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A Metabolic Variation in *Penicillium spiculisporum* Lehman

II. Purification and Some Properties of the Enzyme Synthesizing (—)-Decylcitric Acid

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An enzyme catalyzing the condensation between lauryl-CoA and oxalacetate to give (—)-decylcitric acid has been isolated from *Penicillium spiculisporum* Lehman and purified. The enzymatic reaction is independent of free SH groups and metal ions. Condensations are also performed with fatty acyl-CoA derivatives lower than the natural and phenylacetyl-CoA. α-Ketoglutarate or pyruvate react very slowly, as compared with oxalacetate. Kinetic data show a normal behaviour with increasing oxalacetate concentration, but a strong substrate inhibition with increasing lauryl-CoA concentration. The enzyme has a pH optimum above 9, a temperature optimum of 30—35°C, and its sedimentation coefficient at 5°C and infinite dilution in a buffer has been calculated to 4.7 S.

In fungi and lichens, as well as in plants a group of structurally related aliphatic acids has been found. The general structure of these acids is characterized by a long alkyl chain attached to a shorter polycarboxylic moiety. The isolation of two optical isomers of decylcitric acid from a strain of *Penicillium spiculisporum* has recently been reported.[1] Other fungal acids are spiculisporic acid (*Penicillium spiculisporum*), mineoluteic acid (*P. funiculosum*), and agaricic acid (*Fomes officinalis*). Among the acids from lichens are found roccellic acid (*Roccella tinctoria, etc.*), rangiformic acid (*Cladonia rangiformis, etc.*) and caperatic acid (*Parmelia caperata, etc.*).

The close relationship between the chemical structures in this group of compounds indicate a common pathway for their biological formation. An obvious way of biosynthesis, which has been suggested by several authors, would be an aldol condensation between a fatty acid derivative and the appropriate α-keto acid. This reaction, analogous to the citrate synthase (EC 4.1.3.7)-catalyzed formation of citric acid, has been demonstrated for the formation of spiculisporic acid in feeding as well as in enzymatic experiments.[2] During the investigation of the enzyme responsible for the synthesis of spiculisporic acid from lauryl-CoA and α-ketoglutaric acid, it appeared that the organism

lost the capacity to produce this enzyme. However, a hydrophilic compound was formed from lauryl-CoA, if, besides α-ketoglutarate, the dialyzable fraction was added to the incubation solution. The dialyzable factor proved to be aspartic acid, and thin-layer chromatography showed that the product formed had properties similar to spiculisporic acid. Investigation of the culture medium led to the discovery of the decylcitric acids, one of which was identical with the enzymatically formed product. Evidently the crude enzyme preparation contained an aspartate transaminase (EC 2.6.1.1), since oxalacetate could easily replace aspartate and α-ketoglutarate in the incubation mixture. As this culture, which produces the decylcitric acid-forming enzyme, emanates from cultures that earlier had been producers of spiculisporic acid, it was of interest to compare the physical and chemical properties of the two condensing enzymes, especially as their catalytic activities differed only in their specificity for homologous α-keto acids.

In this publication the purification and some properties of the decylcitric acid synthase will be described.

EXPERIMENTAL

Culture conditions. Penicillium spiculisporum Lehman was grown as described elsewhere.[1]

Enzyme isolation. After three days of growth, *i.e.*, at the onset of decylcitric acid production, the mycelia from 12 flasks were filtered off. The cells were carefully washed with 3 l of distilled water and suspended to a final volume of 400 ml in a buffer solution (0.2 M Tris.HCl, 0.2 M NaCl, pH 7.8). This suspension can be stored for several months at −20°C without significant loss of enzyme activity. The thawed mycelium was homogenized, preferably with a Ribi Cell Fractionator at 0−10°C and 20 000 psi. On a smaller scale sand-grinding or the X-press (BIOX AB, Uppsala, Sweden) have also proved effective.

Centrifugation of the homogenate at 27 000 *g* and +4°C for 30 min to precipitate cell debris yielded 260 ml of a crude extract that had a highly active decylcitric acid-synthesizing capacity.

To the enzyme solution was added solid ammonium sulfate to 30 % saturation at 0°C, and the precipitate was discarded after centrifugation. The concentration of ammonium sulfate was further increased to 40 % of saturation in the supernatant fluid. The precipitate formed was removed, and the protein fraction obtained in the interval of 40−50 % of ammonium sulfate saturation was collected. It was redissolved in 20 ml of 0.02 M sodium phosphate buffer, pH 7.8. In this solution of low ionic strength, loss of enzyme activity will occur, but the stability is improved by including mM EDTA and mM mercaptoethanol. Non-protein matter was adsorbed on 4.5 g of calcium phosphate gel (Sigma, wet weight). After removal of the solid material, the clear solution was dialyzed against the same phosphate buffer for 1 h at 0°C. The enzyme was thereafter adsorbed on a short column (diameter 19 mm, height about 30 mm) of hydroxylapatite (3 g Bio-Gel HTP). The column was washed with 2 ml of 0.02 M phosphate buffer, and the protein eluted stepwise with 10 ml 0.05 M phosphate buffer, 15 ml 0.07 M phosphate buffer, and 20 ml 0.1 M phosphate buffer. EDTA and mercaptoethanol were included in all the buffers used and the pH was held at 7.8. The enzyme was mainly recovered in the 0.1 M phosphate buffer. Rapid handling was essential for obtaining good recovery of the enzyme from this column. The volume of the enzyme solution was reduced to 2 ml by ultrafiltration at 0°C. After this step the enzyme could be stored frozen for 10 days, although sometimes loss of activity occurred.

Further purification required several batches of this enzyme preparation. Thus, five batches were pooled and then put on a column of Sephadex G 200 (diameter and height 3.0 and 70 cm, respectively) equilibrated with 0.059 M Tris, 0.041 M NaH_2PO_4 0.1 M

Table 1. Purification of decylcitric acid synthase. Average values from six (*two) typical experiments.

Fraction	Volume ml	Protein content mg/ml	Total EU	Yield %	Specific activity EU/mg	Purification -fold
Crude extract	5×260	7.3	5×51	100	0.027	1
Ammonium sulfate 40–50 %	5×20	12.3	5×33	64	0.133	5.0
Calcium phosphate gel	5×20	9.7	5×25	48	0.127	4.7
Dialysis	5×21	9.1	5×24	46	0.123	4.6
Hydroxylapatite column	5×8	3.0	5×7	13	0.273	10
Freezing and G 200 *	1.5	5.0	5	2	0.623	23

NaCl, 0.1 M Na_2SO_4, 10^{-3} M EDTA, 10^{-3} M mercaptoethanol, pH 7.8. This buffer was also used for the subsequent elution, which can be followed in Fig. 1. The enzyme was eluted at 1.5 V_0. From the figure can also be seen that the enzyme activity coincides well with the major protein fraction, indicating a fairly pure state of the enzyme. The most active enzyme fractions were pooled and concentrated by ultrafiltration to a final volume of 1.5 ml. The purification procedure can be followed in Table 1.

Enzyme assay. 900 µl phosphate buffer (0.1 M Na-phosphate, pH 7.8, 10^{-3} M EDTA, 10^{-3} M mercaptoethanol), 100 µl 0.1 M Na-oxalacetate, 25 µl 0.02 M lauryl-1-^{14}C-CoA (0.1 µC) and 4 µg of the enzyme were incubated at 21°C for 30 min. Boiling for 10 min

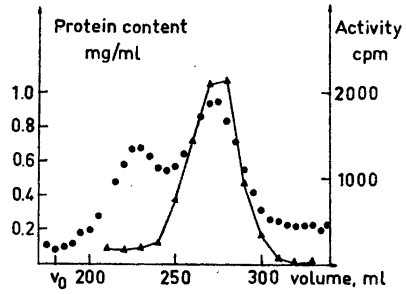

Fig. 1. Elution from Sephadex G 200. The enzyme activity (▲) is given in cpm of decylcitric acid per 10 µl eluate during standard conditions.

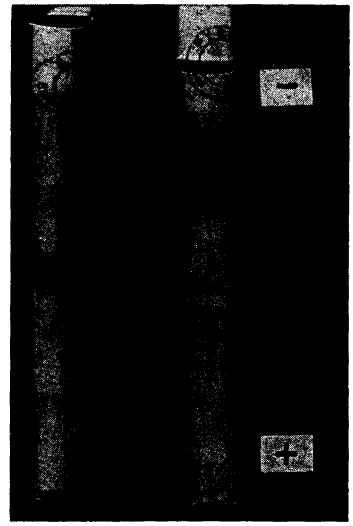

Fig. 2. Electrophoresis of the decylcitrate synthase preparation on 5 % polyacrylamide gel at pH 8.6 and 25 V/cm. 18 µg of protein in 20 µl of buffer has been run for 40 min (at right) and 2 h (at left) and stained with amidoschwarz.

with 100 μl 0.1 M NaOH hydrolyzed remaining thiolester and then, after acidification, lauric acid could be removed by extraction with ligroin (4 × 2 ml). Decylcitric acid was subsequently extracted with 2 ml of ether. The radioactivity was determined in 200 μl of the ether, using 0.5 % BBOT in toluene and measuring in a liquid scintillation spectrometer. The rest of the ether phase was chromatographed on thin-layer for identification (Kieselgel G nach Stahl suspended in 0.1 M oxalic acid, solvent: benzene-dioxane-acetic acid, 90:25:4). Assays with other substrates were performed in the same way, except when using acetyl-CoA and butyryl-CoA; in these cases the incubation mixtures were boiled, acidified, evaporated and extracted with ethanol. The ethanol solutions were chromatographed in three systems together with citric acid.

Purity of the enzyme preparation. a) *Electrophoresis.* A sample of the fraction of maximal enzyme activity from the Sephadex separation was dialyzed against 0.01 M Tris, 0.05 M glycine, pH 8.6 for 1 h. Gel electrophoresis (5 % polyacrylamide) showed a strong protein band without subbands and with minor impurities after staining with amidoschwarz (Fig. 2). b) *Analytical ultracentrifugation.* 1.0 ml of the purified enzyme solution, containing 5.6 mg of protein, was analyzed in a Spinco model E centrifuge adapted with the An—D rotor. The temperature was kept at 5°C and the rotor run at 59 780 rpm. Photos were taken at 8 min intervals, beginning 10 min after reaching maximal speed. The sedimented protein was redissolved and diluted to a concentration of 4.0 mg/ml, and the centrifugation repeated. This procedure was repeated once more, now at a protein concentration of 2.7 mg/ml. The buffer used in the experiments was 0.059 M Tris, 0.041 M NaH_2PO_4. 0.1 M NaCl, 0.1 M Na_2SO_4, 10^{-3} M EDTA, 10^{-1} M mercaptoethanol, pH 7.8. From the Schlieren patterns shown in Fig. 3, the following sedimentation constants have been calculated for the main peak: $s^{5.6}=4.35$, $s^{4.0}=4.41$, $s^{2.7}=4.53$. Extrapolation gave the value for $s_{5,b}°=4.7$ S. A minor decrease (10 %) in enzyme activity was observed in the sedimented protein.

Throughout this investigation protein concentrations have been determined according to the methods of Kalckar [3] and Lowry *et al.*[4] with good agreement between the two methods.

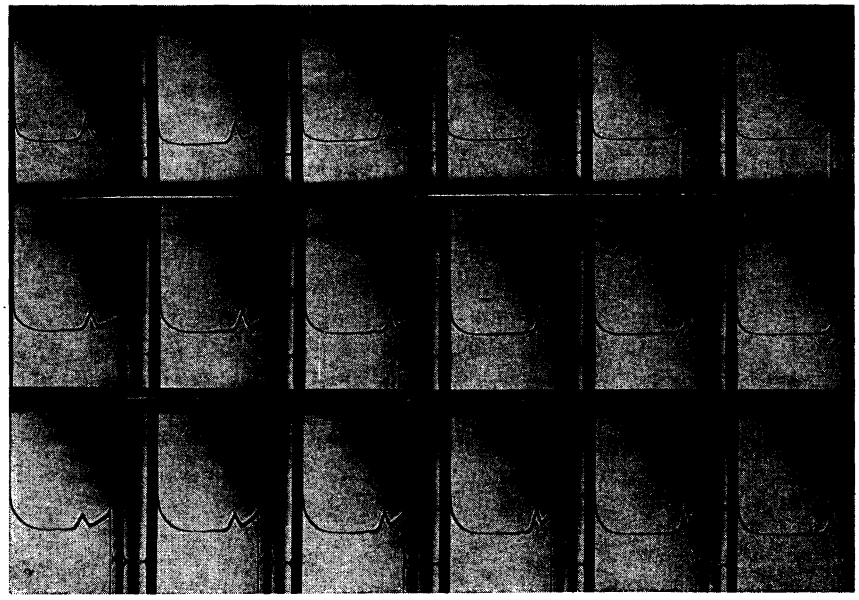

Fig. 3. Schlieren patterns of the decylcitrate synthase preparation. The protein concentrations are 5.6 (upper), 4.0 (middle), and 2.7 mg/ml (lower), and the time interval is 8 min.

Substrates. The Coenzyme A derivatives were prepared from the following ^{14}C-carboxyl-labelled fatty acids, purchased from the Radiochemical Centre, Amersham, England: butyric acid, octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, and phenylacetic acid. The mixed anhydride method of Seubert [5] was used to prepare all derivatives. The keto acids (oxalacetic, α-ketoglutaric, and pyruvic acid) were neutralized to pH 6.5 and diluted to 0.1 M solution before use. *Erythro-* and *threo*-hydroxy-DL-aspartic acids were prepared as described by Hegdcoth and Skinner.[6]

RESULTS AND DISCUSSION

The decylcitrate synthase is a fairly labile enzyme with a half-life of 80 h at 0°C and a concentration of 2 mg/ml in the concentrated buffer described. The inactivation seems to be due to an aggregation of the enzyme molecules to an inactive complex. This is indicated by the appearance in the ultracentrifugational patterns of a protein peak (*s*-value *ca.* 10 S), which increases in proportion in lower enzyme concentration. In spite of lack of accurate knowledge of the viscosity and density of the solution and the partial specific volume of the protein, the measurements of sedimentation constant of the enzyme indicate a value for $s_{20,w}^{\circ}$ of about 5 S corresponding to a molecular weight of the order of 100 000.

The specificity of the enzyme was investigated by substituting ^{14}C-labelled lauryl-CoA in the assay with a series of CoA-derivatives of other ^{14}C-labelled fatty acids. It was found that the following acids could act as substrates in the reaction, although less efficiently: decanoic acid, octanoic acid, butyric acid, acetic acid, and phenylacetic acid. Homologs higher than lauric acid, such as myristic acid and palmitic acid were extremely poor substrates in the reaction. Lacking reference substances the identity of the products formed from the described fatty acids in the condensation with oxalacetate has not been established, except for the formation of citric acid from acetyl-CoA. The results from the experiments have been obtained by observing radioactive spots with expected R_F-values on TLC. Noteworthy is the formation of citric acid and phenylcitric acid from acetyl-CoA and phenylacetyl-CoA, respectively. The rate of citric acid formation, however, is too low to give any reason to believe that the enzyme is identical with the citrate synthase in the organism.

In contrast to the results with the fatty acid moiety, the specificity towards the oxalacetic acid seems to be fairly high; α-ketoglutaric acid and pyruvic acid give rise to less than 10 % of the activity of oxalacetic acid when condensed with lauryl-CoA. As dihydroxymaleic acid is a possible precursor in the formation of mineoluteic acid, this substance was tested as condensation partner. However, it did not yield any reaction product, but when replacing oxalacetate with *erythro*-hydroxyaspartate or especially *threo*-hydroxyaspartate, radioactive condensation products different from mineoluteic acid were obtained, when using the crude enzyme preparation and pyridoxal phosphate as amino group acceptor.

The activity of the enzyme was not inhibited by thiol reagents such as iodoacetamide and *p*-chloromercuribenzoate or EDTA, which exclude either the participation of SH groups in the reaction or the requirement of dissociable metal ions. On the other hand, without affecting the reaction, the enzyme is stabilized by the presence of SH compounds such as mercaptoethanol or

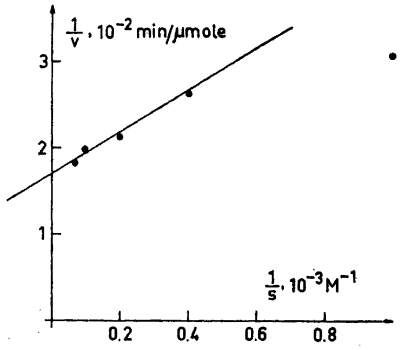

Fig. 4. Rate dependence of oxalacetate concentration. Incubation mixture: lauryl-CoA-1-^{14}C 0.5 μmole, phosphate buffer pH 7.8 90 μmoles, EDTA 0.9 μmole, mercaptoethanol 0.9 μmole, enzyme 4 mU (4 μg of protein), Na-oxalacetate 1—15 μmoles, distilled water 1.0 ml. Incubations were made for 0, 5...30 min and treated as described in the text. The rate was taken from the average of values showing linear dependence of time.

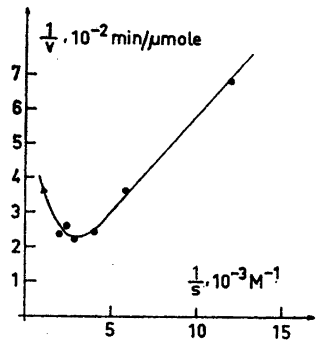

Fig. 5. Rate dependence of lauryl-CoA concentration. The point marked ▲ originates from an experiment at an other time and thus an other specific enzyme activity, but the rate value has been corrected with a simultaneous series of the standard assay to the same number of EU as the rest of the curve. Incubation data are the same as in Fig. 4 except for: Na-oxalacetate 10 μmoles and lauryl-CoA-1-^{14}C 0.1—1 μmole.

cystein; an exception to this is free coenzyme A, which causes a strong inhibition of the reaction.

The rate of reaction was maximal at 30—35°C. The enzyme exhibits a broad pH spectrum in which alkaline pH favours the reaction rate, the pH optimum being above 9.

Because of the lability of the enzyme and the tedious assay method, only limited kinetic investigations have been performed. Fig. 4 shows the linear rate-dependence of oxalacetate concentration in a Lineweaver-Burk diagram. A K_m of 1.2×10^{-3} M was found for oxalacetate under the conditions given in the figure. The rate-dependence of lauryl-CoA concentration is shown in Fig. 5 and in this case substrate inhibition is observed.

Some attempts have been made to study the mechanism of the citric acid formation catalyzed by citrate synthase (Srere,[7] Eggerer,[8] and others), a reaction formally identical with the formation of decylcitric acid. In these experiments no exchange of proton occurred in the methyl group of acetyl-CoA, when oxalacetate was excluded from the incubation mixture, indicating that no free enolate ion ought to be formed during the reaction. This mechanism could very well fit into the stereospecific reaction of decylcitrate synthase as well. As described above, (—)-decylcitric acid is formed in the enzymatic synthesis, which rules out the occurrence of a free carbanion as an intermediate in the reaction. The decylcitric acid contains two assymetric centers, one at the carbon atom carrying the alcohol group, the other at the neighbouring carbon carrying a single proton. Because of the readiness of isomerization in pyridine, it is believed that the (+) and (—) forms of decylcitric acid differ in the configuration around the latter carbon atom.

Other reactions in which the same mechanism presumably is involved have been studied in cell-free solutions. As an example can be mentioned the formation of propylmalate from valeryl-CoA and glyoxalate.[9] Another secondary product from moulds, to which basically a similar mechanism of formation can be ascribed, is glauconic acid as indicated by the labelling experiments done by Bloomer *et al.*[10]

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