Phytochemistry, 1972, Vol. 11, pp. 1281 to 1285. Pergamon Press. Printed in England.

DISC ELECTROPHORESIS OF GLYCOLLATE OXIDIZING ENZYMES

B. GRODZINSKI and BRIAN COLMAN
Department of Biology, York University, Downsview, Ontario, Canada

(*Received* 26 *July* 1971, *in revised form* 19 *October* 1971)

Abstract—Glycollate oxidizing enzymes from a number of higher plant tissues, green and blue-green algae and rat liver were studied by disc electrophoresis in polyacrylamide gels. A method for the detection of enzyme activity in polyacrylamide gel is described. Glycollate oxidase isolated from tobacco, spinach, barley and corn leaves and from castor bean endosperm migrated as two components which were both active in the presence of FMN. The faster migrating component reacted with both glycollate and L-lactate but its activiy with L-lactate was unaffected by the presence of FMN. The slower migrating component reacted only with glycollate in the presence of FMN or FAD. The partially purified enzyme from *Anabaena flos-aquae*, *Oscillatoria* sp. and *Chlorella pyrenoidosa* migrated as a single component, reacted with glycollate and D-lactate, but only slowly with L-lactate and was unaffected by FMN or FAD. Purified rat liver enzyme migrated as a single component which migrated farther than either of the plant enzymes. It was specific for glycollate, reacted slowly with D-lactate and required FAD. These results indicate that the enzyme catalysing the oxidation of glycollic acid in algae differs from that isolated from higher plants and mammalian liver.

INTRODUCTION

AN ENZYME catalysing the oxidation of glycollate is widely found in plant tissues and in some mammalian tissues. Zelitch and Ochoa[1] purified a catalase-free enzyme from spinach leaves which oxidized glycollic and L-lactic acids and showed this to be a flavoprotein with FMN as the prosthetic group. Surveys of a wide variety of plant tissues indicate that this enzyme, glycollate: O_2–oxidoreductase (E.C. 1.1.3.1) is found in the leaves of all higher plants[2,3] and is also operative in castor bean endosperm tissue.[4] Both mammalian liver[5] and kidney tissue[6] appear to contain glycollate oxidase enzymes. These forms appear to function with oxygen or an artificial dye as the hydrogen acceptor.

There are conflicting reports about the presence and nature of this enzyme in algae. However an enzyme is present in a number of green algae[7–10] and in blue-green algae[11] which catalyses the oxidation of glycollate to glyoxyllate. The algal enzyme (i.e. glycollate: DPIP-oxidoreductase) is not activated by the addition of FMN or FAD[8,10,11] but a slight stimulation of activity by FMN has been observed with the enzyme from one blue-green alga[11]. Nelson and Tolbert[10] have shown that the enzyme prepared from several species of green algae oxidizes glycollate and D-lactate, does not couple to molecular oxygen, and is cyanide sensitive. These results indicate that the glycollate oxidizing enzyme isolated from

[1] I. ZELITCH and S. OCHOA, *J. Biol. Chem.* **201**, 707 (1953).
[2] C. O. CLAGETT, N. E. TOLBERT and R. H. BURRIS, *J. Biol. Chem.* **178**, 997 (1949).
[3] C. R. NOLL and R. H. BURRIS, *Plant Physiol.* **29**, 261 (1954).
[4] W. H. TANNER and H. BEEVERS, *Plant Physiol.* **40**, 971 (1965).
[5] E. KUN, J. M. DECHARY and H. C. PITOT, *J. Biol. Chem.* **201**, 269 (1954).
[6] J. C. ROBINSON, L. KEAY, R. MOLINAR and T. W. SIZER, *J. Biol. Chem.* **237**, 2001 (1962).
[7] W. J. S. DOWNTON and E. B. TREGUNNA, *Plant Physiol.* **43**, 923 (1968).
[8] I. ZELITCH and P. R. DAY, *Plant Physiol.* **43**, 289 (1968).
[9] J. M. LORD and M. J. MERRETT, *Biochem. Biophys. Acta* **159**, 543 (1968).
[10] E. B. NELSON and N. E. TOLBERT, *Arch. Biochem. Biophys.* **141**, 102 (1970).
[11] B. GRODZINSKI and B. COLMAN, *Plant Physiol.*, **45**, 735 (1970).

algae (i.e. glycollate dehydrogenase) is different from the enzyme (i.e. glycollate oxidase) of higher plants.

RESULTS

Detection of Enzyme Activity in Polyacrylamide Gel

To further investigate the nature of glycollate oxidizing enzymes, the behaviour of purified preparations of glycollic acid oxidase from spinach, barley, corn and tobacco leaves, castor bean endosperm and rat liver, and partially purified preparations of glycollate: DPIP oxidoreductase from two species of blue-green algae and one green alga *Chlorella pyrenoidosa,* were studied by disc electrophoresis in polyacrylamide gels.

After electrophoresis the position of enzyme activity in the gel was detected by coupling glycollate or lactate oxidation to the reduction of either NTB, through PMS or DPIP. The DPIP stain revealed enzyme activity as a pale or colourless zone on a blue background and this remained visible for 15 min approx. The advantage of using this stain

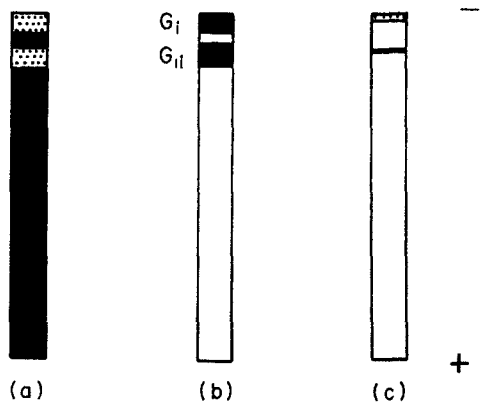

FIG. 1. THE DETECTION OF SPINACH GLYCOLLIC ACID OXIDASE ACTIVITY IN POLYACRYLAMIDE GELS AFTER DISC ELECTROPHORESIS, BY THE REDUCTION OF (a) DPIP OR (b) NTB AND THE DETECTION OF PROTEIN ON SIMILAR GELS (c) WITH AMIDO BLACK STAIN.

first is that the active zone could be marked and the same gel could then be treated with amido black or the NTB stain. The dark bands which appeared after treatment with the tetrazolium stain superimposed the area of the gels in which DPIP reduction had occurred (Fig. 1). The best staining results were obtained with the tetrazolium stain when the gel was incubated in the staining mixture, at 25°, in the dark immediately after electrophoresis. When a gel was stained with amido black for protein, a dark band was always visible in a position which corresponded to the section(s) of the gel which had displayed enzymatic activity (Fig. 1).

In order to ensure that the enzymatic activity detected in the gels was due to the action of only a glycollate oxidizing enzyme, control gels were incubated in the staining mixture without added substrate and/or cofactor (e.g. FMN). When a gel with enzyme was heated in a boiling water bath for 5 min after electrophoresis no bands appeared after incubation in the staining mixture with added glycollate and FMN. When an active preparation of the spinach enzyme was examined electrophoretically two bands (i.e. G_I and G_{II}) were always observed upon incubation of the gel with both glycollate and FMN, (Fig. 2a) but when the

substrate was omitted no bands appeared, regardless of the presence of FMN, FAD, or spinach ferredoxin in the incubation mixture. In the presence of glycollate without either FMN or FAD only the faster migrating component (G_{II}) could be detected. Similarly a single band (G_{II}) appeared when a gel was incubated with a staining mixture containing L-lactate, with or without FMN.

The specificity of the enzyme components, detected in the polyacrylamide gels, to a number of alternative substrates was investigated. After incubating gels which contained spinach glycollate oxidase with tetrazolium stain containing glycollate distinct bands appeared after 1 hr of treatment. However, no bands were detected after 4 hr incubation with or without FMN, when glycollate was replaced by any of the following compounds at a concentration of $1{\cdot}0 \times 10^{-2}$ M: D,L-glycerate, tartarate, isocitrate, citrate, succinate, glycine, serine, D,L-alanine, malonate, glyoxylate, formate, acetate, D-lactate, D,L-malate, pyruvate or α-oxoglutarate.

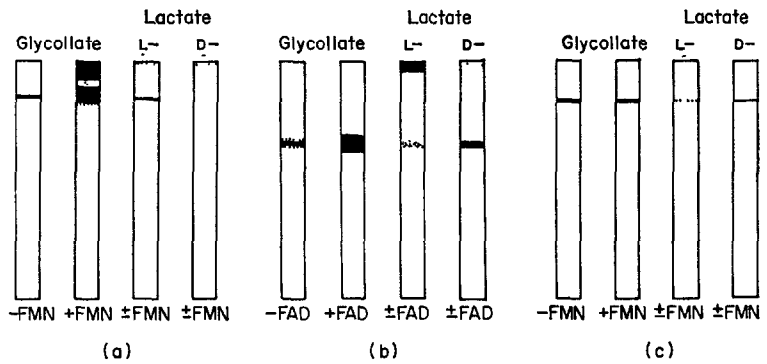

FIG. 2. POLYACRYLAMIDE DISC ELECTROPHORESIS PATTERNS OF GLYCOLLATE OXIDIZING ENZYMES. Electrophoretic patterns obtained using glycollate and lactate as substrate, with and without co-factor, for: (a) purified glycollate oxidase from higher plant tissues, i.e. leaves of spinach, tobacco, barley, and corn, and castor bean endosperm; (b) purified glycollate oxidase from rat liver; and (c) partially purified glycollate: DPIP-oxidoreductase from algae, i.e. *A. flos-aquae*, *Oscillatoria* sp. and *Chlorella pyrenoidosa*. The bands were located using the NTB stain.

Manometric Measurement of Oxidase Activity After Electrophoresis

In an attempt to provide additional evidence that the bands located with the DPIP and NTB stains after electrophoresis of the spinach enzyme were due to the action of the enzyme glycollate: O_2-oxidoreductase (E.C. 1.1.3.1) experiments were carried out to recover oxidase activity from sections of the gels. Marker gels were stained with DPIP or NTB to localize the enzyme activity and unstained gels were cut into zones corresponding to the staining in these marker gels. Each of the zones were separately chopped finely and placed in Warburg flasks containing 0·01 M potassium phosphate buffer and FMN ($1{\cdot}0 \times 10^{-4}$ M). The rate of oxygen uptake was measured manometrically after addition of 50 μmol of glycollate from the side arm of the flask. The results of an experiment with a purified preparation of the spinach enzyme, shown in Table 1, demonstrate that the sections of the gels having the highest glycollate oxidase activity correspond closely to the pattern of bands in the marker gel, and that the recovery of activity from the gel was high. Similar results were obtained with the liver enzyme. In experiments with either spinach or liver enzyme α-

TABLE 1. RECOVERY OF OXIDASE ACTIVITY FROM POLYACRYLAMIDE GELS AFTER ELECTROPHORESIS OF SPINACH GLYCOLLATE OXIDASE

Section of gel	Oxidase activity (initial rate of O_2 uptake μl. min^{-1})	Recovery % of total original activity	% inhibition of activity with 100 μM HPMS
Sephadex G 200	0·52	7·5	92
Corresponding to G_I	2·60	36·3	74
Corresponding to G_{II}	3·45	50·0	88
Top third below G_{II}	0·08	1·2	100
Middle third below G_{II}	0	0	—
Bottom third below G_{II}	0	0	—
Total	6·65	94·0	

Oxidase activity, measured manometrically, of sections of the gel corresponding to those of the NTB-stained gel shown in Fig. 1. The original activity of the enzyme preparation applied to the gel was 6·96 μl O_2 min^{-1}.

hydroxypyridinemethane sulphonate (HPMS), an inhibitor of this enzyme,[12] completely suppressed oxygen uptake by the gel sections which had displayed oxidase activity.

Enzyme Patterns in Various Tissues

An electrophoretic investigation of the glycollate oxidizing enzymes from various sources (Fig. 2) revealed that the nature of the enzyme bands which appeared in the presence of added glycollate was not necessarily the same as that outlined above for the spinach enzyme. Generally, the pattern observed after electrophoresis of the enzyme from each of tobacco, barley, corn, and castor bean endosperm was the same as that of the spinach enzyme. However, the electrophoretic patterns observed for the algal and the liver enzymes were not the same. Although a band (G_{II}) appeared when the enzyme from either a blue-green or a green alga was examined, the intensity of this band was unaltered by the addition of FMN, or FAD to the staining mixture. This single band appeared when D-lactate was used as the substrate and much less distinctly with L-lactate, (Fig. 2c) but did not appear when any of the alternative substrates listed above were tested, with the exception of glyoxylate. However, with glyoxylate, the G_{II} band appeared only after a long period of incubation (approx. 8 hr).

The pattern observed after electrophoresis of the liver enzyme was unlike that of the higher plants, and also unlike that of the enzyme from any of the algae tested (Fig. 2b). Regardless of the level of purity of the liver fraction tested, only one band (G_{III}) appeared in the presence of glycolate as the substrate, and the addition of FAD enhanced both the intensity of this zone and the rate of its appearance on the gel. The band also appeared after staining the gel with D-lactic acid as substrate.

DISCUSSION

The results indicate that the electrophoretic pattern of glycollate oxidase isolated from higher plants differs from that of glycollate dehydrogenase isolated from algae as well as

[12] I. ZELITCH, *J. Biol. Chem.* **224**, 251 (1957).

from mammalian liver glycollate oxidase. The higher plant enzyme displays two bands upon electrophoresis which differ in their requirement for FMN. This observation is consistent with the findings of Frigerio and Harbury[13] who have described two active polymeric forms of spinach glycollate oxidase which also differed in their requirement for FMN. However the enzyme from all three algae investigated appears to migrate as a single protein component under similar experimental conditions and is not stimulated in its activity towards glycollate by the addition of FMN or FAD and reacts with D-lactate more readily than with L-lactate.

These results provide further evidence to support the conclusion of Nelson and Tolbert[10] that the enzyme from green algae is different from that of higher plants and further demonstrates the similarity of the enzymes in the blue–green and green algae.[11]

EXPERIMENTAL

Material. Tobacco (*Nicotiana rustica*), corn (*Zea mays*) and barley (*Hordeum vulgare*) were greenhouse grown and large quantities of spinach (*Spinacea olacea*) were purchased locally. Castor beans were germinated and grown in the dark and used after 5–7 days growth. The blue–green algae *Anabaena flos-aquae* and *Oscillatoria* spp. were grown and harvested as previously described[14] and *Chlorella pyrenoidosa* was grown similarly on the medium of Watt and Fogg.[15] Liver tissue was obtained from freshly killed Sprague–Dawley rats of approx. 150–200 g weight.

Enzyme preparation. Glycollate oxidase from leaves and endosperm tissue was isolated and purified to the stage of calcium phosphate gel absorption by the method of Figerio and Harbury,[13] and that from liver was isolated by the method of Kuhn *et al.*[5] The glycolate oxidizing enzyme from blue–green algae was isolated as described previously and partially purified by precipitation with ammonium sulphate as described by Nelson and Tolbert.[10] The enzyme from *C. pyrenoidosa* was isolated by the method of Nelson and Tolbert[10] but without the use of Triton.

Electrophoresis. The polyacrylamide gels were prepared, run and removed for enzyme or protein assay in a manner similar to that outlined by Clarke.[16] A sample containing 20–100 μg protein was applied to a layer of Sephadex G 200 in water over the polyacrylamide gel. Analytical disc gel electrophoresis was performed with electrolyte buffer pH 8·3, at a temperature between 5 and 10° to minimize loss of enzymic activity, and at a current density of about 4 mA/gel. Variation of the pH of the electrolyte buffer between 7·8 and 8·5 seemed to have little effect upon the nature of the run or the position of the bands.

Detection of enzyme activity. After electrophoresis, a gel was incubated for 20 min at 25° with a freshly prepared mixture comprising 2,6-dichlorophenolindophenol (DPIP) 0·005% w/v, 0·1 M potassium phosphate buffer pH 8·3, $1{\cdot}0 \times 10^{-2}$ M glycollate, 0·01% PMS and 10^{-3} M KCN. The nitroblue tetrazolium (NTB) stain was made up daily, and kept refrigerated in a black bottle until required. It was composed of nitroblue tetrazolium (0·016%); NAD (0·005% w/v); PMS (0·003% w/v); FMN or FAD (10^{-4} M); and $1{\cdot}0 \times 10^{-2}$ M glycollate or other substrate (e.g. lactate). The gel was entirely immersed in approximately 2 ml of the above mixture and incubated in a water bath at 25° in the dark until distinct bands appeared. The temperature of the incubating solution was found to be important for relatively rapid (1 hr) and consistent development of bands. The gels stained with amido black or the NTB were stored for future reference in a weak acetic acid solution (7·5% v/v).

Acknowledgements—The authors are indebted to Dr. B. G. Loughton for his advice on the electrophoretic technique. The work was supported by grants from the National Research Council of Canada.

[13] N. A. Frigerio and H. A. Harbury, *J. Biol. Chem.* **231**, 135 (1958).
[14] A. G. Miller, K. H. Cheng and B. Colman, *J. Phycol.* **7**, 97 (1971).
[15] W. D. Watt and G. E. Fogg, *J. Exptl Bot.* **17**, 117 (1966).
[16] J. T. Clarke, *Ann. N.Y. Acad. Sci.* **121**, 428 (1964).

Key Word Index—Cyanophyta; Chlorophyta; Angiospermae; glycollate oxidase; comparative biochemistry; isoenzymes.