Thiol-dependent degradation of protoporphyrin IX by plant peroxidases

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Received 18 December 1998; received in revised form 9 January 1999

Abstract Protoporphyrin IX (PP) is the last porphyrin intermediate in common between heme and chlorophyll biosynthesis. This pigment normally does not accumulate in plants because its highly photodynamic nature makes it toxic. While the steps leading to heme and chlorophylls are well characterized, relatively little is known of the metabolic fate of excess PP in plants. We have discovered that plant peroxidases can rapidly degrade this pigment in the presence of thiol-containing substrates such as glutathione and cysteine. This thiol-dependent degradation of PP by horseradish peroxidase consumes oxygen and is inhibited by ascorbic acid.

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Key words: Porphyrin; Tetrapyrrole; Degradation; Breakdown; Turn-over

1. Introduction

The tetrapyrrole pathway plays an important role in plants because it leads to hemes, chlorophylls, as well as to a host of biologically important cofactors [1,2]. Protoporphyrin IX (PP) is the last common precursor at the branching point of the tetrapyrrole pathway, with the subsequent chelation of an iron or magnesium in the center of the porphyrin dedicating these molecules to either heme or chlorophyll synthesis, respectively. Most of the steps involved in these pathways have been characterized. The porphyrin biosynthetic pathway is tightly controlled because of the potentially harmful consequences that would occur during unregulated extraplastidic accumulation of photodynamic intermediates. In fact, disruption of this pathway by chemical inhibition of the enzyme protoporphyrinogen oxidase has been successfully used to develop herbicides [3]. While the tetrapyrrole biosynthetic pathway is well understood, little is known of the fate of these pigment intermediates during regular metabolic turn-over and during senescence. Several chlorophyll/porphyrin catabolites have been identified during leaf senescence [4–9]. The involvement of peroxidases in chlorophyll catabolism has been suggested based on the characterization of a catabolite [10], and an unknown enzymatic activity involved in porphyrin degradation has been reported [11].

During our investigations of the metabolism of porphyrins in plants, we have found that horseradish peroxidases (HRP) can oxidize the synthetic porphyrin deuteroporphyrin IX (DP) into chlorins [12–14]. This observation suggests that peroxidase-dependent oxidation of porphyrins may play a significant role in tetrapyrrole catabolism in plants and animals. In this report, we demonstrate that HRP rapidly oxidizes PP into several metabolic derivatives, and that a similar enzymatic activity was detected in cucumber tissues.

2. Materials and methods

2.1. Enzymatic assay and detection

The standard reaction mixture consisting of 100 mM Tris (pH 7.5), 1 mM EDTA, 1 mM reduced glutathione (GSH), 0.02 mg ml^{-1} (3.3 purpurogallin units) HRP type II (Sigma Chemical Co.), and 50 μM PP (Porphyrin Products) in 2.5 ml total volume was modified from Dayan et al. [14]. The conversion of PP was monitored by HPLC. The HPLC consisted of Waters Associates components: Model 717 autosampler, Model 600 controller, and Models 470 fluorescence and 996 photodiode spectrophotometric detectors. The column was a 300×3.9 mm (i.d.) μ-Bondapak C18 reversed-phase column. The solvent system was a linear gradient from 70 to 100% HPLC-grade methanol over 20 min and maintained at 100% methanol for 15 more min. The injection volume was 10 μl.

PP and other porphyrins were detected with fluorescence detector using excitation and emission wavelengths at 400 and 630 nm, respectively. The UV spectra of PP and the breakdown products were observed between 350 and 700 nm with a photodiode array detector. The concentration of PP was calculated using a standard curve established using technical grade PP. Changes in fluorescence were observed with a luminescence spectrometer (Shimadzu Model RF-5301 PC) by scanning fluorescence of the samples between 400 and 700 nm following excitation at 395 nm. All reactions were carried out under dim green light at room temperature unless mentioned otherwise.

2.2. Effect of reducing agents on HRP-catalyzed PP breakdown

The requirement for reducing thiol groups was determined by testing the rate of PP degradation in the presence of 1 mM of either GSH, cysteine, *N*-acetylcysteine, dithiothreitol (DTT), or β-mercaptoethanol in the standard reaction assay. Oxidized glutathione (GSSG) and non-thiol reducing agents, such as ascorbic and citric acid, were also tested at 1 mM. Finally, the inhibition of PP degradation by 10 μM ascorbic acid was tested in the presence of 1 mM GSH. The amount of PP remaining in the assay solution was determined by HPLC as described above.

2.3. Requirement for O_2

The consumption of O_2 occurring during this thiol-dependent PP degradation catalyzed by HRP was determined using a Hansatech DW1 cell connected to an OMS1 oxygen monitoring system. Change in oxygen concentration was determined in the standard reaction assay over 40 s, PP being added 15 s after initiating the reaction. The reaction was carried out at 30°C and in darkness to prevent photodynamic generation of oxygen radicals [15,16].

2.4. Extraction of PP-degrading activity

Cucumber (*Cucumis sativa* L. Straight eight) seedlings were grown for 30 days in growth chambers at 25±2°C under fluorescent lights maintaining a 16-h photoperiod at 400 μmol m^{-2} s^{-1}. Five grams of cucumber leaf tissues were homogenized in 20 ml of cold extraction buffer (2% sucrose in 1 mM EDTA, 1 mM $MgCl_2$, and 100 mM Tris, pH 7.5) for 20 s with a polytron homogenizer (Polytron PT3100). The homogenate was passed through one layer of Miracloth and centrifuged at 50 000×*g* for 20 min at 4°C. The supernatant was collected, adjusted to 2 mg/ml protein according to Bradford [17], and kept at −70°C until used. PP-degrading activity was tested by adding 10 μM PP and 1 mM GSH to 500 μl crude extract of cucumber leaf. The amount of PP remaining in the assay solution was determined by

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PII: S0014-5793(99)00065-4

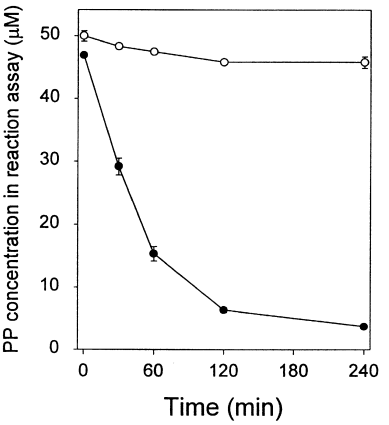

Fig. 1. Time course of HRP-catalyzed PP breakdown in the absence (○) or presence (●) of 1 mM GSH. The reaction assay mixture consisted of 100 mM Tris (pH 7.5), 1 mM EDTA, 0.02 mg/ml (3.3 purpurogallin units) HRP type II, and 50 μM PP. The reaction was carried out in darkness. Each point is the mean of two independent experiments with three observations each ($n = 6$), and the error bars indicate the standard deviation.

HPLC as described above. No loss of activity was detected after 15 days storage at −70°C.

2.5. Statistical analyses

Each experiment consisted of two replications with three observations each, unless indicated otherwise. Data represent means pooled over all experiments ± S.D. ($n = 6$).

3. Results

3.1. Degradation of PP by HRP

Levels of PP decreased rapidly in the presence of HRP and GSH. In a time course study starting with 50 μM PP, degradation of this pigment was nearly complete within 2 h of incubation, and small amounts of PP (< 10%) remained after 4 h incubation (Fig. 1). Only trace amounts of PP remained after overnight incubation (Fig. 2B). The breakdown products generated were much more polar than PP and eluted as a single peak with the solvent front. The UV spectra of the PP breakdown products were identical to that of the PP standard (data not shown). An HPLC chromatogram of the end reaction using a more polar solvent system indicated that at least four major metabolites were generated (Fig. 2C). No degradation was detected over time when GSH or HRP were omitted from the standard reaction assay mixture (data not shown).

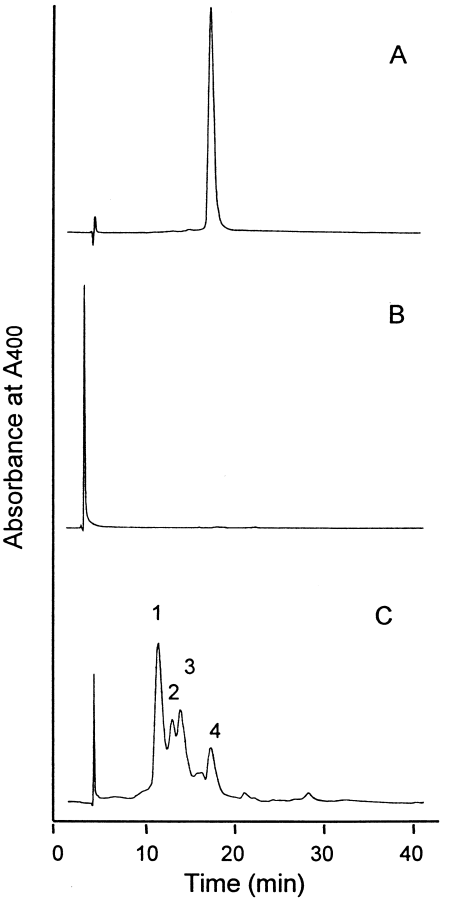

Fig. 2. HPLC chromatograms of the reaction assay at (A) 0 h and (B) 20 h incubation in darkness. HPLC conditions for A and B were a 70:30% MeOH:water to 100% methanol over 20 min. In C, the HPLC gradient was modified to begin with 40:60% MeOH:water to separate the major components of the hydrophilic porphyrin peak eluting with the front in B.

3.2. Requirement for thiol reactive groups

The existence of the peroxidase-dependent degradation of PP was first identified using GSH in the assay. However, GSH could be subsituted with other thiol-containing reducing compounds. While the degradation of PP was more rapid with cysteine or *N*-acetylcysteine, the reaction was slower in the presence of DTT and β-mercaptoethanol (Table 1). Substituting thiol-containing reducing agents with organic acids such as ascorbic and citric acids did not lead to PP degradation (data not shown). In fact, adding 10 μM ascorbic acid to the

Table 1
HRP-dependent degradation of PP in the presence of various thiol-containing compounds after 4 h incubation

Compound	PP remaining (μM)	Degradation (%)
Control	48.0 ± 1.3	4
GSH	6.0 ± 0.6	88
GSSG	45.2 ± 1.7	10
Cysteine	4.7 ± 0.7	91
N-Acetylcysteine	2.9 ± 0.1	94
DTT	25.1 ± 1.1	50
β-Mercaptoethanol	43.4 ± 1.1	13

The reaction assay consisted of 100 mM Tris (pH 7.5), 1 mM EDTA, 0.02 mg/ml HRP and 50 μM PP. Compounds were tested at 1 mM. The reaction was carried out in darkness. Each point is the mean of two independent experiments with three observations each ($n = 6$) followed by the standard deviation. The degradation was calculated based on the initial PP concentration.

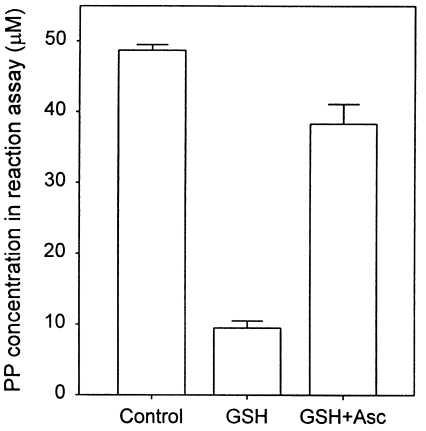

Fig. 3. Inhibitory activity of ascorbic acid (10 μM) in the standard assay after 4 h incubation. The reaction assay mixture consisted of 100 mM Tris (pH 7.5), 1 mM EDTA, 0.02 mg/ml (3.3 purpurogallin units) HRP type II, 50 μM PP, and 1 mM GSH (except for the control where no GSH was added). The reaction was carried out in darkness. Each point is the mean of two independent experiments with three observations each ($n=6$), and the error bars indicate the standard deviation.

standard reaction mixture containing 1 mM GSH dramatically reduced the rate of PP breakdown catalyzed by HRP (Fig. 3).

3.3. Consumption of oxygen during thiol-dependent PP breakdown catalyzed by HRP

Little oxygen consumption occurs during the initial incubation of HRP and GSH (see initial rate during the first 15 s in Fig. 4). However, the concentration of dissolved oxygen in the assay solution decreased rapidly subsequent to the addition of PP to the reaction assay (arrow in Fig. 4). The rate of oxygen consumption remained linear for several minutes and slowly leveled off thereafter.

3.4. Degradation of PP by cucumber leaf crude extract

The ability of plant peroxidases to degrade PP was tested using crude cucumber leaf extracts (Fig. 5). It was necessary to reduce the amount of PP added in the assay because the rate of degradation was much slower than in the standard assay using HRP. PP concentration decreased slowly in the

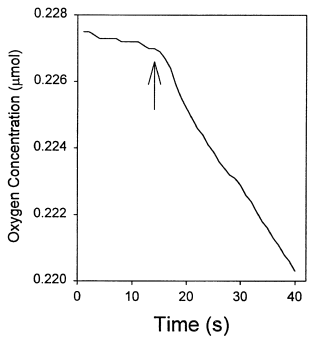

Fig. 4. Oxygen consumption during PP degradation catalyzed by HRP in the presence of 1 mM GSH. The reaction assay mixture consisted of 100 mM Tris (pH 7.5), 1 mM EDTA, 0.02 mg/ml (3.3 purpurogallin units) HRP type II, and 50 μM PP. The reaction was carried out in darkness. The data are a representative example of the oxygen uptake experiment, but the experiment was repeated three times with similar results.

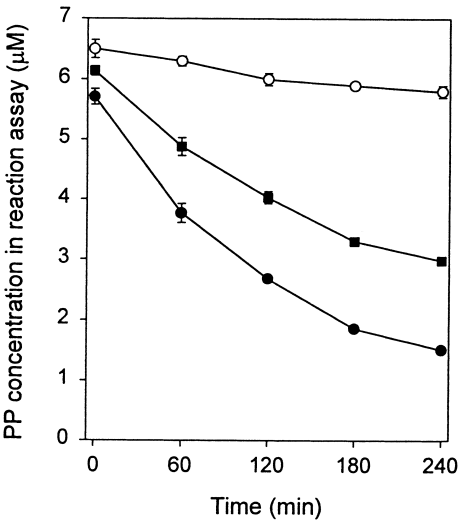

Fig. 5. Degradation of PP in crude cell-free extract of cucumber leaves. ○ = no GSH added to crude extract; ● = 1 mM GSH added to crude extract; ■ = 1 mM GSH and 10 μM ascorbic acid added to crude extract. The assay mixture consisted of 100 mM Tris (pH 7.5), 1 mM EDTA, and 10 μM PP. The reaction was carried out in darkness. Each point is the mean of two independent experiments with three observations each ($n=6$), and the error bars indicate the standard deviation.

absence of exogenously applied GSH. Degradation increased in the presence of 1 mM GSH. Adding ascorbic acid to the assay solution slowed down the thiol-dependent degradation of PP (Fig. 5).

4. Discussion

Following our initial observation that HRP could transform DP into chlorins via an oxidative pathway [12–14], we wanted to determine if HRP could catalyze a similar reaction with PP. It appeared at first that HRP did not react with PP because, unlike with DP, no obvious changes in its UV-Vis spectrum were detected. There was no evidence of oxidation of the porphyrin ring that yielded the typical 'green-colored' chlorin, as was observed with the appearance of a new spectral peak in the 600 nm region, when DP was incubated with HRP and GSH [14]. However, upon HPLC injection, we determined that in the presence of GSH, HRP caused some other transformation on PP. The amount of PP in the assay solution decreased rapidly, and a new peak eluted near the solvent front. While this peak essentially retained the spectral characteristics of PP, it was much more hydrophilic. A similar increase in hydrophilicity was observed when DP was transformed into chlorins in the presence of HRP and reducing thiol groups [14]. The lack of change in the UV spectrum of PP can be attributed to the presence of the vinyl groups on PP. Unlike DP, the vinyl double bonds of PP are more accessible to oxidative degradation than are the conjugated double bonds of the tetrapyrrole backbone. While the oxidation of one of the pyrrole double bonds of DP disrupted the conjugation of the tetrapyrrole that resulted in the appearance of a chlorin peak, oxidation of the vinyl double bond of PP did not affect the porphyrin backbone. As a result, no change in the UV spectrum was observed (data not shown). However, oxidation of the vinyl group dramatically increased the hydro-

philicity of the molecules causing the metabolites to elute much more rapidly (Fig. 2).

The reaction appears to occur via a similar mechanism as the one reported earlier with DP [14]. In particular, this HRP-driven reaction was carried out in the absence of added H_2O_2, requires the presence of reducing thiols, and ascorbic acid was a potent inhibitor. Moreover, no oxygen consumption occurred until PP was added to the reaction mixture.

The ability of peroxidases to oxidize GSH has been reported extensively [18]. Such reactions generate complicated radical reactions that yield highly reactive intermediates that may be able to react directly with porphyrins [19]. PP may even be oxidized via a hydrogen peroxide mediated reaction similar to that reported to occur with heme [20]. Moreover, HRP is known to generate adducts by reacting with thiol-containing substrates and molecules containing accessible vinyl groups [21–23]. While the nature of the PP breakdown products are unknown, our data suggest that the vinyl groups of PP may either be oxidized in a manner similar to that reported on DP [13], or involving thiol adducts similar to those occurring with the terminal vinyl group of eugenol [21,22].

The inhibition of PP degradation observed with ascorbic acid is similar to that reported for the thiol-dependent oxidation of DP [14]. Ascorbic acid has recently been proposed to be the natural substrate of plant peroxidases [24]. As such, it may compete for HRP and prevent peroxidase from utilizing the thiol substrates. In addition, ascorbic acid may act as a quenching agent by reducing any reactive radical intermediates involved in the degradation of PP.

The discovery that plant peroxidases can degrade PP in the presence of suitable reducing thiol substrates has many implications (Fig. 5). Yu et al. [25] reported that an unknown enzymatic activity might be involved in the breakdown of newly synthesized heme in the chloroplast. Various reducing thiols such as cysteine, DTT and β-mercaptoethanol decreased heme synthesis. Their data suggest that active oxygen species are involved in the breakdown of heme. Moreover, ascorbic acid inhibited the degradation of heme. Our results provide an explanation for their observations. We have demonstrated that plant peroxidases can degrade PP in the presence of reduced thiols. Furthermore, we demonstrated that this reaction consumes oxygen and can be inhibited by ascorbic acid. This enzyme system may play an important role in porphyrin turnover.

The breakdown of porphyrins catalyzed by plant peroxidases may also be a key factor in resistance to herbicides that cause PP to accumulate. Herbicides that disrupt the activity of protoporphyrinogen oxidase dramatically affect the pathway and lead to an unregulated accumulation of PP in the cytosol [26–29]. In the presence of light, this photodynamic pigment leads to the formation of highly reactive singlet oxygen believed to be responsible for the herbicidal action of these inhibitors [14]. The photobleaching response is dependent upon the amount of PP accumulating in treated tissues [28–31]. Some plant species are much less susceptible to these herbicides than others [3]. Resistance at the molecular site does not appear to explain these natural differences in sensitivity. Plant peroxidase may protect certain plant species against the accumulation of PP by rapidly breaking down PP, using the thiol-dependent porphyrin degradation pathway such as the one we describe in this report.

Acknowledgements: We are grateful for the excellent technical assistance provided by Stacy N. Allen.

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