Free Rad. Res. Comms., Vol. 17, No. 1, pp. 1-8
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A UNIQUE ASCORBATE PEROXIDASE ACTIVE COMPONENT IN THE CYANOBACTERIUM *Synechococcus PCC 7942 (R2)*

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(Received January 15, 1992; in final form March 25, 1992)

Ascorbate peroxidase active component (APAC) was purified and characterized in *Synechococcus PCC 9742 (R2)* cells. APAC was isolated from freshly harvested cells, by ion exchange chromatography on DEAE cellulose, ultrafiltration through a 3000 dalton cut off filter and high pressure liquid chromatography through a reversed phase C-18 column. APAC was found to be extremely stable to harsh treatments of boiling water for 30 min, acidification to pH 2.0 and proteolytic digestion. A close correlation between activity and iron content of APAC was observed throughout the purification steps. E.S.R. spectrum of APAC showed a resonance line at $g = 4.3$ in the oxidized from. Peroxide reduction by ascorbate decreased the E.S.R. signal, which reappeared upon reoxidation by H_2O_2. The affinities of APAC to H_2O_2 and ascorbate were high (0.38 mM and 0.2 mM, respectively). Amino acid composition analysis of APAC revealed the presence of glutamic acid : glycine : cysteine residues at 2 : 1 : 1 ratio.

KEY WORDS: Ascorbate peroxidase, hydrogen peroxide removal, Fe-complex, *Synechococcus 7942*, cyanobacteria.

INTRODUCTION

Oxygen reactive species are generated concomitantly with oxygenic photosynthesis *in vivo*: superoxide radical is formed as a byproduct of O_2 photoevolution and its partial reduction by photosystem I in the Hill reaction.[1,2] Hydrogen peroxide is produced by superoxide dismutase[3,4] and photorespiration.[5] These oxygen reactive species are extremely toxic to the cells,[6] and the involvement of effective systems for their removal is essential to reduce oxidative damage along with oxygenic photosynthesis. The ascorbate-glutathione pathway, involving ascorbate peroxidase, is the major mechanism for H_2O_2 removal in chloroplasts[7] and in cyanobacteria.[8,9] Plant ascorbate peroxidase was purified and characterized as heme protein with a molecular weight of 30–34 kD for the chloroplast enzyme[10] and 57–72 kD for the cytoplasmic enzyme.[11,12] The chloroplast ascorbate peroxidase was found to be extremely labile

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under oxidative conditions,[13] while the cytoplasmic enzyme was more stable but less active.[10] The present study describes the properties of a unique APAC found in cell free extracts of *Synechococcus 7942*. The currently characterized ascorbate peroxidase form differs from the chloroplastic and cytoplasmic plant type ascorbate peroxidases.

MATERIALS AND METHODS

Cell Growth

Synechococcus PCC 9742 (R2) was cultured in BG-11 mineral medium[14] supplemented with 4 g/l $NaHCO_3$ and 1 g/l KNO_3. Cells were grown at 30°C in 250 ml Erlenmeyer flasks, on a shaker (150 rpm) and illuminated with fluorescent cool white light ($I = 50\,\mu E\,m^{-2}s^{-1}$).

APAC Isolation and Purification

Ten litres of late log phase grown cells (0.5–0.7 OD at 720 nm) were harvested by centrifugation at 3000 *g* for 10 min, washed with phosphate-EDTA buffer (5 mM K_2HPO_4, 5 mM EDTA, pH 7.5). Cells were resuspended in 4 ml phosphate-EDTA buffer containing 1 mM-ascorbate and 20% sorbitol and sonicated (15 Hz, 3 min at 4° C). Soluble cell extract was obtained by centrifugation at 30 000 *g* for 30 min. The supernatent was separated on a DEAF-cellulose column of 1.5 × 40 cm and eluted with 180 ml of the phosphate-EDTA buffer, followed by a gradient of 60–300 mM phosphate buffer containing 5 mM EDTA, 1 mM ascorbate and 20% sorbitol.

APAC Activity and Composition

Ascorbate peroxidase activity was measured spectrophotometrically following H_2O_2 dependent ascorbate oxidation at 290 nm ($E^{mM} = 2.8$ OD) at 25°C, using a Uvicon 810 spectrophotometer.[8] Iron content was determined with a Perkin–Elmer 2380 atomic absorption spectrophotometer. E.S.R. spectrum was determined by Varian E-12 spectrometer. Amino acid analysis was determined with a Biotronic Lc 5000 (515-01) analyzer. Protein content was determined according to Bradford.[16]

RESULTS

It was shown that ascorbate peroxidase played a major role in hydroperoxide removal in *Synechococcus PCC 7942 (R2)*.[17] This study aimed to isolate and purify the cyanobacterial enzyme and compare it with the well characterized ascorbate peroxidase from spinach,[13] Euglena[18] and pea.[19]

APAC Purification

Figure 1(a) shows that ascorbate peroxidase activity, eluted from the DEAE column, does not overlap with the major peak of protein content. The iron content of the eluted fractions was determined and APAC contained a high content of iron, overlapping with its H_2O_2 dependent ascorbate oxidation activity (Figure 1(b)). APAC active fractions were filtered through Amicon 3000 dalton filters and retained their activity and relative iron content.

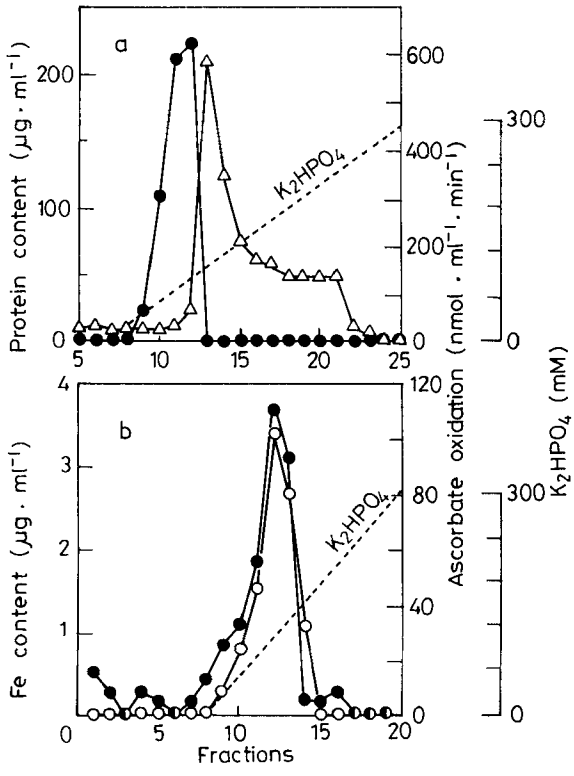

FIGURE 1 Biological activity of APAC and protein or Fe content. Cell sonicate supernatent, collected from 10 litres of *Synechococcus R2* culture at late logarithmic growth phase was loaded on a 1.5 × 40 cm column. The column was developed with 180 ml of 5 mM phosphate-EDTA buffer containing 1 mM ascorbate and 20% sorbitol, pH 7.5, followed by a linear gradient (60–300 mM) of the same buffer. Fractions of 20 ml were collected. Ascorbate oxidation was determined with 0.4 mM ascorbate and 0.2 mM H_2O_2. (a) Ascorbate oxidation (●) and protein content (△). (b) Ascorbate oxidation (●) and Fe content

APAC Stability

APAC was stable to extremely harsh treatments such as: boiling (100°C) for 30 min, acidification at pH 2 for 10 min and chelation with 1 mM EDTA or 100 μM–1 mM desferal for 1 hr at room temperature. APAC activity was not affected by proteolytic enzymes (i.e., 50 μg/ml of *Staphylococcus aureus* V8 protease, proteinase, pronase, thermolysin, trypsin, subtilisin, chymotrypsin and 5 μg/ml of papain). These results suggest that it is unlikely that *Synechococcus (R2)* APAC was the earlier described ascorbate peroxidase enzyme. The involvement of iron in APAC activity is demonstrated by its stabilty to desferal, the high affinity chelator of iron.[20] Figure 2 indicates that the activity was partly lost only upon boiling of APAC in water in the presence of high desferal concentrations.

Biochemical Characterization

The affinity of APAC to ascorbate and H_2O_2 showed apparent Km values of 0.38 mM and 0.2 mM, respectively. Unlike the plant ascorbate peroxidase, no activity was

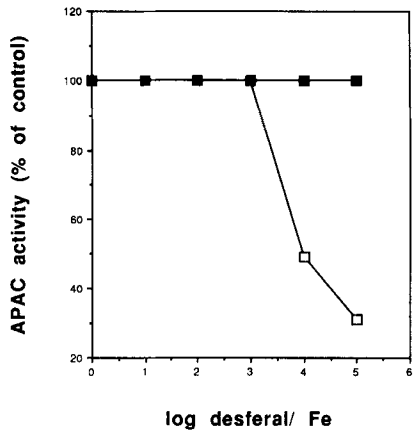

FIGURE 2 The effect of desferal on APAC activity. Active fractions, prepared as described in Figure 1, were incubated with desferal, at different concentration ratios, for 30 min at 25°C (■) or 100°C (□), and analyzed immediately after treatment (100 activity was 250 nmol ascorbate oxidized $\mu g\,Fe^{-1}\,min^{-1}$).

detected with pyrogallol and guaiacol as alternative electron donors. On the other hand, APAC reduced *t*-butyl hyperoperoxide and cummene hydroperoxide in the presence of ascorbate. The optimal pH for APAC activity was between 9.0 and 10.0. APAC flushed with carbon monoxide for 5 min retained full activity. However, it lost 50 percent of its activity in the presence of 4 mM NaCN or 5 mM NaN_3.

Chemical Characterization

Analysis of APAC by E.S.R. spectroscopy showed a single broad resonance line at $g = 4.3$, typical for rhombic Fe^{3+}. Addition of ascorbate resulted in reduction of Fe^{3+} to Fe^{2+}, and decreased the E.S.R. signal, which reappeared upon addition of excess of H_2O_2 as electron acceptor (Figure 3). The influence of the substrates on changes in the iron redox state suggest involvement of the iron in catalysis. APAC was further purified by HPLC separation. Figure 4 shows that the fraction eluted at 4.06 min contained ascorbate peroxidase activity. The amino acid composition of this ascorbate peroxidase active fraction is presented in Table I. It contains almost exclusively the three amino acids: glutamic acid, glycine and cysteine at 2 : 1 : 1 ratio.

DISCUSSION

The high stability and low molecular weight of the cyanobacterial ascorbate peroxidase form led to the assumption that APAC may not be a protein, unlike plant chloroplastic or cytoplasmic ascorbate peroxidase enzyme.[10] A heat stable ascorbate peroxidase fraction, constituting only of a minor part of the total activity was described in pea[11,19] and spinach.[21] APAC consists of a low MW oligopeptide between 300–3000 daltons. This estimation is based on its amino acid analysis containing at least one glycine, glutamate and cysteine tripeptides. 20% of the total pea ascorbate peroxidase activity consists of a non-protein component of less than 10 000 MW.[19] 10% of the total activity of spinach ascorbate peroxidase is a heat-stable factor, which

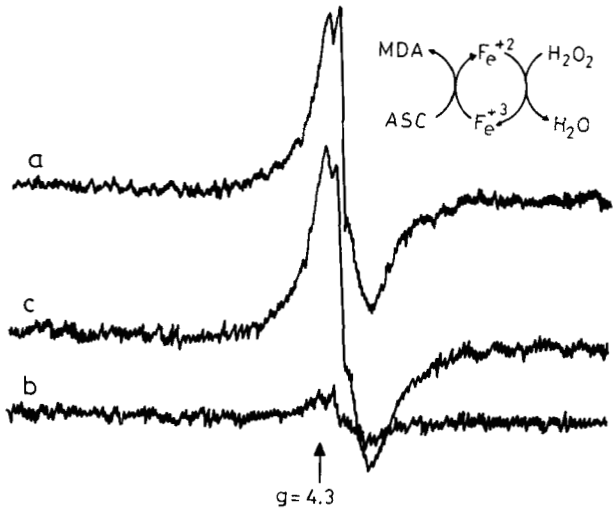

FIGURE 3 E.S.R. spectrum of APAC. Concentrated APAC, prepared as described in Figure 1 and lyophilized, contained 5.6 μg Fe ml^{-1}. The spectrum was obtained at the following set up conditions: 127° K; microwave power 20 mW; microwave frequency 9.194 GH ; receiver gain 2.5 × 10^3; modulation amplitude 4 G at 100 KH . (a) Native APAC, (b) native APAC in the presence of 1.6 mM ascorbate, (c) native APAC in the presence of 1.6 mM H_2O_2. The volume of $g = 4.3$ is marked with an upright arrow.

reacts only with ascorbate as electron donor and is not inhibited by cyanide. The active ascorbate peroxidase fractions from *Synechococcus R2* were eluted from DEAE-cellulose at a low ionic strength of 5 mM phosphate, while the acidity of plant ascorbate peroxidase enzyme was much higher and eluted by higher salt concentrations: 800 mM NaCl in legume root nodules,[22] 400 mM NaCl in Euglena[18] and 60 mM NaCl in spinach.[23] The non-proteinaceous nature of ascorbate peroxidase activity in *Synechococcus R-2* and its stability to harsh treatments suggest the involvement of a low MW metal containing component. Partial inhibition of *Synechococcus R-2* APAC was caused by higher concentrations of cyanide and azide than those needed to inhibit heme proteins, and CO did not inhibit APAC activity at all. The E.S.R. spectrum, the low sensitivity to cyanide or azide, the lack of inhibition by CO and the absence of a heme type absorption spectra (data not shown) excluded the possibility that the iron containing APAC is part of a heme structure. Spinach ascorbate peroxidase is totally inhibited by 1 mM KCN and 0.1 mM sodium azide.[13]

Plant ascorbate peroxidase was found to be a heme iron protein. Nevertheless, it was shown that spinach ascorbate peroxidase was accompanied by a non-heme iron, released from the enzyme under ascorbate depleted conditions.[13,23,24] The finding of this cyanobacterial low MW anti peroxidative activity may correlate with the reported superoxide dismutase active component, composed of a non-proteinaceous Mn-containing complex, observed in *Lactobacillus*.[25]

The amino acid composition of *Synechococcus (R2)* APAC suggests the nature of an oligopeptide similar to glutathione, phytochelatin or metalthionin.[26–28]. A similar APAC isolated from other cyanobacteria, such as *Nostoc PCC-7119* and *Anabaena azollae*, was stable to harsh treatments (data not shown). Extracts from Dunaliela, Retema and Lemna were similar to the plant type ascorbate peroxidase and sensitive

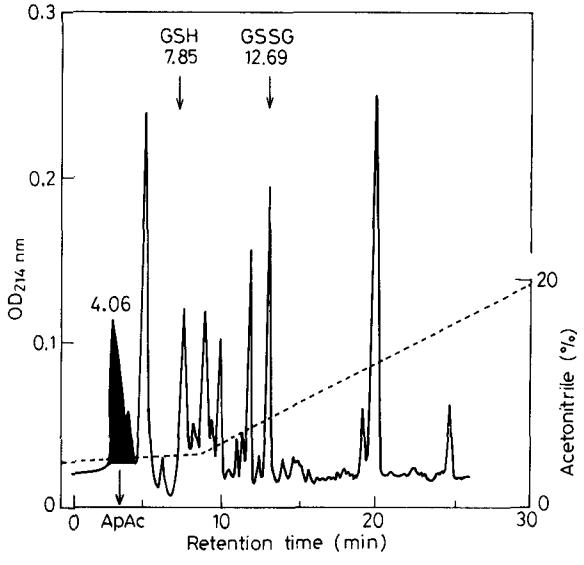

FIGURE 4 HPLC separation of partially purified *Synechococcus R-2* APAC. APAC sample (fractions 11,12 from Figure 1) was filtered through a 3000 dalton Amicon filter, lyophilized and resuspended in water. A sample of 10 μlit was injected into reverse phase C18 column (4.3 × 25 cm). The column was eluted with a linear gradient (1–20%) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Absorbance at 214 nm was measured. The biologically active fractions are shown in black. The retention times of reduced (GSH) and oxidized (GSSG) glutathione are indicated by vertical arrows at top.

to the harsh treatments (data not shown). APAC reacted with ascorbate as electron donor and with H_2O_2 and other organic peroxidases as electron acceptors. Pyrogallol and quaiacol reduced the plant ascorbate peroxidase[13] but not APAC. Optimal pH for APAC was 9.0–10.0, while in the plant ascorbate peroxidase it was 6–7.9.[7,19,21]

The optimal activity of ascorbate peroxidase obtained at 32–34°C was lost at 52°C, while boiled APAC retained its activity. These results lead to the conclusion that ascorbate peroxidase activity in *Synechococcus* is catalyzed by a completely different component than the plant enzyme.[7,10–13,19,21–23] Our finding could be explained by one

TABLE 1
Amino acid composition of APAC[a]

Amino acid	% in sample
Cysteine	18.932
Aspartic acid	2.984
Serine	2.976
Glutamic acid	37.748
Glycine	19.316
Alanine	1.419
Valine	1.590
Lysine	0.776
Ammonia	13.478
Ornithine	0.780

[a] Fractions 11, 12 from Figure 4.

of the following models: (a) the polipeptide and the active component (APAC) are synthesized separately; (b) during the extract preparation an active center (APAC) is detached from the polipeptide; (c) only an active center (APAC) is synthesized by cyanobacterial cells which may be due to the fast response and induction of ascorbate peroxidase activity under oxidative stress conditions. Further studies of these options need to be resolved. The amino acid composition of APAC, its low molecular weight and strong affinity for iron suggest a structure with an iron atom, tightly bound to a glutathione-like or a phytochelatin-like complex.

Acknowledgements

A.R. is a recipient of a Levi Eshcol post doctoral fellowship of the NCRD, Israel. We thank Prof. A. Trebst, Lehrstuhl fur Biochemie der Pflanzen, Ruhr-Universitat of Bochum for his suggestions, Dr.D. Goldfarb, Chemical Physics Department, Weizmann Institute of Science for the E.S.R. analysis and Dr. J. Murder, Agricultural Botany Department, The Hebrew University of Jerusalem for proteolytic enzymes.

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Accepted by Prof. G. Czapski