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Properties of glutamate dehydrogenase in developing legume fruit. I. The effects of chelating agents

M. MARZIAH
Jabatan Biokmia and Mikrobiologi,
Fakulti Sains dan Pengajian Alam Sekitar, Universiti Pertanian Malaysia,
Serdang, Selangor, Malaysia.

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RINGKASAN

Satu penelitian telah dijalan untuk mengkaji kesan-kesan EDTA dan Chelex-100 terhadap pengaktifan glutamat dehidrogenase dari ekstrak kasar bijian Lupinus angustifolius *yang sedang berkembang. Enzim itu tidak terencat oleh Chelex-100, bahkan aktivitinya teransang hingga ke paras melebihi aktiviti yang terdapat dalam kontrol. Glutamat dehidrogenase, sebaliknya, terencat oleh EDTA pada kepekatan 5mM EDTA, teransang pada kepekatan 30mM EDTA, seterusnya terencat kembali pada kepekatan 45mM EDTA.*

SUMMARY

A study was conducted to investigate the effects of EDTA and Chelex-100 on the glutamate dehydrogenase activity from a crude extract of the developing seeds of Lupinus angustifolius. *The enzyme was not inhibited by Chelex-100; instead it was highly stimulated and its activity, when treated with Chelex-100, was higher than that of the control. Glutamate dehydrogenase, however, was inhibited by 5mM EDTA, stimulated at 30mM EDTA, and inhibited again at 45mM EDTA.*

INTRODUCTION

Glutamate dehydrogenase (GDH) is one of the key enzymes involved in amino acid metabolism. Its primary role is in NH_4^+ assimilation, besides, it serves as an important link between the tricarboxylic acid cycle and amino acids. The enzyme catalyses the reaction of 2-oxoglutarate and NH_4^+ to synthesize glutamate:

$$NH_4^+ + \text{2-oxoglutarate} + \text{NAD (P) H} \rightleftharpoons \text{L-glutamate} + \text{NAD (P)}^+ + H_2O$$

In legumes, GDH has been reported to be very active (Atkins *et al.* 1975, Murray and Kennedy, 1980) presumably to cope with the high fluxes of NH_4^+ in the developing seeds.

In spite of the considerable research on this enzyme in plants, there are still areas needing attention and where information is lacking. One of these is interaction of the enzyme with chelating agents, especially EDTA. The mechanism or mechanisms of EDTA inactivation are still unclear.

In the course of investigating the properties of GDH in developing legume fruit, it was found that this enzyme has some peculiar EDTA relationships. This paper reports a study on GDH with EDTA. Chelex-100, a type of chelating agent, was also used as a comparison.

MATERIALS AND METHODS

General Procedure

Plant materials

The plant materials used were obtained from *Lupinus angustifolius* plants grown in naturally Mn-deficient soils in Western Australia. The first three seeds from the main axes were used for all the enzyme studies. This is to ensure that they were all of the same physiological age.

Abbreviations used : EDTA = Ethylene diaminetetra-acetic acid; MSH = 2-Mercaptoethanol; PV = Polivinylprrollidone. TRIS = Trishydroxy methylaminomethane.

Chemicals

All the chemicals and reagents used were of analytical grade. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri; U.S.A.

Experimental details

Crude enzyme preparation

Fresh or frozen seeds were homogenised in a chilled pestle and mortar in two volumes of extraction buffer: it contained 0.1 TRIS-HCl pH 8.0, 1.0mM EDTA, 0.1% MSH (v/v) and 2% insoluble PVP. Fine hydrochloric acid-washed sand (10% HCl v/v) was used to facilitate extraction. The extract was squeezed through 4 layers of muslin, centrifuged at 8,200g for 20 min., and the supernatant was used for all crude extract studies.

Enzyme assays

The incubation mixture contained in a final volume of 1.5ml, 50mM TRIS-HCl pH 8.0 (at 30°), 150mM NH_4Cl 0.07mM NAD (P) H and 5mM 2-oxoglutarate; samples of 0.05ml or 0.10ml of the crude enzyme extract were assayed. The reaction was started by the addition of NH_4Cl and was monitored by the change in optical density at 340nm using a temperature-controlled (30°) Varian automatic recording spectrophotometer, model 635. Linear reaction was maintained for at least 3 min.

Unit of enzyme activity

A unit of enzyme activity is defined as that amount catalysing the oxidation or reduction of 1 umole of coenzyme min^{-1}. Specific activity is defined as units, $(mg\ protein)^{-1}$. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Crude extract studies

pH optimum

Three buffers were employed to determine the pH optimum of the GDH. The buffers used were 50mM TRIS-HCl, 50mM sodium phosphate and 50mM diethanolamine-HCl.

Effects of EDTA and MSH on GDH activities

This experiment was conducted to determine the effects of 1.0mM EDTA and 0.1% MSH used in the extraction medium.

The effects of Chelex-100 an GDH activity.

The effects of Chelex-100, a chelating resin, on GDH activity were examined. The purpose of the study was to chelate any metal ions that may be present in the enzyme extracts. The extracts were treated with Chelex-100 and kept stirring gently at 4° – 5° and at room temperature (25°). Enzyme assays were carried out at different time intervals to determine the point of maximum inhibition.

The effects of EDTA concentration on GDH activities

EDTA used was in solution and the pH was adjusted to 8.0 10mM NaOH. EDTA was added to the reaction mixture just prior to the addition of the last substrate, NH_4Cl, when the reaction rate was started.

The effects of EDTA and incubation items on GDH activity

A time course was set up to determine the optimum incubation time using the lowest EDTA concentration but at the same time giving a maximum inhibition. Final concentrations of 0.5mM, 2.0mM and 5.0mM EDTA were used for different incubation periods.

RESULTS AND DISCUSSION

pH optimum

The NADH- and NADPH-GDH activities had an optimum pH of 8.0 in 50mM TRIS-HCl buffer (Fig. 1). Results of previous workers

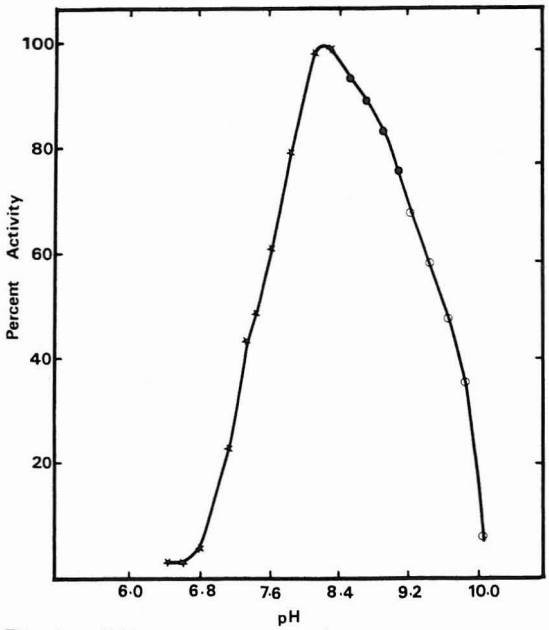

Fig. 1. Effect of pH on the activity of NADH-GDH. The reaction was performed as described in Materials and Methods. The buffers used were 50mM TRIS-HCl (★——★), 50mM sodium phosphate (●——●) and 50mM diethanolamine HCl (○——○).

(King and Wu, 1970; Chou and Splitoesser, 1972) have also shown that the enzyme has a pH optimum of 8.0. The use of TRIS-HCl buffer was found to be most suitable for all subsequent studies because it gave linearity performance and it did not result in precipitation when metal ions were used in the assay mixture in subsequent studies.

Effects of EDTA and MSH on GDH activities

GDH activities (assayed both with NADH and NADPH coenzymes) from lupin seeds were highly stimulated by the addition of MSH in the extraction medium (Table 1). The requirement for a sulfhydryl reagent has been mentioned by several workers for GDH isolated from a variety of plant sources, such as *Urtica dioica* (Welander, 1974), Corn leaves (Bulen, 1956), pea seedlings (Yamasaki and Suzuki, 1969), pumpkin cotyledons (Chou and Splitstoesser, 1972), and *Vigna* cotyledons (Fawole and Boulter. 1977). The addition of 1mM EDTA to the extraction medium did not result in any loss of enzyme activity due to the inhibition effects of EDTA as its concentration in the final assay mixture was too low to influence the enzyme activity.

TABLE 1

The effects of extraction buffers, EDTA and 2-mercaptoethanol on GDH activity in the seeds of *L. angustifolius* CV. UniCrop.

Buffers		Specific activity (nmoles min^{-1}. mg^{-1}. protein)	
		NADH-GDH	NADPH-GDH
0.1M sodium phosphate	pH 7.6	0.09	0.03
0.1M sodium phosphate	pH 8.0	0.09	0.03
0.1M TRIS-HCl	pH 7.6	0.09	0.03
0.1M TRIS-HCl	pH 8.0	0.09	0.03
0.1M sodium phosphate + 1mM EDTA	pH 7.6	1.10	0.04
0.1M sodium phosphate + 1mM EDTA	pH 8.0	1.14	0.04
0.1M TRIS-HCl + 1mM EDTA	pH 7.6	1.12	0.03
0.1M TRIS-HCl + 1mM EDTA	pH 8.0	1.21	0.04
0.1M sodium phosphate + 1mM EDTA + 0.1 % MSH	pH 7.6	4.97	1.82
0.1M sodium phosphate + 1mM EDTA + 0.1 % MSH	pH 8.0	5.80	1.93
0.1M TRIS-HCl + 1mM EDTA + 0.1 % MSH	pH 7.6	5.34	1.83
0.1M TRIS-HC 1 [1] + 1mM EDTA + 0.1 % MSH	pH 8.0	5.81	1.92

The effects of Chelex-100 on GDH activity.

The results of this experiment are shown in *Fig. 2.* The Chelex-treated extract dramatically increased in activity in treatments of 4° and 25°. In fact, in both cases the GDH-activity remained higher than the control. Additionally, there was an increase in enzyme activity in 4° and 25° treatments after 1h. of incubation, but the activity began to decrease after approximately 5h. of incubation, although the activity continued to remain high above the control in all treatments. One possible explanation as to the increase in activity was that Chelex-100 must have removed some divalent cationic inhibitors from the enzyme extracts; additionally, Chelex-100 must not have reached the active site of the enzyme to remove the essential metal ions. The increase in enzyme activity after 1h. of incubation seemed to suggest that the enzyme was not fully activated after extraction.

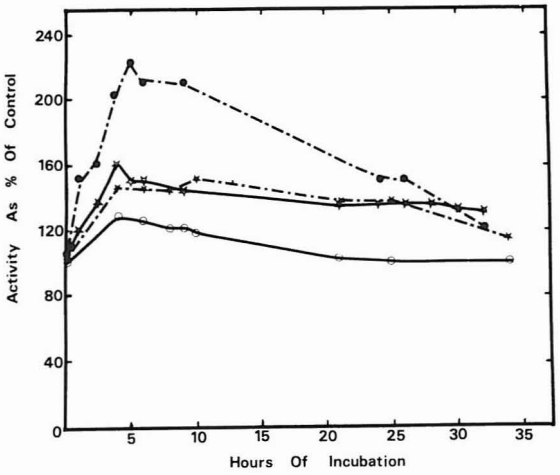

Fig. 2. The effects of Chelex-100 an NADH-GDH activity assayed from the crude extract. The reaction was performed as in Materials and Methods. Incubation without Chelex-100 at 4° (control) (★----★) incubation with Chelex-100 at 4° (●----●), incubation without Chelex-100 at 25° (control (○----○), incubation with Chelex-100 at 25° (☆----☆).

The lowering in enzyme activity that occurred in the later part of the experiment was obviously due to the normal decay of enzyme protein rather than to the chelation effects.

The effects of EDTA on GDH activities

The fact that both the NADH-and NADPH-dependent activities are inhibited by EDTA has been reported for extracts of different plant sources including pumpkin cotyledons (Chou and Splitstoesser, 1972), pea roots (Pahlich and Joy, 1971), pea seedlings (Yamasaki and Suzuki, 1960), *Vigna* cotyledons (Fawole and Boutler, 1971), Soybean cotyledons (King and Wu, 1970), *Lemna minor* (Ehmke and Hartman, 1970; Sheid *et al.*, 1980), Lupin nodules (Stone *et al.*, 1979) and pea cotyledons (Sheid *et al.*, 1980). However, the detailed reports on EDTA inhibitation gave variable responses.

In this study, the results showed that the NADH-dependent activity seemed to be more affected by EDTA inhibition than the NADPH-dependent activity *(Fig. 3).* In an experiment whereby different EDTA concentrations were added to the reaction mixture just prior to the addition of NH_4Cl, it was shown that NADH-GDH activity was completely inactivated at 5mM EDTA, while NADPH-GDH activity was inhibited by 98% at the same EDTA concentration. King and Wu (1970) have reported a similar observation, whereby the NADH-activity was more inhibited by EDTA than the NADPH-activity in soybean cotyledon extracts. An increase in EDTA concentration caused an increase in activities of both forms of GDH. At 30mM EDTA, the maximum increase was 41% and 14% of the total NADPH-GDH and NADH-GDH activities, respectively. A further increase in EDTA concentration to 45mM caused a complete inactivation of NADH-GDH activity and almost a complete inactivation in NADPH-GDH activity. Attempts to reactivate the GDH activity using different metal ions at this point of inhibition were unsuccessful.

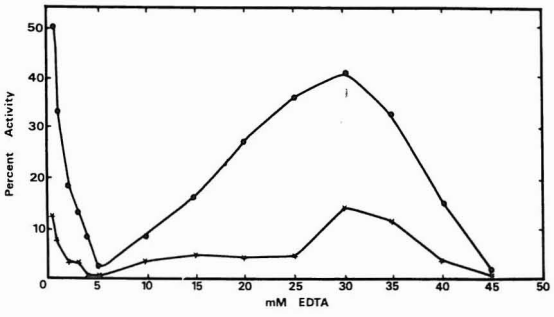

Fig. 3. The effects of EDTA concentrations on the activities of NADH-GDH and NADPH-GDH from the developing seeds of lupins cv. UniCrop. EDTA was not preincubated with the enzyme but was added to the reaction mixture just prior to the addition of the last substrate (NH_4Cl). NADH (★_____★), NADPH (●_____●).

The enzyme is maximally inhibited when incubated with 1mM EDTA in final assay concentration for 30 min. *(Fig. 4)*. This agrees generally with the data obtained from other workers (Yamasaki and Suzuki, 1969; King and Wu, 1979; Pahlich and Joy, 1971; Ehmke and Hartman, 1978). Most papers, however, did not specify the time of incubation. Ehmke and Hartman (1976) reported more than 95% inhibition in *Lemna minor* with only 0.1mM EDTA, whereas in developing lupin seeds, the use of 0.5mM EDTA showed only about 70% inhibition even after 2h. of incubation. However, in a subsequent paper (Ehmke and Hartman, 1978), 1.0mM EDTA used on the enzyme from the same source, *Lemna minor.*

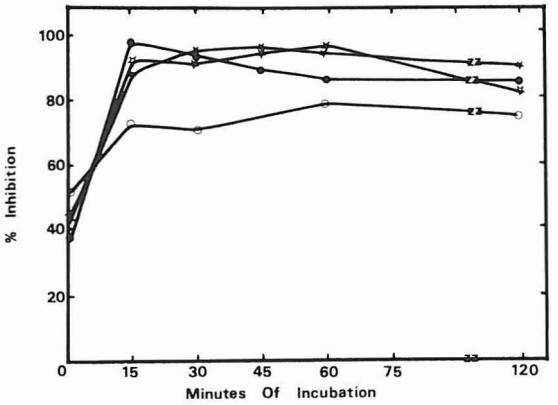

Fig. 4. The inhibitory effects of different concentrations of EDTA on NADH-GDH activity from the developing seeds of lupins cv UniCorp. The crude enzyme extract in the reaction mixture was incubated with EDTA at different time intervals. 0.5mM EDTA (○——○), 1.0mM EDTA (★——★), 2.0mM EDTA (☆——☆), 5.0mM EDTA (●——●).

The increase in enzyme activity when the EDTA concentration was increased has also been reported by Pahlich and Joy (1971), Yamasaki and Suzuki (1969), King and Wu (1970), and Fawole and Boutler (1977), however, the actual mechanism of action is still not known. Pahlich and Joy (1970) speculated that EDTA could interact with the protein causing a conformational change leading to the formation of a new catalytically active form of the enzyme. Alternatively, activation or stimulation at higher EDTA concentrations may have been due to the removal of metal inhibitors by EDTA.

The inactivation of GDH activity and the inablity to stimulate the enzyme activity at a high EDTA concentration has also been reported by Chou and Splitstoesser (1972). In this case, EDTA must cause the removal of essential ions resulting in irreversible changes in the enzyme protein molecule.

CONCLUSION

The degree of GDH inhibition or inactivation by EDTA depends largely on the EDTA concentration used on the time of incubation of the assay mixture. Further results (in subsequent paper) indicated that EDTA inhibition may also be influenced by the interaction of other endogenous cations, and by protein concentrations.

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