

Enzyme Profiles in Seedling Development and the Effect of Itaconate, an Isocitrate Lyase-directed Reagent¹

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ABSTRACT

Changes in levels of isocitrate lyase, malate synthase, and catalase have been investigated during germination of flax (*Linum usitatissimum* L.) in the presence and absence of itaconate. Germination was accompanied by a rapid increase in these enzymes during the first 3 days. The presence of 38 millimolar itaconate inhibited the incidence of seed germination and the growth of embryo axes as well as the appearance of isocitrate lyase but did not alter the levels of malate synthase, catalase, or NADP⁺-isocitrate dehydrogenase. The specific activity for the latter enzyme was constant throughout germination. Oxalate or succinate, each at 38 millimolar, had no effect upon germination of flax seeds. Itaconate did not inhibit the activities of malate synthase, catalase, or NADP⁺-isocitrate dehydrogenase *in vitro* but was a potent noncompetitive inhibitor of isocitrate lyase (K_i :17 micromolar at 30 C, pH 7.6). Itaconate (at 38 millimolar) did not alter the appearance of malate synthase but reduced the incidence of germination, onset of germination, and growth of the embryo axis as well as the specific activity of isocitrate lyase in seedlings of *Zea mays*, *Vigna glabra*, *Glycine hispida*, *Vigna sinensis*, *Trigonella foenumgraecum*, *Lens culinaris*, and *Medicago sativa*. The incidence and onset of germination of wheat seeds were unaltered by the same concentration of itaconate but seedlings did not contain isocitrate lyase or malate synthase. The data suggest that itaconate may be isocitrate lyase-directed in inhibiting the germination of fatty seeds.

Isocitrate lyase (EC 4.1.3.1), a key enzyme of the glyoxylate cycle, is present in a variety of microorganisms (13), germinating fatty seeds (3), and a variety of nematodes (*e.g.* 1, 4). Two enzymes, isocitrate lyase and malate synthase (EC 4.1.3.2), are unique to the glyoxylate cycle. Beevers (3) and Barrett *et al.* (1), respectively, have shown that the function of this cycle correlates with the conversion of lipids to carbohydrate during development of seedlings and of the parasitic nematode *Ascaris lumbricoides*.

Isocitrate lyase activity increases severalfold during the first few days of germination of fatty seeds (3). Isocitrate lyase and malate synthase do not preexist in an inactive form in seeds but are synthesized *de novo* during germination (7, 11). In germinating seeds, enzymes of the glyoxylate cycle are contained in specialized peroxisomes termed glyoxysomes which also contain catalase (EC 1.11.1.6) (3).

The inhibition of isocitrate lyase from *Pseudomonas indigofera* by itaconate (or methylenesuccinate) was first reported by Rao and McFadden (18). In subsequent studies from this laboratory, it has been established that itaconate inhibits by interacting with

the succinate-binding moiety of the active site but that as with succinate binding, prior binding of glyoxylate is required (19, 20, 22, 23, 25). McFadden and Purohit (14) have shown the selective inhibition and reduction in levels of isocitrate lyase by itaconate in *P. indigofera* growing on ethanol. It has also been established (18) that itaconate inhibits isocitrate lyase from *Caenorhabditis elegans* and abolishes development of the larvae of this free-living nematode.

We now describe studies of the effect of itaconate on the appearance of isocitrate lyase during flax seed germination as well as the effect of this compound upon flax isocitrate lyase. Effects of itaconate on isocitrate lyase, malate synthase and germination of seeds from several plant species are also described.

MATERIALS AND METHODS

Chemicals. DL-Isocitric acid (trisodium salt), DTT, itaconic acid, α -toluenesulfonyl fluoride, NADP⁺, ADP, BSA, and sodium glyoxylate monohydrate were obtained from Sigma Chemical Co. All other chemicals used were of analytic quality. In all cases acids such as itaconic acid were neutralized before use.

Plant Materials. The following were used: flax (*Linum usitatissimum*), corn (*Zea mays*), soybean (*Glycine hispida*), green gram (*Vigna glabra*), black eyed bean (*Vigna sinensis*), fenugreek (*Trigonella foenumgraecum*), alfalfa (*Medicago sativa*), lentil (*Lens culinaris*), and wheat (*Triticum aestivum* L.).

Germination and Seedling Growth. Flax seeds variety Varansi local from an Indian cultivar was used for detailed studies. Seeds were germinated essentially as described by Khan *et al.* (9). Seeds were surface-sterilized for 5 s in 0.01% (w/v) HgCl₂ solution, washed with sterile distilled H₂O, and transferred to sterile Petri dishes lined with moistened filter paper and incubated at 25 C.

For the treatment with itaconate, seeds were soaked in an appropriate concentration of the inhibitor for 6 h at 4 C, and the seeds then transferred to Petri dishes containing 10 ml of test solution. Respective controls received the same treatment, except that water was used instead of inhibitor solution. The zero time of the germination period in all of these experiments was 6 h after the initiation of seed imbibition. In separate experiments we have shown that no marked change in enzyme activities was observed during the imbibition period at 4 C. For enzyme assays done on seeds incubated in the presence or absence of itaconate, seeds were selected at random prior to germination. Postgermination assays were conducted only on seeds in which the embryo axis had emerged.

Preparation of Extracts. For the preparation of crude enzyme extract, seedlings were harvested and washed with deionized H₂O followed by glass-distilled H₂O. The seedlings were ground in a chilled mortar in buffer (pH 7.6) (25 C) containing 0.05 M Tris-HCl, 1 mM EDTA, 5 mM Mg²⁺, 1 mM DTT, and 1 mM α -toluenesulfonyl fluoride to give a 10% (w/v) homogenate. The homogenate was squeezed through several layers of cheesecloth and centrifuged at 15,000g for 20 min. All of these operations

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were performed at 4 C. The supernatant was carefully separated from the fatty layer at the top of the centrifuge tube by means of a chilled Pasteur pipette and used without further purification.

Enzyme Assays. Isocitrate lyase activity was assayed as described by Roche *et al.* (21). The assay system contained the following reactants in μmol : K-phosphate (pH 7.6), 200; MgCl_2 , 15; DTT, 5; trisodium DL-isocitrate, 24; and water to a final volume of 2.8 ml. At zero time, 0.2 ml of crude extract was added. After 15-min incubation at 30 C, the reaction was stopped by addition of 0.6 ml of 100% trichloroacetic acid. The tubes were allowed to stand for 1 h at 4 C and centrifuged. A suitable aliquot was diluted to 4.0 ml with distilled H_2O followed by the addition of 0.1 ml 5% (w/v) phenylhydrazine hydrochloride. After thorough mixing, the solution was brought to a boil in 1 min, immediately cooled in an ice water bath for 10 min, and 2.0 ml 12 N HCl added with rapid mixing, followed, after 5 min, by the addition of 0.1 ml 25% (w/v) potassium ferricyanide. After thorough mixing and standing for 15 min, the color intensity was read at 520 nm.

Malate synthase activity was determined at 25 C by the glyoxylate-dependent formation of CoA from acetyl-CoA, measured at 412 nm using 5,5-thiobis-2-nitrobenzoic acid as a color reagent (4). Catalase was assayed at 25 C by the disappearance of H_2O_2 measured at 240 nm (2). NADP⁺-linked isocitrate dehydrogenase activity was measured spectrophotometrically at 25 C at 340 nm (6). An NAD⁺-linked isocitrate dehydrogenase activity could not be detected.

In all cases, 1 unit of enzyme is defined as that amount which either catalyzed the utilization of 1 μmol of substrate or the substrate-dependent formation of 1 μmol of product per min. Specific activity is given by units per mg protein.

Protein Determinations. To a suitable aliquot of the homogenate, 3 ml of 20% (w/v) trichloroacetic acid was added. The precipitate was centrifuged, and washed successively three times with 5 ml each of 0.1 M potassium acetate in alcohol, alcohol, and ether. The washed precipitate was dissolved in 0.1 N NaOH at 60 C. Protein concentrations were determined by the method of Lowry *et al.* (12) using BSA as a standard.

RESULTS

The development of isocitrate lyase activity during the germination of flax seeds is shown in Figure 1. As established earlier by Khan (8), isocitrate lyase was undetectable in ungerminated flax

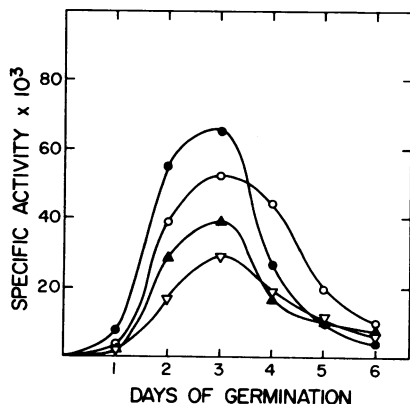


FIG. 1. Effect of different concentrations of itaconate on changes in flax seedling isocitrate lyase activity during germination at 25 C. The seeds were soaked in appropriate concentrations of itaconate (or water) for 6 h at 4 C and then transferred to Petri dishes lined with filter paper containing 10 ml of different test solutions. The Petri dishes were incubated at 25 C for germination. Appropriate aged seedlings were homogenized and assayed for isocitrate lyase activity as described under "Materials and Methods." Control (●); itaconate, 7.5 mM (○); 19 mM (▲); and 38 mM (▽).

Table I. Effect of Salts of Dicarboxylic Acids (at 38 mM) on Growth of Flax Seedlings

Flax seeds were soaked in 38 mM test compound for 6 h at 4 C then transferred to petri dishes containing 10 ml of the same solution for germination at 25 C. Length of the embryo axis was measured at 24-h intervals. Data for itaconate reflect averages of three independent experiments. In each case, 100 seeds were examined.

Day	Length of Embryo Axis (cm)			
	Control	Itaconate ¹	Succinate	Oxalate
1	NM ²		NM	NM
2	0.5	0.2	0.5	0.4
3	2.0	0.5	2.1	2.0
4	4.0	1.0 (necrosis starts)	3.8	3.8
5	9.0	1.2	9.0	9.1
6	10.5	2.0	10.1	10.3
7	13.0	2.0	12.8	12.6

¹ This concentration of itaconate reduced the incidence of germination by 75 to 80% at 3 days so that axis elongation was measured in 20 to 25% of the seeds which germinated. However, the incidence of germination in presence of succinate and oxalate was normal.

² NM = not measurable.

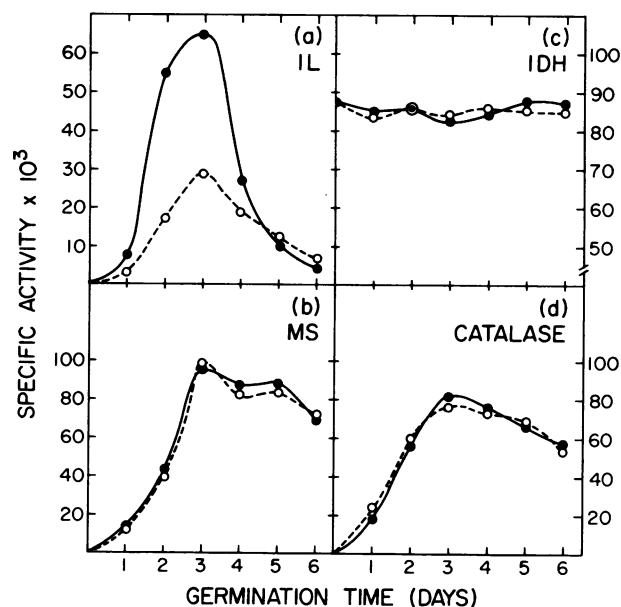


FIG. 2. Effect of itaconate on changes in: (a) isocitrate lyase; (b) malate synthase; (c) NADP⁺-isocitrate dehydrogenase; and (d) catalase activities during germination of flax seeds. Seeds were germinated in the presence or absence of 38 mM itaconate as described in Figure 1. Control profiles and those for 38 mM itaconate are specified by (●—●) and (○—○), respectively.

seeds. The enzyme activity increased rapidly during germination, reached a maximum after 3 days, and slowly declined thereafter. Itaconate at 7.5, 19, or 38 mM reduced the rate of appearance of isocitrate lyase activity during germination. The latter concentration was markedly effective and was used throughout the study, unless otherwise indicated. Gel filtration of extracts on Sephadex G-25 had no effect upon the specific activity of isocitrate lyase, establishing that the reduced level of this enzyme could not be attributed to carryover of itaconate into the assay medium. Table I contrasts the effect of itaconate on growth of the embryo axis of flax seedlings with that of succinate or oxalate, salts of two other dicarboxylic acids known to inhibit the bacterial enzyme but less markedly than itaconate (25). When the seeds were soaked and incubated in the presence of itaconate, the germination incidence and seedling growth rate were markedly arrested whereas neither phenomenon was affected by succinate or oxalate.

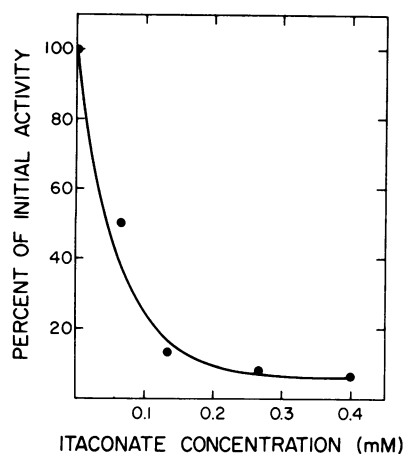


FIG. 3. Inhibition of isocitrate lyase from flax seedlings by itaconate. Three-day-old seedlings were used for enzyme preparation.

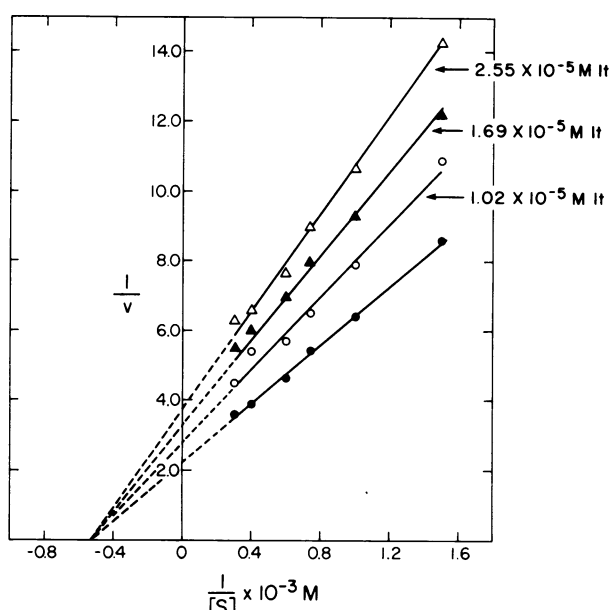


FIG. 4. Double reciprocal plots showing effect of itaconate (It) on isocitrate lyase activity. Seedlings (72-h) were homogenized and used as an enzyme source. Assay was done in duplicate throughout at each substrate concentration.

Changes in the activities of malate synthase, catalase, and NADP⁺-isocitrate dehydrogenase at different stages of germination of flax seeds are compared with those for isocitrate lyase (Fig. 2). Malate synthase and catalase were both absent in ungerminated flax seeds but increased rapidly during germination and peaked at day 3 after germination. The decline in both malate synthase and catalase activities is slower than for isocitrate lyase. There was no change in NADP⁺-isocitrate dehydrogenase activity with germination and it was present even in ungerminated flax seeds (Fig. 2c). There was no effect of itaconate on the profile for malate synthase (Fig. 2b), catalase (Fig. 2d), or isocitrate dehydrogenase activity during germination (Fig. 2c). Neither oxalate nor succinate at 38 mM had any effect upon the appearance and disappearance of isocitrate lyase during germination.

Itaconate strongly inhibited isocitrate lyase at a saturating concentration of D₃-isocitrate, the substrate (Fig. 3). About 87% inhibition was obtained at 0.13 mM itaconate. Malate synthase, catalase, and isocitrate dehydrogenase activities in extracts were not inhibited by the same concentration of itaconate.

Kinetic analysis of isocitrate lyase from flax seedlings showed

that itaconate was a noncompetitive inhibitor (Fig. 4). The inhibition constant (K_i) was 17 μ M.

In Table II, the effect of 38 mM itaconate on appearance of isocitrate lyase and malate synthase activities during germination of various other seeds is shown. The presence of itaconate in the germination medium reduced isocitrate lyase activity but had no effect on malate synthase appearance in all seeds except wheat, in which these enzymes could not be detected. The data also indicate that the effect of itaconate is reversible in some cases at least, because after 72 h of germination of fenugreek or alfalfa the specific activity of isocitrate lyase was higher than after 36 h. In Table III, it is established that 38 mM itaconate delayed the onset of germination and reduced the incidence of germination and seedling growth within the same array of seeds in which the level of isocitrate lyase was reduced. In contrast, 7.5 mM itaconate had little or no effect upon germination and seedling growth (Table III) and 38 mM oxalate or succinate had no effect upon germination (data not shown).

DISCUSSION

In previous research on isocitrate lyase from *P. indigofera*, numerous compounds were surveyed for inhibitory activity including a variety of analogs of glyoxylate and succinate. In that study itaconate was the strongest inhibitor examined (18). Itaconate at lower concentrations (up to 25 μ M) was an uncompetitive inhibitor but became noncompetitive with respect to isocitrate at 50 μ M (18, 25), probably as a consequence of the reaction mechanism (25). The apparent K_i was 7 μ M at pH 7.7 at 30 C (25). Under corresponding conditions the K_i values for two nematode enzymes, both of which were inhibited noncompetitively, were 7 and 19 μ M (17). The flax enzyme is similarly inhibited ($K_i = 17 \mu$ M at pH 7.6, 30 C).

In the present research the changes in levels of isocitrate lyase, malate synthase, catalase, and NADP⁺-isocitrate dehydrogenase have been studied in dark-grown flax seedlings. At a concentration of 38 mM in the germination medium itaconate markedly reduces the incidence of germination and elongation rate of the embryo axis as well as the appearance of isocitrate lyase activity in those seeds which germinate (Fig. 1 and Table I) but has no effect upon the levels of malate synthase, catalase, and NADP⁺-isocitrate dehydrogenase. The latter observations serve as an internal control and establish that itaconate does not exert its effect by generally reducing the level of active enzymes. Similar effects of itaconate

Table II. Effect of Itaconate on Appearance of Isocitrate Lyase and Malate Synthase during Germination of Various Seeds

Seeds were treated and germinated in a similar manner as in Table I. The 36- and 72-h-old seedlings were homogenized and enzyme activities were assayed as described under "Materials and Methods."

Seeds	% Reduction ¹ by 38 mM Itaconate	
	Isocitrate Lyase	
	36 h	72 h ²
Corn		71
Green gram		75
Soybeans		47
Black-eyed beans		58
Fenugreek	53	36
Alfalfa	72	39
Lentil		100
Wheat	Activity not detected	

¹ Reduction in specific activity.

² Specific activity of malate synthase was unaffected in seedlings of corn, green gram, soybeans, black-eyed beans, fenugreek, and alfalfa after 72-h germination in the presence of 38 mM itaconate. Malate synthase could not be detected after 36- or 72-h germination of wheat.

Table III. Effect of 38 mM Itaconate on Germination and Growth of Various Seeds

Seeds ¹	Time after Imbibition					
	24 h		48 h		72 h	
	% of Control Germination ¹	% of Control Embryo Axis ²	% of Control Germination	% of Control Embryo Axis ²	% of Control Germination	% of Control Embryo Axis ²
Corn	NM ³	NM	31 (69)	0 (100)	31 (69)	27 (100)
Soybean	NM	NM	40 (74)	0 (100)	40 (74)	40 (100)
Black-eyed bean	NM	NM	50 (70)	0 (100)	50 (70)	45 (91)
Fenugreek	0 (80) ⁴	0 (40)	60 (80)	40 (80)	60 (80)	30 (103)
Green gram	40 (100)	33 (100)	80 (100)	20 (100)	80 (100)	35 (94)
Alfalfa	0 (70)	NM	15 (100)	0 (100)	15 (100)	13 (100)
Lentil	0 (100)	NM	0 (100)	0 (100)	0 (100)	0 (106)
Wheat	100 (100)	NM	100 (100)	0 (100)	100 (100)	50 (109)

¹ Fifty seeds were used in each case except for alfalfa and fenugreek in which case 100 seeds were observed.

² Percent of control embryo axis length represents observations made on at least 15 seeds in each group. Lengths of control axes in cm for the three germination times of 24, 48 and 72 h were for: corn, NM, 0.4, 1.5; soybean, NM, 0.2, 1.0; black-eyed bean, NM, 0.2, 1.1; fenugreek, 0.5, 1.5, 3.0; green gram, 0.6, 2.5, 3.4; alfalfa, NM, 0.8, 1.5; lentil, NM, 1.0, 1.7; and wheat, NM, 0.2, 1.2.

³ Not measurable.

⁴ Parenthetical values show the effect of 7.5 mM itaconate.

upon isocitrate lyase and malate synthase are obtained with all other germinating plant seeds tested except wheat (Table II). In all but the latter the incidence of germination, onset of germination and rate of elongation of the embryo axis are arrested by incubation of the seeds in the presence of 38 mM itaconate. In contrast 38 mM itaconate has no effect upon the incidence of germination and relatively little effect upon growth of wheat seedlings (Table III) in which we could not detect isocitrate lyase or malate synthase. The data suggest that catalysis by isocitrate lyase is correlated with seedling development which correlates in turn with function of the glyoxylate cycle (3).

In related studies in our laboratory, itaconate at a similarly high concentration (20 mM) inhibited growth of *P. indigofera* on ethanol but not on glucose. Of nine enzymes (including isocitrate lyase and malate synthase), which function uniquely during growth on ethanol, itaconate inhibited and reduced the level of isocitrate lyase only (14). The function of isocitrate lyase in nematode development has been implied (1, 16, 17). In recent research on the nematode *C. elegans* it has been found that 15 mM itaconate almost completely abolishes axenic growth and is much more toxic than oxalate, maleate and succinate (17).

Considering the results with bacteria and nematodes as well as the present data with various seedlings, it is possible that itaconate is indeed an isocitrate lyase-directed reagent but that it is only effective at relatively high concentrations in the growth medium. Of special significance is the possibility that itaconate regulates both the levels and activity of isocitrate lyase activity. Such a possibility is not without precedence. For example in *Salmonella typhimurium*, charged histidyl t-RNA both inhibits and represses the formation of ATP phosphoribosyl transferase, the enzyme at the branch point in the pathway leading to histidine (10). The apparent discrepancy between the concentration dependence of the effect of itaconate *in vivo* and *in vitro* could be the result of transport or of metabolic modification to less inhibitory forms. In *P. indigofera*, itaconate is apparently not metabolized (14). On the other hand in some pseudomonads (5, 15) and in animal tissues (24), itaconate is catabolized to pyruvate and acetyl-CoA via citramalyl-CoA. It is perhaps significant that in the present research the marked diminution in isocitrate lyase activity observed after 36 h of germination of fenugreek and alfalfa in the presence of itaconate had been partially reversed by 72 h.

In summary, itaconate appears to be an isocitrate lyase-directed reagent in terms of its effect upon seed germination but further research will be required to elucidate its exact mode of action.

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