Serological Characterization of the Glycolate-oxidizing Enzymes from Tobacco, *Euglena gracilis*, and a Yellow Mutant of *Chlorella vulgaris*

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ABSTRACT

An antiserum to tobacco glycolate oxidase has been prepared by injection of the purified enzyme into rabbits. Double gel diffusion tests between the antiserum and purified antigen and also with a crude tobacco preparation gave a single immunoprecipitation band. Crude extracts of *Euglena gracilis* Z Klebs, containing glycolate dehydrogenase, and of *Chlorella vulgaris* 211-11h/20, containing glycolate oxidase, also formed single bands with the tobacco antiserum. The algal bands were identical and showed partial identity with the tobacco band. The antiserum inhibited the glycolate oxidase activities of the tobacco and *Chlorella* extracts but did not affect *Euglena* glycolate dehydrogenase activity.

All higher plants so far examined have been shown to contain an enzyme for the oxidation of glycolate. The enzyme, glycolate oxidase (glycolate:oxygen oxidoreductase E.C. 1.1.3.1.), is a FMN-flavoprotein and catalyzes the transfer of electrons from glycolate to O₂, forming glyoxylate and H₂O. Artificial dyes such as DCPIP and methylene blue can function as electron acceptors in the reaction (3, 8), and the enzyme may, under certain conditions, donate electrons to quinones (5, 11, 12).

Glycolate-oxidizing enzymes have also been detected in several unicellular green algae (3, 15, 19, 28). The enzyme prepared from *Euglena gracilis* Z. Klebs, *Chlorella pyrenoidosa* 211/8p, and *Chlamydomonas reinhardtii* Danglard (—) 90, all from the Cambridge Culture Collection, functions with DCPIP as electron acceptor but does not link to oxygen (3). Similar results were obtained with enzyme preparations from *E. gracilis* and *Chlamydomonas reinhardtii* (—) 90 from the Indiana Algal Culture Collection and with *Aetobacteriia mediterranea* (19, 20). Furthermore, a glycolate-oxidizing enzyme with no O₂ requirement has been found in the blue-green algae *Anabaena flos-aquae* and an *Oscillatoria* species (9). No requirement for FMN was observed in the green algal and *A. flos-aquae* preparations, although a slight enhancement by FMN was noted with the *Oscillatoria* extract (3, 9, 20, 28). This enzyme found in the above algae is referred to in the literature as glycolate dehydrogenase. Neither the higher plant nor the algal enzymes described show an absolute specificity for glycolate. The former can also oxidize L-lactate but not the D-isomer (1). The algal enzymes readily use D-lactate as a substrate but only slowly attack the L-form (10, 20).

An algal glycolate-oxidizing enzyme showing a mixture of the criteria described has been characterized. Intact cells and enzyme preparations from green *Chlorella vulgaris* 211-11h (Algal Culture Collection, Göttingen) and the chlorophyll-free mutant *Chlorella vulgaris* 211-11h/20 (25) show a glycolate-dependent oxygen consumption, similar to that of higher plants (14). However, the addition of FMN does not affect enzyme activity *in vitro*, and both the D- and L-lactate isomers are oxidized, at approximately equal rates (6, 14).

The present investigation was carried out to characterize further plant glycolate-oxidizing enzymes from a serological approach, using an antiserum to the pure tobacco enzyme. Effects of this antibody to glycolate oxidase on immunoprecipitation and inhibition of the homologous enzyme are reported. The antibody has also been tested against enzyme preparations from *E. gracilis*, taken as an example of the algal glycolate dehydrogenase against *Chlorella vulgaris* 211-11h/20, which exhibits glycolate oxidase activity.

MATERIALS AND METHODS

Plant Material. *Nicotiana tabacum* L. cv. John Williams' Broadleaf was grown as described previously (23). *Euglena gracilis* Z. Klebs was cultured phototrophically with air (3) on the inorganic medium of Cramer and Myers (2). The yellow mutant *Chlorella vulgaris* 211-11 h/20 was grown as reported previously (14), on a glucose medium (13).

Enzyme Preparations. All procedures were performed at 0 to 4 C.

Tobacco. One hundred grams of leaves (8–10 weeks old) were washed, and their midribs were removed. The leaves were homogenized with 200 ml of 50 mM tris-HCl buffer, pH 8.0, containing 0.25 mM sucrose, for 30 sec in a Braun mixer. After filtration through a Schleicher and Schüll 6206 II ½ paper, the suspension was centrifuged at 600g for 20 min. The supernatant was then spun at 18,000g for 45 min, and the resulting supernatant was retained.

*Euglena*. Exponential phase cells were harvested by centrifugation at 2,000g for 5 min, washed twice in 0.1 M K₂HPO₄, and disrupted by passage through a French pressure cell (18) at 4 tons/cm². The extract was spun at 17,000g for 30 min, and the supernatant was decanted and used for experimental purposes.

1 Abbreviations: DCPIP: 2,6-dichlorophenolindophenol; DEAE: diethylaminoethyl.
Chlorella. Harvesting and washing of log phase cells were carried out as for Euglena. The algae were disrupted by ultrasonication, and a dialyzed ammonium sulfate enzyme preparation was obtained exactly as described previously (14).

Assays. The oxidation of glycolate was followed by measuring O₂ consumption, the reduction of DCPIP as an artificial acceptor, and glyoxylate formation.

Oxygen uptake in the dark was measured by Warburg manometry as detailed earlier (24) and also by the use of a Pt-Ag Rank oxygen electrode, maintained at 25 C. The reduction of DCPIP was followed by measuring the decrease in extinction at 600 nm. The assay was performed in 3-ml Thunberg cuvettes of 1 cm light path; additions were as described by Lord and Merritt (16). The cuvettes were evacuated and flushed six times with O₂-free N₂ to provide anaerobic conditions. The rate of glyoxylate formation from glycolate was followed by measuring the formation of glyoxylate phenylhydrazide. Additions were made to a cuvette in the following order (final volume 3 ml): 200 μmoles of potassium phosphate buffer, pH 8.2; 10 μmoles of cysteine·HCl; 10 μmoles of phenylhydrazine·HCl at pH 6.8; 0.1 μ mole of FMN; enzyme; and 30 μmoles of glycolate to start the reaction. The rate of increase in extinction at 324 nm was measured and related to a glyoxylate phenylhydrazide calibration curve. Spectrophotometric assays were performed at room temperature with a Zeiss MQ II spectrophotometer.

Purification of Tobacco Glycolate Oxidase. Enzyme measurements during purification were performed with the oxygen electrode. All purification procedures were performed at 0 to 4 C in a cold room.

Homogenization and First (NH₄)₂SO₄ Precipitation. An 18,000 g supernatant was obtained as already described in the crude tobacco enzyme procedure, starting from 3 kg of leaves and 6 liters of grinding medium. (NH₄)₂SO₄ precipitation was performed with 1.5-liter batches. Solid (NH₄)₂SO₄ was added to 25% saturation, and the mixture was stirred for 45 min. It was then centrifuged at 18,000 g for 45 min. (NH₄)₂SO₄ was added to the supernatant to 35% saturation, and the mixture was spun as before. The pellet was resuspended in the minimum amount of 50 mM K₂HPO₄, and the suspension was dialyzed, in darkness, for 4 hr against 5 liters of 10 mM phosphate.

DEAE-cellulose Chromatography and Second (NH₄)₂SO₄ Fractionation. Two 25-× 2.5-cm columns of Whatman DE 22 standard DEAE-cellulose were prepared and equilibrated with 10 mM K₂HPO₄. Washing with about 3 liters of phosphate was then necessary to eliminate a positive Lowry protein reagent reaction in the eluate. The dialyzed enzyme was applied to the first column and eluted with 10 mM K₂HPO₄. The first 200 ml of Lowry reagent-negative eluate was pooled and applied to the second column, which was eluted as before. The first 200 ml of the second eluate were pooled and diluted to 400 ml with 10 mM K₂HPO₄. (NH₄)₂SO₄ was then added to 30% saturation. Centrifugation was performed as before, and a second fraction (30-80%) was prepared. The latter precipitate was resuspended in 20 ml of 50 mM K₂HPO₄ and dialyzed overnight against 5 liters of 10 mM phosphate.

Sephadex G-200 Chromatography. The dialyzed 30 to 80% fraction was applied to a 2.5-× 85-cm Sephadex G-200 column previously equilibrated with 10 mM K₂HPO₄. The column was eluted with the same buffer at a rate of 0.5 ml/min, and 6.5-ml fractions were collected. Those fractions containing the highest specific enzyme activity were retained for electrophoretic analysis.

Electrophoresis. The polyacrylamide gels were prepared, run, and removed for protein analysis according to the Davis procedure (7). Samples contained 50 to 100 μg of protein; 2 μM potassium phosphate buffer, pH 8.0, served as the electrolyte; and separation was carried out with 2% (w/v) amido black in 7% (v/v) acetic acid for 4 hr followed by overnight washing in several changes of 7% (v/v) acetic acid.

Immunological Procedures. Two milliliters of fraction 28, containing 0.62 mg of protein were emulsified 1:1 with Disco complete Freund's adjuvant and injected subcutaneously into the hindfoot of a rabbit. After 4 weeks, a booster injection, consisting of the same amount of antigen without the adjuvant, was given into the ear vein. Blood was withdrawn 1 week later for serum preparation. An untreated rabbit provided null serum as a control. Sera were stored at -16 C. Characterization of the antiserum obtained and comparative immunological analyses were performed using the Ouchterlony double diffusion technique (21), in agarose. Agarose A 37 was obtained from Indubiose L.Industrie Biologique Francaise S. A., Genevilliers, France. Protein was determined by the Lowry method (17) with crystalline bovine serum albumin as a standard.

RESULTS

A summary of the purification of tobacco glycolate oxidase is given in Table I. A specific activity almost the same as that of the enzyme crystallized by Frigerio and Harbury (8) was measured in fraction 27 (Table I). Electrophoresis revealed a major band and a second diffuse band (Fig. 1). This may be consistent with the demonstration of two higher plant glycolate oxidase components of different electrophoretic mobility (10). Fraction 28 was selected for immunization since, although the second faint upper band was just visible, this fraction had the highest protein content (Table I).

Characterization of Antiserum in the Ouchterlony Test. A single precipitation band was consistently formed between the purified tobacco enzyme and the antiserum (Fig. 2). Double diffusion of the antiserum and a crude 18,000 g tobacco supernatant yielded a sharp narrow band (Fig. 3A). No precipitation bands were observed between the enzyme and null serum (Figs. 2 and 3A). The single bands resulting from comparative double diffusion of the purified enzyme and 18,000 g tobacco supernatant against the antiserum showed, in Ouchterlony's terminology (22), interference, no inhibition, slight deviation, and a partial fusion (Fig. 3B). Since the antiserum formed a single band with the homologous antigen (Fig. 2) and, significantly, with the crude tobacco supernatant (Fig. 3A), then Fig. 3B is interpreted to indicate that the compared re-
Identical antigenic determinants. Preparations of *E. gracilis* Z. Klebs and *Chlorella vulgaris* 211-11 h/20 both reacted with the tobacco antiserum in the Ouchterlony test (Fig. 3, C and D). Complete fusion between the algal enzyme-antiserum bands occurred, owing to the presence of identical antigenic determinants. Interference, deviation, and partial fusion of both algal bands with the tobacco-antiserum band was observed (Fig. 3, C and D), indicating that the tobacco and algal preparations also contained identical antigenic components.

**Effects on Enzyme Activity.** Under the conditions shown in Figure 4, the antiserum completely inhibited tobacco glycolate oxidase, measured as oxygen uptake. DCPIP reduction and glyoxylate formation by the tobacco extract were also inhibited (Table II). Inhibition of glycolate-dependent oxygen consumption in preparations of the *Chlorella vulgaris* mutant was also observed. For example, a 30% inhibition is shown in Figure 5, although a 50% inhibition, under the same conditions, was also recorded. Reduced rates of DCPIP reduction and glyoxylate oxidase by homologous antiserum. The center well contained purified enzyme (fraction 28). 1: null serum; 2: antiserum. Photographed before staining.

![Figure 1](image1.png) Polyacrylamide disc electrophoresis of Sephadex G 200 fractions showing highest specific activity of tobacco glycolate oxidase. Fractions are numbered 27 to 30 from left to right. All columns were loaded with 80 to 100 μg of protein. Fraction 28 (2nd from left) was taken for immunization.

![Figure 2](image2.png) Immunoprecipitation of purified tobacco glycolate oxidase from null serum. The center well contained purified enzyme (fraction 28). 1: null serum; 2: antiserum. Photographed before staining.

![Figure 3](image3.png) Immunoprecipitation of tobacco and algal glycolate oxidizing enzymes by antiserum to tobacco glycolate oxidase. A: 18,000g tobacco supernatant in center well; 1: antiserum; 2: null serum (after staining). B: Antiserum in center well; 1: purified tobacco glycolate oxidase; 2: 18,000g tobacco supernatant (after staining). C: Antiserum in center well; 1 and 4: 17,000g *Euglena* supernatant; 2: 18,000g tobacco supernatant; 3: *Chlorella* supernatant (15), taken before staining. D: C, after staining.

![Figure 4](image4.png) Inhibition of tobacco glycolate oxidase by antiserum. The assay consisted of 200 μmoles of K2HPO4, pH 7.8, 0.1 μmole of FMN, 4 μmoles of KCN, and 18,000g tobacco supernatant (containing 2.5 mg of protein). Thirty μmoles of neutralized sodium glycolate were added as indicated, followed by 0.1 ml of null serum or antiserum. Final volume 3.0 ml. Taken from an oxygen electrode trace.
Table II. Effect of Tobacco Glycolate Antiserum on Glycolate-dependent DCPIP Reduction and Glyoxylate Phenylhydrazone Formation by Tobacco and Algal Extracts

All assays are run in the range of linear concentration dependence of enzyme preparations. For the DCPIP assay, 3.0 mg of tobacco protein, 0.3 to 3.0 mg of Euglena protein, and 6.25 mg of Chlorella protein were tested with 0.1 ml, up to 0.4 ml, and 0.2 ml, respectively, of null serum and antiserum. The same amounts of sera were used in the phenylhydrazone assays, which contained 0.5 mg of tobacco protein, 0.3 to 3.0 mg of Euglena, and 6.8 mg of Chlorella protein. The specific activities defined as moles of glycolate oxidized per mg of protein per hr in the controls without antiserum or null serum were: tobacco, 4; Euglena, 2.1; Chlorella, 0.4.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Net Inhibition by Antiserum¹</th>
<th>Glyoxylate phenylhydrazone formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>100</td>
<td>63</td>
</tr>
<tr>
<td>E. gracilis Z Klebs</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chlorella vulgaris 211-11 h/20</td>
<td>61</td>
<td>50</td>
</tr>
</tbody>
</table>

¹ Compared with rates measured with full reaction mixtures and corrected for null serum.

Fig. 5. Effect of antiserum on glycolate-dependent oxygen uptake by Chlorella extracts. The reaction was measured in Warburg flasks as before (14), with 0.1 ml of null serum or antiserum.

ate formation confirmed the inhibitory effect of the antiserum on the Chlorella vulgaris enzyme (Table II). The partial inhibition of the Chlorella enzyme is a function of the ratio of enzyme to antiserum, and increasing the ratio caused a greater degree of inhibition.

The antiserum was also tested against the glycolate dehydrogenase of E. gracilis. As already reported (3), no glycolate-dependent O₂ uptake by this enzyme occurred (Fig. 5). However, DCPIP reduction and glyoxylate phenylhydrazone formation were measured, at rates similar to those previously found (4), with specific activities, for the two methods, of 2.1 and 0.65 µmoles of glycolate oxidized per mg of protein per hr in the present work. No net inhibition of the Euglena enzyme by the antiserum was observed (Table II), even in assays containing the maximal obtainable ratio of antiserum to enzyme.

DISCUSSION

An antiserum to tobacco glycolate oxidase was obtained by injection of the purified enzyme into rabbits, as evidenced by the single immunoprecipitation band obtained with the purified antigen and with the crude tobacco enzyme preparation (Figs. 2 and 3A). It is possible that some antigenic determinants of the purified enzyme which react with the antiserum are not accessible in the 18,000g supernatant, which may explain the incomplete fusion and spur shown in Figure 3A. A diffuse precipitate, tending to disappear upon staining, was sometimes present behind the band formed between antiserum and crude tobacco enzyme (Fig. 3 C and D). This may be due to the association of enzyme molecules with different particle sizes in the crude preparation, which would imply a range of slower diffusion rates through the gel and different distances covered before meeting the antiserum.

On the basis of the Ouchterlony test, the glycolate-oxidizing enzymes of the two algae contain identical serological determinants and are serologically related, but, are not fully identical with the tobacco glycolate oxidase (Fig. 3, C and D). Figures 3, C and D, clearly shows that the algal antigens have determinants in common with the tobacco preparation. However, the tobacco enzyme contains additional determinants which do not occur in the algal enzyme preparations from Euglena and Chlorella. The antibodies to these additional determinants diffuse through the precipitate produced by the reaction of the antibodies with the Euglena or Chlorella preparations, yielding behind it, upon reaction with the tobacco antigen, the well known spur phenomenon (22). Hence, Figure 3, C and D, shows that both the Euglena and Chlorella preparations contain only part of the determinants of the tobacco protein. Whereas the algal proteins are serologically identical, the algal enzymes show only partial serological identity with the tobacco enzyme. In the case of nonidentity both precipitation lines should have completely crossed.

The fact that the activities of the tobacco and Chlorella enzymes were inhibited by the antiserum (Figs. 4 and 5) implies that both enzymes have at least one accessible binding site to antibodies. At such a site the enzyme activity is apparently sensitive to antibody binding, and in both cases the resulting antigen-antibody complex has no or only impaired enzyme activity. On the other hand, the Euglena preparation yields an identical precipitation band in the Ouchterlony test with the Chlorella preparation even though its activity is not affected by the binding of antibodies to the antigen (Fig. 3, C and D, Table II). One speculative explanation of this phenomenon is that the binding of antibodies to the Chlorella protein or to the tobacco protein results in a conformational change which affects the enzyme activity, whereas binding of the antibodies to the Euglena protein does not lead to such a change. Whether or not this explanation proves to be correct, our experiments show that two enzymes, one of which functions as a dehydrogenase and the other as an oxidase, can be fully identical serologically.

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LITERATURE CITED


