA radical was obtained by subtracting normalized elements of the primary radical.

compartments during stress and development [12,13]. The results shown here provide the mechanistic basis for the redox coupled H_2O_2 scavenging system of such compartments in vascular plants.

4.2. Zinc-stabilized radicals as oxidized products of the GuPX reaction

The spin stabilization technique used by Kalyanaraman et al. [17] for the identification of catecholamine radicals was also effective in revealing CGA radicals at physiological pH (Figs. 3 and 4). During the oxidation of CGA in the H_2O_2-HRP system, two types of free radicals with distinct lifetimes were detected when Zn^{2+} was present to complex the semiquinone structures (Fig. 4). The primary radical was a relatively short-lived 'open-chain' form derived from the one-electron oxidation of the parent CGA. This species slowly decayed and was replaced by a persistent secondary radical which was stable in solution for more than 30 min. The decay kinetics of the primary radical, as well as the more extended hyperfine interaction apparent in the spectrum of the secondary radical, are consistent with the presence of a secondary cyclized product, as has been previously proposed for the HRP-catalyzed oxidation of epinephrine [17]. We consider the secondary radical to be an *o*-semiquinone derived from cyclization of the *cis*-isomer of the primary radical. This product would be structurally related to coumarins which are normally synthesized from cinnamic acid. Further oxidation of the secondary radical would give rise to a complex mixture of quinones as well as polymerized products [11]. Together with a structural analysis of the intermediates in the reaction, further investigations are required to confirm the degradation mechanism.

4.3. Physiological implications of spin stabilized phenoxyl radicals in plants

An important implication of the present study is that metal ions may influence the nature of plant phenolics in vivo by altering the lifetime of phenoxyl radicals as the oxidized products. In general, radicals derived from redox active phenolics are toxic to living systems because of their ability to propagate radical chain reactions in cells [27]. In fact, *o*-dihydroxy phenolics show anti-herbivore activity under certain conditions due to their actions as prooxidants [28]. In addition to Zn^{2+} used in this study, Al^{3+}, Cd^{2+}, Ca^{2+} and Mg^{2+} have been reported to stabilize *o*-semiquinones in both aqueous and non-aqueous media [17]. It is of interest to consider whether Ca^{2+} and Mg^{2+} endogenously act as radical stabilizers. Although the stabilizing roles of divalent cations in the plant cell apoplast and cell membranes are well known, we consider it plausible that these cations could also promote oxidative damage, due to stabilization of phenoxyl radicals, under conditions of oxidative stress. Indeed the toxicity of heavy metals, such as Al^{3+}, Cd^{2+} and Zn^{2+} retained in the root apoplast, could be explained in this way.

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