CRYSTALLIZATION AND SUBUNIT COMPOSITION OF CITRATE LYASE OF *RHODOPSEUDOMONAS GELATINOSA*

F. GIFFHORN and G. GOTTSCHALK
Institut fur Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Gottingen, FRG

Received 3 October 1978

1. Introduction

Previously, a purification procedure for citrate lyase (EC 4.1.3.6) of *Rhodopseudomonas gelatinosa* has been described [1] which because of the instability of the enzyme in some purification steps and of the low yields is not suited for the preparation of large amounts of citrate lyase. In connection with studies on the enzymes which modify citrate lyase by acetylation or deacetylation [2,3] it was necessary to prepare large amounts of enzyme. The procedure used for this purpose is reported here. It involves 2 very effective crystallization steps and yields preparations with a specific activity much higher than measured with purified citrate lyases from other sources. Sodium dodecylsulfate gel electrophoresis revealed the presence of 3 types of subunits in the enzyme as has been shown for citrate lyase from other microorganisms [4–7].

2. Materials and methods

2.1. *Growth of bacteria*

Rhodopseudomonas gelatinosa DSM 149 was grown in 20 l carboys anaerobically in the light for 36 h. The medium contained 30 mM sodium citrate as carbon source, otherwise it was composed as in [2]. The cells were harvested with a Cepa continuous centrifuge, and the cell paste was stored at –20°C.

2.2. *Electrophoresis*

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in tubes and vertical slabs using 7.5% gels as in [8]. The electrophoresis was carried out in 50 mM sodium phosphate buffer (pH 7.0) + 0.1% SDS at a current of 6 mA/tube for 3.5 h and a power of 10 W for 6 h for the slab, respectively. Sample preparation: Citrate lyase crystals were washed twice with 1 ml 0.3 M ice-cold sodium phosphate buffer (pH 7.0). The sediment was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and adjusted to 1 mg protein/ml. The solution was then incubated with 2% SDS and 1% 2-mercaptoethanol for 3 min at 100°C. After staining with Coomassie blue the gel was scanned for protein A_{609} with a Vitatron TLD 100. Protein determinations were performed according to [9].

2.3. *Enzyme assay*

Cells of *R. gelatinosa* used for enzyme purification contained citrate lyase in the inactive HS-form. For determination of enzyme activity it was necessary to acetylate the enzyme chemically with acetic anhydride [10]. The test system was described [1].

Citrate lyase from *Streptococcus diacetilactis* was purified by the method in [11].

2.4. *Materials*

DEAE-Cellulose (DE 52) was purchased from Whatman Biochem., Maidstone, Kent and Alumina C γ gel from Serva, Heidelberg. The chemicals and enzymes were from E. Merck, Darmstadt or from Boehringer, Mannheim.

3. Results

3.1. *Purification and crystallization of citrate lyase*

Unless otherwise specified all steps were carried

out at 0–4°C. Frozen cells, 120 g, were suspended in 360 ml 75 mM potassium phosphate buffer (pH 7.2) containing 3 mM $MgCl_2$ and 1 mM dithioerythritol (DTE). The cells were disrupted by passing the suspension through a French press at 9500–13 500 N/cm^2. Cell debris was removed by centrifugation at 20 000 × *g* for 45 min, and the supernatant (cell extract) was used for purification of citrate lyase.

A 1% (w/v) solution of cetyltrimethylammonium bromide (CTAB) 0.4 vol., was added dropwise to the cell extract with stirring. The precipitation was removed by centrifugation at 20 000 × *g* for 45 min and the supernatant was subjected to precipitation with Alumina C γ gel. Alumina C γ gel (20 mg dry wt/ml) 0.5 vol., was carefully mixed with the supernatant, and the precipitate was removed by centrifugation as above. DTE was added to the supernatant to give 1 mM final conc., then the supernatant was concentrated at room temperature with the hollow fiber system DC 2 HF 10 (Amicon, N. V., Oosterhout) to 100 ml.

The protein solution from the above step was applied to a column (5 cm diam × 25 cm) of DEAE-cellulose which had been equilibrated against the same buffer as used for cell extract preparation. The column was washed with 1200 ml of this buffer and citrate lyase was eluted with 0.1 M potassium phosphate buffer (pH 7.2) containing 0.1 M KCl, 3 mM $MgCl_2$ and 1 mM DTE at 80 ml/h flow rate. Fractions, 6 ml, were collected and citrate lyase eluted with fractions 55–75.

The pooled fractions from chromatography on DEAE-cellulose (120 ml containing 2.8 mg protein/ml) were concentrated in an ultrafiltration cell using an Amicon filter PM 10. At ~5 ml the highly concentrated protein solution became turbid and crystals appeared. Centrifugation in a laboratory centrifuge at 3000 rev./min for 5 min yielded a yellowish sediment which contained 90% of the total citrate lyase activity. It was washed 3 times with each 1 ml ice-cold 0.3 M potassium phosphate buffer (pH 7.0).

Crystal fraction I contained considerable amounts of amorphous material and was subjected to recrystallization. It was dissolved in 10 ml 0.1 M potassium phosphate buffer (pH 7.2) containing 3 mM $MgCl_2$ and 1 mM DTE. After incubation at 30°C for 15 min undissolved material was removed by centrifugation (10 min at 3000 rev./min). The supernatant was kept in an ice-bath and KCl was added to give 0.1 M final conc. During concentration in a 10 ml ultrafiltration cell, crystallization started when ~7 ml was reached. After concentration to 3 ml the crystals were collected by centrifugation, washed once with 1 ml ice-cold buffer as above and suspended in 2 ml 3 M ammonium sulfate. This preparation could be stored at 4°C for 8 months with no detectable loss of activity.

Crystals of the final preparation are shown in fig.1. The purification procedure is summarized in table 1.

3.2. *Reinvestigation of subunit composition*

The specific activity of citrate lyase prepared by the above procedure was 3-times higher than that obtained with the previous procedure. Since the presence of 2 types of subunits with mol. wt 61 000 and 30 000 was reported for the previous preparation [1] the crystalline enzyme preparation was also subjected to SDS–PAGE. It is apparent from fig.2 that citrate lyase of *R. gelatinosa* contains 3 types of subunits as reported for this enzyme from *Enterobacter aerogenes* [4–6] and from *S. diacetilactis* [7,12]. The 3 protein bands corresponded to mol. wt 55 600, 31 600 and 11 400, respectively (fig.3). In order to establish the reliability of our procedure the electrophoresis was run with citrate lyase from *S. diacetilactis* as a marker protein. Values of 53 700, 35 500 and 12 000 were obtained for the subunits.

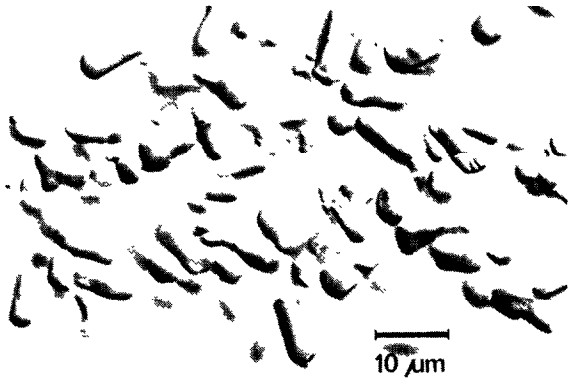

Fig.1. Photograph of citrate lyase crystals using a Leitz photomicroscope with the Leitz-interference contrast T.

Table 1
Purification scheme of citrate lyase of *Rhodopseudomonas gelatinosa*

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Spec. act. (U/mg)	Yield (%)
Cell extract	440	13 200	19 320	1.5	100
CTAB precipitation	520	7920	17 380	2.2	90
Alumina C γ gel	660	2680	15 180	5.7	79
DEAE-cellulose	120	336	12 800	38	66
Crystal fraction I	1.5	50.4	9750	193	51
Crystal fraction II	2.0	26	9300	358	48

4. Discussion

The property of citrate lyase from *R. gelatinosa* to crystallize from a partially purified, concentrated protein solution allowed the preparation of homogeneous citrate lyase in large amounts. The enzyme obtained had spec. act. 360 U/mg protein which was much higher than obtained with the previous purification procedure or reported for the citrate lyases from *S. diacetilactis* and *Ent. aerogenes*. A possible reason for this could be that this purification procedure did not involve gel filtration steps. During purification of citrate lyase from *R. gelatinosa* these steps were always associated with a considerable decrease of the yield [1], and the final enzyme preparations presumably contained considerable amounts of denatured enzyme.

SDS–PAGE showed that citrate lyase of *R. gelatinosa* is also composed of 3 types of subunits. This subunit composition conforms to the model of the hexameric structure of citrate lyase as proposed [1] on the basis of electron microscopic studies.

Acknowledgements

The excellent technical assistance of Mrs Anita Kuhn is gratefully acknowledged. We wish to thank Dr K. Mendgen for taking the photograph. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

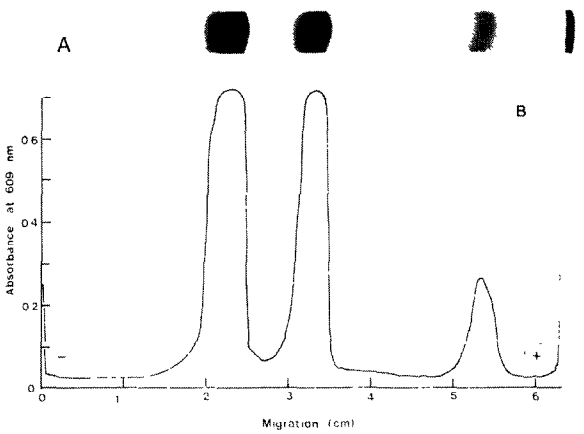

Fig.2. SDS–polyacrylamide gel electrophoresis of crystalline citrate lyase (A) and absorbance scan of the gel stained with Coomassie blue (B). 30 μg of enzyme were subjected to electrophoresis.

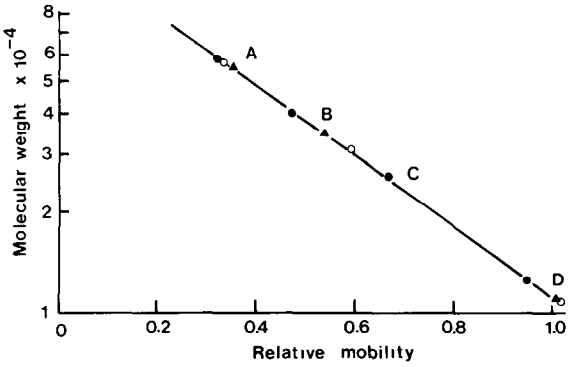

Fig.3. Determination of the molecular weight of the subunits of citrate lyase by vertical slab gel electrophoresis. The calibration curve for a 7.5% gel was obtained using the method in [8]. The molecular weight of the marker proteins was: A, catalase (58 000); B, aldolase (40 000); C, chymotrypsinogen A (25 700); D, cytochrome *c* (12 500). (•) Marker proteins; (○) subunits of citrate lyase from *R. gelatinosa*; (▲) subunits of citrate lyase from *S. diacetilactis*.

References

[1] Beuscher, N., Mayer, F. and Gottschalk, G. (1974) Arch. Microbiol. 100, 307–320.
[2] Giffhorn, F. and Gottschalk, G. (1975) J. Bacteriol. 124, 1052–1061.
[3] Antranikian, G., Giffhorn, F. and Gottschalk, G. (1978) FEBS Lett. 88, 67–70.
[4] Singh, M., Carpenter, D. E. and Srere, P. A. (1974) Biochem. Biophys. Res. Commun. 59, 1211–1218.
[5] Carpenter, D. E., Singh, M., Richards, E. G. and Srere, P. A. (1975) J. Biol. Chem. 250, 3254–3260.
[6] Dimroth, P. and Eggerer, H. (1975) Eur. J. Biochem. 53, 227–235.
[7] Singh, M. and Srere, P. (1975) J. Biol. Chem. 250, 5818–5825.
[8] Weber, K., Pringle, J. R. and Osborn, M. (1972) Methods Enzymol. 25, 3–27.
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[10] Buckel, W., Buschmeier, V. and Eggerer, H. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1195–1205.
[11] Kummel, A., Behrens, G. and Gottschalk, G. (1975) Arch. Microbiol. 102, 111–116.
[12] Bowien, S. and Gottschalk, G. (1977) Eur. J. Biochem. 80, 305–311.