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PROTEINASES AND THE INSTABILITY OF ISOCITRATE LYASE IN EXTRACTS OF DEVELOPING FLAX SEEDLINGS

BRUCE A. MCFADDEN and BERTOLD HOCK*

Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164, U.S.A.; *Lehrstuhl für Botanik, Technische Universität München, D-8050 Freising-Weihenstephan, West Germany

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Key Word Index—*Linum usitatissimum*; Linoceae; *Citrullus vulgaris*; Cucurbitaceae; seedlings; flax isocitrate lyase; flax proteinases; flax enzyme instability.

Abstract—In extracts of flax seedlings 4 days after imbibition, isocitrate lyase activity is unstable in comparison to that in extracts from 2.5-day seedlings or to malate synthases analysed at several stages of development. This instability in extracts of 4-day seedlings is especially pronounced when a large number of seedlings is homogenized per unit volume of Tris–Mg^{2+}–EDTA–dithioerythritol buffer. However, isocitrate lyase can be stabilized when the resultant homogenate is diluted soon after seedling breakage. The pronounced instability in more concentrated extracts is not due to inadequate buffering by the homogenization medium, nor can it be due to polyphenols because added polyvinylpyrrolidone has no effect. Mixing of a heated supernatant from concentrated extract with dilute unheated extract yields the units of stable isocitrate lyase expected in the dilute extract, ruling out stoichiometric inactivation by a heat-stable component. The pronounced instability is attributed to the action of proteinases. A theoretical model assuming a decay process that is first order in isocitrate lyase and first-order in one or more proteinases is in reasonable agreement with the results. Malate synthase and $NADP^+$-isocitrate dehydrogenase are much more stable in concentrated extracts prepared from 4-day flax seedlings. Isocitrate lyase is stable in concentrated extracts of 5-day watermelon seedlings, which is a developmental stage analogous to that for 4-day flax seedlings.

INTRODUCTION

In developing flax seedlings, the specific activity of isocitrate lyase (EC 4.1.3.1) increases from zero to a maximum three days after imbibition and subsequently declines [1]. The developmental profiles for isocitrate lyase and malate synthase (EC 4.1.3.2) correspond to that for the conversion of lipid to carbohydrate, suggesting that the glyoxylate cycle is functional in this conversion which is presumably essential to growth of flax seedlings.

There are indications in the literature that proteinases contribute to the disappearance of isocitrate lyase in oil-rich seeds such as those from the sunflower [2], castor bean [3], pine [4] and flax [5, 6]. Our previous research has established that the disappearance of isocitrate lyase during germination of flax seeds is prevented by 25 μM cycloheximide and partially prevented by actinomycin D at concentrations above 20 μg/ml. Isocitrate lyase was much more stable in extracts of 3-day seedlings than in those from 4-day seedlings whereas malate synthase, catalase, and isocitrate dehydrogenase ($NADP^+$; EC 1.1.1.42) were stable in the latter [5]. These observations suggested that m-RNA for an isocitrate lyase-directed proteinase is synthesized and translated in seedlings at about the third day of germination.

Recently flax-seedling proteinases have been characterized by gel electrophoresis during seed germination. One major and one minor EDTA-inhibited proteinase appeared 4 days after imbibition and the addition of EDTA spared isocitrate lyase in extracts of 4-day seedlings [6]. We now describe a novel approach in probing the mechanism of disappearance of an enzyme *in vitro* and results which also imply the proteolytic destruction of isocitrate lyase in extracts of 4-day flax seedlings.

RESULTS

Effect of dilution on the stability of flax isocitrate lyase

In extracts incubated at 37°, isocitrate lyase, which was largely confined to cotyledons, was progressively less stable as the germination time was prolonged from 2 to 4 days (Table 1). Of particular interest was the fact that the mixing of equal volumes of extracts of 4 day cotyledons and embryonic axes at 2° yielded an activity/seed of 103.6 at t_0 (37°) that was about 60% higher than the value obtained by adding the isocitrate lyase content in cotyledons plus embryo axes per seed (56.2 + 4.1). This suggested that some inactivation of isocitrate lyase in the undiluted extract of cotyledons had occurred before t_0 and that it was dependent upon cotyledon protein concentration. Upon reviewing our unpublished data for 4-day flax seedlings, we found numerous examples of this, i.e. isocitrate lyase was much less stable in protein-rich extracts. For example, the enzyme was much more stable in extracts derived from breakage of a smaller number of cotyledons per unit volume. This was true regardless of the buffering capacity of the homogenization medium (pH 7.80 at 22°) between Tris concentrations of 0.05–0.4 M and ruled out the possibility that the effect of dilution was one of pH adjustment. After breakage of 99 2.5-day seedlings in 9 ml of the breakage buffer containing 0.4 M Tris, the pH of the extract was 7.83 (at 22°). In all experiments to be described, the homogenization buffer

Table 1. Stability of isocitrate lyase at 37° in extracts of 35 flax seedlings

Seed portion	Time (min) at 37°†	Activity*/seed in seeds germinated for 46 hr	68 hr	88 hr
Cotyledons	0	35.8	62.3	56.2
	30	35.2	39.5	21.6
	60	35.3	31.0	11.9
	120	—	13.4	6.6
	150	33.2	—	—
Embryo axes	0	1.6	6.6	4.1
	30	1.6	7.0	—
	60	1.8	6.7	3.0
	120	—	4.8	—
	150	1.6	—	1.3
0.5 Cotyledons + 0.5 embryo axes‡	0	38.4	78.0	103.6

*Expressed as nmol of isocitrate-dependent glyoxylate/min at 30°.

†Incubation at 37° was started within 15 min of preparation of the 15 000 *g* supernatant from homogenate prepared as described with buffer containing 0.05 M Tris, pH 7.6 at 22°.

‡One volume of the 15 000 *g* supernatant from the homogenate of cotyledons (from 35 seeds) was mixed with one volume of the analogous supernatant from the homogenate of embryo axes prior to incubation at 37°. Each tissue had been homogenized in 9 ml of breakage buffer.

(pH 7.80 at 22°) contained 0.4 M Tris and the other components specified.

Effect of PVP on the stability of flax isocitrate lyase

In additional experiments with 4-day seedlings the preparation of cotyledon extracts in the presence of 6.7% (wt/vol.) of polyvinylpyrrolidone had no effect on the stability of isocitrate lyase in concentrated extracts. This reagent is known to complex strongly with polyphenols at pH values below the pK_as of phenols [7]. Thus the participation of polyphenols in the inactivation of isocitrate lyase observed in highly concentrated seedling extracts was ruled out.

A model for proteinase-catalysed disappearance of flax isocitrate lyase

To accommodate the observed protein-dependent decay the following model was hypothesized:

isocitrate lyase (IL) + proteinase(s) → inactive (or less active) IL + proteinase(s)

for which the rate dependence would be:

$$(1)\quad -\frac{d[IL]}{dt} = [IL]\,(k_1\,[\text{proteinase}_1] + \ldots\ldots + k_n\,[\text{proteinase}_n])$$

in which the product of [IL] and each term containing a proteinase concentration reflects the action of a given proteinase (through n proteinases) contributing to the disappearance of IL. When rewritten, (1) becomes:

$$(2)\quad -\frac{d[IL]}{dt} = [IL]\sum_{i=1}^{i=n}(k_i[\text{proteinase}]_i)$$

This model predicts that the decay of IL should be first order in [IL] and that x-fold dilution of the extract should lead to a resultant decay that is $(1/1+x)^2$ as fast as that for undiluted extract. In consequence, isocitrate lyase would actually be markedly stabilized by dilution of the extract.

Data depicted in Fig. 1 for 4-day seedlings establish that isocitrate lyase decayed rapidly at 2° in cotyledon extracts derived from breakage of 99 seedlings and that this decay was greatly reduced by dilution of extracts as soon as possible after breakage. At both dilutions tested, the decay was first order in isocitrate lyase. The initial approximate rates of decay (estimated from Fig. 1) are 0.3 and 1.5 units/hr for the extract three-fold and one-fold diluted, respectively. Whereas the first value should be 25% as large as the second if the model is correct, it is 20% as large. Thus the data are in reasonable accord with the model. On the other hand, the approximate initial rate of decay of isocitrate lyase in the undiluted extract (Fig. 1) was 19 units/hr. Thus the observed rate ratio for 1/2-diluted to undiluted extract was 0.079. Clearly, this is a significant deviation from the predicted ratio of 0.25 which could indicate the need for a different or modified model.

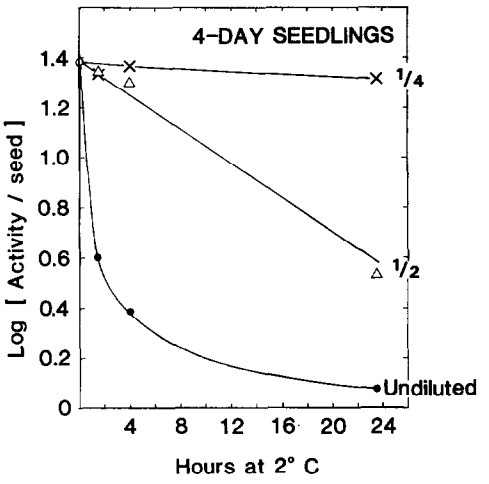

Fig. 1. Log of isocitrate lyase activity/cotyledon pair plotted against time of incubation of flax extracts at 2°. Cotyledons from 99 4-day seedlings were homogenized as described and dilutions with 1 volume ($\frac{1}{2}$) or 3 volumes ($\frac{1}{4}$) of cold breakage buffer were made after filtration through cheesecloth within 5 min of breakage. The supernatants after centrifugation as described were used for assay. The time elapsed is the time between completion of homogenization and assay.

Other results (not presented) in which cotyledons from 15 4-day seedlings were broken in 9 ml of homogenization buffer established that isocitrate lyase was completely stable during the 24-hr incubation period at 2° (cf. data for 99 seedlings shown in Fig. 1).

A test for stoichiometric inactivation of isocitrate lyase in concentrated flax extracts

Collectively these results suggested that one or more proteinases catalyse the degradation of isocitrate lyase in 4-day flax seedlings. Because of the biphasic decay of isocitrate lyase (Fig. 1), there remained the possibility, however, that there is a stoichiometric inactivation of this enzyme by a non-proteolytic, heat-stable component at the high extract concentrations tested and that the complex between this component and isocitrate lyase is dissociated by dilution. To test this possibility a cotyledon extract from 99 4-day seedlings (in 9 ml of homogenization buffer) was prepared and an aliquot portion immediately heated to 100° for 5 min. After centrifugation, the supernatant was added in a large volume excess to the analogous extract of 15 4-day seedlings. The resultant isocitrate lyase level was identical with that in a control in which homogenization buffer was substituted for the supernatant from heat treatment. Thus stoichiometric inactivation of the type considered could not have accounted for the rapid initial inactivation rate (Fig. 1).

Effect of homogenization medium on the stability of flax isocitrate lyase

Other experiments established that isocitrate lyase derived from cotyledons of 4-day flax seedlings was more stable in a homogenization buffer containing bovine serum albumin and sucrose. The composition of that buffer (pH 7.5) was: 0.25 M sucrose, 0.1% bovine serum albumin, 10 mM KCl, 1 mM $MgCl_2$, 1 mM disodium EDTA, 10 mM dithioerythritol and 50 mM HEPES.

Comparative stability of flax isocitrate lyase and malate synthase

In contrast to the results for isocitrate lyase, malate synthase was stable during 5.3 hr-incubation at 2° in extracts prepared by breakage of cotyledons from either 99 or 15 4-day flax seedlings in 9 ml of homogenization buffer (data not shown).

In experiments with 2.5-day old seedlings, isocitrate lyase in concentrated cotyledon extracts prepared from 99 flax seedlings (Fig. 2) was comparatively stable at 2° (cf. Fig. 2 and Fig. 1) but was further stabilized by one-fold dilution. In contrast, malate synthase was reasonably stable in the same extracts over the entire incubation period of 11.5 hr at 2° and the stability was unaffected by one-fold dilution of concentrated extracts (Fig. 2).

Stability of enzymes in concentrated extracts of watermelon cotyledons

In marked contrast to the instability of isocitrate lyase in flax-seedling extracts, this enzyme was extremely stable in concentrated cotyledon extracts from 5-day watermelon seedlings. When 24 or four cotyledon-pairs were homogenized in 9 ml of the Tris buffer used for breakage of flax seedlings, there was virtually no loss of isocitrate

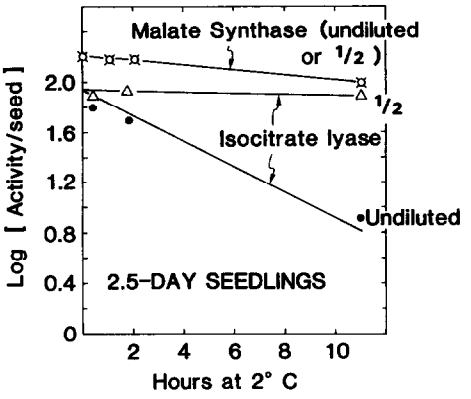

Fig. 2. Log of isocitrate lyase and malate synthase activity/cotyledon pair plotted against time of incubation of flax extracts at 2°. Cotyledons from 99 2.5-day seedlings were homogenized, filtered, diluted and assayed as described for Fig. 1 and in the text. The time elapsed is the time between completion of homogenization and assay.

lyase, malate synthase and $NADP^+$-isocitrate dehydrogenase during 27-hr incubation at 2° (Table 2). In the more concentrated extract, the protein concentration was the same as that in analogous extracts (of 99 cotyledon-pairs) of flax seedlings. The isocitrate lyase content is known to peak in cotyledons of 3- to 4-day watermelon seedlings and to decline rapidly thereafter [8].

DISCUSSION

The development of the present theoretical model for proteolysis of isocitrate in flax seedling extracts arose from the puzzling observation that this enzyme was more stable in dilute extracts. Indeed, it was consistently found that 15 4-day seedlings contained as much total isocitrate lyase activity as 99 seedlings when both batches of seeds

Table 2. Stability of enzymes at 2° in extracts of watermelon cotyledons after 5-day growth

Specific activity of enzyme*	Incubation time at 2° after extract preparation:		
In 48 cotyledons	50 min	4 hr 4 min	27 hr
Isocitrate lyase	28.9	32.6	28.5
	81 min	4 hr 33 min	27.6 hr
Malate synthases	141	136	140
	67 min	4 hr 13 min	27.5 hr
$NADP^+$-isocitrate dehydrogenase	16.6	15.5	14.8
In 8 cotyledons	62 min	4 hr 10 min	27 hr
Isocitrate lyase	36.9	38.9	34.9
	90 min	4 hr 33 min	27.6 hr
Malate synthase	125	138	135
	78 min	4 hr 25 min	27.5 hr
$NADP^+$-isocitrate dehydrogenase	17.1	20.9	18.8

*Expressed as nmol/min/mg protein.

were assayed 30 min after homogenization. When assays were performed on suspensions 2 min after breakage of 99 seedlings, the isocitrate lyase content was increased to 40% of theoretical [unpublished observations]. These results led to the hypothesis that there was very rapid degradation of isocitrate lyase in protein-rich extracts of 4-day seedlings and that this could be markedly reduced by dilution of these extracts. In developing a theoretical model, it was recognized that dilution of extracts would simultaneously and equivalently lower the concentration of both isocitrate lyase and proteinase(a). Thus for a kinetic process that is first order in each, the resultant decay of isocitrate lyase would be drastically reduced, i.e. by the square of the dilution factor. The results (Fig. 1) conform to this model when two dilutions are compared. In contrast, malate synthase in the same concentrate extract of 4-day flax seedlings is stable at 2°.

The present observation that a molecule may be stabilized in a crude extract by dilution would presumably apply to any biological extract in which a macromolecule is being enzymatically degraded. Thus the principle of dilution to stabilize a component may have wide application. We stress this because in preparative biochemistry concentrated extracts are often sought as the starting point in fractionating a desired macromolecule such as an enzyme. This ostensibly sound approach may actually be counterproductive if the extracts are rich in one or more enzymes that degrade the macromolecule of interest. As an example taken from the present work, should purification of isocitrate lyase from flax seedlings be the objective, it would be highly desirable to start with more dilute extracts and, preferably, of 2.5-day seedlings (Fig. 2).

In the present research, it has been found that isocitrate lyase is enriched by 9–22-fold in cotyledons compared with radicles in developing flax seedlings. Moreover, the enzyme becomes progressively more unstable in extracts prepared from 2-, 3- and 4-day seedlings (Figs 1 and 2 and unpublished observations). This may be related to the specific activity profile for isocitrate lyase during germination which shows a peak at 3 days [1].

The present data suggesting that flax isocitrate lyase is degraded by proteinases in crude extracts is in marked contrast to the stability of this enzyme in extracts of watermelon seedlings, even when highly concentrated. This comparison suggests that flax seedling proteinases are especially potent and stresses the variability that exists among fat-storing dicotyledenous tissues from different angiosperms.

EXPERIMENTAL

Materials. Flax seeds (*Linum usitatissimum*), variety Vitagold, harvested near Freising-Weihenstephan were used throughout this research. After surface sterilization with 0.1% $HgCl_2$, seeds were washed × 3 with sterile H_2O and sown in 9-cm Petri dishes (35/dish) containing 0.8% sterile agar. Plates were wrapped in aluminum foil and incubated in the dark at 25°. At designated times they were harvested, the embryo axes (consisting of radicles and hypocotyls) removed (and analysed where indicated) and the cotyledons retained and homogenized at 2° in 9 ml of homogenization buffer within 30 min after storage at 2°. The buffer (pH 7.8, 22°) contained 0.4 M Tris–HCl, 5 mM $MgCl_2$, 1 mM disodium EDTA and 1 mM dithioerythritol. Suspensions were broken in a Virtis 60 K homogenizer at 45 000 rpm in a small Virtis vessel immersed in ice using two 30 sec treatment times. After filtration of the suspension through 2 layers of cheese cloth, it was normally centrifuged for 15 min at 15 000 *g* (2°) in a SS-34 Sorvall rotor. The supernatant was removed by Pasteur pipet to minimize contamination by lipid and stored at 2°.

Watermelon seeds (Stone Mountain variety) were obtained from Vaughan's Seed Co. in Ovid, Michigan and embryos removed, surface sterilized and germinated at 30° [8]. They were then homogenized in the same medium used for flax seeds.

Enzyme assays. All assays were performed with an Eppendorf spectrophotometer at 30°. Isocitrate lyase was assayed by measuring the formation of the phenylhydrazone of glyoxylate at 334 nm [9]. Malate synthase was assayed continuously by measuring the glyoxylate-dependent liberation of 5-thio-2-nitrobenzoate (from the disulphide) at 405 nm as a function of coenzyme A formed from acetyl CoA [10]. $NADP^+$-isocitrate dehydrogenase was assayed spectrophotometrically at 334 nm [1].

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