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GLUTAMATE DEHYDROGENASE ISOENZYMES IN *RICINUS COMMUNIS* SEEDLINGS

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Abstract—Castor bean seedling glutamate dehydrogenase isoenzymes are not artifacts. The isoenzymes have different salting out properties and they utilize NAPD to differing extents, but they have the same isoelectric point of pH 6·2. Tissue specific patterns occur but the patterns are the same between genotypes. The GDH isoenzymes are probably of functional significance in castor bean seedlings.

INTRODUCTION

THE isoenzymatic nature of glutamate dehydrogenase (L-glutamate-NAD oxidoreductase, E.C. 1.4.1.3), or GDH, has been well documented in higher plants.[1–5] The enzyme is of central importance in nitrogen metabolism, as well as being a critical link between nitrogen and carbohydrate metabolism. Regulatory properties for the enzyme have been described from a number of microorganisms as well as beef liver.[6–8] Pahlich and Joy described changes in the reversibility of the enzyme reaction of GDH purified from pea roots; they suggested that the plant enzyme may also have regulatory properties.[9]

The purpose of this research was to analyze GDH isoenzymes from castor bean seedlings to obtain evidence concerning the functional significance of these isoenzymes in plants. Stewart and Beevers analyzed the movement of nitrogen from endosperm to cotyledon in castor bean seedlings and showed that the sole form of nitrogen transported was L-glutamine.[10] This means high levels of glutamic acid and glutamine synthesis should occur in the endosperm, and high levels of degradation of the two compounds in the cotyledon. If the isoenzymes have any significance pertaining to the direction of the enzyme reaction, isoenzyme pattern differences should be seen in the two tissues. In addition, preliminary analysis was performed on the slowest and fastest migrating electrophoretic forms (isoelectric focusing, ammonium sulfate precipitation, and cofactor dependence).

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[9] PAHLICH, E. and JOY, K. W. (1971) *Can. J. Biochem.* **49,** 127.
[10] STEWART, C. R. and BEEVERS, H. (1967) *Plant Physiol.* **42,** 1587.

RESULTS AND DISCUSSION

Typical isoenzyme patterns from seedling extracts of castor beans are described in Fig. 1a. The same multiple isoenzyme pattern was detected from 40 individual seedling genotypes of the variety examined. It is apparent, then, that the isoenzymes of GDH in these plants are not a result of allelic interactions, as in the case of corn esterase and alcohol dehydrogenase,[11] since no segregants were detected. The slowest and fastest migrating bands (*S* and *F*) were sliced from gels and subjected to a second electrophoretic separation. The isoenzymes migrated as before (Fig. 1a). Cotyledon and endosperm GDH isoenzyme patterns are seen in Fig. 1b. Cotyledon extracts possessed greatest stainability in the *S* isoenzyme; in endosperm extracts the *F* band stained most intensely. In order to develop methods for the

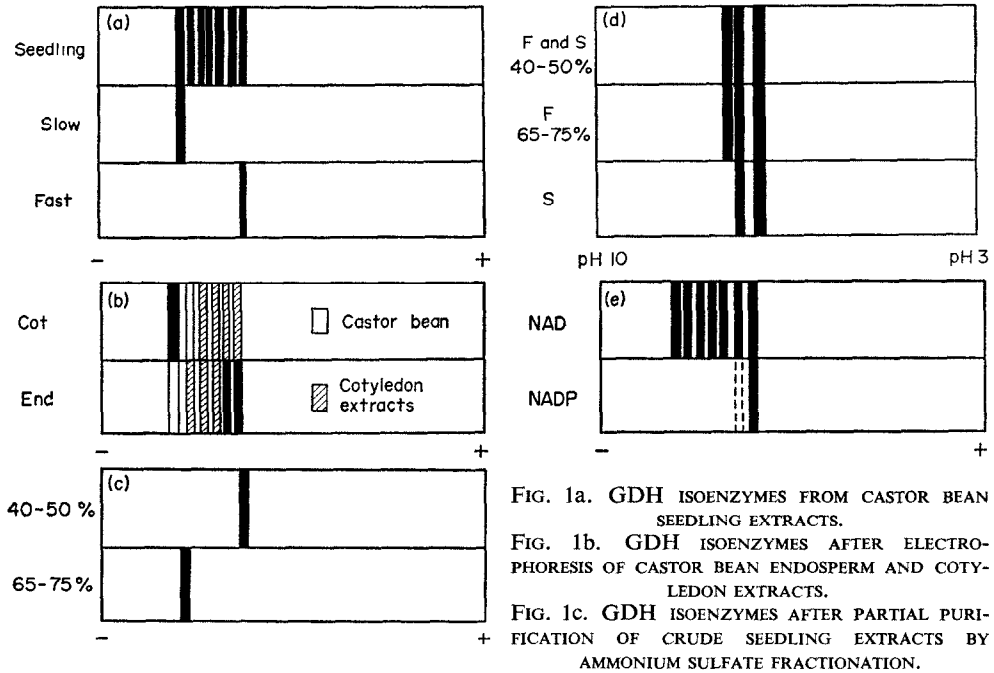

FIG. 1a. GDH ISOENZYMES FROM CASTOR BEAN SEEDLING EXTRACTS.
FIG. 1b. GDH ISOENZYMES AFTER ELECTROPHORESIS OF CASTOR BEAN ENDOSPERM AND COTYLEDON EXTRACTS.
FIG. 1c. GDH ISOENZYMES AFTER PARTIAL PURIFICATION OF CRUDE SEEDLING EXTRACTS BY AMMONIUM SULFATE FRACTIONATION.
FIG. 1d. GDH ISOENZYMES AFTER ISOELECTRIC FOCUSING OF AMMONIUM SULFATE FRACTION OF WHOLE SEEDLING EXTRACTS.
FIG. 1e. GDH ISOENZYMES AFTER ELECTROPHORESIS OF CASTOR BEAN SEEDLING EXTRACTS

separation and purification of the *F* and *S* isoenzymes, the effect of ammonium sulfate concentration on their precipitation was studied. Preliminary results indicated that the fastest migrating isoenzymes were precipitated by a low salt concentration, and the slowest bands by the greatest concentration. More detailed analysis revealed that the *F* band could be separated by precipitating twice in a 40–50% range of ammonium sulfate concentration, and the *S* band in a 65–75% concentration (Fig. 1c). There was no alteration in the electrophoretic mobility of these single isoenzymes, even after storage for 6 months at −20°. Isoelectric focusing was performed to determine if there were differences in the isoelectric

[11] SCANDALIOS, J. G. (1969) *Biochem Genet.* **3**, 37.

points between the two isoenzymes separated by ammonium sulfate fractionation. The results (Fig. 1d) indicated two bands for each fraction, with one band in common. There were no significant differences in the isoelectric points of any of the peaks, all were isolated in a region of approximately pH 6·2. The presence of different bands with seemingly identical isoelectric points has been explained by other authors.[12] Analysis of cofactor dependence indicated that the *F* band possessed both NAD and NADP activity while the *S* band showed virtually no NADP-dependent staining (Fig. 1e). These results were obtained from the analysis of crude extracts and the ammonium sulfate fractions of whole seedlings.

The following tentative conclusions can be drawn. First, GDH isoenzymes in castor beans are physically stable and are not artifacts of the methods of separation, since neither re-electrophoresis, ammonium sulfate precipitation, or long-term storage altered the electrophoretic characteristics of the isoenzymes. Pahlich has published evidence that pea GDH isoenzymes are actually the same molecule in different conformational states.[13] If this is true for castor bean GDH is it clear that the conformers are extremely stable. Secondly, the GDH isoenzymes appear to have functional significance. The consistent tissue-specific patterns, as illustrated in seedling cotyledons and endosperm, correlate with known metabolic activities in these tissues.[14]

The presence of the *F* isoenzyme in the endosperm (where synthesis of glutamic acid and glutamine occurs) suggests that the *F* isoenzyme functions in an anabolic capacity, and the *S* isoenzyme is responsible for catabolic activity. The *F* isoenzyme has NAD and NADP-dependent activity, while the *S* isoenzyme stains only with NAD. An established principle for pyridine nucleotide-dependent dehydrogenase in eukaryotic cells is that anabolic (and energy requiring) activity requires NADPH, and catabolic (energy yielding) activity requires NAD, the reduced NAD then feeding the electron transport system.[15] Finally, the results of Kanamori *et al.* that the fastest migrating GDH isoenzyme in rice was induced by the addition of ammonium, support this hypothesis.[16]

It is hypothesized that these isoenzymes may be of specific functional significance in plants. Analysis of GDH patterns may be of assistance in the analysis of certain plant-cell systems such as tobacco teratoma[17] and wild carrot[18] where GDH activity has been thought as critical in events leading to differentiation. The partial purification of separate GDH isoenzymes should open the way to a detailed analysis of differences between isoenzymes; an examination of single isoenzyme kinetics would constitute a more critical test of the hypothesis raised in this article.

EXPERIMENTAL

Castor bean seeds (*Ricinus communis* L., var. Early Spineless), obtained from Dr. Oved Shifriss, were germinated on moist towelling (30° and in darkness) for 5 days prior to analysis. Extraction was performed at 3°; centrifugation was for 20 min at 15000 *g*. The seedlings were ground in a pestle and mortar with 0·1 M $KHCO_3$ soln at pH 8·0 (25%, w/v), then the slurry was immediately centrifuged. The supernatant was mixed with sucrose (final concn 20% w/v) and immediately subjected to electrophoresis. For analysis of separated endosperm and cotyledons, these tissues were carefully separated and washed repeatedly with dist. H_2O prior to grinding. $(NH_4)_2SO_4$ fractionation of the crude whole seedling extracts was performed

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after mixing with aq. sols of protamine sulfate (Sigma) to a final concentration of 0·1% (w/v) protamine sulfate. The mixture was centrifuged and the supernatant was subjected to precipitation by slowly mixing for 15 min with a soln of ammonium sulfate in 0·1 M $KHCO_3$ saturated and kept at 3°. After 15 min the solns were centrifuged and mixed with additional $(NH_4)_2SO_4$ soln to give the desired concentration. The ppts were dissolved in *ca.* 3 ml of the extraction soln and dialysed against the same soln to remove $(NH_4)_2SO_4$. Disc electrophoresis was performed on 5·25% polyacrylamide gels.[19] Isoelectric focusing was performed in polyacrylamide gels following procedures described by Wrigley.[20] The samples were mixed with sucrose (final 20%, w/v) and added to the tops of gels in both procedures. *Ca.* 200 μg of extracted protein were subjected to analysis. Protein concentrations were determined colorimetrically,[21] using bovine serum albumin as the standard. Prior to polymerization, both acrylamide monomer and bis-acrylamide were recrystallized.[22] GDH activity was visualized in the gels by the precipitation of formazan. Gels were incubated in solutions of 6 mg NAD, 0·25 mg phenazine methosulfate, and 1·5 mg MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diaphenyl tetrazolium chloride) in 10 ml 0·1 Tris-HCl, pH 8·6. Neutralized sodium glutamate was added to give a final concentration of 0·15 M. Staining for the NADP-dependent GDH activity was essentially the same as for NAD, with the same cofactor concentration and the use of 0·1 M $KHCO_3$ buffer (pH 8·0). Gels were photographed, and scanned in a Gilford No. 2400 spectrophotometer at 590 nm with gel scanning attachment. All chemicals for gel staining were obtained from Sigma

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