Hypothesis

Ascorbate is the natural substrate for plant peroxidases

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Abstract Ascorbate-dependent detoxification of hydrogen peroxide by guaiacol-type peroxidases is increased considerably in the presence of 3,4-dihydroxyphenolic compounds, suggesting that ascorbate is the natural substrate for many types of peroxidase in situ and not just the ascorbate-specific peroxidases. The ascorbate-dependent destruction of hydrogen peroxide in the more acidic cellular compartments such as the vacuole may be an important function of such non-specific peroxidases. The stress-induced production of phenolic compounds would render the guaiacol peroxidases in other less acidic-cellular sites effective as ascorbate-dependent H_2O_2-detoxifying enzymes.

Key words: Poplar; Ascorbate peroxidase; Horseradish peroxidase; Caffeic acid; PAGE; Oxidative stress

1. Introduction

Peroxidases (EC 1.11.1.7) are widely distributed in the plant kingdom. They have been isolated and characterized from many other sources [1,2]. Enzymes of the guaiacol-peroxidase type are generally directed to the vacuole and apoplast via the endoplasmic reticulum. While the basic chemical reactions of such peroxidases, the reduction of H_2O_2 by a large array of hydrogen donors, is well established, the function of these isoenzymes and their differential regulation remain largely unknown [3]. Thus, peroxidases have been associated with an ever-increasing number of physiological processes. These include leaf and flower abscission, aging and senescence, apical dominance, cold tolerance, dormancy, fruit development and ripening, germination and early development, reaction and resistance against parasitism [2]. More specifically, where isoenzyme forms have been studied, cationic peroxidases have been implicated in auxin metabolism, whereas anionic peroxidases are thought to participate in lignification [4].

The ascorbate peroxidases (APXs) are involved in the detoxification of hydrogen peroxide both within the cell and in the apoplast [5,6]. Along with the cytochrome *c* peroxidases the APXs belong to the Class I peroxidase family which have been distinguished from the classical plant peroxidases which fall in Class III [7]. Ascorbate-dependent peroxidase activity was first described in the thylakoid membranes [8] but was subsequently found to exist in both soluble cytosolic and plastidic forms [9–11]. APX activity has been found to be present in all higher plant species examined so far [12] and has been isolated from *Euglena gracilis* [13], spinach [14], soybean root nodules [15], pea [16] and tea leaves [17]. Like horseradish peroxidase (HRP), APX is a haem-containing protein [13,14] but its primary structure is more similar to the yeast cytochrome *c* peroxidase than HRP [17,18]. Frequently, APXs have been considered to be functionally distinct from the extensively studied HRP [12]. There are several major differences between these enzymes. The family of peroxidases to which HRP belongs are glycoproteins [19] whereas APX is not [11]. Guaiacol peroxidases can use a range of substrates as electron donors while APXs are often considered to be highly specific for ascorbate as an electron donor [14]. However, APX will use guaiacol and also other substrates [11,13–16]. Indeed, the *Euglena* enzyme catalyses the oxidation of pyrogallol at a 2-fold higher rate than that of ascorbate [13]. APXs from some cyanobacteria can reduce a variety of organic hydroperoxides as well as H_2O_2 and hence may protect the cell membrane from the lipid peroxides produced during degradation of unsaturated fatty acids [20]. APXs are inactivated by *p*-chloromercuribenzoate but guaiacol peroxidases such as HRP are not [21]. The chloroplast APXs are very sensitive to inhibition in the absence of ascorbate [11]. The cytosolic forms are less sensitive and the guaiacol-type peroxidases are not inhibited in the absence of ascorbate [21]. All APXs and guaiacol peroxidase can oxidise ascorbate, pyrogallol and guaiacol [21], hence, the assay of hydrogen-peroxide-dependent oxidation of ascorbate (or pyrogallol or guaiacol) cannot be used as a specific assay for APX or guaiacol peroxidase respectively. Amako et al. [21] have described separate specific assays for these enzymes based on their relative sensitivities to *p*-chloromercuribenzoate.

Intact chloroplasts contain a high level of peroxidase activity to detoxify H_2O_2. The chloroplast peroxidases can use a variety of substrates [22]. The extent to which this activity is related to ascorbate-dependent activity rather than general guaiacol peroxidase is unknown but using *p*-chloromercuribenzoate Amako et al. [21] were only able to detect ascorbate-dependent peroxidase activity in intact spinach chloroplasts. This does not appear to be the case for chloroplasts from all plant species (P. Guilleman, personal communication).

2. Ascorbic acid as a substrate for peroxidases

Ascorbate-dependent peroxidase activity was first recog-

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Abbreviations: ESR, electron spin resonance; HRP, horseradish peroxidase; IEF, isoelectrofocusing; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

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nized in the 1920's by Szent-Györgyi. This activity was found to have a pH optimum of 4.3 [23] and, hence, ascorbic acid was not considered to be an important substrate of plant peroxidases [1–3]. It is now recognized that the ascorbate-dependent peroxidases of plants fulfil a similar function to the glutathione peroxidases of animal cells in detoxifying hydrogen peroxide and also other peroxides in some cases [12,24]. These peroxidases have a pH optimum at pH 7.0 [11,13–16].

The water-soluble apoplastic guaiacol peroxidases will catalyse the H_2O_2-dependent oxidation of phenolic compounds such as coniferyl alcohol. This oxidation is prevented and even reversed in the presence of ascorbic acid [25]. In this situation, hydrogen peroxide is reduced with the phenolic compound acting as the primary reducing agent and ascorbate acting as the secondary and ultimate reductant. Hence, the apoplastic peroxidases can scavenge H_2O_2 and oxidise ascorbate without sustained oxidation of the intermediate phenolic compound. While the guaiacol peroxidases such as HRP prefer phenolic compounds as substrates, they can become effective ascorbate-dependent H_2O_2-scavenging enzymes if ascorbate is available in sufficient concentrations.

The K_m values we obtained for the ascorbate-dependent peroxidase activity of HRP with respect to ascorbic acid and H_2O_2 were 308 ± 18 μM and 31 ± 7 μM, respectively. When the enzyme was pre-incubated in the presence of small quantities (2–200 nM) of caffeic acid, chlorogenic acid or other 3,4-dihydroxyphenolic compounds, which contain the caffeic acid moiety, the ability of HRP to utilise ascorbic acid as a co-substrate was increased considerably (Table 1). If the enzyme was incubated with 3,4-dihydroxyphenolic compounds and H_2O_2 alone no peroxidase activity was detected. The increased affinity of the enzyme for ascorbic acid in the presence of higher concentrations of phenolic compounds (1–2 μM) appeared to be due to turnover of these compounds. ESR experiments confirmed oxidation of ascorbate to the monodehydroascorbate radical, catalyzed by the HRP. ESR spectra were recorded at ambient temperature using a Bruker ER-420 X-band spectrometer and 0.4 mm i.d. quartz flat cells for the aqueous samples. Spectrometer settings were: microwave frequency 9.53 GHz, microwave power 1 mW, modulation 0.5 G, field sweep 100 G, field setting 3400 G, scan time 4 min, gain 2.5×10^6. Assay conditions were as described by Yamazaka et al. [2]. The formation of the monodehydroascorbate radical was identified by its characteristic hyperfine splitting value $a(H) = 1.76$ G and g-factor = 2.0052. If the enzyme had been pre-incubated with caffeic acid or chlorogenic acid, the ESR signal of the monodehydroascorbate radical disappeared faster than in the absence of caffeic acid equivalent to the time when ascorbic acid became limiting as a substrate.

Table 1
Effect of different phenolic compounds (20 nM) on ascorbate-dependent destruction of H_2O_2 by horseradish peroxidase (type I)

Phenolic compound	Stimulation	3,4-dihydroxyphenolic compound
Caffeic acid	+	+
Chlorogenic acid	+	+
Cinnamic acid	–	–
Coumaric acid	–	–
Ferulic acid	–	–
Sinapic acid	–	–
Catechin	+	+
Quercetin	+	+
Rutin	+	+

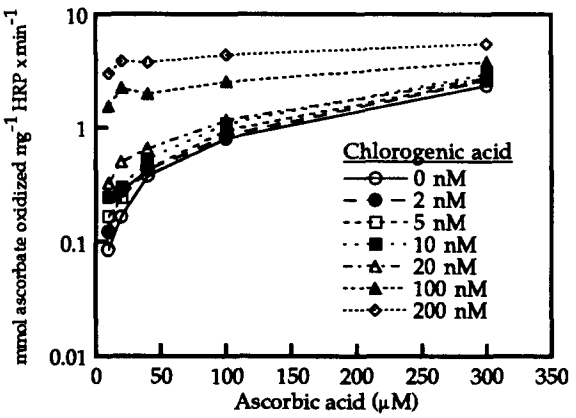

Fig. 1. The effect of low concentrations of chlorogenic acid (2–200 nM) on levels of ascorbic acid-dependent HRP type I activity at different concentrations of ascorbic acid and constant concentrations of H_2O_2 (0.25 mM).

A comparison of the kinetic constants of APX [11,13–16] and those obtained for HRP at pH 7.0 reveals that measured rates of reaction of the enzymes with ascorbate and hydrogen peroxide are remarkably similar (Fig. 1). This indicates that ascorbic acid may be a much more important substrate for general plant peroxidases than has been considered to date and may contribute to the detoxification of hydrogen peroxide. The low pH optimum of the enzyme would indicate that they would be most effective in the acid compartments of the cell such as the vacuole and the apoplastic space [23]. In addition, such peroxidases may use ascorbic acid efficiently as a hydrogen donor inside the thylakoid membranes where the concentration of ascorbic acid is high [26].

Environmental stresses increase the formation of 3,4-dihydroxyphenolic compounds [27–29]. In the presence of 3,4-dihydroxyphenolic compounds, that contain a caffeic acid moiety, HRP will use ascorbic acid as a reductant (Table 1). This may be related to the ability of ascorbic acid to reduce o-quinone derivatives [30–31]. Hence, in stress situations peroxidases may contribute to H_2O_2 destruction in cellular compartments with neutral pH values. Thus caffeic acid will protect tobacco cells against paraquat-mediated lipid peroxidation [32].

3. Activity staining on PAGE gels

Using p-chloromercuribenzoate to distinguish ascorbate-specific peroxidase activity from that guaiacol-type peroxidases Amako et al. [21] were able to identify three isoforms of APX activity in extracts from tea leaves. They conclude that APX is the major peroxidase in leaves but not in roots. We have used a method of activity staining on gels (see legend to Fig. 2 for method) after non-denaturing PAGE and isoelectric focusing that is essentially similar to that described by Mittler and Zilinskas [33]. Three major bands and one minor band of ascorbic acid-dependent peroxidase activity were observed in extracts from poplar leaves using this stain subsequent to non-denaturing PAGE on 10–30% gradient gels [34]. The staining procedure for guaiacol-type peroxidase activity using phena-

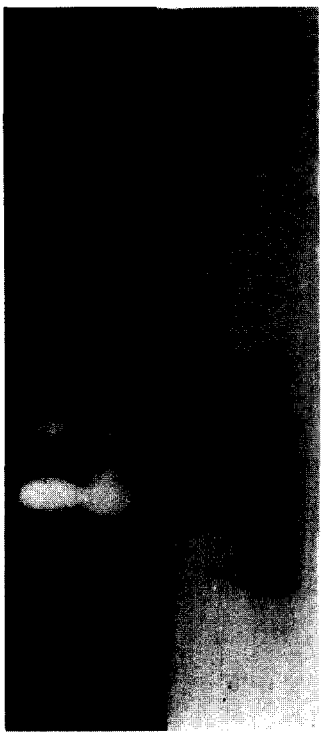

Fig. 2. Isoelectrophoretogram of types I and II HRP identified by the ascorbate-specific peroxidase stain (A,B) and by the general peroxidase stain (C,D). The amounts of protein per track was 50 mg. For detection of non-specific peroxidase activity, the gel was immersed in 100 ml of staining solution containing 1.5 M sodium acetate buffer (pH 5.5) containing 50 mg 3-amino-9-ethylcarbazole and 5 g of antipyrine and 2 ml of 1% (w/v) $MnCl_2$ and 150 ml of 30% hydrogen peroxide were added. This solution was prepared fresh directly before use each day. The gel was incubated in this medium until red bands appeared against a clear background (approximately 10 min). For ascorbate-specific peroxidase activity, gels were soaked in 0.25 M sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 10 min for detection of ascorbate-specific peroxidase activity on the gels. The solution was then changed to one containing 0.25 M sodium phosphate buffer (pH 7.0), 2.0 mM ascorbate and 2 mM hydrogen peroxide. The gel was washed once with 0.25 M sodium phosphate buffer (pH 7.0) and immersed in 25 ml of staining solution containing 2 mM 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide and 10 mM phenazone methosulphate. The latter step must be performed in the dark and the gel incubated in darkness for approximately 10 min until colourless bands appear on a dark blue/green background.

zine and 3-amino-9-ethylcarbazole was able to detect two of these bands. In the poplar leaf extracts one major band of peroxidase (equivalent to the ascorbate-dependent activity) was observed after IEF and also present in the non-specific stain together with other minor (>4) bands of peroxidase activity. Both activity staining methods have similar pitfalls because of the intrinsic capacity of both APX and guaiacol peroxidase to use a variety of substrates the ascorbate-dependent activity stain will also detect other peroxidases normally considered to be of the guaiacol-peroxidase type if sufficient protein is loaded onto the gels. Mittler and Zilinskas [33] found that detection of the latter activity was possible at a loading of 0.2 units of activity and our studies agree with this value. This value may depend on phenolic compounds such as caffeic acid are associated with the peroxidase activity.

Two types of commercially-available HRP were tested for ascorbate-dependent peroxidase activity. Many of the bands of activity detected by the general peroxidase method also displayed peroxidase activity when supplied with ascorbic acid alone as substrate (Fig. 2). To confirm the identity of the ascorbate-specific peroxidase activity, we transferred the protein following activity staining to nitrocellulose and probed the filters with antibody specific to tea leaf APX. Antibodies raised to the tea leaf APX antibodies were found to cross-react with all the isoenzyme forms of tea APX but not with the tea or spinach leaf guaiacol peroxidase [11]. The antiserum raised against the tea APX cross-reacted with both spinach thylakoid-bound APX and tea cytosolic APX [11,35]. In contrast, polyclonal antibodies to the pea cytosolic APX did not cross-react with the chloroplast isoforms [36]. Using the tea leaf APX-specific antibody produced in Professor Asada's laboratory [11], four bands were detected in the poplar leaf extract equivalent to the positions of the bands of activity demonstrated by non-denaturing PAGE (Fig. 3). However, in our hands, this antibody also cross-reacted with the major band in both forms of commercially-available HRP (Fig. 3). This suggests that although these proteins are not immunologically related, they do have common epitopes.

4. The functional perspective

We suggest that APXs and guaiacol-type peroxidases may not be as mutually exclusive in their functional capacities as previously considered and that, in certain circumstances, HRP may function as an APX and vice versa. This adds a degree of flexibility to the antioxidant defences and may facilitate an important regulatory switch in metabolism between lignin biosynthesis in the cell wall and the scavenging of excess H_2O_2 in the apoplast.

On the basis of our observations and those of others [30,31] we are drawn to the conclusion that ascorbic acid is the natural substrate for many types of peroxidase including those currently considered to have no known physiological substrate. Indeed, if guaiacol-type peroxidases are present in the thylakoid membranes ascorbate-dependent destruction of H_2O_2 in the acidic lumen may be an important function on 'non-specific' peroxidases. Other important roles may accrue such as regulation of H_2O_2-dependent events in the apoplastic space. One example may be the oxidative burst associated with the hypersensitive response from the plasmalemma [37–39] in which H_2O_2 plays a key role. This is a powerful modulator of structure and function in response to an immediate threat to metabolism and viability. One result of the oxidative burst is the rapid insolubilization of pre-existing hydroxyproline-rich structural proteins in the plant cell was as a result of H_2O_2-mediated cross-linking. This process precedes the expression of transcription-dependent defences [37,38] and renders the cell wall resistant to digestion by microbial enzymes. In addition H_2O_2 produced in this way functions as a local trigger of programmed cell death in challenged cells and as a diffusible signal for the induction of genes encoding cellular protectants in adjacent cells [39]. The spread of the signal could be regulated to some degree by the participation of cell wall peroxidases which could use ascorbate as substrate in the presence of phenolic com-

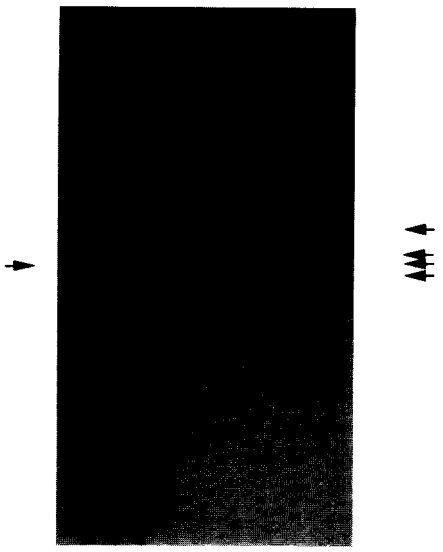

Fig. 3. Western blot of types I and II HRP (A and B, respectively) and poplar leaf extracts (C,D) probed with antibody to tea leaf APX. The amounts of protein loaded on the gels were 100 mg per well in the case of the poplar leaf extract and 50 mg protein in the case of HRP. Proteins were transferred to nitrocellulose and probed with antisera raised against either tea leaf APX or HRP (Sigma Chemical Co., St Louis, MO, USA).

pounds [22,30,31,40,41]. The stress-mediated induction of secondary compounds may in this way aid the elimination of H_2O_2 [27,31].

Ascorbate has been shown to be an important component of the apoplastic space in leaves [30,31,40–45] and is present at concentrations in the millimolar range. In the apoplast, ascorbate is known to regulate cell wall-associated enzymes. For example, it inhibits the peroxide-mediated oxidation of coniferyl alcohol by reducing the radical products of the reaction [30]. Ascorbate itself is oxidized in this process and the initial substrate for peroxidase is regenerated such that net oxidation of coniferyl alcohol is observed only after the oxidation of all the ascorbate present [25,41]. The monodehydroascorbate produced in this way may fulfil other regulatory roles since monodehydroascorbate-dependent electron transport by enzymes on the plasmamembrane has been implicated in growth and other processes [46–49].

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