Metabolism of Hydrogen Peroxide in *Euglena gracilis* z by L-Ascorbic Acid Peroxidase

Shigeru SHIGEOKA, Yoshihisa NAKANO and Shozaburo KITAOKA
Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 591, Japan

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*Euglena gracilis* was found to contain a peroxidase that specifically requires L-ascorbic acid as the natural electron donor in the cytosol. The presence of an oxidation–reduction system metabolizing L-ascorbic acid was demonstrated in *Euglena* cells. Oxidation of L-ascorbic acid by the peroxidase, and the absence of ascorbic acid oxidase activity, suggests that the system functions to remove H₂O₂ in *E. gracilis*, which lacks catalase.

O₂-utilizing organisms possess enzymes capable of destroying toxic intermediates of O₂ metabolism. Catalase, peroxidases and superoxide dismutases are such enzymes ordinarily functioning to scavenge the toxic substances. *Euglena gracilis* lacks catalase (Graves *et al.*, 1971; Lord & Merrett, 1971; Asada *et al.*, 1977) and the method by which this organism disposes of H₂O₂ has not been elucidated. Cytochrome c was claimed to have a peroxidative activity (Brown *et al.*, 1975), but the view has apparently not gained wide acceptance as a convincing explanation.

In the course of studying the metabolism of L-ascorbic acid in *E. gracilis*, we have found that it is oxidized by the action of a peroxidase that specifically requires L-ascorbic acid as the natural electron donor. This peroxidase appears to be the sole agent destroying H₂O₂ in *Euglena* cells.

**Materials and Methods**

Heterotrophic cultures of *E. gracilis* were obtained by culturing the protozoon at 26°C in Koren & Hutner (1967) medium under illumination (2000lx) or in the dark, and photoautotrophic cultures were obtained in the pH6.8 salt medium described by Cramer & Myers (1952).

Harvested cells (4 x 10⁸) were washed with 25 mM-veronal buffer, pH 6.8, containing 30% (w/v) sucrose, and disrupted in 5 ml of the same buffer by sonication (10 kc) for a total of 2 min with seven rest intervals of 15 s each. The supernatant obtained by centrifuging the sonicated cells at 20000g for 15 min was used as the enzyme source.

L-Ascorbic acid peroxidase was assayed at 32°C (optimum temperature) in a reaction mixture (2 ml) containing 25 mM-veronal buffer, pH 6.2 (optimum pH), 0.4 mM-sodium L-ascorbate, 0.11 mM-H₂O₂ and enzyme, by measuring the decrease in *A*₂₈₅. Peroxidase activity with pyrogallol (Nakarai Co., Kyoto, Japan) as an artificial electron donor was assayed by the method of Chance & Maehly (1955). Catalase (EC 1.11.1.6) was assayed by the method of Abei (1974). L-Ascorbic acid oxidase (EC 1.10.3.3) was assayed by measuring the decrease in *A*₂₈₅ (Attridge, 1974) and also by measuring O₂ consumption with an oxygen electrode (Strothkamp & Dawson, 1977); the reaction mixture (2 ml) contained 50 mM-potassium phosphate buffer, pH 6.0, 1 mM-sodium L-ascorbate and enzyme. Dehydro-L-ascorbic acid reductase (EC 1.8.5.1) was assayed by a method similar to that for assaying glutathione peroxidase (EC 1.11.1.9) (Sies & Moss, 1978); the reaction mixture (2 ml) contained 50 mM-potassium phosphate, pH 6.5, 2.5 mM-dehydro-L-ascorbic acid, 2.5 mM-reduced glutathione, 0.2 mM-NADPH, glutathione reductase (1 unit; Sigma) and enzyme. This enzyme was also assayed by the method of Foyer & Halliwell (1977). Monodehydro-L-ascorbic acid reductase (EC 1.6.5.4) was assayed by the method of Marre & Arrigon (1958). Total L-ascorbic acid was determined by the method described by Shigeoka *et al.* (1979a).

For studying the subcellular distribution of peroxidase activity, a cell homogenate was obtained by partial trypsin digestion of pellicle followed by mild mechanical destruction (Tokunaga *et al.*, 1976a; Shigeoka *et al.*, 1979b), and then submitted to linear sucrose-density-gradient centrifugation (Yokota *et al.*, 1978; Yokota & Kitaoka, 1979a). Succinic semialdehyde dehydrogenase as a marker for mitochondria (Tokunaga *et al.*, 1976b, 1979) and NADPH–cytochrome c reductase as a marker for microsomal fraction (Omura & Takesue, 1970), mitochondria (Yokota & Kitaoka, 1979b) and chloroplasts (Lazzarini & Pietro, 1962) were assayed by the reported methods. Chlorophyll and protein
were determined respectively by the methods of MacKinney (1941) and Lowry et al. (1951) (with bovine serum albumin as a standard).

Disc electrophoresis in polyacrylamide gel was performed as described by Davis (1964) with the use of 7.5% polyacrylamide gels. Electrophoresis was carried out in a cold-chamber by using a constant current (2 mA/gel), with Bromophenol Blue as migration marker. The distribution of the peroxidase activity with L-ascorbic acid or pyrogallol as an electron donor was determined on 1 mm slices of the gel column.

Results and Discussion

Table 1 shows activities of the enzymes related to the oxidation–reduction system of L-ascorbic acid in the crude extract of *E. gracilis*. A very high activity was found for the peroxidase that utilized L-ascorbic acid only and not compounds such as NADH, NADPH, cytochrome c, glutathione and palmitic acid as the natural electron donor. The extract contained some monodehydro-L-ascorbic acid reductase and dehydro-L-ascorbic acid reductase activities, but no L-ascorbic acid oxidase. Scheme 1 illustrates the oxidation–reduction system of L-ascorbic acid in *E. gracilis* based on the results of the present experiments and those with plants (Mapson & Moustafa, 1956; Halliwell, 1978). Monodehydro-L-ascorbic acid, the primary oxidation product of L-ascorbic acid, undergoes a non-enzymic dismutation, giving dehydro-L-ascorbic acid and L-ascorbic acid (Yamazaki et al., 1960). The monodehydro compound is also reduced to form L-ascorbic acid by the action of monodehydro-L-ascorbic acid reductase, the presence of which in *E. gracilis* was demonstrated in the present experiments. Reduction of dehydro-L-ascorbic acid to L-ascorbic acid proceeds by the action of dehydro-L-ascorbic acid reductase, thus completing the oxidation–reduction cycle.

In *E. gracilis* this oxidation–reduction system starts by the oxidation of L-ascorbic acid catalysed by L-ascorbic acid peroxidase, with H₂O₂ as the oxidant; this is a characteristic difference from higher plants in which L-ascorbic acid is oxidized by L-ascorbic acid oxidase, with O₂ as the oxidant (Dawson et al., 1975). Since only one peak of peroxidase activity with either L-ascorbic acid or pyrogallol as electron donor was detected in the gel column, a single protein appears to be responsible for the peroxidative activity. As reported in detail elsewhere this peroxidase has many unique properties and apparently belongs to a new type of peroxidase (Shigeoka et al., 1979d). Groden & Berk (1977, 1979) have reported the occurrence of an L-ascorbic acid-specific peroxidase in spinach chloroplasts which decomposes H₂O₂. The *Euglena* peroxidase with L-ascorbic acid and pyrogallol as electron donors was found to be located only in the

Table 1. Activities of the enzymes related to the oxidation–reduction system of L-ascorbic acid in *E. gracilis*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(μmol/min per 10⁹ cells)</th>
<th>(nmol/min per mg of protein)</th>
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<tbody>
<tr>
<td>L-Ascorbic acid oxidase</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>L-Ascorbic acid peroxidase</td>
<td>78.4</td>
<td>768.4</td>
</tr>
<tr>
<td>Monodehydro-L-ascorbic acid reductase</td>
<td>2.1</td>
<td>20.5</td>
</tr>
<tr>
<td>Dehydro-L-ascorbic acid reductase</td>
<td>9.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>Nil</td>
<td></td>
</tr>
</tbody>
</table>

Scheme 1. Oxidation–reduction system of L-ascorbic acid in *E. gracilis*

Key to reactions: (1) L-ascorbic acid peroxidase; (2) monodehydro-L-ascorbic acid reductase; (3) non-enzymic reaction; (4) dehydro-L-ascorbic acid reductase. GSSG and GSH, oxidized and reduced glutathione.
cytosol (Fig. 1); it was not present in mitochondria, chloroplasts and microsomal fractions. A similar centrifugation with 30–60% sucrose concentrations showed that the enzyme was not present in the microbody fraction either. Dehydro-L-ascorbic acid reductase was also detected in the cytosol, as in spinach leaves (Foyer & Halliwell, 1976).

Initiation of the oxidation–reduction system of L-ascorbic acid in *E. gracilis* by the peroxidase indicates that this system serves to destroy H$_2$O$_2$ generated in the cells. The L-ascorbic acid peroxidase activity is much higher than those of the L-ascorbic acid-regenerating enzymes in the recycling system. The high peroxidase activity together with the large amount of L-ascorbic acid in the cells (Shigeoka et al., 1979a,c) suggests that L-ascorbic acid peroxidase and L-ascorbic acid are used for immediate and efficient detoxification of H$_2$O$_2$. Groden & Berk (1979) have found a similar situation in spinach chloroplasts in which the L-ascorbic acid-consuming activity is 5–25 times as high as the regenerating activity. The maximum rate of H$_2$O$_2$ generation was reported to be about 10 nmol/min per mg of protein in rat liver (Bovers et al., 1972) and about 10 nmol/min per mg of protein in spinach chloroplasts (Asada et al., 1974). If these values are applied to *Euglena* cells containing all organelles common to both samples, the maximum H$_2$O$_2$-generation rate is about 20 nmol/min per mg of protein. It is about 2.6% of the activity of L-ascorbic acid peroxidase and 18% of those of L-ascorbic acid-regenerating enzymes. Accordingly the H$_2$O$_2$-generating rate is the limiting factor of the oxidation–reduction cycle of L-ascorbic acid, controlling the oxidation of L-ascorbic acid. This indicates that this cycle is sufficiently operative as the recycling system. The cell extract contained no catalase (Table 1), confirming results of previous papers (Graves et al., 1971; Lord & Merrett, 1971; Asada et al., 1977). The occurrence of the peroxidase only in the cytosol indicates the presence of a unique defence mechanism against O$_2$ toxicity in this protozoon.

A H$_2$O$_2$-generating system should exist in *E. gracilis*, since superoxide dismutase activity was detected in the cells (Asada et al., 1977; Kanematsu & Asada, 1979). The activity of superoxide dismutase was reported to be 10.3 units/mg of protein in photoautotrophic cells, 6.0 units/mg in photoheterotrophic cells and 3.5 units/mg in heterotrophic cells (in the dark), indicating that the enzyme activity is affected by growth conditions. The contents of total L-ascorbic acid and activities of L-ascorbic acid peroxidase in *Euglena* cells grown under the corresponding conditions were 4010 nmol/10$^9$ cells and 328.0 μmol/min per 10$^9$ cells (photoautotrophic), 940 nmol/10$^9$ cells and 78.4 μmol/min per 10$^9$ cells (photoheterotrophic) and 185 nmol/10$^9$ cells and 6.9 μmol/min per 10$^9$ cells (heterotrophic) respectively. The results show that the cellular L-ascorbic acid content and L-ascorbic acid peroxidase activity vary in parallel with the change in superoxide dismutase activity and thus the generation of H$_2$O$_2$.

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References


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