CRYSTALLIZATION AND SOME PROPERTIES OF THE CITRATE CLEAVAGE ENZYME OF RAT LIVER

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Recently, knowledge on the physiological significance of the citrate cleavage enzyme has increased, especially with regard to its role in lipogenesis (Spencer et al., 1964) and in gluconeogenesis (D'Adamo, Jr. and Haft, 1965). Moreover, in support of its physiological significance, it has been shown that its activity in rat liver varies in close relation with changes in various physiological conditions. For example, it decreases sharply on induction of diabetes, during fasting or after hypophysectomy and is greatly elevated by administration of a high carbohydrate diet or insulin (Kornacker and Lowenstein, 1964; Spencer et al., 1964; Takeda et al., 1964; Abraham et al., 1964).

Srere partially purified the citrate cleavage enzyme from the soluble fraction of chicken liver and studied some of its properties (Srere, 1959; Srere, 1961; Srere and Bhaduri, 1964). However, no further purification of this enzyme has been achieved because of its instability and nothing is known about the nature of the rat liver enzyme despite of its metabolic significance.

The present paper describes the purification and crystallization of the citrate cleavage enzyme from rat liver and some of its properties.

Wistar strain rats, weighing about 200 g, were used throughout. They were maintained on a high carbohydrate diet, which consisted of 63% sucrose,

30% casein, 4% salt mixture, 2% cellulose powder, 1% vitamin mixture and 0.1 % choline chloride, for 3 days before being killed. This diet resulted in about a 10-fold increase in the level of the citrate cleavage enzyme in the liver. The enzyme activity was measured at 37° by the hydroxamate method (Srere, 1961) in steps 1, 2 and 3, and by the spectrophotometric method (Srere, 1959) in the subsequent steps.

Purification of the citrate cleavage enzyme

Step 1. Preparation of the crude extract ——— Fresh livers (380 g), from rats which had been fed on a high sucrose diet, were homogenized in 2 volumes of 0.25 M sucrose containing 0.02 M tris buffer, pH 8.0, using a Potter-Elvehjem glass homogenizer. The supernatant fluid (crude extract) was obtained by centrifugation at 15,000 x g for 30 minutes and then at 105,000 x g for 30 minutes.

Step 2. 1st ammonium sulfate fractionation ——— The crude extract was diluted to about 20 mg protein per ml and ammonium sulfate was added to 25% saturation. The precipitate formed was removed by centrifugation and discarded. Further ammonium sulfate was then added to the supernatant to 45% saturation. The resulting precipitate was collected by centrifugation and dissolved in 0.01 M tris buffer, pH 7.8. The enzyme solution was dialyzed overnight against 30 volumes of the same buffer.

Step 3. DEAE-cellulose column chromatography ——— The dialyzed enzyme was diluted with the same buffer, to give 15 mg protein per ml, and then run into a column containing 1.2 g DEAE-cellulose per 100 mg protein, which had been equilibrated with 0.005 M tris buffer, pH 7.8. Elution was effected by a continuous gradient of KCl in 0.02 M tris buffer, pH 7.4, with an initial concentration of 0.02 M in the mixing chamber and 1 M in the reservoir. The eluate was collected in 20-ml fractions at a flow rate of 4 ml per minute. Active fractions were pooled and dialyzed overnight against 30 volumes of 0.005 M tris buffer, pH 7.2.

Step 4. Alumina CY gel treatment ——— Step 3 enzyme was adjusted to pH

6.8 with 1 M acetate buffer, pH 5.0, and then mixed with an amount of alumina CY gel equivalent to that of the protein. After stirring for 15 minutes, the mixture was centrifuged and the supernatant fluid was discarded. The precipitate was washed twice with 0.02 M potassium citrate, pH 7.0, and then the enzyme was eluted with three 200 ml portions of 0.1 M potassium citrate, pH 7.0.

Step 5. 2nd ammonium sulfate fractionation ——— The eluate was fractionated with ammonium sulfate at pH 7.4. The fraction precipitated between 30 and 45% saturation was dissolved in a small volume of 0.002 M tris buffer, pH 7.0, and dialyzed overnight against 200 volumes of the same buffer.

Step 6. Brushite column chromatography ——— The dialyzed enzyme was applied to a column of brushite (10 ml brushite per mg protein) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. After washing the column with a bed volume of the same buffer, elution was carried out with 0.07 M potassium phosphate buffer, pH 7.2, at a flow rate of about 1 ml per minute. Fractions having a specific activity of over 3 were collected.

Step 7. Sephadex G-200 treatment ——— Step 6 enzyme was precipitated by addition of ammonium sulfate to 50% saturation and dissolved in a small volume of 0.01 M tris buffer, pH 7.4, containing 0.1 M KCl. Then the enzyme was applied to a column of Sephadex G-200 (equivalent to 30 volumes of the enzyme solution) equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of about 5 ml per hour. Eluent was collected in 2-ml fractions and active fractions with the same specific activity were combined. The purification of the enzyme is summarized in Table I.

Crystallization of the enzyme was carried out as follows. Step 7 enzyme was precipitated by addition of ammonium sulfate to 50% saturation. After centrifugation, the precipitate was extracted successively with 45, 40, 35, 30 and 25% ammonium sulfate solution containing 0.01 M tris buffer, pH 7.8, 0.01 M mercaptoethanol and 0.01 M potassium citrate. Each extract was

TABLE I

Purification of the Citrate Cleavage Enzyme of Rat Liver

Step	Protein mg	Total activity* units	Specific activity** x 10^2	Recovery
1. Crude extract	24150	1852	7.67	100
2. 1st ammonium sulfate fractionation	8450	1528	18.08	82.5
3. DEAE-cellulose column chromatography	1375	1024	74.47	55.3
4. Alumina CY gel treatment	640	977	152.7	52.8
5. 2nd ammonium sulfate fractionation	339	788	232.4	42.5
6. Brushite column chromatography	106	401	378.3	21.6
7. Sephadex G-200 treatment	31	165	532.3	8.9

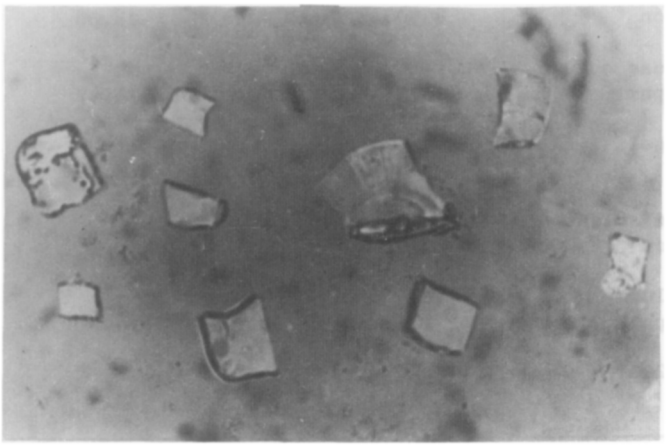
* One unit of enzyme is defined as the amount of enzyme that forms 1 µmole of acetohydroxamate or oxidizes 1 µmole of NADH per minute.
** Specific activity is defined as the number of units per mg protein.

stored at 4°. After several days, small colorless plates had formed from the extract with 30% ammonium sulfate (Fig. 1). The specific activity of the crystalline enzyme was still the same after crystallization.

Properties of the citrate cleavage enzyme

The ultracentrifugal pattern of the enzyme purified as described above exhibited only one sharp and symmetrical boundary (Fig. 2). The homogeneity of the preparation was further confirmed by electrophoresis on cellulose acetate strips. The sedimentation constant, $s_{20,w}$, was found to be 13.5 S and the diffusion coefficient, $D_{20,w}$, was 2.62 x 10^{-7} cm^2/sec. Assuming a partial specific volume of 0.75, the molecular weight of the enzyme was calculated to be approximately 500,000.

After the 1st ammonium sulfate fractionation, the enzyme was very labile on storage. However, its inactivation could be prevented by the addition of 0.01 M mercaptoethanol together with 0.001 M $MgCl_2$. Therefore, all the purification steps were performed in the presence of these two agents.



1. Crystals of the citrate cleavage enzyme.

Fig. 1. Crystals of the citrate cleavage enzyme.

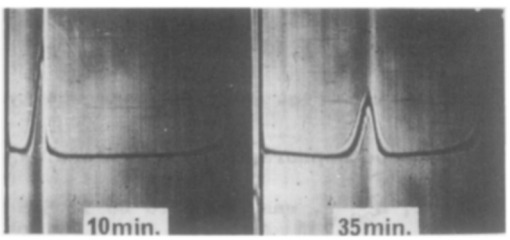


Fig. 2. Sedimentation pattern of the citrate cleavage enzyme. The protein concentration was 0.6%. The photographs were taken at the times indicated after reaching 51,200 r.p.m.

The enzyme was specific for citrate and showed no activity towards cis-aconitate, isocitrate and tricarballylate. Fluorocitrate inhibited the reaction with citrate competitively. ATP was also necessary for the reaction, and GTP and UTP were inactive. The Km values at pH 7.4 were 6.2 x 10^{-4} M for citrate and 2.5 x 10^{-4} M for ATP.

Details of results on the properties of the crystalline citrate cleavage enzyme will be published elsewhere.

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