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Purification and Properties of Carnitine Acetyltransferase from Citric Acid Producing *Aspergillus niger*

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ABSTRACT

Carnitine acetyltransferase was purified from the citric acid producing *A. niger* mycelium with a protein band showing a relative molecular weight of 77,000 and a pH optimum of 7.3. The K_m values for the purified enzyme for acetyl-CoA and for carnitine were 0.1 m*M* and 1 m*M*, respectively. Carnitine acetyltransferase was located both in the mitochondria and in the cytosol. Both mitochondrial and cytosolic enzyme were purified using ammonium sulfate precipitation, Mono Q and Superose 12 separation. Regarding the localization, except for maximum velocity, there were no differences observed in substrate specificity and inhibition. Inhibition of the enzyme with micromolar concentrations of Cu^{2+} could contribute to a greater citric acid biosynthesis. Carnitine acetyltransferase can be considered as an enzyme necessary for the transport of acetyl groups through mitochondrial membrane in both directions.

Index Entries: Carnitine acetyltransferase; citric acid; *Aspergillus niger;* enzyme kinetics; inhibition.

INTRODUCTION

Carnitine acetyltransferase (CAT) catalyzes the reversible transfer of short chain acyl groups between CoA and carnitine, so that they can cross intracellular membranes. Much is known about acetyl group transfer in

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mammalian cells, particularly liver (*1,2*). In various strains of yeasts, the highest activities of CAT were determined for *Candida utilis* and *Saccharomyces cerevisiae* and lower activities, down to 3% of the highest values were found in lipid-accumulating strains of yeasts *Candida curvata* and *Lipomyces starkeyi* (*3*).

Much work has been done on purification of CAT from the alkane assimilating yeast *Candida tropicalis* (*4,5*). Ueda and coworkers separated and characterized peroxisomal and mitochondrial carnitine acetyltransferases purified from alkane-grown *Candida tropicalis* (*6,7*).

For *Aspergillus niger* it was reported that ATP:citrate lyase (ACL) was the enzyme responsible for acetyl-CoA formation (*8*). Our previous work with *Aspergillus niger* has demonstrated that CAT was also responsible for the transport of acetyl groups across the mitochondrial membrane even in a greater extent than ACL (*9*). It has also been observed that during intensive citric acid production the specific activity of CAT was lowered (*9*). In the present paper we report the results about the isolation procedure of CAT from cytosolic and mitochondrial compartment and describe their characteristics.

MATERIALS AND METHODS

Cultivation of *Aspergillus niger*

Aspergillus niger strain A60 MZKIBK (NRLL 2270) was grown in the medium under the conditions described by Jernejc and coworkers (*10*). After 72 h of fermentation the mycelium was removed by suction filtration.

Preparation of Cell Free Extracts

Mycelium washed with 0.1*M* Tris-HCl buffer was ground under liquid nitrogen. Ground mycelium was transferred to an extraction buffer (1.2*M* ammonium sulfate in 0.1*M* Tris [hydroxymethyl] aminomethane-hydrochloride buffer pH 7.5) according to Kohlhaw and Tan-Wilson (*11*), and disrupted by using a Braun (Melsungen FRG) homogenizer (10 × 7 s). Homogenate was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was used as the crude cell free extract.

Isolation of Mitochondrial and Cytosolic Carnitine Acetyltransferase

For obtaining coupled mitochondria the method described by Watson and Smith (*12*) with some modifications was used. Mycelium was initially washed with an isotonic solution (0.25*M* saccharose, 1 m*M* EDTA, and 50 m*M* Tris-HCl, pH 7.4), grinded with 0.5 mm glass beads in the isotonic solution by a Braun homogenizer for 20 s. The homogenate was centrifuged at 3000*g* for 30 s to remove beads and unbroken mycelium.

The supernatant was further centrifuged at 8000*g* for 15 min at 40°C, which resulted in sedimentation of intact mitochondria. Supernatant was used as cytosolic fraction. After twofold washing with isotonic solution the mitochondrial pellet was resuspended in 0.1*M* Tris-HCl buffer pH 7.4 and sonicated for 1 min using a W-385 ultrasonicator (Ultrasonics, Inc). The sonicate was centrifuged at 12,000*g* for 20 min at 4°C and the supernatant was used as mitochondrial extract.

Enzyme Assay

CAT (EC 2.3.1.7) was assayed spectrophotometrically by following the formation of CoA-SH, which forms a yellow adduct with 5,5′-dithiobis (2-nitro benzoic acid) according to the method described by Kohlhaw and Tan-Wilson (*11*). For determination of V_{max} values 10-fold concentration of K_m values have been taken. To calculate specific activities, proteins were measured according to the method of Bradford (*13*) using crystalline bovine serum albumin as standard.

Purification of Carnitine Acetyltransferase

Crude cell free extract from the whole *A. niger* mycelium was further elaborated. Ammonium sulfate was added to the final concentration of 65% saturation. The mixture was stirred for 30 min at 4°C and then centrifuged at 12,000*g* for 15 min and the supernatant discarded. Precipitated proteins were resuspended in 0.02*M* Tris-HCl buffer pH 8.0 and dialyzed overnight against the same buffer. The sample was then concentrated in an Amicon ultrafiltration cell using a YM 30 membrane filter. The concentrate obtained was applied to a Mono Q 5/5 column (Pharmacia) connected to FPLC system and eluted with 20 mL of a 0–0.5*M* linear gradient of NaCl in 0.2*M* Tris-HCl buffer pH 8.0 at a flow rate of 1 mL/min. Fractions showing CAT activity were pooled and concentrated. Further purification was done on a Pharmacia Superose 12 HR 10/30 column and eluted with 0.1*M* Tris-HCl buffer pH 8.0 at a flow rate of 0.5 mL/min.

To the separated mitochondrial and cytosolic fractions, ammonium sulfate was added to obtain 30% saturation. Mixtures were stirred on ice bath for 30 min and then centrifuged at 12,000*g* for 15 min. Pellets were discarded and ammonium sulfate added to the supernatant to a final saturation of 65% and stirred on ice for another 30 min. After centrifugation at 12,000*g* for 15 min pellets were resuspended in 0.02*M* Tris-HCl buffer pH 8.0 and dialyzed against the same buffer. Further purification of each mitochondrial and cytosolic CAT was done in the same manner as with the whole cell enzyme extract.

Electrophoresis

A 12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed, proteins were silver stained and molecular mass determined by using standards with known molecular weights (Sigma LMW standards) (*14*).

Table 1
Purification Steps of *Aspergillus niger* Carnitine Acetyltransferase

Purif. step	Total protein, mg	Total activity, mU/mL	Yield, %	Specific activity, mU/mg	Degree of purific., fold
Cell free extract	107.10	8782	100	82	1
$(NH_4)_2SO_4$ (30–65%)	23.30	7933	90	340	4
Mono Q	8.02	3465	39	432	5
Superose 12	0.98	1044	12	1065	13

RESULTS AND DISCUSSION

Level of Carnitine Acetyltransferase Activity

As reported previously, the specific activity of CAT measured in a crude enzyme preparation of the whole *A. niger* cells increased slowly until day 3 of fermentation (*9*). The increase was more pronounced under citric acid nonaccumulating conditions. In a citric acid accumulating process after three days the specific activity dropped and retained low values till the end of the fermentation, while with nonaccumulating one after a small drop, the activity increased again to high values (*9*).

Purification of Carnitine Acetyltransferase

Since the highest specific activity of CAT in citric acid producing mycelia was observed at the day 3 of cultivation, 3-d-old mycelia was taken for extraction and purification of CAT. The enzyme was purified in three steps: After ammonium sulfate precipitation ionic chromatography was used. Final purification was done by gel filtration. The initial specific activity of 82 mU/mg protein was increased to 1065 mU/mg protein with a 13-fold purification (Table 1).

Separation of Cytosolic and Mitochrondrial Carnitine Acetyltransferases

Respective preparations from fungal mycelium were subjected to purification as described under Materials and Methods. Figure 1 shows elution patterns of cytosolic CAT. Cytosolic as well as mitochondrial enzyme were precipitated with ammonium sulfate (30–65% saturation), eluted from Mono Q in an interval of 0.04–0.14*M* NaCl in 0.02*M* Tris-HCl buffer pH 8.0. Concentrated fractions with CAT activity were applied to Superose 12. In the fraction eluted at 11.5–12.0 mL a single peak showed CAT activity. By using Sigma low molecular weight markers for gel filtration, apparent M_R of the enzyme was estimated to be 77,000.

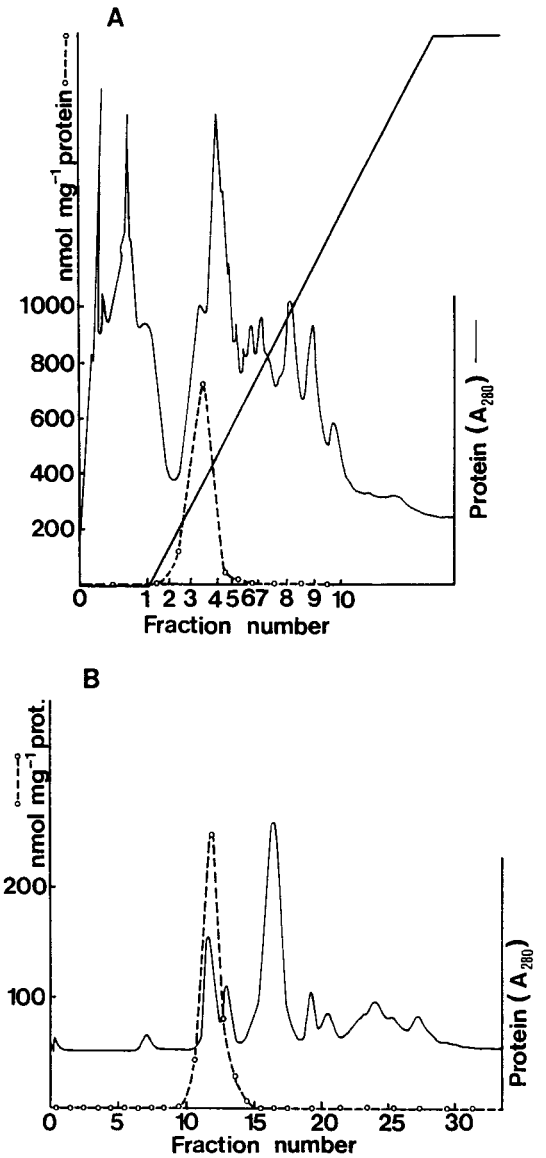

Fig. 1. Elution patterns of cytosolic carnitine acetyltransferase from Mono Q (**A**) and Superose 12 column (**B**).

Properties of Carnitine Acetyltransferase

For the purified enzyme pH optimum of 7.3 was determined. The pH curve showed a steep decrease toward lower pH values while up to a pH of 8.5 the activity decreased slowly. Similar results were observed with *Candida tropicalis* with a pH optimum of 7.2 (*15*). For mammalian carnitine acetyltransferases the pH optima lie in a more alkaline range (with a peak at pH 8.7 for human liver CAT and a similar value was also found in pig heart) (2).

Table 2
Properties of Cytosolic and Mitochondrial Carnitine Acetyltransferase

Property	Cytosolic CAT	Mitochondrial CAT
V_{max} (mU/mg)	67	178
Optimum pH	7.3	7.3
K_m acetyl-CoA (m*M*)	0.11	0.09
K_m carnitine (m*M*)	1.10	1.00
Inhibitors	Cu, Mg, Ca, Mn	Cu, Mg, Ca, Mn

K_m was 1 m*M* for carnitine and 0.1 m*M* for acetyl-CoA, which is in accordance with that found in *Saccharomyces cerevisiae* (*16*), whereas K_m values for the enzyme isolated from animal origin (pigeon, pig, mouse, rat) were substantially lower (*17*). Divalent cations Mg^{2+}, Ca^{2+}, Mn^{2+}, and Cu^{2+} caused different degrees of inhibition. Noncompetitive inhibition with all cations tested was observed in m*M* concentrations (K_i determined was 1.5 m*M* for Mg^{2+}, 2.6 m*M* for Ca^{2+}, and 5.0 m*M* for Mn^{2+}), while copper inhibited in u*M* amounts (K_i was 4.6 u*M*). It was reported that sulfydryl ligating dications such as Zn^{2+} and Hg^{2+} are inhibitors of bovine and pig heart CAT (*17*). Minor effects were noted for Ca^{2+} and Mg^{2+} on the bovine enzyme, whereas Zn^{2+} inhibited in micromolar concentrations. Unlike mammalian CATs, yeast enzyme is sensitive to both Ca^{2+} and Mg^{2+} (*18*). With *A. niger* it was reported that addition of minor amounts of Cu^{2+} to the production media (*10*) and lower specific activity of CAT (*9*) enhanced citric acid biosynthesis. So inhibition of CAT with Cu^{2+} could contribute to higher citric acid yields.

As seen in Table 2 for isolated carnitine acetyltransferases, both cytosolic and mitochondrial ones, except for their specific activity, there was nearly no difference in their properties being the same as for the whole cell enzyme.

On SDS-PAGE each enzyme preparation was found to consist of a single band with relative molecular weight of 39,000 (Fig. 2), what suggested that the enzyme with M_R of 77,000 as determined by gel filtration was split into two equal subunits.

For rat liver CAT it was reported that the enzyme from the mitochondrial fraction consisted of two polypeptides (36.5 kDa and 27 kDa) as determined by SDS-PAGE, but only one polypeptide (67.5 kDa) was observed when protease inhibitors were added to the extract (*19*). For *C. tropicalis* it was also reported that CAT consisted of two subunits (*5,6,18*). They obtained molecular weights ranging from 52–60 kDa for whole cell, mitochondrial, and peroxisomal CATs. In spite of many similarities between cytosolic and mitochondrial enzymes found in *A. niger* cells, such as substrate specificity, pH optimum, inhibition with divalent cations, there could be some differences in the isoelectric point and amino acid compo-

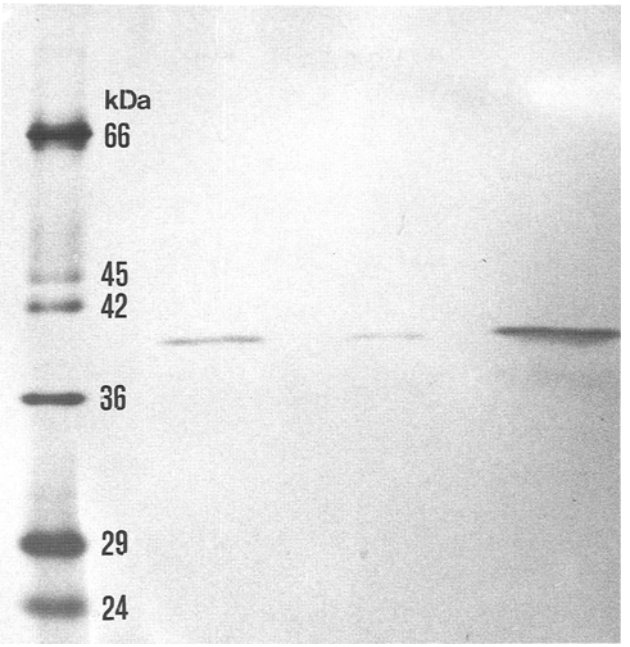

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified enzymes. Lane 1, molecular weight markers (LMW, Sigma); Lane 2, enzyme purified from cytosol; Lane 3, enzyme purified from mitochondrial fraction and Lane 4, enzyme purified from whole fungal cells.

sition as it was observed for *C. tropicalis* (*6*). However, only one protein band was reported for mammalian CAT with a molecular weight of 60.5 kDa for human liver CAT and 62 kDa for the beef heart enzyme (*2*).

In all experiments CAT was isolated from citric acid producing *A. niger* grown in a substrate that forces the fungus to accumulate and excrete high amounts of citric acid. Tricarboxylic acid cycle enzymes are located in mitochondria. This suggested that acetyl-CoA necessary for the synthesis of citrate in mitochondria is supplied from the cytosol by the transport via acetylcarnitine shuttle mediated by carnitine acetyltransferase. This speculation seems possible since CAT isoenzymes have been found to be localized both in cytosol and in mitochondria.

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