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INHIBITION OF GLUTAMATE DECARBOXYLASE IN EXTRACTS OF THE SLIME MOLD *PHYSARUM POLYCEPHALUM*

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Abstract—Ammonium sulfate fractions of supernatants from *Physarum polycephalum* browned rapidly at room temp. This browning was accompanied by complete inactivation of glutamate decarboxylase. Fractions exhibiting low activity and poor yields were obtained by decreasing plasmodium–buffer ratios of homogenates. Total activity of these fractions was increased by gel-filtration with Sephadex G-150. Samples of a crude slime mold pigment preparation inhibited supernatant activity by 42%. Since the enzyme was being inactivated either by phenols or their oxidation products, EDTA, cysteine, and Polyclar AT were added to extracting media to increase enzyme recovery. With their aid the enzyme was purified more than 15-fold by ammonium sulfate fractionation and gel-filtration with Sephadex G-200.

INTRODUCTION

A GLUTAMATE decarboxylase (E.C. 4.1.1.15) from *Physarum polycephalum* was recently identified in supernatants prepared from lyophilized plasmodia.[1] Subsequent attempts to fractionate these supernatants with ammonium sulfate yielded preparations that browned rapidly and were completely devoid of activity. An active fraction was ultimately obtained when the plasmodium–buffer ratio of the original homogenate was reduced from 10 g/100 ml to 4 g/100 ml. The specific activity of this ammonium sulfate fraction was twice that of the supernatant and the yield was only 26%. A gradual browning of the active material occurred, accompanied by a progressive inactivation of the enzyme. This inactivition was partially reversed and the enzyme separated from yellow pigments by chromatography on Sephadex G-150. These pigments, which are present in slime mold extracts and in ammonium sulfate precipitates obtained from them contain phenolic components which are probably oxidized by the polyphenol oxidase of the extracts.[2] As polyphenols and their oxidation products react with enzymes,[3] these observations suggested that the inactivation of glutamate decarboxylase was associated with the yellow pigments or their oxidation. To test this, various combinations of ethylenediaminetetra-acetic acid (EDTA), cysteine and Polyclar AT (an insoluble polyvinylpyrrolidone) were added to the homogenizing medium of separate samples of lyophilized plasmodium in order to inhibit polyphenol oxidase, remove quinones, and absorb phenolic compounds, respectively (Table 1). Pellet material centrifuged from plasmodium homogenized in buffer began to brown in less than 30 min at room temp. Pellets containing EDTA exhibited distinct browning after 90 min. A distinctly different gray appearance but no browning was observed after 15 min for material sedimented from homogenates with cysteine and with cysteine

[1] C. NATIONS and R. M. ANTHONY, *Can. J. Biochem.* **47**, 821 (1969).
[2] J. W. DANIEL, in *Cell Synchrony* (edited by I. L. CAMERON and G. M. PADILLA), p. 117, Academic Press, New York (1966).
[3] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).

plus Polyclar AT. None of the additives had any effect on the glutamate decarboxylase of plasmodial homogenates but did increase the activity of ammonium sulfate fractions precipitated at 60% saturation and dialyzed 8 hr against their respective media. Enzyme recovery in these fractions was enhanced more than 10% by EDTA. Cysteine augmented the effect of EDTA by almost 30%. Polyclar AT reduced yield slightly. Temperature was an important factor. The 44·3% yield obtained when buffer alone was employed at 0–4° was 18% more than previous yields at 25–30°. Assay conditions listed for polyphenol oxidase by Miller *et al.*[4] and by Guest and Horowitz[5] reveal that this enzyme should be quite active at 25–30°.

TABLE 1. GLUTAMATE DECARBOXYLASE ACTIVITIES OF SUPERNATANTS AND AMMONIUM SULFATE FRACTIONS FROM *Physarum polycephalum*

Homogenizing medium*	Activity†/ml supernatant	Total supernatant activity	Activity/ml fraction	Total activity of fraction	Yield (%)
B	30·8	3080	76·0	1375·6	44·3
B, EDTA	32·0	3200	92·8	1761·1	55·0
B, EDTA, CSH	30·1	3010	143·6	2541·0	84·0
B, EDTA, CSH, P	30·1	3010	127·0	2336·1	77·6

* B = pyridoxal phosphate 2 mM in 0·1 M phosphate buffer, pH 5·8; EDTA = ethylenediaminetetraacetic acid 3 mM; CSH = cysteine 6 mM; P = Polyclar AT.
† μl. CO_2/10 min.

These results indicate that a plasmodial component inactivates glutamate decarboxylase when concentrated into protein preparations during fractionation. Samples of a crude pigment preparation were found to inhibit supernatant activity by 42% when added in a 10 mg/ml ratio. The pigment had been extracted with 80% aq. acetone from 7 g defatted[6] plasmodium. The extract was heated to 85°, centrifuged, and dried *in vacuo*. The yield was 240 mg. Anhydrous solvents including acetone, butanol, and methanol were ineffective as extracting media.

Conventional techniques were applied to the purification of glutamate decarboxylase using EDTA, cysteine and Polyclar AT to decrease the inactivation. 96% of supernatant activity with a 4·2-fold purification was recovered in an ammonium sulfate fraction precipitated between 30–55% saturation (Table 2). Further purification was achieved on a Sephadex

TABLE 2. STEPS IN THE PURIFICATION OF SLIME MOLD GLUTAMATE DECARBOXYLASE*

Fractions	Specific activity (mU/mg protein)	Total activity	Yield (%)
Supernatant	46	28,869	100
$MnCl_2$, 30–55% $(NH_4)_2SO_4$	194	27,902	96
Sephadex G-200 (6·5–7·0 ml fraction)	715	6,929	24

* Values recorded are averages of near identical results from three repetitions.

[4] W. H. MILLER, M. F. MALLETTE, L. J. ROTH and C. R. DAWSON, *J. Am. Chem. Soc.* **66**, 514 (1944).
[5] H. GUEST and N. H. HOROWITZ, *J. Gen. Microbiol.* **18**, 64 (1958).
[6] R. K. MORTON, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 25, Academic Press, New York (1955).

G-200 column (Fig. 1). The most active fraction collected contained 24% of the activity recovered and a specific activity 15·3 times greater than that of the supernatant. Total recovery was 86·4%. Active fractions were eluted from the column immediately after the void volume, indicating that the molecular weight of the slime mold enzyme may dwarf that from bacteria and mouse brain.[7,8] Although no stabilizing effects have been demonstrated for Polyclar AT it removes some pigment from supernatants and thus represents a purification step. Yield is not reduced by its addition to dilute (2·5 g/100 ml) homogenates.

These results demonstrate that glutamate decarboxylase from *P. polycephalum* can be purified by conventional techniques in the presence of EDTA and cysteine. The observations

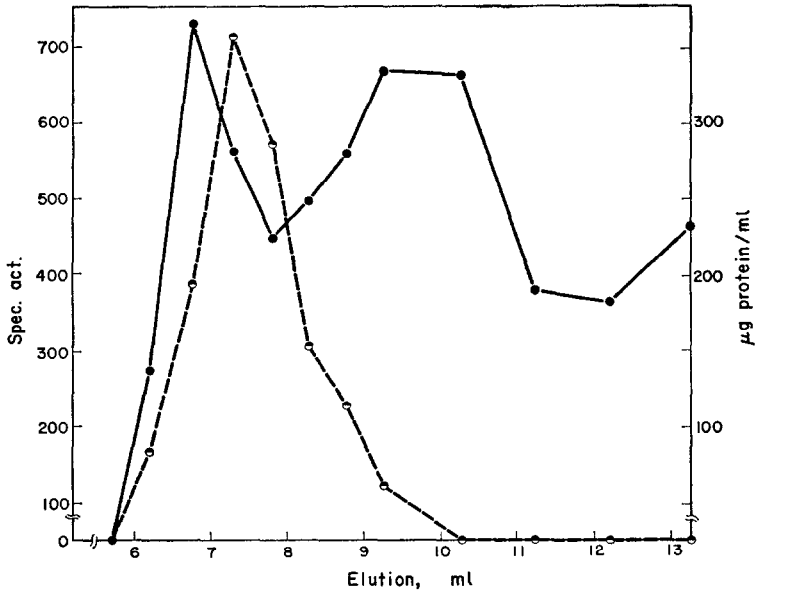

FIG. 1. CHROMATOGRAPHY OF SLIME MOLD GLUTAMATE DECARBOXYLASE ON SEPHADEX G-200.

o——o Spec. act.; •——• protein. (Column dimensions: 0·9 × 30 cm; eluted with 0·1 M phosphate buffer, pH 5·8, containing pyridoxal phosphate 2 mM, cysteine 6 mM, and EDTA 3 mM. Flow rate: 0·1 ml/min. Spec. act. denotes mμmoles glutamate decarboxylated/min/mg protein.)

suggest that the browning reactions of slime mold preparations are analogous to those complicating enzyme extraction from higher plants. This information is of potential significance to studies of slime mold biochemistry. Relatively few enzymes have been demonstrated for this organism. Treatments inhibiting the formation of protein–phenol complexes may permit the study of many slime mold enzymes that have not previously been detected. Evidence is currently being sought to determine whether the enzyme is inactivated by the phenolic component associated with pigment, by a quinone resulting from polyphenol oxidase activity or by a condensed polyphenol formed as a result of oxidation.

[7] R. SHUKUYA and G. W. SCHWERT, *J. Biol. Chem.* **235**, 1658 (1960).
[8] J. P. SUSZ, B. HABER and E. ROBERTS, *Biochem.* **5**, 2870 (1966).

EXPERIMENTAL

Plasmodial fractions were assayed for glutamate decarboxylase activity at 36° by conventional Warburg techniques. The assay procedure consisted of 0·5 ml of enzyme and 1·5 ml of the appropriate homogenizing medium in the main chamber of the Warburg flask, and 0·5 ml of 0·1 M monosodium glutamate, pH 5·8, in the side arm. After equilibration, substrate was combined with enzyme and 10-min readings were recorded for 1 hr. A control vessel with buffer substituting for substrate permitted correction for CO_2 evolved in the absence of glutamate. The gas phase was nitrogen.

Preparation of Extracts

Lyophilized plasmodia were prepared and stored as previously described. Homogenizing media consisted of pyridoxal phosphate 2 mM in 0·1 M potassium phosphate buffer, pH 5·8, plus selected additives. Various combinations of EDTA (3 mM), cysteine (6 mM) and Polyclar AT (6 g) were included in the homogenizing medium (100 ml) of separate 4-g samples of dry plasmodium. Homogenates were centrifuged at 12,800 *g* for 30 min. The rate of browning of pellet material was observed by placing them at room temp. on paper towels, or by covering them with 20 ml of homogenizing medium. $(NH_4)_2SO_4$ was added to each supernatant to 60% saturation. The resulting precipitates were collected by centrifugation, resuspended in 10-ml aliquots of homogenizing media and dialyzed for 8 hr against 500 ml of their corresponding medium. The dialyzed preparations were centrifuged at 12,800 *g* for 15 min and allowed to incubate for 30 min at room temp. prior to assay. All operations were at 0–4° unless otherwise indicated.

Glutamate Decarboxylase Purification

A homogenate was prepared with mortar and pestle by grinding 2·5 g dry plasmodium and 4 g hydrated Polyclar AT in 100 ml of buffer containing pyridoxal phosphate, EDTA and cysteine in the concentrations listed above. It was centrifuged at 36,000 *g* for 30 min. Nucleic acids were precipitated from the resulting supernatant by slow addition of 4 ml 1 M $MnCl_2$. The $(NH_4)_2SO_4$ fraction sedimented between 30–55% saturation was resuspended in 10 ml of homogenizing medium and dialyzed 3 hr against 500 ml of medium. A 0·2 ml sample of the dialyzed preparation was applied to a 0·9 × 30 cm Sephadex G-200 column. Fractions were eluted with homogenizing medium. The flow rate was 0·1 ml/min. All steps were performed at 0–4°. One unit of enzyme is that amount which mediates the decarboxylation of 1 μmole of glutmate in 1 min. Specific activity is defined as milliunits of enzyme per milligram of protein. Protein determinations were by the procedure of Lowry *et al.*[9] Corrections for the influence of pigment on these determinations were made by preparing blanks from heat-treated (85°, 30 min) and centrifuged samples of all pigmented fractions. The effectiveness of this procedure was confirmed by comparing values for yield based on total protein with those based on total ml. Polyclar AT was provided by General Aniline and Film Corp., Dyestuff and Chemicals, New York, N.Y., U.S.A.

[9] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).