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SOME PROPERTIES OF GLUTAMATE DEHYDROGENASE FROM PEA SEEDLINGS

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Abstract—Glutamate:NAD oxidoreductase, deaminating (E.C. 1.4.1.2) was partially purified from pea seedlings. Optimum pHs for the reductive amination and oxidative deamination were 7·5 and 10·0 respectively. Michaelis constants were $1{\cdot}6 \times 10^{-3}$ M for glutamate and $4{\cdot}1 \times 10^{-5}$ M for NAD^+. Activity was inhibited by *p*-chloromercuriphenylsulfonic acid (PCMPSA), phenylmercuric acetate, *o*-phenanthroline, ethylenediamine tetraacetate (EDTA), zincon and other metal-binding reagents. Inhibition by PCMPSA was reversed by reduced glutathione. Meanwhile, the activity was much decreased by dialysis against EDTA solution and its activity was restored by the addition of several divalent cations.

INTRODUCTION

GLUTAMATE dehydrogenase (GDH) has been isolated, purified and characterized to various degrees from a number of higher plant sources. Bulen[1] demonstrated that GDH from maize leaves is a thiol enzyme. Damodaran and Nair[2] reported GDH from pea seedings but were unable to purify their preparation. On the other hand, Davies[3] showed that GDH occurred in the mitochondrial fraction from the pea epicotyl. However, detailed biochemical studies have not yet been carried out on the GDH from higher plants and this paper deals with some properties of GDH from pea seedlings.

RESULTS

General Properties

The pH optimum for the reductive amination of α-ketoglutarate was 7·5, whereas the optimum for the oxidative deamination of glutamate was found to be 10. This value is higher than that of *Neurospora* (9·5)[4] and beef liver (8·5).[5] The Michaelis constants (K_m), by statistical calculation, are $1{\cdot}6 \times 10^{-3}$ M for glutamate and $4{\cdot}1 \times 10^{-5}$ M for NAD^+, respectively.

Inhibition by Thiol Reagents

The enzyme was inhibited by PCMPSA and phenylmercuric acetate (PMA). As shown in Table 1, even if one of the inhibitors were preincubated with enzyme, inhibition was not extensive. However, if the enzyme was preincubated with NAD^+ or glutamate before the

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[2] M. DAMODARAN and K. R. NAIR, *Biochem. J.* **32**, 1064 (1938).
[3] D. D. DAVIES, *J. Exptl Botany* **7**, 203 (1956).
[4] C. S. STACHOW and B. D. SANWAL, *Biochem. Biophys. Acta* **139**, 294 (1967).
[5] H. J. STRECKER, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 2, p. 220, Academic Press, New York (1955).

TABLE 1. PROTECTION BY NAD, GLUTAMATE AND GSH OF PEA GDH ACTIVITY AGAINST INHIBITION BY THIOL REAGENTS

		Order of addition of reagents				
		I	I	NAD	GA	GSH
		NAD	20 min	I	I	I
		GA	NAD+GA	10 min	10 min	10 min
				GA	NAD	NAD+GA
Thiol reagent (mM)		Percentage inhibition				
PCMPSA	0·1	75	75	65	63	26
PMA	0·1	54	54	44	47	20

Standard assay conditions were used; NAD reduction. Final concentration of GSH was 10^{-3} M. I, GSH and GA are thiol reagents, reduced glutathione and sodium glutamate respectively. 20 min and 10 min indicated the time interval between the additions of reagents. Temperature 24°.

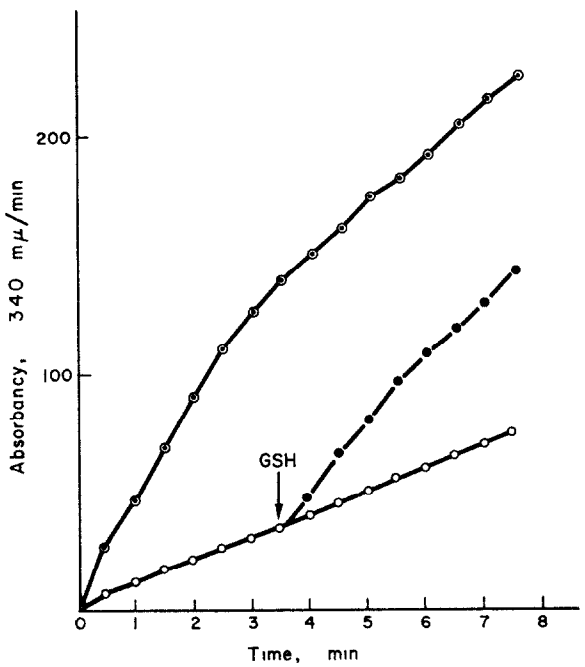

FIG. 1. REACTIVATING EFFECT OF GSH ON THE PCMPSA-INHIBITED PEA GDH.

⊙ Without PCMPSA and GSH. ○ Inactivated enzyme by PCMPSA. ● Inactivated enzyme plus GSH. GSH was added at the time indicated by the arrow. Final concentration: PCMPSA 10^{-4} M. GSH, 10^{-3} M. Other conditions were similar to the standard assay.

thiol reagent was added, it was partially protected against inhibition. On the other hand, the enzyme which had been inactivated by preincubation with PCMPSA reactivated almost completely by addition of 10 moles of reduced glutathione per mole of PCMPSA (Fig. 1).

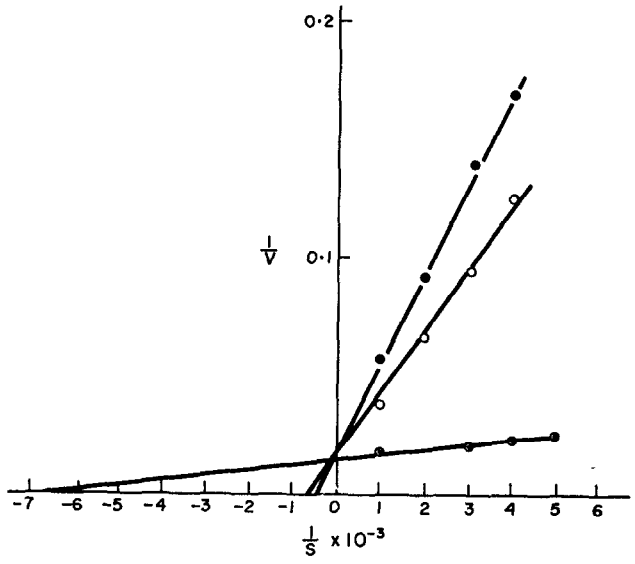

FIG. 2. LINEWEAVER–BURK PLOTS FOR DIFFERENT CONCENTRATION OF PCMPSA AT VARIOUS GLUTAMATE CONCENTRATIONS. STANDARD ASSAY CONDITIONS WERE USED.

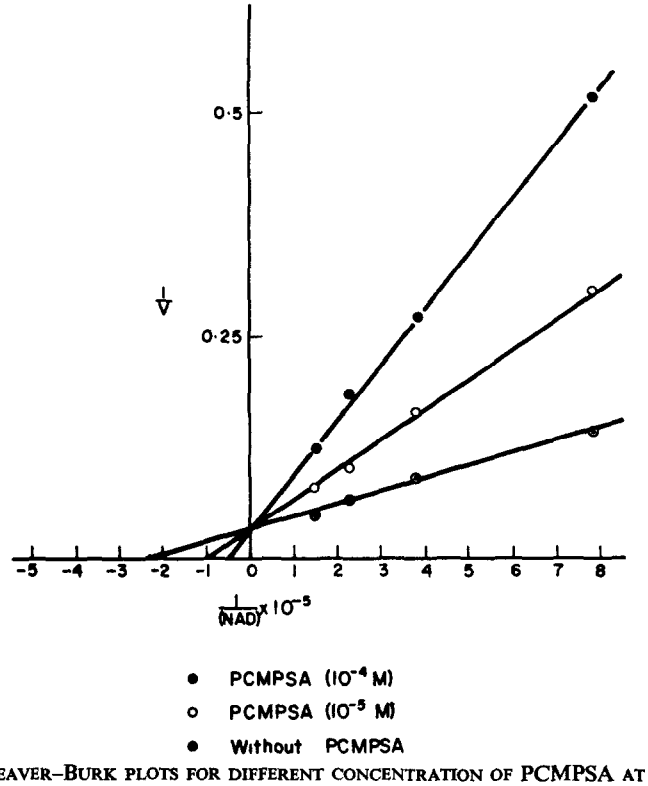

FIG. 3. LINEWEAVER–BURK PLOTS FOR DIFFERENT CONCENTRATION OF PCMPSA AT VARIOUS NAD CONCENTRATIONS. STANDARD ASSAY CONDITIONS WERE USED.

Double reciprocal plots of the resulting data (Figs. 2 and 3) revealed that PCMPSA apparently was competitive with NAD^+ or glutamate. These results indicated that the enzyme possesses sulfhydryl groups essential for its activity.

Effect of Some Metal-Binding Agents

The addition of several metal-binding reagents such as *o*-phenanthroline, $\alpha\alpha'$-dipyridyl, EDTA, zincon, ferron, Nitroso-R-salts and 8-hydroxyquinoline to the reaction cuvette all reduced the enzymatic activity of pea GDH (Table 2). The relative activity is shown in Fig. 4

TABLE 2. INHIBITION OF PEA GDH BY SOME METAL-BINDING AGENTS

Agents	Final concentration (mM)	Inhibition (%)* NAD reduction	Inhibition (%)* NADH oxidation
o-Phenanthroline	1	23	25
Disodium ethylene-diamine tetraacetate	1	28	71
Ferron	0·36	65	—
Ferron	0·33	—	†
2,3-Dimercaptopropanol	1	7	14
$\alpha\alpha'$-Dipyridyl	1	7	22
Sodium diethyldithio-carbamate	1	7	—
Zincon	0·36	60	—
Zincon	0·33	—	14
Cupferron	1	12	—
Nitroso-R-salt	0 36	25	—
Nitroso-R-salt	0 33	—	10
8-Hydroxyquinoline	0 36	17	—
Potassium thiocyanate	1	10	—

* Enzyme was preincubated with metal-binding agents for 10 min. Standard assay conditions were used at 24° in phosphate buffer (pH 8·0).

† No inhibition, but instead 37% stimulation in enzyme activity.

for *o*-phenanthroline and EDTA. In this experiment, the high concentration of EDTA caused some stimulation, but the mode of action is unknown. In an attempt to produce a metal-free apoenzyme, the enzyme solution (6 mg), containing 0·1 ml of 2-mercaptoethanol (0·01 M), in a Visking sac which had been soaked in 0·01 M 2-mercaptoethanol solution, was dialysed for 20 hr against 100 ml of 0·1 M tris-HCl buffer solution (pH = 6·3) containing 0·1 M EDTA and 0·01 M 2-mercaptoethanol.

Then, the dialysate was again dialysed for 48 hr against 3 l. of 0·1 M tris-HCl buffer solution (pH 6·8) containing 0·01 M 2-mercaptoethanol (in three changes). All procedures were carried out at 0–4°. After dialysis to remove excess EDTA, 70 per cent inhibition was obtained, and then the reactivation was achieved not only with Zn^{2+} but also with Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and MoO_4^{2-} (Table 3). It was postulated that EDTA complexes with, but does not remove, the essential metal component (if any) of the enzyme protein.

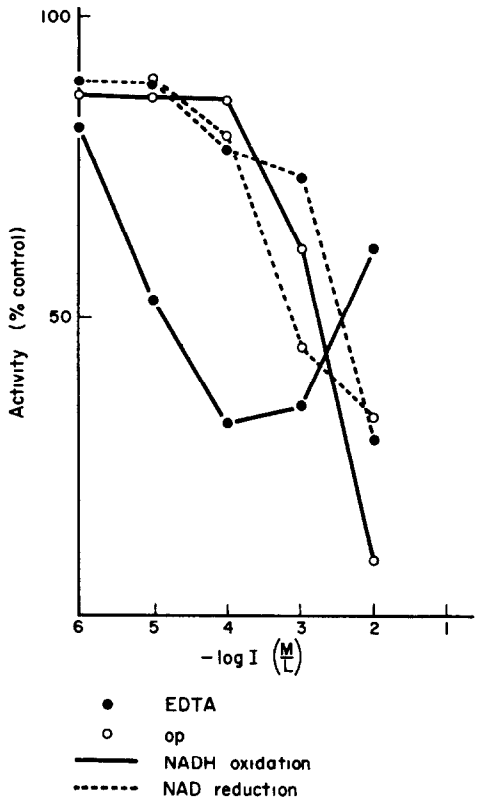

FIG. 4. INHIBITION BY EDTA AND *o*-PHENANTHROLINE. STANDARD ASSAY CONDITIONS WERE USED. REACTION WAS STARTED BY THE ADDITION OF ENZYME SOLUTION.

TABLE 3. STIMULATING EFFECT OF VARIOUS METAL SALTS ON THE ACTIVITY OF PEA GDH INACTIVATED BY DIALYSIS AGAINST EDTA SOLUTION

	Addition							
	None	$ZnSO_4$	$FeSO_4$	$Co(CH_3COO)_2$	$MgSO_4$	$MnCl_2$	$CaCl_2$	Na_2MoO_4
	(Percentage inhibition)							
NADH oxidation	72	21	25	23	52	24	7	30
NAD reduction	65	65	72	54	46	57	52	72

Standard assay conditions were used, except for the following conditions; inactivated enzyme was preincubated (10 min) with metallic salts before the addition of coenzyme and glutamate. Final concentrations of metallic salts were 5×10^{-5} M. Tris-HCl buffer solution (0·1 M, pH 8·0) was used. Temperature 26°.

DISCUSSION

The results suggest that pea GDH is a thiol enzyme as is the preparation from maize leaves,[1] though less sensitive to PCMPSA and PMA than the pea alcohol dehydrogenase.[6]

[6] Y. SUZUKI, *Phytochem.* **5**, 761 (1966).

The competitive action of PCMPSA against either glutamate or NAD^+ may suggest a certain intimate relation of the thiol group in the enzyme with glutamate and/or NAD^+.

Although Bulen[1] reported that cyanide did not reduce the GDH activity from maize leaves, Gilmanov *et al.*[7] reported that diethyldithiocarbamate completely inhibited the enzyme of maize root. On the other hand, only EDTA, among several chelating agents tested, inhibited significantly *Neurospora* GDH.[4] Present work shows that pea GDH is sensitive to several metal-binding agents such as *o*-phenanthroline, EDTA and so on. *o*-Phenanthroline is a typical inhibitor for the liver GDH, which contains zinc, since its inhibition may be completely reversed by the addition of $ZnCl_2$.[8] However, pea GDH did not show such a characteristic.

When the enzyme was dialysed against EDTA solution and almost all of the unbound EDTA removed by further dialysis, the addition of Zn^{2+} caused remarkable reactivation of the reductive amination reaction. However, several other divalent cations also reactivate the inactivated enzyme (Table 3).

It has been reported that EDTA inhibits elastase (E.C. 3.4.4.7) which is a non-metalloenzyme.[9] However, the concentration of EDTA for the elastase inhibition was much higher than the case of pea GDH.

These results, therefore, suggest that pea GDH is a metalloenzyme; however, it is not at present clear whether zinc is involved or not.

EXPERIMENTAL

Enzyme Preparation

The peas (*Pisum sativum*, Alaska) were soaked for 15 hr in running tap water at 17° and, after incubation for 48 hr in a dark incubator at 23°, the seedlings were harvested. The seedlings thus obtained (750 g) were blended in a Waring blender for 1 min with 1500 ml of a cold solution, consisting of 0·2 M sucrose, 0·05 M phosphate buffer (pH 7·0) and 0 005 M EDTA The macerate, squeezed through two layers of cotton cloth, was centrifuged for 10 min at 5500 × *g*. The supernatant was centrifuged for 30 min at 15,000 × *g* to give a pellet. The pellet was resuspended in a cold solution which consisted of 0·25 M sucrose, 0 04 M phosphate (pH 7·0) and 0·002 M EDTA, and then re-centrifuged. The pellet thus obtained was treated with 40 ml of 1% digitonin suspension. After agitating for 10 min, it was centrifuged at 15,000 × *g* for 30 min. To the clear supernatant was added 0·27 ml of protamine sulphate solution (mg/ml) and the mixture was agitated for 30 min. The supernatant, produced by centrifugation, was salted out with $(NH_4)_2SO_4$ to 40% saturation. After 1 hr, the supernatant, produced by centrifugation, was treated again with $(NH_4)_2SO_4$ to 55% saturation. After 2 hr, the precipitate was collected and then suspended in 10 ml of 0·1 M phosphate (pH 8) and dialysed for 48 hr against 15 l. of 0·01 M phosphate (pH 7·0) with five changes. After centrifugation, the supernatant was treated with alumina C_γ gel[10] at a concentration of 70 mg/mg protein. The suspension was stirred for 10 min and the gel was recovered by centrifugation and the enzyme was eluted from the gel by stirring with 5 ml of 0·1 M phosphate (pH 8 0) for 1 hr and then again with 5 ml of 0·2 M phosphate (pH 8·0) for 1 hr. The combined eluate (10 ml) was dialysed overnight against 5 l of 0·005 M phosphate (pH 7 0). All operations were followed at 0–4°. The procedure of purification is summarized in Table 4.

Enzyme Assays

The pea GDH activity was assayed by measuring spectrophotometrically the initial rate of oxidation of NADH or reduction of NAD^+ at room temperature (22°, except where stated otherwise). The standard assay system was as follows: in the case of NADH oxidation, 0·1 ml of enzyme solution, 0·2 ml of 0·2 M sodium α-ketoglutarate, 0 2 ml of 1·5 M $(NH_4)_2SO_4$, 1·0 ml of 0·1 M phosphate (pH 8·0), 0·5 ml of NADH solution

[7] M. K. GILMANOV, V. I. YAKOVLEVA and V. L. KRETOVICH, *Dokl. Akad. Nauk SSSR* **175**, 949 (1967).
[8] S. J. ADELSTEIN and B. L. VALLEE, *J. Biol. Chem.* **233**, 589 (1958).
[9] F. LAMY, C. P. CRAIG and S. TAUBER, *J. Biol. Chem.* **236**, 86 (1961).
[10] S. P. COLOWICK, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 97, Academic Press, New York (1955).

TABLE 4. SUMMARY OF PURIFICATION PROCEDURE

Procedure	Total volume (ml)	Units/ml	Total units	Protein (mg/ml)	Specific activity (units/mg)	Recovery (%)
Digitonin extract	40	200	8000	5·9	33·8	—
Protamine sulphate treatment	38	180	6840	4·9	36·7	85·5
Ammonium sulphate precipitate (40–55%)	13	300	3900	3·3	90·9	48·8
Alumina C_γ eluates	10	380	3800	1·2	316·6	47·5

Standard assay conditions were used; NAD reduction.

(2 mg/ml) and 1·0 ml of deionized water. In the case of NAD^+ reduction, 0·2 ml of enzyme solution, 0·2 ml of 0·1 M sodium glutamate, 1·0 ml of 0·1 M phosphate (pH 8·0), 0·5 ml of NAD solution (2 mg/ml) and 1·1 ml of deionized water were used. Unit activity was arbitrarily defined as that causing 0·01 increase of absorptivity at 340 nm in 1 min, calculated from the change between the 30- and 60-sec readings. Specific activity was expressed as units per mg of protein. The enzyme protein was determined by the method of Kalckar.[11]

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[11] H. M. KALCKAR, *J. Biol. Chem.* **167**, 461 (1947).