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CITRATE SYNTHASE AND MALATE DEHYDROGENASE FROM TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; citrate synthase; citrate metabolism.

Abstract—The purification of citrate synthase and malate dehydrogenase from tomato fruit is described. Citrate synthase has a total M_r of approximately 104 000 and a subunit M_r of approximately 50 000, indicating a dimer of similar sized subunits. K_m values for oxaloacetate and acetyl-CoA are 19 and 18 μM respectively. 5,5′-Dithiobis-(2-nitrobenzoic acid) is a potent inhibitor of enzyme activity; indole acetic acid neither enhances or inhibits activity. Thermal inactivation studies suggest the absence of isoenzymes; one substrate, oxaloacetate, confers complete protection against inactivation whereas the other, acetyl-CoA, affords none. The product, citrate, confers limited protection but only at high concentrations (> 100 mM). Isoelectric focussing studies of MDH revealed four isoenzymes with isoelectric points of 4.17, 4.35, 4.50 and 4.70. Isoenzymes from the cytosol were separated from the mitochondrial form by Percoll fractionation and required completely different conditions for elution from matrex gel orange A. The enzyme consists of two subunits of similar M_r 76 000.

INTRODUCTION

Tomato fruit ripening involves *de novo* synthesis of enzymes, probably controlled by ethylene [1–3] in addition to the reduction of specific chloroplast mRNA species [4–6]. Concurrently, large changes occur in the specific activities of several citric acid cycle and related cytosolic enzymes. These changes, not mediated by ethylene, involve an increase in the specific activity of NADP-linked malic enzyme concomitant with decreases in the specific activities of cytosolic malate dehydrogenase and mitochondrial citrate synthase, NAD-linked isocitrate dehydrogenase and malate dehydrogenase. The specific activities of these enzymes fall by 60% during ripening and then stabilize at this new level. As discussed elsewhere [7], one method of determining whether modification or proteolytic breakdown is involved in these changes is to quantify the amount of enzyme protein (independent of activity measurements) present at different stages of ripening, using antibodies raised against purified citric acid cycle enzymes. As a step towards that goal we here report the purification of tomato fruit citrate synthase (CS) and malate dehydrogenase together with some properties of the enzymes.

RESULTS

Purification of citrase synthase

Citrate synthase (EC 4.1.3.7) from a microbial source has been purified successfully using matrex gels [8]. The binding efficiency of several of these gels for CS from tomato fruit is shown in Table 1 which indicates that red A is the matrex of choice, although, if yield is sacrificed, orange A is suitable. Citrate synthase from tomato fruit could not be eluted specifically using CoA and oxaloacetate from any of the matrex gels. Ion-exchange using DEAE-cellulose and affinity chromatography on ATP-Sepharose proved ineffective methods for purifying CS. In the former case, the enzyme bound but no activity was eluted and in the latter, no binding occurred although CS from pigeon breast bound to the same column. Citric synthase from tomato fruit was purified by a combination of ammonium sulphate fractionation and chromatography using matrex gel red A and Sephacryl S-200. The purification is summarized in Table 2. Gel permeation chromatography of the purified enzyme in the presence of markers of known M_r yielded a total M_r for CS of approximately 104 000 and SDS polyacrylamide gel electrophoresis indicated that the protein was a dimer with similar size sub-units of M_r 50 000. In a previous paper [9] we reported the 60% fall in specific activity of CS from ripening tomato fruit, and in an attempt to determine whether this fall was due to the loss of one or more

Table 1. The binding efficiency of Amicon matrex gels for citrate synthase from *Lycopersicon esculentum*

Matrex gel	% of total activity loaded that was eluted using 0.5 M KCl in 50 mM Mops, pH 7.8	% of total protein loaded that was eluted using 0.5 M KCl in 50 mM Mops, pH 7.8
Green A	35	40
Blue A	80	27
Blue B	7	11
Red A	85	15
Orange A	40	4

1 mg of protein in a volume of 1 ml of crude supernatant was applied to each column. Protein was eluted as described.

Table 2. Purification scheme for citrate synthase

Stage	Protein (mg)	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
Crude extract	2101	3820	993	0.47		100
Ammonium sulphate 50–70% saturation	537	96	556	1.03	2.2	56
Matrex gel red A	24	24	288	12.0	25.5	29
Sephacryl S-200	1.87	4.8	202	108.0	230	20

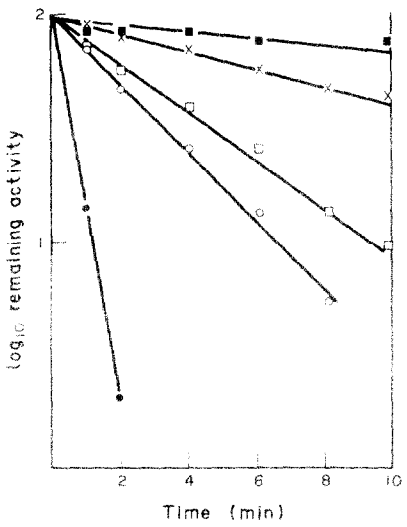

Fig. 1. Thermal inactivation of purified citrate synthase. Inactivation of citrate synthase from *L. esculentum* incubated at the following temperatures: (x——x), 40°; (□——□), 47.5°; (○——○), 50° and in the presence of 1 mM OAA (■——■), 47.5° and 10 mM acetyl-CoA (●——●), 47.5°.

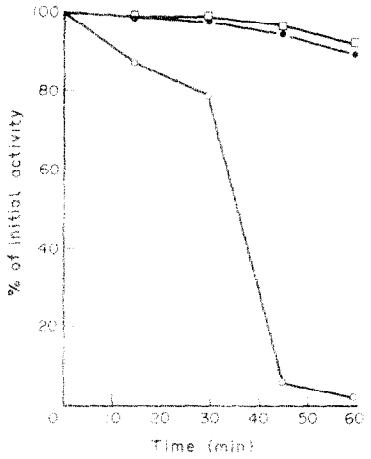

Fig. 2. Inhibition of citrate synthase by DTNB (○——○). Citrate synthase from *L. esculentum* in the presence of 50 mM Mops plus 5 mM DTNB; (●——●), in the presence of an 50 mM Mops; (□——□), pig heart citrate synthase in the presence of 50 mM Mops plus 5 mM DTNB.

isoenzymes, thermal inactivation studies were performed on the purified CS. Figure 1 shows that all the semi-log plots were monophasic indicating the probable lack of isoenzymes and, in addition, that 1 mM oxaloacetate protected the enzyme against thermal inactivation, while 10 mM acetyl-CoA enhanced the rate of inactivation compared with the control. The data from an identical experiment using crude tomato homogenate gave similar results, suggesting that isoenzymes had not been lost during purification.

Sarkissian [10] reported that CS from bean hypocotyl was activated and modified in molecular size by the plant growth substance IAA. We examined the effect of IAA on CS from tomato fruit at two concentrations and found no evidence of enhancement or inhibition of activity. The inactivation of CS by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is shown in Fig. 2. An identical amount of pig heart CS showed no inhibition in the presence of DTNB. The activity of CS from tomato fruit was inhibited by ATP competitively with respect to acetyl-CoA and non-competitively with respect to oxaloacetate. NADH (0.1 mM–1.0 mM) did not inhibit the activity of CS from tomato fruit.

Purification of malate dehydrogenase

Malate dehydrogenase was separated from contaminating malic enzyme (EC 1.1.1.40) activity by ammonium sulphate fractionation, MDH precipitating in the 50–70% fraction and malic enzyme in the 30–50% fraction. In addition, MDH was eluted from matrex gel red A in two distinct fractions: 80% of the activity applied was eluted at a KCl concentration of 130 mM, while the remaining 20% was eluted at 220 mM KCl. This latter fraction co-eluted with citrate synthase activity. MDH was further purified by anion-exchange chromatography on a Mono Q column. All activity was recovered in three consecutive fractions. These fractions were pooled, dialysed and applied to a column containing matrex gel blue A which has been equilibrated with 20 mM Mops, pH 8.0. After elution the fractions containing MDH were concentrated and loaded onto an SDS-polyacrylamide gel. The results indicated a single band with an estimated M_r of 40,000. Samples were run on a 5% non-denaturing polyacrylamide gel and four protein bands coincided with four bands which could be stained for MDH activity. No other protein bands were visible.

Malate dehydrogenase which was dialysed and subjected to isoelectric focussing (IEF) on an LKB column gave four peaks of activity with pI values of 4.17, 4.35, 4.50

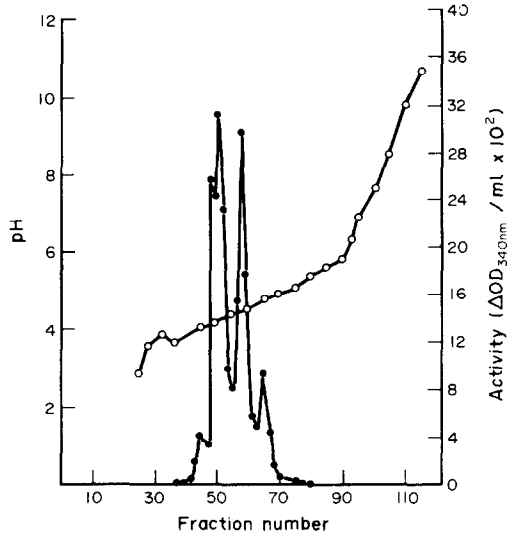

Fig. 3. Resolution of malate dehydrogenase isoenzymes by iso-electric focussing. Elution profile of malate dehydrogenase isoenzymes from an LKB iso-electric focussing column (100 ml). The pH gradient is shown by the open circles and malate dehydrogenase activity by the closed circles. Twenty-four thousand units were added in a total of 2 ml and 22 780 units were recovered. Intially, 500 V were applied to the column; this was gradually increased to 2000 V. Focussing was completed when the current stabilized at its lowest level.

and 4.70 (Fig. 3). During isoelectric focussing, 95% of the contaminating CS activity was lost but 90% of the MDH activity was retained. The peak at 4.70 was relatively small compared to the other peaks. The M_r of MDH was estimated by gel filtration on Sephadex G-100 to be approximately 76 000.

DISCUSSION

On the basis of the absence of a biphasic inactivation curve, the thermal inactivation studies suggest that only one isoenzyme of CS is present in tomato fruit. Zehler *et al.* [11] showed that the glyoxysomal and mitochondrial isoenzymes of CS from *Ricinus communis* have different thermal inactivation characteristics. The mitochondrial isoenzyme lost 50% activity within 10 min at 45° whereas the glyoxysomal isoenzyme was completely stable at 47°. Thus the thermal inactivation profile of CS from *Lycopersicon esculentum* is similar to that of the mitochondrial form of CS from *Ricinus communis* but differs from that of the glyoxysomal isoenzyme. Some plant CS enzymes are inactivated by DTNB as is CS from *Lycopersicon esculentum*. Although the same concentration of DTNB had no effect on pig heart CS, there was a rapid inactivation of CS from tomato fruit. The inactivation was slow for 30 min and then, within a short period, there was complete loss of activity. The inactivation of CS from *Phaseolus vulgaris* [12] followed a similar trend; complete inactivation in the presence of 0.1 mM DTNB took 90 min, while similar concentrations of mercuric acetate and silver nitrate inactivated the enzyme complete within 5 min. In the presence of excess dithiothreitol (DTT), 60% reactivation occurred in the case of mercuric acetate and silver nitrate; however, DTT failed to reactivate the enzyme that had been treated with DTNB. Srere *et al.* [13] found that CS from mango fruit was inhibited by several sulphydryl-blocking reagents including DTNB, Hg^{2+} and paramercuri-benzoate, but that excess DTT only reactivated enzyme which had been treated with the latter two inactivators. This may be because the slow inactivation by DTNB causes a conformational change such that the enzyme dissociates into its subunits or steric hindrance may prevent DTT reducing the disulphide bridges. Sarkissian [10] reported the inactivation and modification of CS from bean shoot and cauliflower mitochondria. However, Brock and Fletcher [14] could find no activation when they repeated the work. In the present study there was no evidence of activation by IAA. In contrast to the findings of Sakamoto *et al.* [15], using castor bean tissue, no inhibition of CS by citrate or malate was detected. However, the concentrations of citrate and malate used by Sakamoto *et al.* were high (20 mM and 84 mM respectively) and probably not physiological. The protection afforded by oxaloacetate against thermal inactivation was almost complete. Srere [16] proposed that the binding of oxaloacetate to CS induces a conformational change which increases the stability of the enzyme and aids proton abstraction from acetyl-CoA.

All eukaryotic citrate synthases examined to date have been found to be inhibited by adenine nucleotides, normally in the order ATP ADP AMP. Similar findings were made in the case of tomato CS. However, there are strong arguments against the idea that the modulation of CS activity by adenine nucleotides is of any physiological significance [17].

The M_r and subunit composition of CS from tomato fruit closely resemble those of other eukaryotic citrate synthases, with the exception of the enzyme from mango fruit [13] which has a total M_r of 60 000. The enzyme is therefore small and distinct from the 'large' enzyme found in certain prokaryotes [18]. Citrate synthase from tomato fruit appears similar in its physical and chemical characteristics to the enzyme from many other sources. In its sensitivity to sulphydryl-blocking reagents, it resembles several other plant citrate synthases which in this respect differ from their animal counterparts. The four isoenzymes of MDH found in the isoelectric focussing study are in agreement with the work of Hobson [19]. One of these isoenzymes was mitochondrial and could be separated from the others by Percoll fractionation. Elution of the mitochondrial isoenzyme from matrex gel orange A was by salt only; in contrast, the cytosolic isoenzymes could be eluted specifically from the same gel by 1 mM malate plus 1 mM NADH.

The homogeneous MDH from *L. esculentum* has an M_r of approximately 76 000 and is a dimer of similar sized sub-units. This compares with a size of 76 000 for the mitochondrial enzyme from the cotyledons of *Citrullis vulgaris*, which has also been shown to be a dimer of identical sub-unit sizes [20].

EXPERIMENTAL

Materials. Fruit were supplied by Long Ashton Research Station, Tanyard Nurseries, Lower Weare, Somerset and Stourbank Nurseries, Wimborne, Dorset. Matrex gels were supplied by Amicon and Mono Q and Sephacryl S-200 by Pharmacia. All other chemicals were from Sigma Chemical Co.

Extraction of enzyme. To extract fruit with a considerable amount of free organic acid, an extraction buffer was used which contained 100 mM 3-(*N*-Morpholino)propane sulphonic acid (Mops) and 100 mM *N,N*-bis(2-hydroxyethyl)glycine (Bicine) with 3 mM EDTA to maintain the pH at 8.0; 1% solid polyvinylpyrrolidone was added after maceration. All extractions were held at 4.0° and all glassware and centrifuge tubes were precooled. Washed samples of tomato fruit were cut into small pieces and homogenised at full speed for 15 sec in a Waring blender containing the extraction buffer at a 1:1 ratio (w/v). The homogenate was strained through four layers of muslin and centrifuged at 20 000 *g* for 15 min. The supernatant was used for enzyme assays.

Enzyme assays. Citrate synthase was assayed by the method of ref. [21]. The assay mixt. contained 50 mM Mops, 0.2 mM acetyl-CoA, 0.1 mM DTNB and 50 μl of extract, all at pH 7.9. The reaction was started by the addition of oxaloacetate (to 0.2 mM) to make a total vol. of 1.0 ml. The change in *A* was followed at 412 nm and a molar absorption coefficient of 13.6×10^3 mol/cm was used to calc. activity. One unit of activity was taken as that amount of enzyme catalysing the liberation of 1.0 μmol of CoA/min under assay conditions. Malate dehydrogenase was assayed spectrophotometrically at 340 nm. The assay mixture contained 50 mM Mops, pH 8.0, 0.4 mM NADH and 10 μl of extract. The reaction was started by the addition of oxaloacetate to give a final concn of 0.2 mM in a total vol. of 1.0 ml. One unit of activity was taken as the amount of enzyme catalysing the oxidation of 1 μmol of NADH per min under assay conditions. Protein was determined by the method of ref. [22].

Molecular mass estimation using gel filtration. Gel filtration was performed on a column of Sephacryl S-200 (2.5 × 100 cm) or G-100 (2.5 × 100 cm). The S-200 column was equilibrated with 50 mM Bicine at pH 7.9 and a peristaltic pump was used to provide a flow rate of 5 ml/h. The G-100 column was equilibrated with the same buffer at pH 8.0 at a flow rate of 7 ml/hr. Fractions (1 ml) were collected and the M_r calibration standards used were: ferritin (450 000), catalase (124 000), aldolase (158 000), bovine serum albumin (68 000), chymotrypsinogen (25 000) and cytochrome *c* (12 500). In later experiments ribonuclease (13 400) was substituted for Cytochrome *c*, as the latter protein given anomalous results.

Electrophoresis. SDS polyacrylamide gel electrophoresis was used to give an estimate of the relative M_r of the sub-units of CS. The method of ref. [23] was used to produce a 7.5% gel in slabs (14 × 16 × 1.5 cm). Protein markers and their M_r values were: trypsin inhibitor (21 500), RNA polymerase (39 000), bovine serum albumin (68 000) and the mean of RNA polymerase B (155 000) and B' (165 000). Gels were run at 3 mA/well. Native gels were run as described previously [24]. Activity staining of MDH in native gels was by the method of ref. [25].

Matrex gel affinity chromatography. Gels were washed with 6 M urea and 0.5 M sodium hydroxide and equilibrated with 50 mM Mops, pH 7.8. Protein was washed with the same buffer after application and eluted with 0.5 M KCl or 100 μM CoA + 100 mM oxaloacetate, in 50 mM Mops, pH 7.8. Gel red A was washed with (a) 100, 200 or 500 μM of both oxaloacetate and CoA together, (b) 100 μM of both acetyl-CoA and citrate, and (c) 100 μM oxaloactate + 100 μM CoA + 100 μM IAA. Only 0.5 M KCl was successful in eluting CS.

Fast protein liquid chromatography. After dialysis against the start buffer (1,3-bis[tris(hydroxymethyl)methylamino]propane) (bis tris propane), pH 7.5, MDH was applied to a mono Q column HR5/5 and eluted with a linear sodium chloride gradient, 0–500 mM over 45 ml, at a flow rate of 1.5 ml/min.

Thermal stability studies. At each temperature, 16 μg of enzyme were withdrawn and assayed for citrate synthase activity after 1,2,4,6,8 and 10 min. Citrate was used at final concentrations of 100, 10 and 1 mM, oxaloacetate at 10 and 1 mM and acetyl-CoA at 10 mM.

Activation and inhibition studies. IAA was used at final concentrations of 40 μM and 4 nM with substrate concentrations of $10 \times K_m$ – $0.5 \times K_m$. DTNB was used at a final concentration of 5 mM. K_m values were determined by varying one substrate concentration from 200–6.25 μM whilst keeping the other at saturating concentration.

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