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IDENTIFICATION OF A THYLAKOID PEROXIDASE OF BARLEY WHICH OXIDIZES HYDROQUINONE

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; chloroplasts; peroxidase; photooxidative stress; quinone oxidation; thylakoid.

Abstract—The oxidation of hydroquinone by two peroxidases (EC 1.11.1.7) fractions obtained from barley leaves has been studied. The results indicated that thylakoid contains hydroquinone peroxidase which may be distinguished from soluble peroxidase by apparent K_Ms, influence of pH on activity and zymographic assays. Appropriate controls indicated that thylakoid peroxidase was not a contamination from other cellular fractions. Thylakoid peroxidase may be solubilized by Triton-containing buffers but not by buffers without detergent and can also be distinguished of other guaiacol peroxidases (as cell wall peroxidase) and of thylakoid ascorbate peroxidase (EC 1.11.1.11) by zymographic assays and stability and kinetic properties. The reduced form of quinone electron carrier of thylakoids may be substrates of HQ peroxidase. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Phenolic compounds are very reactive and easily subjected to oxidation, substitution and coupling reactions [1]. In addition to a large variety of phenolic compounds stored in the vacuole or in cell wall space, hydroquinones involved in photosynthetic electron transport could be substrates of peroxidases. Hydroquinones are oxidized by peroxidases to the semiquinone radical, which is very unstable and rapidly transformed to paraquinones, dimers or polymers, depending on the substitution pattern [2, 3]. As chloroplast superoxide dismutase (EC 1.15.1.1) produces H_2O_2 [4] but the organelle does not contain catalase (EC 1.11.1.6) [5], one obvious question is whether a plastid peroxidase could oxidize hydroquinones.

In this report, we studied the capacity of soluble and thylakoid fractions obtained from barley leaves to oxidize hydroquinone (HQ) to *p*-benzoquinone (BQ). Kinetic properties and zymographic patterns of soluble and Triton solubilized thylakoid peroxidases were compared.

RESULTS

Characterization of the oxidation of hydroquinone by barley leaf peroxidase fractions

The oxidation of HQ by peroxidases can be followed spectrophotometrically, since the BQ end-product has a characteristic absorption band in the region 235–268 nm [6]. While a certain chemical oxidation of HQ to BQ by H_2O_2 took place in the absence of enzyme (Fig. 1B), thylakoid fraction (Fig. 1A) and soluble fraction (not shown) strongly stimulated the formation of BQ in the incubation medium in a reaction strictly dependent on the presence of H_2O_2 (Fig. 1C). The appearance of an isosbestic point at $\lambda = 268$ nm in the consecutive spectra suggested that there was a constant formation of BQ (A increase at 250 nm) from HQ (A decrease at 290 nm).

These observations, together with the inability of thylakoid fraction (Fig. 1C) and soluble fraction (not shown) to oxidize HQ in the absence of H_2O_2, and the fact that it was not possible to determine polyphenol oxidase (EC 1.14.18.1) activity in barley protein fractions using 4-methylcatechol (4-MC) as substrate [7], suggest that the oxidation of HQ to BQ must be attributed to peroxidase activities.

Thylakoid peroxidase activity was assayed in the fraction solubilized with 1.5% Triton. No peroxidase activity could be solubilized with potassium phosphate buffer (without Triton) from pelleted thylakoid membranes.

In order to characterize the peroxidative oxidation of HQ by thylakoid and soluble barley peroxidases, the oxidation rate (estimated from the increases in A at 250 nm) was determined at different pHs and H_2O_2 and HQ concentrations.

Variation of the pH of a 100 mM Tris-acetate buffer in the 5.0–8.0 range did not significantly modify the

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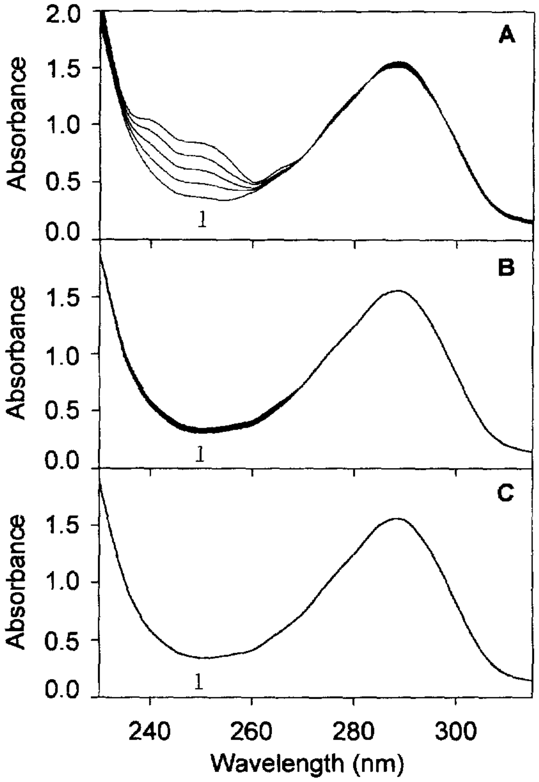

Fig. 1. A. Spectrophotometric recording of the oxidation of 0.5 mM hydroquinone by 10 μl thylakoid peroxidase and 0.5 mM H_2O_2 at 25° in the presence of 50 mM potassium phosphate buffer at pH 7. Consecutive scans were recorded each two min after the addition of enzyme (scan 1). **B**. Control in the absence of enzyme. **C**. Control in the absence of H_2O_2.

HQ oxidation rate by thylakoid peroxidase activity (Fig. 2B) (around 0.3 μmol min^{-1} mg protein^{-1}). Soluble peroxidase activity (Fig. 2A) showed a maximum of activity (around 1.8 μmol min^{-1} mg protein^{-1}) at pH 7.0 which was only reduced to 75% at pH 3.0. In contrast, the HQ oxidation rate by thylakoid peroxidase activity at pH 3.0 was lower than 15% of the rate at pH 5.0 to 8.0. For kinetic purposes, and due to the neutral pH of the two compartments (chloroplasts and cytosolic medium), we used pH 7.0 in all the subsequent kinetic studies.

Except for progressive inhibitions at high substrate concentrations (above 10 times K_M), the dependence of the HQ oxidation rate on a variation in the H_2O_2 and HQ concentrations showed, in both cases, Michaelis–Menten-type kinetics for both soluble and thylakoid peroxidase activities. Although valid K_M values cannot be defined for oxidations catalyzed by peroxidases [3], as Table 1 shows, apparent K_Ms for HQ were similar in thylakoid and soluble peroxidases (50 and 60 μM, respectively) while thylakoid peroxidase showed lower apparent K_M (40 μM) than soluble peroxidase (70 μM) for H_2O_2.

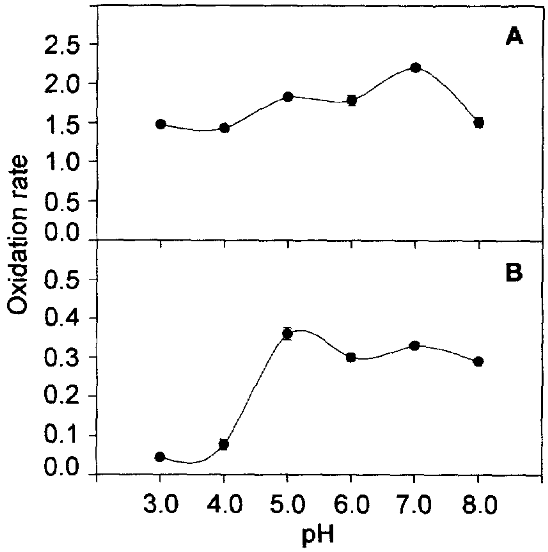

Fig. 2. Dependence of the hydroquinone oxidation rate on the pH of a reaction medium composed of 0.5 mM HQ, 0.1 mM H_2O_2 and 100 mM Tris-acetate buffer of variable pH (3.0–8.0 range), for soluble (**A**) and thylakoid (**B**) peroxidase. Oxidation rates are expressed as μmol min^{-1} mg protein^{-1} of Q produced.

Table 1. Kinetic properties of thylakoid and soluble peroxidases

Enzyme fraction	K_M (μM)		V_{max} μmol of Q min^{-1} mg protein^{-1})
	HQ	H_2O_2	
Thylakoid	50	40	0.3
Soluble	60	70	1.8

Screening of barley peroxidase isoenzymes capable of oxidizing HQ

In addition to differences in the effect of pH on activity and in apparent K_M for H_2O_2, thylakoid and soluble peroxidase activities with HQ could be distinguished by staining peroxidase isoenzymes separated by electrophoresis in polyacrylamide gels. Fig. 3 shows the band patterns of peroxidases. For comparison, zymograms with 4-methoxy-α-naphthol (MN) as substrate and of ascorbate peroxidase were also performed. Under the assay conditions, four HQ peroxidase isoenzymes (Fig. 3 HQ) could be distinguished (those of the highest and the lowest migration rate showing high activity) in soluble fraction. The thylakoid fraction only showed one activity of low migration rate clearly distinguishable from the still lower migration rate of the lowest mobility soluble isoenzyme. Migration differences between these thylakoid and soluble activities were more apparent for

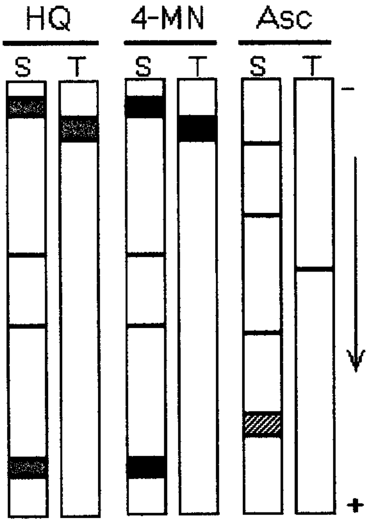

Fig. 3. Comparative isoenzyme pattern of soluble (S) and thylakoid (T) peroxidases after staining with 4-methoxy-α-naphthol (MN), hydroquinone (HQ) and ascorbate (Asc). 40 μg proteins was loaded per lane. Arrow indicates the direction of protein migration during the electrophoresis.

longer migration times, when fast migrating bands of soluble fraction left the gel (not shown). The isoenzyme patterns were identical to those obtained with MN (Fig. 3 MN), the latter being a specific substrate of peroxidases not oxidizable by other heme-proteins [8]. Entirely different isoenzyme patterns were obtained with ascorbate as substrate (Fig. 3 Asc). The single middle migration rate activity detected with ascorbate in thylakoid had no counterpart in thylakoid HQ-MN peroxidase activities.

Thylakoid HQ peroxidase could also be distinguished from ascorbate peroxidase by stability: after two weeks stored at −20° virtually all original ascorbate-dependent activity (0.03 μmol of ascorbate oxidized min^{-1} mg $protein^{-1}$ in barley thylakoids) was inactivated while 60% of the original HQ peroxidase remained. Additionally, while thylakoid ascorbate peroxidase was quickly inactivated after 20 min at 0–5°, HQ peroxidase remained active.

Barley cell wall peroxidase showed similar activities at pH 4 (5.76 ±0.65 μmol min^{-1} mg $protein^{-1}$), 5.0 (5.63 ±0.33 μmol min^{-1} mg $protein^{-1}$) and 7.0 (5.26 ± 1.01 μmol min^{-1} mg $protein^{-1}$), while at pH 4.0 thylakoid peroxidase showed only around 20% of the activity at pH 7.0 (Fig. 2B). Moreover, in contrast to cell wall peroxidase, which retained more than 60% activity after dialysis, essentially all thylakoid HQ peroxidase was lost after a 3-hs dialysis against buffer H. Zymographic analysis of cell wall peroxidase (results not shown) gave similar bands in soluble peroxidase (Fig. 3), indicating again that the activity solubilized with Triton was genuine thylakoids and not a contamination by cell wall nor soluble peroxidase.

DISCUSSION

Though only protein sequencing would provide final evidence, isoenzyme pattern, kinetic parameters, pH dependence of activity and stability indicate that HQ peroxidase solubilized with Triton from barley thylakoid is different from soluble peroxidase. The last may include several of the well known plant cytosolic and extracellular peroxidases [3]. HQ peroxidase activity in Triton-solubilized fraction from thylakoid is low (0.3 μmol min^{-1} mg $protein^{-1}$ in barley thylakoids) when compared with the specific activity of peroxidase in soluble fraction (1.8 μmol min^{-1} mg $protein^{-1}$), but significantly higher than that of the ascorbate peroxidase described in chloroplasts [9] (0.03 μmol min^{-1} mg $protein^{-1}$ measured in barley).

Isoenzyme patterns (Fig. 3) and stability clearly indicate that thylakoid HQ peroxidase of barley is different of ascorbate peroxidase. A guaiacol peroxidase, probably also localized in chloroplasts, has been recently [10] studied in spruce needles.

Barley thylakoid HQ peroxidase shows low K_Ms for H_2O_2 (40 μM) and HQ (50 μM) which raises the question of its role in chloroplasts. Preliminary results indicated that dithionite-reduced commercial vitamin K and plastoquinone are substrates of barley thylakoid peroxidase (data not shown). These results suggest that HQ peroxidase could counterbalance the increase of H_2O_2 concentration (produced for example under photooxidative stress) by diverting electrons to H_2O_2 from photosynthetic electron transport chain at the level of quinone transporters. Thus, HQ peroxidase, as ascorbate peroxidase, could act as a mechanism of defense against photooxidative stress by scavenging H_2O_2 in chloroplasts which lack catalase [5].

Photooxidative stress arises from the harmful effects of active oxygen species, such as H_2O_2 and superoxide anion and hydroxyl radicals, which react with lipids, pigments, proteins and nucleic acids [11]. In order to prevent oxidative stress, organisms have evolved a complex antioxidant system composed of non-enzymic and enzymic mechanisms which scavenge active oxygen species [4, 11, 12]. High light intensity and low CO_2 fixation rates increase the levels of the reduced forms of photosynthetic electron carriers [13], which favour the production of active oxygen species, such as superoxide anion (O^{-}_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH) [14] initiated by one-electron transfer from reduced carrier to O_2.

When, under high light intensity and low CO_2 fixation rate, the concentration of H_2O_2 increases, HQ peroxidase acting on reduced quinone electron carriers of thylakoid would have a double effect against photooxidative stress by lowering both H_2O_2 concentration and the levels of reduced forms of electron carriers. Accordingly, our preliminary results (not shown) indicated that the specific activity of barley thylakoid HQ peroxidase was 46 ± 20% higher when leaf segments were incubated at 22° under 46 W m^{-2} white light than when they were incubated under 22 W m^{-2} white light during 20 hr.

EXPERIMENTAL

Reagents

Hydroquinone (HQ) was obtained from BDH Chemicals. H_2O_2 was obtained from Merck and 4-methoxy-α-naphthol (MN) from Aldrich. Protein Assay Kit was obtained from Bio–Rad Laboratories. All other chemicals used in this work were the best available commercial products.

Plant materials

Barley (*Hordeum vulgare* L cv. Hassan) was grown on vermiculite in a controlled growth chamber as previously described [15] for 2 weeks.

Leaf segments (10 g) were homogenized for 6 sec at 0–5° in an Omni-mixer (Sorvall, USA) with 3 vols of a freshly prepared isotonic buffer that contained 50 mM K.Pi (pH 7.0), 1 mM L-ascorbic acid, 1 mM EDTA and 0.33 M sorbitol (buffer E) supplemented with 50 mg/ml polyvinylpyrrolidone. The homogenate was strained through 4 layers of muslin and centrifuged at 200 *g* for 5 min. The supernatant was centrifuged at 2500 *g* for 10 min. The new supernatant was the soluble fraction (around 0.25 mg protein ml^{-1}) and the pellet containing intact chloroplasts [16], free from soluble or mitochondrial contamination [17] was washed with buffer E (45 ml). The new chloroplast pellet was resuspended in 1.5 ml of buffer E without sorbitol (buffer H) at 0–4° with gentle shaking during 6 min and it was centrifuged for 15 min at 4500 *g*. The thylakoid pellet was resuspended in 1 ml of buffer H and used immediately for solubilization of thylakoid-bound peroxidase. This activity was solubilized from the membraneous fraction by adding 10% (w/v) Triton X-100 to make a final 1.5% sol and gently stirred for 45 min. The suspension was centrifuged at 20 000 *g* for 30 min. The supernatant (around 0.9 mg protein ml^{-1}) was the Triton-solubilized thylakoid fraction. Cell wall peroxidase was isolated as described in ref [18].

Kinetic assays

Unless otherwise noted, the spectrophotometric assay of peroxidase was performed at 25° in a 1-ml assay vol. containing 0.5 mM HQ, 0.1 mM H_2O_2 and 50 mM K.Pi buffer at pH 7.0. After mixing, the enzymatic reaction was initiated by adding 10 μl of enzyme. The increase in *A* 250 nm over a time period of 1 min was measured, the *A* increase always being linear with respect to time. The oxidation rate was expressed as μmol of product formed min^{-1} using a $\epsilon = 1.9 \times 10^4$ M^{-1} cm^{-1} for p-benzoquinone [3].

The assay of polyphenol oxidase activity using 4-methylcatechol (4-MC) as substrate was carried out as described in [7]. The assay of ascorbate peroxidase was carried out as described in [19].

Zymographic assays

Peroxidase isoenzymes of soluble and thylakoid fractions of leaf barley was separated by native polyacrylamide electrophoresis essentially as described in ref [20] except that Na dodecyl sulfate (SDS) was omitted and the carrier buffer contained 2 mM ascorbate.

Electrophoretic separation was performed at 4° using 10% polyacrylamide gels. Staining was performed by following standard methods with MN [8], HQ [3] or ascorbate [21] as substrates.

Protein conc. was measured by the method of ref [22] with a Protein Assay Kit (Bio–Rad) using BSA as standard.

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