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Metabolism of *trans*-Aconitic Acid in Maize[1]

I. PURIFICATION OF TWO MOLECULAR FORMS OF CITRATE DEHYDRASE

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ABSTRACT

***Trans*-aconitate synthesis via citrate dehydrase was determined in crude extracts of maize (*Zea mays* L.) coleoptiles. Two molecular forms of this enzyme were purified by substrate-specific elution from DEAE-cellulose, ammonium sulfate precipitation, and gel filtration. Each molecular form migrates as a single band in isoelectric focusing. Gel filtration and sodium dodecyl sulfate electrophoresis provided evidence that one enzyme form is composed of four 80,000-dalton subunits while the other is composed of two 60,000-dalton subunits. There was no evidence of proteolytic conversion of the large to the small molecular weight form when the former was incubated with either the 15,000*g* supernatant or with proteases. The data indicate that the two molecular forms of citrate dehydrase are isozymes.**

Trans-Ac[2] is the predominate organic acid in grasses, often present in excess of 1% of the dry matter (5, 6, 17, 18). Interest in this acid stems from its suspected role in grass tetany (3, 5, 9, 11). Burau and Stout (5) postulated that *trans*-Ac was a causal agent since maximum concentration in range grasses coincided with increased incidence of tetany. Bohman *et al.* (3) induced tetany in beef animals by feeding *trans*-Ac. The severity and frequency of the disorder was increased by supplementing the diet with KCl. Kennedy (9) found that *trans*-Ac caused a marked reduction in plasma Mg in sheep. This reduction was associated with a temporary increase in urinary Mg. Since replenishment of plasma Mg from either soft tissue or bones occurs slowly, severe depletion of plasma Mg usually leads to tetany (9). Bohman *et al.* (3) concluded that a feed containing greater than 1% *trans*-Ac (dry matter basis) is potentially toxic to ruminants. Maize herbage, as well as many forage and range grasses, may exceed this level (5, 17).

Trans-Ac metabolism in higher plants has been investigated by MacLennan *et al.* (14), MacLennan and Beevers (13), and Altekar *et al.* (1). The substrate for *trans*-Ac was not identified by these studies. In *Pseudomonas* species, *trans*-Ac is synthesized from *cis*-Ac via aconitate isomerase (Fig. 1). Rao and Altekar (16) partially purified this enzyme from fluorescent *Pseudomonas* species. Klinman and Rose (10) achieved a 45-fold purification from *Pseudomonas putida.*

Clark (6) suggests that *trans*-Ac production in maize is via a K-dependent enzyme. Purification of two molecular forms of K-dependent citrate dehydrase (Fig. 1) which utilize citrate as substrate for *trans*-Ac synthesis is reported herein. This is believed to be the first report of the existence of citrate dehydrase.

MATERIALS AND METHODS

Enzyme Activity in 15,000*g* Supernatant. *Trans*-Ac production in the crude extract was determined with reaction times of 10, 30, and 120 min using either 5 mM citrate or 5 mM *cis*-Ac with and without 100 mM KCl as substrate. *Trans*-Ac produced was fractionated by ion exchange chromatography and measured by GLC (2). Evidence for the reverse reaction was found using 5 mM *trans*-Ac as substrate.

Assay for Citrate Dehydrase Activity. The enzyme was K-activated by incubating 50 μl enzyme for 30 min at room temperature in 1.0 ml KCl buffer (100 mM KCl, 0.1 mM EDTA, and 50 mM Tris-HCl [pH 7.2]). *Trans*-Ac synthesis was then initiated by addition of 0.95 ml KCl buffer containing 20 mM citrate. The amount of *trans*-Ac synthesized in 30 min was determined by the increase in A_{240} (15).

Electrophoresis. Purity of enzyme preparations was established by isoelectric focusing in 5% polyacrylamide gels, pH gradient 3 to 10 (3). Molecular weights of the subunits of CD I and CD II were measured by SDS-polyacrylamide electrophoresis using BSA, chymotrypsin, papain, and pepsin as standards (19).

Protein Determination. The protein concentration in extracts was estimated by the method of Lowry *et al.* (12) using BSA as a standard.

Purification of Citrate Dehydrase. All procedures were conducted at 0 to 4°C.

Step 1. Preparation of 15,000g Supernatant. Corn kernels (cv DeKalb XL-64) were germinated in darkness, 20°C, in seed trays moistened with distilled H_2O. Six days after planting, 300 g coleoptile tissue were ground in a mortar with 200 ml extracting buffer (50 mM Tris-HCl [pH 7.2], 50 mM KCl, and 0.1 mM EDTA), strained through cheesecloth, and centrifuged at 15,000*g* for 20 min.

Step 2. Elution from DEAE-Cellulose with K-Citrate. DEAE-cellulose was prepared by suspending 10 g in 100 ml distilled H_2O and pelleting by centrifugation at 5,000*g* for 15 min. Two hundred ml 15,000*g* supernatant were added to the prepared cellulose and stirred for 30 min. The protein-saturated cellulose was then pelleted by centrifugation, resuspended in 100 ml extracting buffer, and again centrifuged. The final pellet was resuspended for 5 min in 100 ml of substrate-specific eluate (25 ml Tris-HCl [pH 7.2], 0.1 mM EDTA, and 10 mM K-citrate). The enzyme was conveniently recovered in the supernatant after centrifugation.

Step 3. Ammonium Sulfate Precipitation. Proteins in the supernatant of Step 2, precipitating within the range of 60 to 80% saturated ammonium sulfate, were pelleted by centrifugation at 33,000*g* for 30 min. The pellet was suspended in 2.0 ml of 50 mM Tris-HCl (pH 7.2) and 0.1 mM EDTA.

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[2] Abbreviations: *trans*-Ac, *trans*-aconitic acid; *cis*-Ac, *cis*-aconitic acid; CD I, large mol wt form of citrate dehydrase; CD II, small mol wt form of citrate dehydrase.

Table I. *Purification of Citrate Dehydrase*

Purification Step	Volume	Activity	Protein	Specific Activity	Purification	Recovery
	ml	*μmol/min*	*mg*	*μmol/min·mg protein*	*-fold*	*%*
1. 15,000*g* supernatant	200	0.467	10,800	4.3×10^{-5}	1	100
2. 10 mM K-citrate eluate	100	0.388	4.3	0.092	2,120	83
3. 60–80% saturated $(NH_4)_2SO_4$	2	0.373	2.1	0.177	4,090	80
4. Gel filtration						
a. CD I (fractions 48–51)	4	0.075	0.2	0.373	8,640	16
b. CD II (fractions 60–64)	5	0.223	1.4	0.136	3,160	48

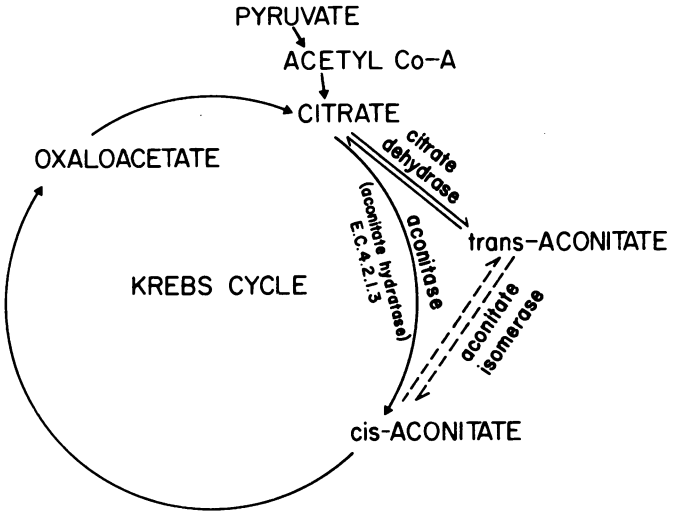


FIG. 1. Model for *trans*-aconitate metabolism.

Step 4. Gel Filtration. The suspended proteins from Step 3 were applied to a Bio-Gel A-5m column (2 × 30 cm) previously equilibrated with the same buffer, flow rate 4 ml/h. Serial 1-ml fractions were collected and assayed for enzyme activity. Specific activity is expressed as μmol of *trans*-Ac/min·mg protein. The Bio-Gel A column was calibrated for mol wt determinations with the following compounds: Blue Dextran 2000, urease, BSA dimer, BSA monomer, pepsin, and tyrosine.

RESULTS AND DISCUSSION

Enzyme Activity in 15,000*g* Supernatant. *Trans*-Ac production from citrate by enzymes in the crude extract was not observed in the absence of KCl. With KCl, introconversion of *trans*-Ac and citrate occurred within 30 min without measurable *cis*-Ac production (Fig. 2, A and B). With *cis*-Ac as substrate, *trans*-Ac production was not observed within the first 30 min (Fig. 2C). The appearance of *trans*-Ac thereafter is attributed to the presence of citrate produced from *cis*-Ac via aconitase (Fig. 1). These results indicate that citrate is the substrate for *trans*-Ac synthesis in maize.

Purification of Citrate Dehydrase. Purification steps are summarized in Table I. Citrate dehydrase bound to DEAE-cellulose at 50 mM KCl, but it was possible to elute the enzyme using its specific substrate, K-citrate (Step 2). Specific activity was increased from 4.3×10^{-5} to 0.092. With isoelectric focusing, only three protein bands were detected in the eluate. Further purification via ammonium sulfate precipitation removed one of the bands, increasing the specific activity to 0.177 (Table I, Step 3).

Gel filtration of the proteins in the ammonium sulfate precipitate revealed two molecular forms of citrate dehydrase (Fig. 3). Fractions 48 to 51 are designated CD I while fractions 60 to 64 are designated CD II. Each protein appeared as a single band with isoelectric focusing (4).

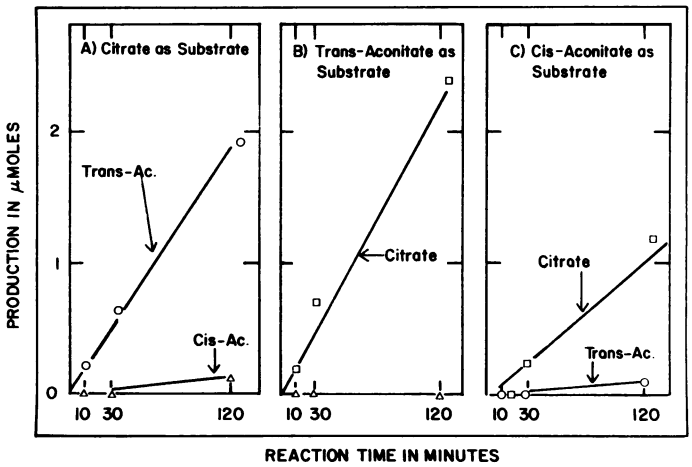


FIG. 2. Interconversion of citrate, *trans*-Ac, and *cis*-Ac by the crude extract as influenced by substrate. Production of *trans*-Ac, citrate, and *cis*-Ac was determined by GLC using 5 mM citrate (A), *trans*-Ac (B), or *cis*-Ac (C) as substrate at pH 7.2 with 100 mM KCl.

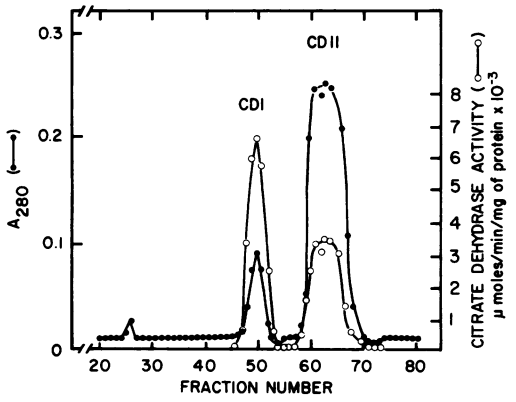
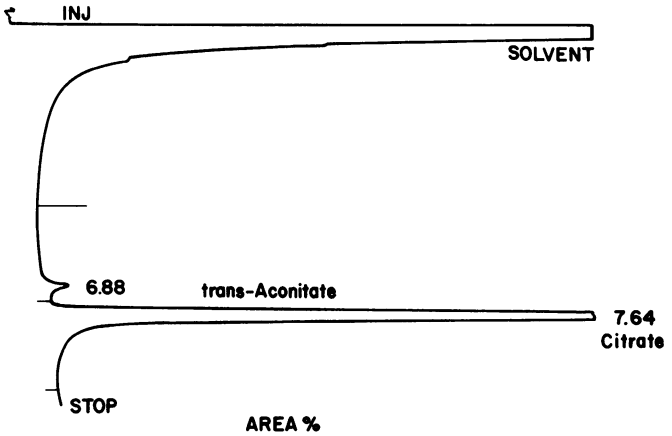


FIG. 3. Elution profile with Bio-Gel A-5m column of CD I and CD II. Proteins fractionated by K-citrate elution and ammonium sulfate precipitation were eluted with 50 mM Tris-HCl (pH 7.2) and 0.1 mM EDTA, flow rate 4 ml/h.

Substrate Specificity. With citrate as substrate, *trans*-Ac was the only acid produced in 24 h as determined by GLC (Fig. 4). Conversely, citrate was the only acid produced when *trans*-Ac was the substrate. Interconversion between the two acids occurred only in the presence of K, and neither *cis*-Ac nor isocitrate were utilized as substrate.

Molecular Weights and Subunit Composition. Gel filtration indicated that CD I and CD II had mol wt of 316,000 ± 11,000 and 124,000 ± 8,000 daltons, respectively (Fig. 5). With SDS electrophoresis, each molecular form migrated as a single band with mol wt of subunits being 81,000 ± 5,000 and 59,000 ± 3,000 daltons, respectively. These differences discount the possibility



AREA %

RT	TYPE	AREA	
6.88	T	11634	3.598
7.64	TM	311730	96.4

FIG. 4. GLC tracing of organic acids produced from citrate.

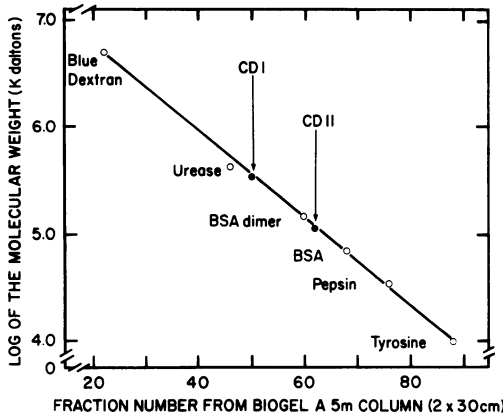


FIG. 5. Mol wt of CD I and CD II as determined by gel filtration.

that CD I is an aggregate of CD II (7).

Evidence against Proteolytic Conversion of CD I to CD II. To assess the possibility that a protease in the 15,000*g* supernatant converts CD I to CD II, 1.0 ml supernatant was incubated 24 h with and without 50 μg purified CD I. After incubation, the proteins in each treatment were separated by gel filtration. Citrate dehydrase activity in fractions corresponding to CD II did not increase, indicating that CD I was not converted to CD II. Similarly, limited proteolytic digestion by either chymotrypsin or papain failed to show evidence of such conversion.

The most reliable criterion for establishing that different molecular forms of an enzyme are isozymes is to demonstrate that they are coded on separate genomes. Such information is not yet at hand for citrate dehydrase. However, evidence that CD I and CD II are isozymes is provided by the following: (a) similar substrate specificity; (b) differences in molecular weights of subunits; (c) no proteolytic conversion; (d) different kinetics; and (e) intracellular compartmentation. Items d and e are the subject of a separate paper in which we attempt to explain the correlation between leaf K and *trans*-Ac accumulation in maize (6).

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