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ISOLATION AND PROPERTIES OF WATERMELON ISOCITRATE LYASE

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Key Word Index—*Citrullus vulgaris*; Cucurbitaceae; glyoxylate cycle; watermelon isocitrate lyase; seed germination.

Abstract—The glyoxylate cycle enzyme, isocitrate lyase (EC 4.1.3.1) was purified from cotyledons of *Citrullus vulgaris* (watermelon). The final preparation, which had been 97-fold purified with a specific activity of 16.1 units/mg protein in a yield of 36%, was homogeneous by gel- and immunoelectrophoretic criteria. The tetrameric enzyme had: a molecular weight of 277 000, a sedimentation coefficient of 12.4 s, and a K_m for D_s-isocitrate equal to 0.25 mM. Isocitrate lyase from this source is not a glycoprotein as shown by total carbohydrate content after precipitation by trichloroacetic acid of the purified enzyme. Reduction of the enzyme with thiols increased activity and maximal activity was obtained with at least 5 mM dithiothreitol. EDTA partially substituted for thiol in freshly isolated enzyme. Watermelon isocitrate lyase was also protected against thermal denaturation at 60° for at least 1 hr by 5 mM Mg^{2+} plus 5 mM oxalate. Oxalate was a competitive inhibitor with respect to isocitrate (K_i: 1.5 μM, pH 7.5, 30°).

INTRODUCTION

Isocitrate lyase (EC 4.1.3.1) is an important regulatory enzyme in the anaplerotic glyoxylate cycle, which is present in a number of organisms ranging from bacteria to fat-rich seedlings and certain classes of nematodes [1]. The cycle in fat rich seedlings and nematodes has been shown to be essential for the conversion of fat stores into carbohydrates during germination or embryogenesis. Isocitrate lyases have been highly purified from the seedlings of cucumber [2], flax [3] and castor bean [4].

In order to study the catalytic and regulatory functions of this enzyme from higher plants, we originally used the flax enzyme [3, 5]; however, this enzyme is relatively unstable. Furthermore, aspects of the reported fractionation have proven to be variable and the purified enzyme has varied in specific activity from 2 μmol glyoxylate formed/min (units)/mg protein to 20 units/mg protein after identical enzyme purifications and from seeds identically germinated. These problems prompted us to study the enzyme from other seedlings such as that from watermelon (*Citrullus vulgaris*). These seeds have an advantage over those from flax in that they (1) have much larger cotyledons which are therefore easier to handle, (2) provide a significantly higher frequency of germination, and (3) afford a richer base of information with respect to glyoxysome biogenesis. Glyoxysomes have proven to be difficult to recover from flax seedlings because the seed coats are covered with mucilage.

Although isocitrate lyase from watermelon cotyledons has been purified earlier [6], we now describe an improved purification which yields a gel-electrophoretically homogeneous, stable enzyme that has an approximately ten-fold higher specific activity. We also report for the first time a number of molecular and catalytic properties of watermelon isocitrate lyase.

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RESULTS

Isocitrate lyase purification

The purification of watermelon isocitrate lyase is summarized in Table 1. Enzyme was obtained after 97-fold purification in a yield of 36%. Over several purifications, the specific activity of the final preparation varied between 13 and 16 units/mg of protein. The final preparation (Table 1) was completely stable to storage at −120° for at least one month. The specific activities for our crude extract and final preparation were approximately ten-fold higher than those obtained earlier [6] for enzyme from the same source, recalculated in terms of our units. The lower value obtained in earlier work may have reflected proteolysis as proteinase inhibitors were not employed in that purification.

When the crude extract was prepared in the presence of 0.2 mM α-tolylsulfonyl fluoride (an inactivator of serine proteinases) in the isolation buffer, only 50% of the starting units of enzyme activity were recovered after ammonium sulfate fractionation (data not shown). In contrast, the use of this reagent at 1 mM in the isolation buffer afforded quantitative recovery of enzyme units as shown in Table 1.

Isocitrate lyase did not bind to DEAE-cellulose at pH 7.5 in Tris buffer. This is contrary to observations for the enzyme from other higher plants, i.e. flax seedlings [3] and cucumber cotyledons [2]. In our case, all the activity eluted during the buffer wash.

The final preparation sedimented as a single symmetrical peak with an $S_{20,w}$ value of 12.4×10^{-13} sec^{-1} (protein concentration: 0.14%), had a K_m for D_s-isocitrate of 0.25 mM and a turnover number of 18.5 moles sec^{-1} · mole of active $site^{-1}$.

Criteria of purity

The purity of isocitrate lyase was established by SDS-

Table 1. Purification of isocitrate lyase

Isolation step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield	Purification
Crude extract	1488	248	0.17	(100)	(1)
26–41 % $(NH_4)_2SO_4$ precipitate	144	245	1.7	99	10
Sepharose 6B (concentrated)	33	155	4.6	63	28
DEAE-Cellulose buffer wash (concentrated)	5.6	90	16.1	36	97

polyacrylamide gel electrophoresis (compare lanes 2, 3, 4 and 5, Fig. 1) as well as immunologically. Antibody raised against purified isocitrate lyase showed only a single precipitin line against the crude extract from *Citrullus vulgaris* and this revealed immunological identity with that of purified isocitrate lyase. This provided evidence for the absence of other antigenically significant proteins in the final preparation. The enzymes from *Citrullus vulgaris* (watermelon) and *Linum usitatissimum* (flax) showed antigenic cross-reactivity but nonidentity as seen from the precipitin formed after double diffusion against antibody to the watermelon enzyme and the spur at the junction of precipitins for wells 2 and 3 (Fig. 2). The appearance of two precipitin lines corresponding to well A4 may have been the result of degradation of the enzyme as the preparation was old and had lost all enzyme activity. The purified isocitrate lyase preparation from watermelon also showed a single precipitin line on immunoelectrophoresis using the antiserum prepared in rabbits and control sera showed no reaction (not shown).

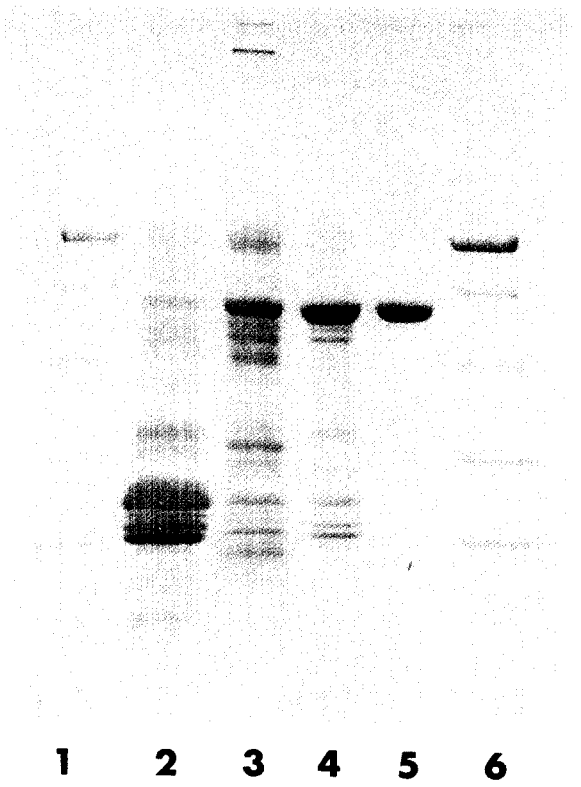

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis and Coomassie-blue staining reflecting different steps of purification (lanes 2–5) and protein standards (lanes 1 and 6). Fifteen μl each of: 2-fold diluted crude extract (lane 2), the 26–41 % $(NH_4)_2SO_4$ precipitate (lane 3), the Sepharose 6B peak fraction (lane 4) and the DEAE-cellulose peak fraction (lane 5) were subjected to electrophoresis on a 4–20 % gradient polyacrylamide gel in the presence of SDS until the marker dye ran out. The protein standards (and M_r values) from Bio-Rad Laboratories, Richmond, CA, USA were: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Molecular weight

After chromatography of *ca* 1200 μg of purified isocitrate lyase on Sephacryl 300, the molecular weight of the watermelon enzyme was 277 000 (Fig. 3a). The subunit molecular weight was 64 000 by linear regression analysis (Fig. 3b). We infer that the enzyme is a tetramer as seen for isocitrate lyases from all the sources purified to date.

Amino acid analysis

The amino acid composition of isocitrate lyase from *Citrullus vulgaris* is shown in Table 2. The $S\Delta n$ values for a comparison with the enzyme from *Neurospora crassa* [7] have been calculated on the basis of all residues as described by Cornish-Bowden [8]. The value of 7397 suggests that there are no apparent sequence homologies between the higher plant and fungal enzyme. However, the latter contains *ca* 16 residues more per subunit than the watermelon enzyme.

The $S\Delta n$ analysis is more effective in correlating amino acid compositions with sequence homologies when test proteins are of closely similar chain length. When the subunits are of appreciably different size as in the case of isocitrate lyase from *Citrullus vulgaris* and *Pseudomonas indigofera* (M_r: 63 000 and 51 000, respectively), the $S\Delta Q$ analysis [9] is more valid. The parameter, $S\Delta Q$, equals $S(X_{i,j} - X_{k,j})^2$ where X_j is the mole percent of a given amino acid (excluding half-cystine and tryptophan), and the subscripts i and k identify the two proteins that are being compared. According to Marchelonis and Weltman, $S\Delta Q$ was greater than 100 for unrelated proteins and was less than 50 for related proteins. The values for the comparison of isocitrate lyase from *Citrullus vulgaris* with the enzyme from *N. crassa* or *P. indogofera* are 24 and 31, respectively. Thus, this analysis suggests that sequence homologies may exist between the watermelon, fungal and pseudomonad enzymes.

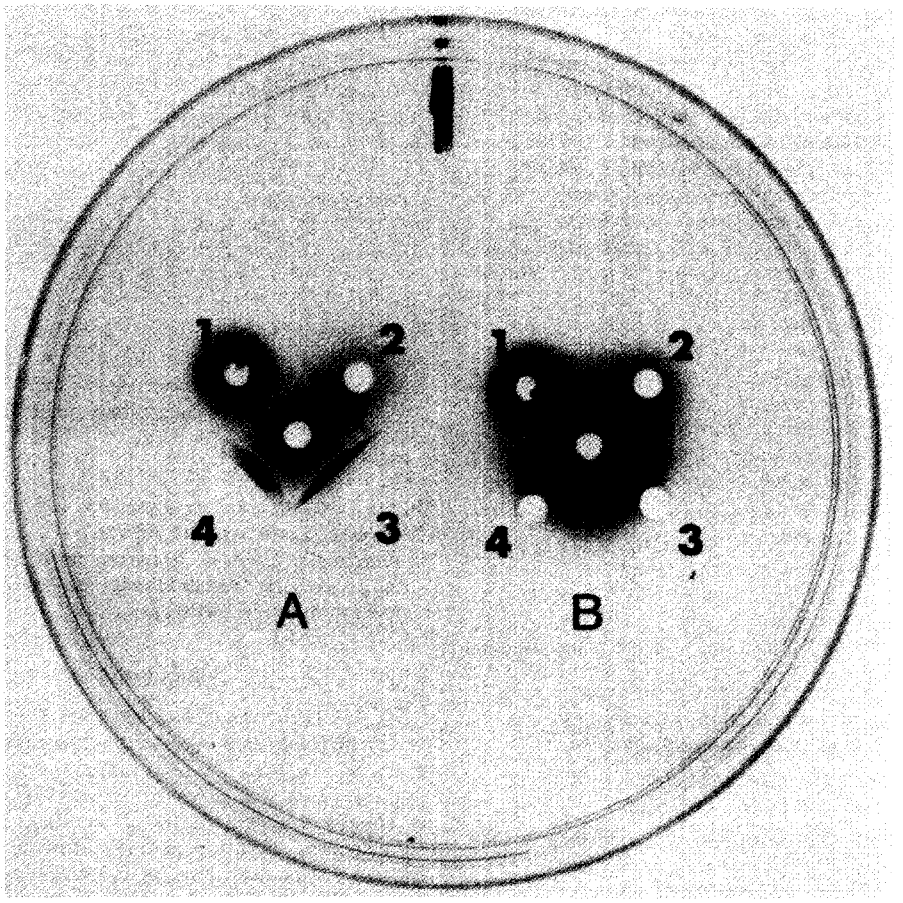

Fig. 2. Ouchterlony double diffusion of *C. vulgaris* crude extract (1), *L. usitatissimum* (flax) crude extract (2) and purified *C. vulgaris* isocitrate lyase (3,4) against antibody to purified *C. vulgaris* isocitrate lyase (A) or pre-immune serum (B). Precipitin lines were developed at 4°. A single precipitin line was visible in A1, but disappeared during the 0.15 M sodium chloride wash for staining.

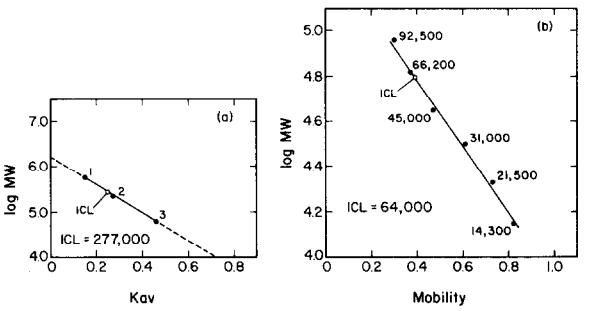

Fig. 3. (a) Molecular weight of *C. vulgaris* isocitrate lyase estimated on a Sephacryl 300 column (1.5 × 56.5 cm). Standards (and MWs) were: 1, spinach RuBP carboxylase/oxygenase (540000); 2, catalase (250000) and 3, hemoglobin (68000). (b) The mobilities of standards (○) and *C. vulgaris* isocitrate lyase (○) after SDS-PAGE in a gradient gel formed from 4–20% acrylamide (Fig. 2c, lanes 5 and 6). Standards are from top to bottom in order of decreasing MW.

Carbohydrate content

The results of carbohydrate content analysis on purified watermelon isocitrate lyase as well as control proteins are shown in Table 3. The loss of carbohydrate from isocitrate lyase upon 30-minute precipitation by cold trichloroacetic acid suggests that the enzyme had non-covalently bound carbohydrate which was not removed by extensive dialysis. A comparison with glycoproteins serves to emphasize this point and to make the precipitation a valid methodology. It should also be noted that the apparent carbohydrate content of dialysed isocitrate lyase was variable between different batches of purified enzyme.

Thiol requirement

Freshly isolated watermelon enzyme required a thiol compound for maximum activity. Either 2-mercaptoethanol or dithiothreitol met this requirement and 5 mM dithiothreitol supported maximal activity. With freshly isolated enzyme, EDTA could partially substitute for 2-mercaptoethanol as for other isocitrate lyases [3, 10].

Thermostability with oxalate

As with the enzyme from other sources [1], oxalate was a linear competitive inhibitor of the watermelon enzyme with a K_i of 1.5 micromolar (data not shown). Isocitrate lyase from *C. vulgaris* was exposed for varying times to

Table 2. Amino acid composition of watermelon isocitrate lyase

Amino acid	Extrapolated or maximum value (nmol)	Nearest integral number of amino acids per 277 000-D molecule of protein*
Asp	58.5	228
Thr	46.1	180
Ser	45.0	176
Glu	73.4	287
Pro	20.5	80
Gly	44.6	174
Ala	68.3	267
Cys	9.5	37
Val	33.0	129
Met	12.7	50
Ile	30.4	119
Leu	49.9	195
Tyr	18.6	73
Phe	27.1	106
Lys	37.0	144
His	16.8	66
Arg	42.1	164
Trp	—	32

*Values corrected from 95% recovery based on norleucine used as an internal standard.

Table 3. Carbohydrate content of watermelon isocitrate lyase and established glycoproteins

Protein		% carbohydrate: After dialysis	% carbohydrate: After TCA precipitation
Isocitrate lyase	1.	3.2	Not determined
(*C. vulgaris*)	2.	5.4	0.0
	3.	1.3	0.0
Ovalbumin		2.5	2.4
Transferrin		2.1	2.0
Avidin		6.5	7.0

temperatures of 60° in the presence and absence of oxalate. Figure 4 shows the results. In the absence of oxalate, isocitrate lyase was rapidly inactivated. However, 5 mM oxalate almost quantitatively protected the enzyme from heat denaturation in the present studies. Similar results were obtained with 25 or 50 mM oxalate (data not shown). It is of interest to note that when the enzyme was not fully reduced with 5 mM DTT prior to incubation with 5 mM oxalate, only 60% of the activity was protected over a 45-min exposure at 60° as opposed to more than 90% for the fully reduced enzyme. This observation points towards a conformational change on reduction which enhances oxalate binding or in some manner renders the oxalate-enzyme complexes less susceptible to thermal denaturation.

Our efforts to use heat treatment in the presence of oxalate to purify the enzyme were unsuccessful. When

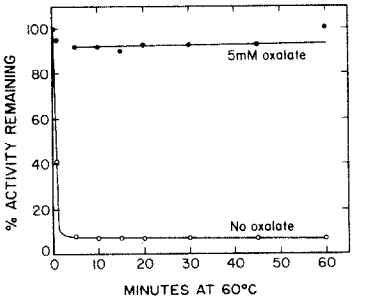

Fig. 4. Protection of *C. vulgaris* isocitrate lyase against heat denaturation by 5 mM oxalate. Enzyme was treated as described in text. Similar results as above were obtained with 25 mM and 50 mM sodium oxalate, but are omitted for clarity.

50 mM oxalate and heating at 65° for 15 min was used, 60% of the activity was recovered in the supernatant. However, there was no apparent purification as seen by specific activity measurements as well as by SDS-polyacrylamide gel electrophoresis.

DISCUSSION

In this report we have described the purification of isocitrate lyase from a higher plant source, cotyledons of *Citrullus vulgaris*. The final preparation obtained in the highest yield ever reported is very stable and has an essentially constant turnover number. Inclusion of a serine protease inhibitor, α-tolylsulfonyl fluoride at 1 mM during purification, results in almost quantitative recovery of isocitrate lyase at the ammonium sulfate precipitation step. The use of 0.2 mM inhibitor gives much poorer recovery. Clearly, proteolysis in crude extracts contributes both to lower yields and variable specific activities from developing cotyledons. Giachetti *et al.* [11] have described instability of this enzyme from *Pinus pinea* in crude extracts. We suggest the routine inclusion of α-tolylsulfonyl fluoride during the purification of isocitrate lyase from higher plants.

Watermelon isocitrate lyase does not bind to DEAE-cellulose at pH 7.5 in Tris buffer unlike the flax [3] and cucumber enzyme [2, 12]. Electrophoresis of the purified watermelon enzyme on 6% polyacrylamide gel exhibited two species, one major and one minor [unpublished observation], both of which contained activity. However, the watermelon enzyme chromatographs as a single species in contrast to multiple forms observed from *Lupinus* [13] and *Linum* [3]. Since our preparation does not bind to DEAE-cellulose, any differences in anionic charge which would have otherwise been highlighted during salt elution remain a possibility. We need more data to say conclusively whether there is more than one form of the *C. vulgaris* enzyme. It must be stressed, however, that multiple forms observed in other research may have arisen from proteolysis occurring after cell breakage.

Our enzyme preparation is pure on the basis of gradient polyacrylamide gels containing SDS and homogenous on the basis of immunoelectrophoresis. The molecular weight values of the enzyme and derived subunits are in good accord with those reported for isocitrate lyase from other higher plants, e.g. flax, 264 000 and 67 000 [3] and

cucumber, 260 000 and 64 000 [12] but in stark contrast with those for the enzyme from castor bean of 140 000 and 35 000 [4]. Since no proteinase inhibitors were used in purifying the castor bean enzyme, the low molecular weights may reflect proteolysis in crude extracts. Like the enzyme from all sources studied to date, isocitrate lyase from *C. vulgaris* appears to be a tetramer made up of identical or closely similar subunits.

The present enzyme preparation has a very high turnover number of 18.5 moles of substrate·sec^{-1}·mole of active site^{-1}, assuming one active site per monomer. In comparison, the cucumber enzyme which has been highly purified in two different laboratories has a turnover number that is an eighth [12] to a third as high [2]. Only flax isocitrate lyase has a higher turnover number (of 21.8; see ref. [3]) but is much less stable than the watermelon enzyme; moreover, we have experienced difficulties in obtaining reproducible fractionation of the flax enzyme.

Whether isocitrate lyase is a glycoprotein or not is highly significant, as it is a protein packaged in glyoxysomes. The higher plant enzyme is not synthesized as a higher molecular weight precursor [14] and thus may not be a glycoprotein [15]. There are conflicting reports in literature, however, regarding the glycoprotein nature of isocitrate lyase from higher plants. Frevert and Kindl [12] have suggested that the cucumber enzyme is a glycoprotein, based on (a) Schiff staining after SDS-polyacrylamide gel electrophoresis, (b) periodate oxidation followed by NaB[^{3}H]$_4$ labeling, and (c) incorporation of [^{3}H]glucosamine in immunoprecipitable material. However, Riezman *et al.* [14] claim that the enzyme from the same source is not a glycoprotein. Their results are based on failure to specifically label isocitrate lyase with ^{125}I-Con A and gas chromatographic analysis of the purified protein. We conclude that the watermelon enzyme is not a glycoprotein because, unlike results with glycoproteins, mild trichloroacetic acid treatment removes all the sugar residues from purified isocitrate lyase indicating a non-covalent inter-action (Table 3). Further, our efforts to stain isocitrate lyase from glyco-protein using the Schiff-periodate stain referred to by Frevert and Kindl [12] also give negative results under conditions where ovalbumin stained positively [unpublished observation].

To summarize, isocitrate lyase has been purified in excellent yield from watermelon cotyledons and some of its physical and catalytic properties have been described. The enzyme is extremely stable and has a high and constant turnover number. The availability of this enzyme opens the way to studies of the active site and other catalytic parameters. Moreover, the availability of monospecific antibodies will enable studies of the regulation of enzyme levels during cotyledonary development as well as the effect of light on isocitrate lyase activity during germination of watermelon seedlings [16].

EXPERIMENTAL

Materials. Watermelon seeds (Stone Mountain variety) were obtained from Vaughn Seed Co., Ovid, Michigan. Sepharose 6B was purchased from Pharmacia Fine Chemicals, DEAE-cellulose (DE 52) from Whatman and XM50 ultrafiltration membranes from Amicon Corp. Molecular weight standards for SDS-polyacrylamide gels were obtained from Bio-Rad Laboratories and Freund's adjuvants from DIFCO. All other chemicals used were of the highest grade available.

Seed germination. Seed coats were removed from watermelon (*Citrullus vulgaris*) seeds and the embryos stored at 4° until required for germination. In this condition, the embryos remained viable for more than 6 months. Prior to germination, embryos were washed for 5–10 sec with 0.1% $HgCl_2$ soln. This was followed by extensive washing (4–5 washes) with distilled water to remove the $HgCl_2$. They were then laid out on 14 cm glass petri plates containing 50 ml of 0.8% agar. About 25–30 embryos were spread per plate, the plate wrapped in aluminum foil, and incubated in the dark at 23°. After 2.5–3 days of germination [16], radicals were removed and the cotyledons used for enzyme extraction.

Enzyme assays. Isocitrate lyase was assayed by a discontinuous method [17] with some modifications. A suitable aliquot portion of enzyme was diluted in assay buffer (50 mM MOPS, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT) to a volume of 0.95 ml. To this was added 0.1 ml of a 1.6% (w/v) phenylhydrazine HCl solution and the assay started with 0.05 ml of 20 mM D, L-isocitrate. After incubation at 30° for the desired time, the reaction was stopped with 0.5 ml of 12 N HCl. To this was added 0.1 ml 8% $K_3Fe(CN)_6$. After thorough mixing, the color intensity was read at 535 nm. The control was treated identically except that HCl was added prior to isocitrate addition. This assay was at least twice as rapid as the method of Roche *et al.* [17] but with a similar extinction for the colored product. One unit of enzyme activity was the amount that formed one micromole of glyoxylate per minute under conditions of the assay.

Protein estimation. Protein was estimated [18] with corrections for Tris buffer [19] and for thiols [20].

Purification of isocitrate lyase. Step 1: *Crude extract.* After removing the seed coat, about 300 pairs of cotyledons were washed in isolation buffer (50 mM Tris–Cl, pH 7.5 at 4°, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mM α-tolylsulfonyl fluoride). The washed cotyledons were then homogenized in a total of 50–60 ml of this buffer using 3 × 30 sec pulses at maximum setting in a Sorval Omni mixer (model no. 17105). The homogenate was centrifuged at 27 000 *g* at 4° for 20 min and the supernatant passed through glass wool and recentrifuged. After one more identical passage and recentrifugation, all fatty material was removed. This is referred to as the 'crude extract'. All subsequent steps were carried out at 2–5° unless otherwise mentioned. *Step* 2: *Ammonium sulfate fractionation.* The crude extract was brought to 26% saturation by slowly adding 0.14 g of solid $(NH_4)_2SO_4$ per ml of soln at 4°. After stirring for 30 min, the precipitate was removed by centrifugation at 20 000 *g* for 20 min. The supernatant was adjusted to 41% of saturation by the addition of 0.088 *g* of solid $(NH_4)_2SO_4$ per ml of soln. After stirring as before, the precipitate was similarly removed. The precipitate which contained most of the isocitrate lyase activity was taken up in 7–10 ml of TEMB buffer (50 mM Tris–Cl, pH 7.5, at 4°, containing 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol). *Step* 3: *Chromatography on Sepharose 6B column.* The 26–41% $(NH_4)_2SO_4$ precipitate was put on a 2.5 × 93 cm column of Sepharose 6B pre-equilibrated with TEMB buffer. The column was run at a flow rate of 15–20 ml/hr and 4-ml fractions were collected. A suitable aliquot was assayed for isocitrate lyase activity. The fractions showing activity were pooled and concd through an Amicon XM50 ultrafiltration membrane using 20 atm nitrogen pressure. The final volume of the pooled and concentrated Sepharose 6B fractions was kept between 5 and 10 ml. *Step* 4: *Chromatography on DEAE-cellulose column.* The concd Sepharose 6B fractions were put on a 2.5 × 37 cm DEAE-cellulose column (DE 52) which had been pre-equilibrated with TEMB buffer. The column was subjected to a buffer wash at a

flow rate of 15–20 ml/hr and 4 ml fractions were collected. All isocitrate lyase activity was eluted off immediately after the flow-through volume. A gradient of up to 0.5 M NaCl in TEMB yielded no additional enzyme activity. The fractions showing isocitrate lyase activity were pooled and concd through an Amicon XM50 ultrafiltration membrane as described. The enzyme in 2–3 ml was stored in this form in 0.5 ml aliquot portions at −120°.

Molecular weight of isocitrate lyase and its subunits. The molecular weight of watermelon isocitrate lyase was estimated by gel filtration on a Sephacryl 300 column (1.5 cm × 56.5 cm) which had been pre-equilibrated with TEMB buffer. Purified isocitrate lyase was chromatographed on the same column which had been previously calibrated. The molecular weight of subunits was estimated on a 4–20% polyacrylamide gradient gel containing 0.2% sodium dodecyl sulfate [21]. The gels run in a Tris–glycine–SDS buffer system were calibrated with standard proteins in a BioRad LMW kit.

Production of antiserum to purified isocitrate lyase. Antiserum was raised in a pair of rabbits using some modifications in the technique of Lamb *et al.* [2]. Rabbits were injected subcutaneously with 350 μg and 100 μg of the purified enzyme preparation in 0.5 ml complete Freund's adjuvant with a week between the two injections. Rabbits were then bled 10 and 17 days after the second injection. A booster injection containing 100 μg of the purified enzyme in 0.5 ml incomplete Freund's adjuvant was given a week after the second bleeding and the rabbits bled 7 and 14 days thereafter. Ten days prior to the final bleeding, the rabbits were given another booster injection containing 100 μg of the purified enzyme in 0.5 ml Freund's incomplete adjuvant. All bleedings were from the ear artery, using xylene as an irritant. The blood was allowed to clot for 2–3 hr at 4° before centrifuging at 5000 *g* for 10 min. Sera were checked for immunoglobulin production by Ouchterlony double diffusion in 0.8% Noble Agar. Control sera were obtained prior to immunization.

A crude immunoglobulin fraction was prepared by precipitation with 0.25 g/ml $(NH_4)_2SO_4$. The pellet was removed by centrifugation at 20000 *g* for 20 min, resuspended in a volume of water equal to the serum volume, and dialysed overnight against 500-fold excess water with two changes at 4°. The dialysis was continued against a 100-fold excess of 0.05 M potassium phosphate, pH 7.5, containing 0.15 M NaCl for 6 hr at 4°. The dialysed fraction was divided into 1 ml aliquots and stored frozen at −20°.

Immunodiffusion and immunoelectrophoresis. Ouchterlony double diffusion was carried out in 0.8% Nobel Agar in water. Plates were incubated in a moist chamber at room temperature or 4° for the precipitin line to develop.

Immunoelectrophoresis was carried out to assess the purity of the antigen. Ethanol-washed slides for immunoelectrophoresis were prepared from 4 ml of 1.5% (w/v) Nobel Agar in 0.05 M sodium barbital, pH 8.6, containing 0.02% (w/v) sodium azide. 7 μl of purified isocitrate lyase or the crude cotyledonary extract was placed in the central well and electrophoresis was carried out at 3 mA/slide for 4 hr in 0.05 M sodium barbital, pH 8.6. After electrophoresis, slots were made and filled up with 150 μl of the appropriate immunoglobulin. The slides were then incubated in a moist chamber at 4° for 24–48 hr, during which precipitins became visible.

For staining, the immunoelectrophoresis slides were washed extensively in 0.15 M NaCl. The washing was done for 36 hr with several saline changes at 4°. The slides were then rinsed in a 1% glycerol solution for 15 min at room temperature and dried in a 45° oven for 6 hr. Staining was done for 5 min with 1% Amido Black in H_2O–MeOH–HOAc (6:3:1) followed by destaining in the same medium.

Amino acid analysis. Isocitrate lyase from *C. vulgaris* was exhaustively dialysed against 0.1 M NaCl prior to amino acid analysis. Aliquots of the dialysed enzyme containing 70 μg protein were transferred to hydrolysis vials having norleucine as an internal standard. The contents were lyophilized, 0.5 ml of 6 N HCl was added to each vial, and the vials were degassed and sealed under N_2. The hydrolysis was carried out for 24, 48 and 72 hr at 110° and all hydrolysates were analyzed with a Beckman 121 MB automatic amino acid analyser [22]. Half-cystine was determined as cysteic acid and memthionine as methionine sulfone [23]. Tryptophan content was determined spectrophotometrically in 0.1 N NaOH [24] using the tyrosine content determined by amino acid analysis.

Carbohydrate content. The carbohydrate content of purified isocitrate lyase was determined by the phenol–sulfuric acid method [25] using D-glucose as a standard. Purified isocitrate lyase (0.5–1.0 mg) was dialysed against 1000-fold excess water with four changes at 4° and analysed for total carbohydrate content. The same amount of protein was precipitated with 10% trichloroacetic acid on ice for 30 min and the precipitate removed, washed once with cold 5% trichloroacetic acid, 3–4 times with deionized water and then analysed for total carbohydrate content. Glycoproteins like ovalbumin, avidin and human transferrin were also subjected to identical treatment and served as positive controls.

Thermostability and the effect of oxalate. To determine the thermostability of purified isocitrate lyase in oxalate, the enzyme was first diluted into MEMD buffer (50 mM morpholinopropanesulfonic acid, pH 7.5 at 4°, containing 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM dithiothreitol) which had been adjusted to 5 mM in DTT. The enzyme was incubated at 30° for 10 min in this buffer to reduce it completely. Equal volumes of the reduced enzyme and the appropriate sodium oxalate soln (prepared in MEMD and adjusted to pH 7.5) were added together and kept in a 60° water bath. The final enzyme concentration was kept between 0.10–0.16 units/ml (10 μg protein/ml). Aliquot portions of 50 μl were removed between 0 and 60 min, chilled on ice, diluted 40-fold with MEMD buffer and then an appropriate aliquot assayed for remaining isocitrate lyase activity at 30°. Residual activity was expressed as a percentage of the zero-time control kept throughout on ice but assayed at 30°. The final oxalate concentrations were 0, 5, 25 and 50 mM.

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