The Distinctive Features of Ascorbate Peroxidase in Dormant *Crocus sativus* L. Corm

Lila Ghamsari[1] and Ezzatollah Keyhani[1,2]
[1]Institute of Biochemistry and Biophysics, University of Tehran, 13145 Tehran
[2]Laboratory for Life Sciences, 19979 Tehran
Iran

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Abstract

The presence of ascorbate peroxidase activity was investigated in an extract prepared from dormant *Crocus sativus* L. corm. The enzymatic activity was measured by following spectrophotometrically the oxidation of sodium ascorbate at 290 nm, in the presence of hydrogen peroxide, in citrate-phosphate buffer, upon addition of the extract. After correction for ascorbate auto-oxidation, the extract exhibited ascorbate peroxidase activity with an optimum pH of 8.0. At that pH, K_m was 0.15 ± 0.02 mM for ascorbate, and 0.05 ± 0.005 mM (three times less) for hydrogen peroxide. Apparent V_{max}, calculated per mg protein in the extract, was 54 ± 6 µM.min^{-1} for ascorbate and 205 ± 15 µM.min^{-1} (four times more) for hydrogen peroxide. The catalytic efficiency (per mg protein in the extract) was 0.36 ± 0.08 min^{-1} for ascorbate and 4.1 ± 0.6 min^{-1} for hydrogen peroxide, thus eleven times that for ascorbate. The pseudo-first order rate constant for ascorbate as the varying substrate was 0.0089 ± 0.0006 min^{-1} and it was 0.5 ± 0.06 min^{-1} (fifty five times higher) for hydrogen peroxide as the varying substrate. Thus, ascorbate peroxidase activity was detected in dormant *C. sativus* L. corm extract. The enzyme obeyed simple Michaelis-Menten kinetics with either hydrogen peroxide or ascorbate. But, surprisingly, in contrast to other ascorbate peroxidases as well as to other hemoproteins, it was insensitive to up to 100 mM cyanide, 100 mM azide or 100 mM aminotriazole. This may correspond to a specific requirement of metabolic control activity of this enzyme in dormant corm.

INTRODUCTION

Peroxidases and catalases are efficient scavengers of hydrogen peroxide produced by oxidative stress (Castillo, 1992). Peroxidases activity increases under environmental stress (Gaspar et al., 1991), enabling the cell to resist against the formation of H_2O_2. Peroxidases produced by plants are present in multiple isozymic forms. They exhibit little substrate specificity and are able to oxidize a variety of organic substrates and dyes. The biological role of peroxidases is thought to include lignification, cross-linking of cell wall polysaccharides, regulation of cell elongation (Gaspar et al., 1991), wound defense and plant hormone metabolism (Campa, 1991).

Heme peroxidases contain a single polypeptide chain, about 300 residues in length with a single non-covalently bound heme. Welinder classified peroxidases into three categories based on sequence alignments and biological origin (Welinder, 1992). Class I, the intracellular peroxidases, includes yeast cytochrome *c* peroxidase (CCP) and a number of plant and bacterial peroxidases found in the cytosol or chloroplasts. Secretory fungal peroxidases form class II. These enzymes are monomeric glycoproteins with four conserved disulfide bridges and two conserved calcium sites. The secretory plant peroxidases, or classical plant peroxidases, form class III. These also are monomeric glycoproteins with four conserved disulfide bridges and two calcium ions, although the placement of the disulfides differs from the class II enzymes.

Ascorbate peroxidase (L-ascorbate: hydrogen-peroxide oxidoreductase, EC 1.11.1.11) exhibits a high specificity for ascorbic acid as electron donor. In plants, ascorbate peroxidase functions as scavenger of hydrogen peroxide (H_2O_2), thereby protecting plant cells from the deleterious effect of H_2O_2 generated as a by-product of

respiration and photosynthesis (Dalton, 1991). The reaction catalyzed by ascorbate peroxidase can be summarized as follows:

2 ascorbate + H_2O_2 → 2 monodehydroascorbate + 2 H_2O

The reverse reaction regenerating ascorbate is catalyzed by monodehydroascorbate reductase. A spontaneous reaction of 2 monodehydroascorbate may also occur that produces ascorbate and dehydroascorbate. This is followed by coupled reactions involving dehydroascorbate reductase and glutathione reductase in the ascorbate-glutathione pathway, an important defense against activated oxygen species in higher plants and some cyanobacteria (Dalton, 1995; Asada, 1994).

An ascorbic acid-dependent H_2O_2-scavenging pathway during germination has been subjected to considerable investigation and cytosolic ascorbate peroxidase was found to rapidly increase during germination (Klapheck et al., 1990; Kakmak et al., 1993) as well as during postgermination by light (Morimura et al., 1999).

MATERIALS AND METHODS

Dormant saffron (*Crocus sativus* L.) bulbs were used throughout these studies. They showed neither roots nor shoots. Extracts were prepared from bulbs weighing each between 3 and 6 g, by homogenization in phosphate buffer 0.1 M, pH 7.0, containing 0.02% phenylmethanesulfonyl fluoride as protease inhibitor. After centrifugation at 4,000 g for 3 min, then at 10,000 g for 5 min, a clear, transparent supernatant termed 'crude extract' was obtained and used for our studies. Protein concentrations were determined by the Lowry method.

Peroxidase activity was determined by following the oxidation of o-dianisidine at 460 nm, guaiacol at 470 nm, pyrogallol at 430 nm and ascorbate at 290 nm with extinction coefficients 11.3 $mM^{-1}cm^{-1}$, 26.6 $mM^{-1}cm^{-1}$, 2.47 $mM^{-1}cm^{-1}$ and 2.8 $mM^{-1}cm^{-1}$, respectively. Assays were carried out at room temperature (≈ 22-25ºC), in the presence of 0.3 mM H_2O_2, using Aminco DW2 and Milton Roy spectrophotometers. Results were average of 3 different experiments conducted on crude extracts from 3 different batches of bulbs.

The rate constant for the reaction between the enzyme-substrate complex and the electron donor molecule, k_4, was calculated according to (Chance and Maehly, 1955) as follows (Keyhani et al., 1999). Under our experimental conditions, given the respective final concentrations of H_2O_2 and of ascorbate, k_4 could be expressed as follows:

$k_4 = 1/a_0e \times \Delta x/\Delta t$

where $\Delta x/\Delta t$ is the rate of substrate disappearance over the measured time interval, a_0 is the initial concentration of donor and e is the concentration of enzyme (which was expressed as mg protein in the sample).

The pH activity curve was determined using a citrate-phosphate-borate buffer system (range 3-10) at a concentration of 0.1 M.

The effect of peroxidases inhibitors, namely azide, cyanide, 3-amino-1,2,4-triazole (aminotriazole), was studied by assaying the enzymatic activity after preincubation of the extract with various concentrations of a given inhibitor for 5 minutes, at room temperature.

RESULTS AND DISCUSSION

Sequence analysis of all plant ascorbate peroxidases indicates 7 types of ascorbate peroxidase (Jespersen et al., 1997). These include two types of cytosol soluble ascorbate peroxidase (cs1, cs2), three types of cytosol, membrane-bound ascorbate peroxidase (cm1, cm2, cm3) where one is bound on the matrix side of the glyoxysomal membrane while the location of the other two is unknown, and two types of chloroplast ascorbate peroxidase (chs, cht).

The ascorbate peroxidase reported in this paper is the cytosolic one since the centrifugation method eliminates all membrane-bound organelles. On the other hand, since cs2 seems to be rare (Jespersen et al., 1997), presumably only the cytosolic ascorbate peroxidase cs1 is present in our soluble fraction.

Figure 1 shows the rate of oxidation of ascorbate by saffron corm extract at different pHs, ranging from 4.0 to 10.0. One optimum peak was found at pH 8.0.

Figures 2 and 3 show the Michaelis-Menten plots for H_2O_2 (Figure 2) and for ascorbate (Figure 3) respectively as variable substrate. The K_m values found for H_2O_2 and ascorbate were respectively 0.05 ± 0.005 mM and 0.15 ± 0.02 mM.

Various isoperoxidases have been detected in *C. sativus* dormant corms (Keyhani et al., 2000). Table 1 compares the K_m and V_{max} values for ascorbate with those for other substrates utilized by various saffron corm peroxidases such as pyrogallol, o-dianisidine and guaiacol. The lowest K_m value, 0.15 mM, was obtained for ascorbate and the highest value, 3.3 mM, was obtained for guaiacol. Ascorbate peroxidase showed high affinity for both ascorbate and H_2O_2, but its affinity for H_2O_2 was three times higher than that for ascorbate.

Table 2 compares the sensitivity to inhibitors of various dormant saffron corm extract peroxidases. The activity of ascorbate peroxidase in the presence of 1 μM cyanide or azide was unchanged compared to the control. Only 5 to 10 % inhibition was observed with 100 μM cyanide or azide. In contrast 100 μM cyanide or azide severely inhibited guaiacol peroxidase. Pyrogallol and o-dianisidine peroxidases were inhibited by only 25 to 30 % in the presence of 100 μM azide, but by 85 % in the presence of 100 μM cyanide. Data showed that in dormant corm ascorbate peroxidase was insensitive to azide and cyanide compared to other isoperoxidases. Even in the presence of 100 mM inhibitors (including aminotriazole) dormant saffron corm ascorbate peroxidase still showed significant residual activity (90%). In contrast ascorbate peroxidase from Japanese radish root was sensitive to 100 μM cyanide. 95 % of enzyme activity was inhibited at this concentration of cyanide. The pH profile obtained for ascorbate peroxidase in saffron with optimum at pH 8.0 was also different from the pH optimum for ascorbate peroxidase of Japanese radish root (single peak at pH 6.0) (Ohya et al., 1997).

CONCLUSIONS

Ascorbate peroxidase activity was detected in *C. sativus* dormant corm extract. The enzyme involved was presumably cytosolic since organelles and membranes were eliminated by centrifugation during the extract preparation. The pH activity profile indicated a single peak at pH 8.0, suggesting a single enzyme. To date, two types of cytosolic plant ascorbate peroxidases have been reported, one of them seemingly rare. We thus concluded that the enzyme detected in our system corresponds to the cytosolic plant peroxidase type cs1. The kinetic parameters, K_m, V_{max}, k_4 (rate constant), and catalytic efficiency (expressed per mg protein in the extract) were determined and reported. Finally, the sensitivity to inhibitors of the enzymatic activity was investigated and the results showed a surprising resistance to inhibitors such as cyanide, azide and aminotriazole compared to other peroxidases.

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Tables

Table 1. Kinetic parameters for the peroxidase activity found in dormant *Crocus sativus* L. corm extract in the presence of various substrates (electron donors)

Substrate	K_m (μM)	V_{max} [nmol.min^{-1}(mg prot)^{1}]	k_4[(1)] (x 10^5)	catalytic efficiency[(2)] (min^{-1})
Ascorbate (pH 8.0)	0.15	54.0	14.0	0.36
Pyrogallol (pH 7.0)	0.8	47.0	5.9	0.06
o-dianisidine (pH 6.5)	0.25	30.0	12.0	0.12
guaiacol (pH 6.5)	3.3	4.5	0.14	0.001

[(1)]Expressed in L.min^{-1}(mg prot)$^{-1}$
[(2)]Expressed per mg extract prot

Table 2. Sensitivity to inhibitors of peroxidase activities detected in *Crocus sativus L.* dormant corm.

Inhibitor	Relative activity (% control)			
	Ascorbate (pH 8.0	Pyrogallol (pH 7.0)	(pH 7.0) (pH 6.5)	Guaiacol (pH 6.5)
None	100	100	100	100
Azide				
1 μM	100	85	100	65
10 μM	98	85	100	50
100 μM	95	75	70	10
Cyanide				
1 μM	100	95	90	70
10 μM	95	65	55	40
100 μM	90	15	15	5

Figures

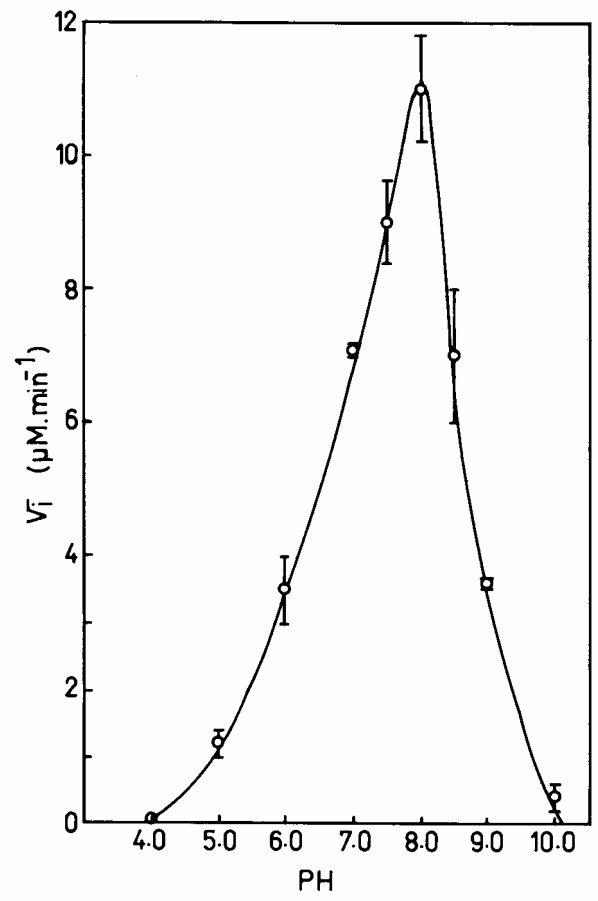

Fig. 1. pH activity profile of the ascorbate peroxidase activity detected in *C. sativus* dormant corm extract.

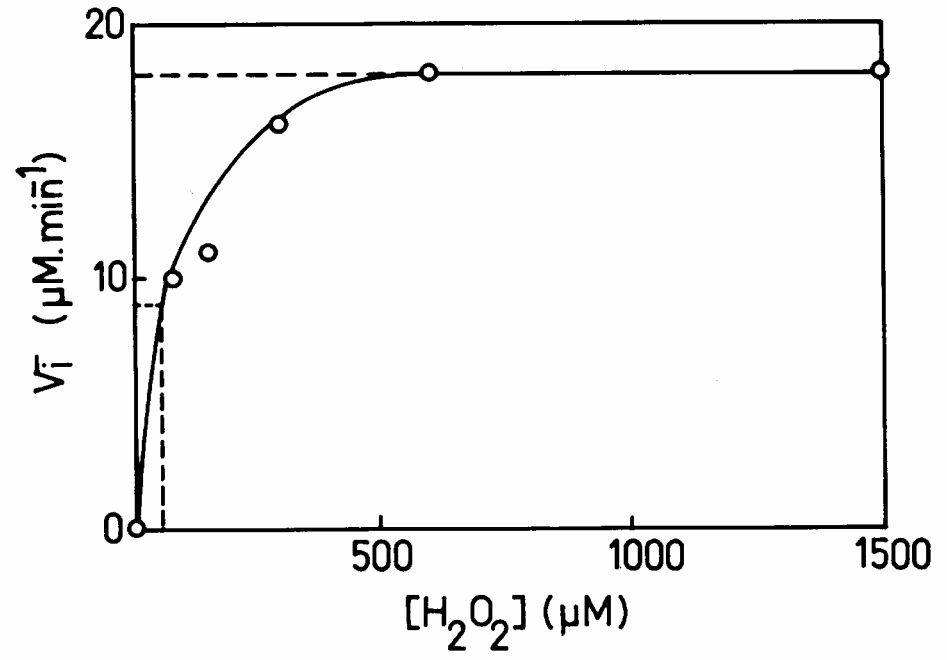

Fig. 2. Michaelis-Menten plot of *C. sativus* ascorbate peroxidase detected in dormant corm extract as a function of hydrogen peroxide concentration. Ascorbate concentration was kept at 0.5 mM.

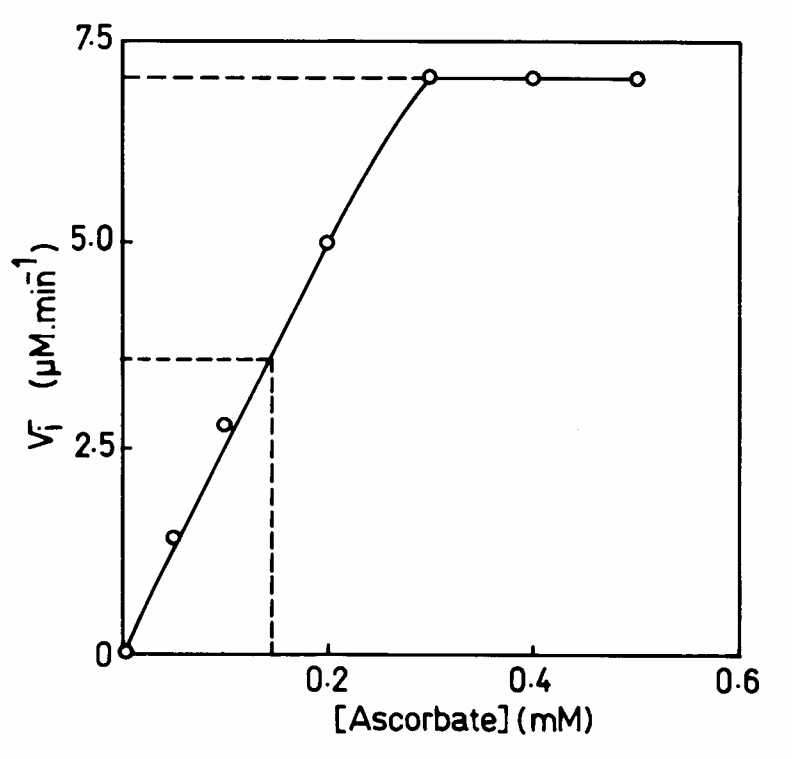

Fig. 3. Michaelis-Menten plot of *C. sativus* ascorbate peroxidase activity detected in dormant corm extract as a function of ascorbate concentration. Hydrogen peroxide concentration was kept at 1.6 mM.