Phytochemistry, 1973, Vol. 12, pp. 1249 to 1254. Pergamon Press. Printed in England.

PURIFICATION AND PARTIAL CHARACTERIZATION OF CITRATE SYNTHASE FROM *PHASEOLUS VULGARIS* MITOCHONDRIA

GERALD A. GREENBLATT and IGOT V. SARKISSIAN
Department of Biology, Texas A&M University, College Station, Texas, TX 77843, U.S.A.

(*Received* 2 *August* 1972. *Accepted* 10 *January* 1973)

Key Word Index—*Phaseolus vulgaris*; Legumiosae; bean; citrate synthase, sulfhydryl groups; *p*-hydroxymercuribenzoic acid.

Abstract—Citrate synthase (E.C. 4.1.3.7) has been isolated from bean mitochondria by an improved procedure. The purified enzyme had a specific activity of 50. In most respects (e.g. sedimentation constant, K_ms, pH sensitivity and ionic strength inhibition) the enzyme is similar to that prepared from mammalian sources. The feature distinguishing the plant enzyme from the others was its inhibition by several sulfhydryl reagents. The substrates conferred either complete protection (acetyl coenzyme *A*) or partial protection (oxalacetic acid) against the inhibition. Dithiothreitol (DTT) was capable of partially reversing the inhibition. The efficacy of DTT varied with the sulfhydryl reagent and was inversely related to the period of incubation of the enzyme with the reagent.

INTRODUCTION

CITRATE synthase, from many sources, has been intensively studied for a number of years. It has been shown that a number of compounds are capable of inhibiting the enzyme activity. End-products of the Krebs' cycle, such as ATP,[1] and other nucleotides[2] can act as feedback inhibitors; substrate analogues[3,4] can act as competitive inhibitors with both substrates or with CoASAc alone, and the enzyme is affected by the nature of its ionic environment.[5] However, until recently, it has been assumed that the enzyme from all higher organisms was unaffected by sulfhydryl reagents.[6,7] Only the bacterial enzyme, which also differs from the others in respect to MW, has been shown to be affected by such reagents.[8] A recent report,[9] indicated that citrate synthase preparation from mango fruit, does in fact react with sulfhydryl reagents.

The purpose of the present study was to improve the isolation procedure of citrate synthase from plants and to evaluate the sulfhydryl nature of this enzyme. The procedure described here routinely yields a preparation with the highest specific activity yet reported for plant tissues (see for example Ref. 10). Some kinetic properties of the enzyme and

[1] JANGAARD, N. O., UNKELESS, J. and ATKINSON, D. E. (1968) *Biochim. Biophys. Acta* **151,** 1225.
[2] WEITZMAN, P. D. J. (1966) *Biochim. Biophys. Acta* **128,** 213.
[3] SRERE P. A. (1963) *Biochim. Biophys. Acta* 77, 693.
[4] LEE, L. P. K. and KOSICKI, G. W. (1967) *Biochim. Biophys. Acta* **139,** 195.
[5] WU, J. and YANG, J. T. (1970) *J. Biol. Chem.* **245,** 3561.
[6] SRERE, P. A. (1965) *Biochem. Biophys. Res. Commun.* **18,** 87.
[7] SARKISSIAN, I. V. (1970) in *Proc. VII Int. Conf. Plant Growth Subst.* (CARR, D. J., ed.), Springer, Berlin.
[8] WRIGHT, J. A. and SANWAL, B. D. (1971) *J. Biol. Chem.* **246,** 1689.
[9] SRERE, P. A., PAVEKLA, S. and DAS, N. (1971) *Biochem. Biophys. Res. Commun.* **44,** 717.
[10] BOGIN, E. and WALLACE, A. (1969) in *Methods in Enzymology* (LOWENSTEIN, J. M., ed.), Vol. 13, p. 19, Academic Press, New York.

additional evidence on the sulfhydryl nature of bean citrate synthase are presented. The fact that two plant citrate synthases independently show sulfhydryl reactivity may indicate that citrate synthase from plant tissue is quite different from the animal enzyme.

RESULTS

An outline of the purification procedure is shown in Table 1. The major improvement involves DEAE-cellulose chromatrogaphy. The extensive washing of the column with 20 mM potassium phosphate buffer removes most of the contaminating malate dehydrogenase prior to desorption of the citrate synthase with the phosphate gradient. The 100 mM phosphate buffer and DTT in the eluant tubes also substantially improved enzyme recovery. Citrate synthase in the final preparation had a specific activity of about 50; malate dehydrogenase was about 32.

TABLE 1. PURIFICATION OF CITRATE SYNTHASE FROM BEAN HYPOCOTYL (200 g)

Step	Volume (ml)	Units/ ml	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Sonicated mitochondria	17·8	2·2	10	0·22	100	1
$(NH_4)_2SO_4$ from 0 45–0 80	3 5	7·9	4 1	1 7	70	7·7
DEAE-cellulose chromatography	1·3	6 2	0 12	51	20·4	232

Activities were determined with 75 μM CoASAc and 100 μM OAA.

Upon electrophoresis on cellulose acetate membranes, the preparation showed three protein bands, the most cathodic of which contained citrate synthase. Two more anodic bands both contained malate dehydrogenase (Fig. 1).

The calculated sedimentation constants of the plant and mammalian enzyme, were 6·5*S* and 6·3*S* respectively. This is in reasonable agreement with a previous report of 6·2*S*[11] for

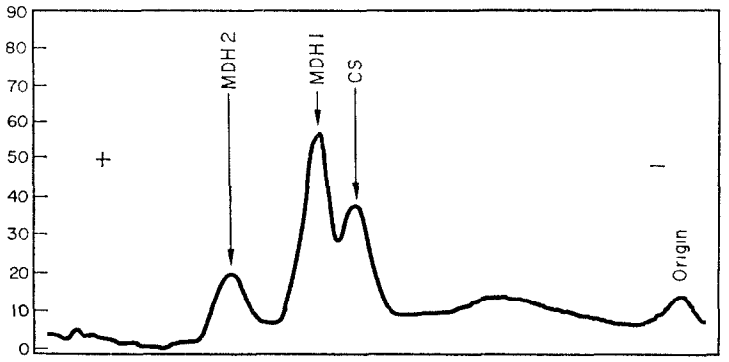

FIG. 1. ELECTROPHEROGRAM TRACE OF THE ENZYME PREPARATION CONTAINING CITRATE SYNTHASE (CS) AND MALATE DEHYDROGENASE (MDH1 AND MDH2).
0·5 μl of solution (0·34 μg protein) was applied to the origin. Other conditions are as described in Experimental.

[11] SINGH, M., BROOKS, G. C. and SRERE, P. A. (1970) *J Biol. Chem.* **245**, 4636.

the mammalian enzyme; therefore, we assume that the bean citrate synthase has a MW about equal to that of the mammalian citrate synthase or about 10^5 daltons. The half-life of the purified bean enzyme in 0·1 M K phosphate buffer, pH 7·5 with 1 mM DTT was about 6 weeks at 0°.

Properties of Bean Citrate Synthase

Kinetics. The usual Michaelis–Menten relationship is valid when the reaction was measured as a function of CoASAc and OAA. A Lineweaver–Burk plot gave a straight line for both substrates, the apparent K_m values for CoASAc and OAA being 28 and 12 μM respectively. The enzyme exhibited maximal activity at about pH 7·8 in 20 mM phosphate buffer. The pH optimum is broad ranging from about 7·4 to 8·1, maximal activity in phosphate buffer, pH 7·5, occurring at a concentration of 40–50 mM. A 40% reduction in activity occurred in 200 mM buffer whereas a 60% reduction occurred with very dilute buffer (actually 0·1 mM).

Effect of sulfhydryl reagents. Small aliquots of the dialyzed enzyme preparation were incubated with various sulfhydryl reagents at 20°. Portions of the mixtures were then assayed for activity with time. Bean citrate synthase is rapidly inhibited by *p*-hydroxymercuribenzoate (*p*-HMB). About 90% of the activity was lost within 5 min with concentrations of $3{\cdot}56 \times 10^{-4}$ M and greater. Lower concentration of *p*-HMB also inhibited the

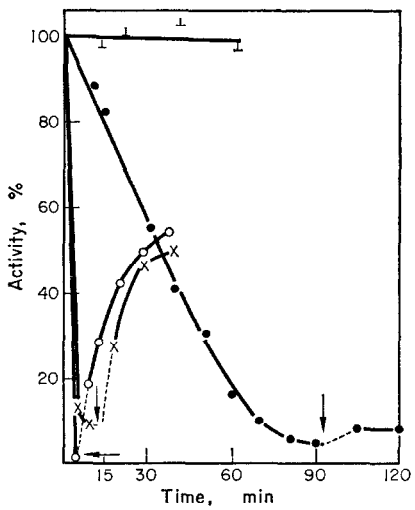

FIG. 2. INHIBITION OF THE ENZYMATIC ACTIVITY OF CITRATE SYNTHASE BY VARIOUS SULFHYDRYL REAGENTS.
The mixtures contained the following amounts of dialyzed enzyme and reagents: 1·1 units with $7{\cdot}2 \times 10^{-4}$ M DTNB, ●; 1·1 units with $7{\cdot}2 \times 10^{-4}$ M NEM, ⊥; 0·6 units with $1{\cdot}5 \times 10^{-4}$ M mercuric acetate, ×; 0·214 units with 3×10^{-5} M silver nitrate, ○. The mixtures were assayed at the indicated times using 50 μM CoASAc and 70 μM OAA. The arrows indicate the time of addition of 500 molar excess DTT.

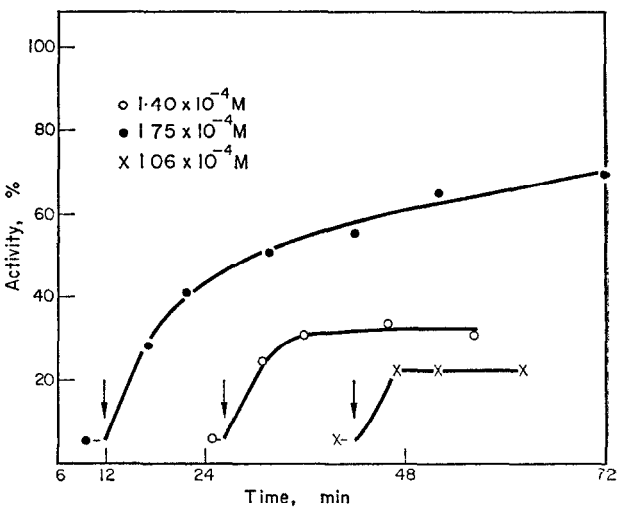

FIG. 3. REGAIN, BY DTT, OF ENZYMATIC ACTIVITY OF *p*-HMB TREATED CITRATE SYNTHASE. THE DIALYZED ENZYME (20·4 μg protein) WAS INCUBATED WITH *p*-HMB TO ACHIEVE THE FINAL VOLUME OF 0·036 ml.

The arrows indicate the time of addition of 400 M excess of DTT. The mixtures were assayed at the indicated times using 50 μM CoASAc and 70 μM OAA.

enzyme but time was required to approach this level of inhibition. Silver nitrate and mercuric acetate also rapidly inhibited the enzyme (Fig. 2). The effect of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was different: the enzyme was inhibited to about the same extent as with the previously mentioned sulfhydryl reagents but the time required for this inhibition was much longer (90 min). *N*-Ethyl maleimide (NEM) had essentially no effect.

Innibition by *p*-HMB was partially reversed with the addition of excess DTT (Fig. 3). Only a slight reversal of the DTNB inhibited enzyme was found (Fig. 2). The duration of enzyme incubation with the sulfhydryl reagents influenced the extent of the DTT reversal. Mixtures requiring a relatively long time for inhibition showed the lowest reversal of inhibition when treated with excess DTT. Also expectably less reversal by DTT occurred when an identical mixture of enzyme and sulfhydryl reagent was allowed to react for a longer time. The enzyme was protected against *p*-HMB inhibition by both of its substrates. The protection was complete with CoASAc (5×10^{-4} M) and only about 60% effective with OAA (5×10^{-4} M).

DISCUSSION

The integrated electropherogram (Fig. 1) suggests that this preparation of citrate synthase is about 30% pure. The specific activity of the homogeneous enzyme may thus be about 165, in agreement with previous work done with the crystalline mammalian enzyme.[11] The bean enzyme also exhibits other properties similar to the latter. Its MW and the effect of pH and ionic strength on its activity are very similar.[5,11] The apparent K_ms reported here are somewhat different than those previously found, but this may be due to differences in ionic strength of the various assay mixtures.[12]

The startling dissimilarity between this enzyme and those isolated from mammalian sources is with respect to sulfhydryl reagent sensitivity. Both this enzyme and that prepared from mango fruit obviously contain essential–SH groups. However, the incomplete reversal of the action of the sulfhydryl reagents by DTT is difficult to interpret at this time. Mercaptide formation is a reversible reaction and the addition of an excess of a thiol should regenerate the –SH group(s) of the enzyme and restore activity. The failure to achieve complete reversal by DTT may be simply due to the composition of the solution with respect to pH and concentration and type of anions.[13] Alternatively, it may mean that some irreversible precipitation has occurred similar to the situation with pig heart citrate synthase[6] or that the sulfhydryl reagents are dissociating the enzyme into subunits[14] which are very labile.

We are presently extending this work by attempting to purify the enzyme to homogeneity from bean and other readily available plant tissues. Future studies will then be addressed toward a complete analysis of the nature of the reactive sulfydryl group(s).

EXPERIMENTAL

The enzyme was extracted from mitochondria of 5·5-day-old etiolated bean seedlings (*Phaseolus vulgaris*, L., var. Burpee's Stringless Greenpod) grown in vermiculite at 20°. The hypocotyl hook sections (1–2 cm) were excised, rinsed with H_2O and immediately chilled. All subsequent steps, unless noted otherwise, were done at 1–4°.

[12] MORIYAMA, T. and SRERE, P. A. (1971) *J. Biol. Chem.* **246,** 3217.
[13] MADSEN, N. B. (1963) in *Metabolic Inhibitors* (HOCHSTER, R M. and QUASTEL, J H , eds.), Vol. II, p. 119, Academic Press, New York.
[14] SMITH, G. D. and SCHACHMAN, H. K. (1971) *Biochemistry* **10,** 4576.

Preparation and sonication of mitochondria. The tissue was ground with mortar and pestle in 50 mM K phosphate buffer, pH 7·5, 0·45 M sucrose, and 1 mM EDTA. The brei was filtered through nylon cloth (mesh *ca.* 50 μ), and the residue re-extracted in half of the original vol. of fresh grinding medium and filtered. The combined filtrate was centrifuged 18 min at 2500 *g*. The pellet was discarded and the supernatant was centrifuged 15 min at 23000 *g*. The pelleted mitochondria were suspended in 60 ml of the grinding medium and centrifuged 8 min at 37000 *g*. The washed mitochondrial pellet was suspended in 16 ml of 20 mM K phosphate buffer, pH 7·5, containing 10 mM DDT and sonicated at −5° by ten 30-sec bursts over a 10 min period using Bronwell Biosonic II equipped with a 3 mm probe. The suspension was centrifuged for 10 min at 37000 *g*; the supernatant was assayed for citrate synthase, and diluted with the sonicating medium to 0·4 units citrate synthase per ml.

Ammonium sulfate fractionation. Solid $(NH_4)_2SO_4$ was slowly added with stirring to 45% saturation, the pH being kept at 7·5 with additions of KOH. The mixture was stirred for 30 min after the last $(NH_4)_2SO_4$ addition and centrifuged for 10 min at 37000 *g*. The supernatant was then brought to 80% $(NH_4)_2SO_4$ saturation, stirred for 30 min, and then centrifuged for 10 min at 37000 *g*. The resulting precipitate was dissolved in 3 ml of 20 mM K phosphate buffer, pH 7·5. At this point, the enzyme was stable at 2° for several days.

Column chromatography. The enzyme solution was desalted on a Sephadex G50 column (1·5 × 10 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7·5. Active fractions were directly applied to a DEAE-cellulose column (1·5 × 10 cm) previously equilibrated with 2 mM K phosphate buffer, pH 7·5. The enzyme was washed onto the column with a small portion (*ca.* 5 ml) of 20 mM K phosphate buffer, pH 7·5. The column was eluted with 150 ml of the same buffer at a hydrostatic pressure of 60 cm. The eluting buffer was then replaced by a shallow convex ionic strength gradient. The initial concentrations of buffer in the mixing chamber (56 ml) and in the reservoir were 20 and 60 mM respectively. The eluant was collected in tubes containing DDT in 1 M K phosphate buffer, pH 7·5 to make a final concentration of DDT and buffer of 1 and 100 mM respectively. All fractions containing 0·5 units per ml or more of the enzymatic activity were pooled and condensed under N_2 pressure in an Amicon ultrafiltration cell equipped with an XM50 membrane. This preparation was either used directly or was extensively dialyzed against 100 mM phosphate buffer, pH 7·5 to remove the DTT prior to use.

Electrophoresis. The purified enzyme was electrophoresed on a cellulose acetate membrane in a Beckman microzone cell (R-101). The buffer was 30 mM K phosphate, pH 6·5. A constant voltage of 300 V was applied for 27 min. The protein on half of the membrane was fixed and stained with 0·05% Coomassie brilliant blue in 6% TCA for 10 min. The membrane, after rinsing in 5% HOAc, was air-dried and protein was measured with a Beckman densitometer (R-110). The areas on the other half of the membrane corresponding to the protein peaks were excised, eluted with 50 mM phosphate buffer, and assayed for citrate synthase and malate dehydrogenase activity.

Ultracentrifugation. The purified enzyme was layered on a 5–25% linear sucrose gradient in 0·1 M K phosphate, pH 7·5 and centrifuged in an International B60 ultracentrifuge (Rotor No. SB-405). After centrifugation (16 hr at 50 000 rpm) the gradients were fractionated (0·1 ml/fraction) and assayed for activity. Catalase was used as an internal marker and assayed according to the procedure supplied by Sigma Chemical Co. Duplicate tubes were run using pig heart citrate synthase and the calatalase marker. Sedimentation constants were determined according to Martin and Ames.[15]

Enzyme assays. Citrate synthase activity was determined by measuring the rate of disappearance of the thioester bond of CoASAc at 233 nm at 25°: additions were made to the cuvette in the following order: H_2O, K phosphate buffer, pH 7·5, enzyme, CoASAc and OAA. The OAA was freshly prepared and brought to pH 7·5 with 0·5 M Tris base. Unless specified, the final K phosphate concentration was 100 mM. Calculations were based on a combined extinction coefficient (233 nm) of $5{\cdot}4 \times 10^3$ M^{-1} cm^{-1}.[16] A unit of citrate synthase is that amount of enzyme capable of utilizing 1 μmol of CoASAc per min at 25°. Specific activities are expressed per mg protein. Determination of protein in relatively crude enzyme preparations were performed according to Warburg and Christian,[17] whereas the modified Folin test,[18] using bovine serum albumin as a standard, was used for highly purified preparations. The enzyme preparation was also assayed for malate dehydrogenase activity (E.C. 1.1.1.37). Malate dehydrogenase activity was measured at 340 nm in an assay modified from Davies.[19] Additions to the 1 ml reaction mixture were made in the following order: H_2O, K phosphate buffer, pH 7·5, enzyme, NADH and OAA. The final buffer concentration was 100 mM. Substrate levels were 100 μM NADH and 500 μM freshly prepared OAA, pH 7·5. The extinction

[15] MARTIN, R. G. and AMES, B. N. (1961) *J. Biol. Chem.* **236,** 1372.
[16] KOSICKI, G. W. and SRERE, P. A. (1961) *J. Biol. Chem.* **236,** 2557.
[17] WARBURG, O. and CHRISTIAN, W. (1941–42) *Biochem. Z.* **310,** 384.
[18] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[19] DAVIES, D. D. (1969) in *Methods in Enzymology* (LOWENSTEIN, J. M., ed.), Vol. 13, p. 148, Academic Press, New York.

coefficient used for NADH was $6{\cdot}22 \times 10^3$ M^{-1} cm^{-1}.[20] A unit of malate dehydrogenase is that amount of enzyme capable of oxidizing 1 μmol of NADH per min at 25°.

Biochemicals. Acetyl-coenzyme-*A* (lithium salt), oxalacetic acid, dithiothreitol, NADH, and sucrose were obtained from Nutritional Biochemicals. Enzyme-grade $(NH_4)_2SO_4$ was bought from Mann Research Laboratories DEAE cellulose (exchange capacity—0·68 mequiv./g) was obtained from Bio-Rad Laboratories. Sephadex G50, p-hydroxymercuribenzoate (Na salt), *n*-ethyl maleimide, 5,5′-dithiobis (2-nitrobenzoic acid), Coomassie brillant blue, bovine serum albumin (fraction IV), bovine liver catalase (thymol free) and porcine heart citrate synthase were obtained from Sigma Chemical Co. All other chemicals were reagent grade. Glass dist. H_2O was used exclusively.

Acknowledgements—This work was supported by grants GB-7618 from the National Science Foundation and A-338 from the Robert A. Welch Foundation of Houston, Texas. G A. Greenblatt is post doctoral fellow of the Robert A. Welch Foundation

[20] THORNE, C. J. R. and KAPLAN, N. O. (1963) *J. Biol Chem.* **238,** 1861.