

# Tricarboxylic Acid Cycle Enzymes of the Ectomycorrhizal Basidiomycete, *Suillus bovinus*

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In crude cell extracts of the ectomycorrhizal fungus, *Suillus bovinus*, activities of citrate synthase, aconitase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase, and malate dehydrogenase have been proved and analyzed. Citrate synthase exhibited high affinities for both its substrates: oxaloacetate ( $K_m = 0.018$  mM) and acetyl-CoA ( $K_m = 0.014$  mM). Aconitase showed better affinity for isocitrate ( $K_m = 0.62$  mM) than for citrate ( $K_m = 3.20$  mM). Analysis of isocitrate dehydrogenase revealed only small maximum activity ( $60 \text{ nmol} \times \text{mg protein}^{-1} \times \text{min}^{-1}$ ), the enzyme being exclusively NADP<sup>+</sup>-dependent. Using the artificial electron acceptor dichlorophenol indophenol, activity and substrate affinity of succinate dehydrogenase were rather poor. Fumarase proved Fe<sup>2+</sup>-independent. Its affinity for malate was found higher ( $K_m = 1.19$  mM) than that for fumarate ( $K_m = 2.09$  mM). High total activity of malate dehydrogenase could be separated by native PAGE into a slowly running species of (mainly) cytosolic (about 80%) and a faster running species of (mainly) mitochondrial origin. Affinities for oxaloacetate of the two enzyme species were found identical within limits of significance ( $K_m = 0.24$  mM and  $0.22$  mM). The assumed cytosolic enzyme exhibited affinity for malate ( $K_m = 5.77$  mM) more than one order of magnitude lower than that for oxaloacetate. FPLC on superose 12 revealed only one activity band at a molecular mass of  $100 \pm 15$  kDa. Activities of 2-oxoglutarate dehydrogenase and of succinyl-CoA synthetase could not be found. Technical problems in their detection, but also existence of an incomplete tricarboxylic acid cycle are considered. Metabolite affinities, maximum activities and pH-dependences of fumarase and of malate dehydrogenase allow the assumption of a reductive instead of oxidative function of these enzymes *in vivo*.

## Introduction

*Suillus bovinus*, a common basidiomycete in temperate climates, is a frequent mycorrhizal partner of various coniferous trees. Being often in contact with one of the most forested species, *Pinus sylvestris*, it is interesting not only for scientific understanding of a wide-spread symbiotic system, but also with respect to commercial timber production.

As a result of analyzing the basic metabolism of this fungus, recently we reported on existence of

the complete sequence of glycolytic enzymes (Kowallik *et al.*, 1998). This indicated initial breakdown of carbohydrates for energy and metabolite supply by an ubiquitous route in living matter. Fructose-6-phosphate kinase and hexokinase exhibited very low maximum *in vitro* activities, which in addition showed common regulation of this pathway. There was also a remarkable low activity of aldolase. This corresponds to results with various heterotrophic organisms ranging from the protozoon *Tetrahymena* through insect muscles and tissues of molluscs to those of vertebrates (see Gäde, 1983). But in spite of the low activity and of no particular high affinity towards its substrate, in no case this enzyme has been proven to be of regulatory significance. Highest activity was found for triosephosphate isomerase, which is also

**Abbreviations:** DCPIP, dichlorophenol indophenol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; PMS, phenazine methosulfate.

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known for other heterotrophic sources (Lambeir *et al.*, 1987).

We extended our work on *Suillus bovinus* by investigating the further metabolism of the glycolytic end product, pyruvate. The assumption was tested first that complete oxidation via the tricarboxylic acid cycle might be involved. Activity of some of its enzymes have been reported for mycorrhizal fungi (Niederpruem, 1965; Hascaylo, 1973; Martin *et al.*, 1987, 1988; Jakobsen, 1991; Griffin, 1994, see also Hampp *et al.*, 2000), but to our knowledge no complete sequence has been published.

The results of this study indicate existence of most, but not all of the eight enzymes. It is discussed, whether the organism uses only several steps of the tricarboxylic acid cycle or whether we did not succeed in detecting activity of two of its enzymes.

## Material and Methods

Axenic cultures of *Suillus bovinus* (L. ex Fr.) O. Kuntze, Boletaceae, were used. Mycelia were isolated from fruiting bodies collected at Sennefriedhof Bielefeld by Dr. U. Röder, Department of Ecology, University Bielefeld.

### Growth conditions

Mycelia were grown in suspension cultures in liquid medium according to Kottke *et al.* (1987) containing 7.6 mmol/l  $\text{NH}_4$ -ions as nitrogen and 55 mmol/l (= 10 g/l) glucose as carbon sources. pH of the medium was set to 5.0 at start. It dropped with time, mainly because of uptake of ammonium ions, but never left the range for optimum growth (see Kowallik *et al.*, 1998). The autoclaved (5 min at 1.2 bar) medium was inoculated with suspended hyphae and the resulting suspension filled in sterilized (4 h at 150 °C) culture tubes with an inlet for aeration at their bottom. Length of tubes was 45 cm, Ø 4 cm. For growth, tubes were placed in a water bath of 25 °C in the dark and the suspensions were aerated continuously with compressed air.

### Preparation for enzyme analysis

3–4 days after inoculation, mycelia were harvested on filter paper in a Buchner funnel. The

resulting pellet was washed with water 3 times and resuspended in 0.1 M phosphate buffer pH 7.5 (1/5 w/v). Cells were broken by grinding with sea sand in a mortar under cooling with liquid nitrogen. After separation from sand and large fragments by filtration through 4 layers of cheese cloth, the resulting homogenates were centrifuged for 20 min at 20000×g and 4 °C (Sorvall RC-5 Superspeed Refrigerated Centrifuge). The resulting supernatants were used as crude extracts. In some cases, the sediments were analyzed for enzyme activities, in addition. They were suspended in the above buffer containing 0.1% Triton TX 100.

### Isolation of mitochondria

A slightly modified method of Teague and Henny (1973) was used. Washed hyphae, dissolved in 5 volumes of ice-cold 25 mM TRIS-buffer pH 7.6 containing 300 mM mannitol, 1 mM EDTA, 10 mM  $\text{K}_2\text{HPO}_4$ , 5 mM  $\text{MgCl}_2$ , 4 mM mercaptoethanol, 0.1% bovine serum albumin (w/v) and – to prevent foam – traces of octanol, were broken in a Waring blender (30 sec, high speed). The resulting homogenate was passed through 4 layers of cheese cloth and the filtrate cleaned further by low speed centrifugation (500×g, 10 min). From the resulting supernatant mitochondria were separated by centrifugation at 5000×g for 10 min. They were further purified by washing with large volumes of the above buffer including 2 repeated centrifugation cycles (500×g, 10 min and 5000×g, 10 min). Mitochondria were broken by two methods, either by osmotic shock (sediments were dissolved in hypotonic phosphate buffer, 20 mM, pH 7.6) or by freezing and thawing (sediments, dissolved in 100 mM phosphate buffer pH 7.6 at 0 °C, were exposed to –20 °C for 30 min and brought back to 0 °C thereafter). In both cases, resulting suspensions were finally cleaned from membrane fractions by centrifugation at 20000×g for 15 min.

### Enzyme assays

All enzyme assays, based on literature as indicated below, were optimized for the *Suillus* crude extract; i.e., maximum *in vitro* activities were determined. Optimum concentrations for substrates and cofactors, optimum pH, and appropriate concentrations of auxiliary enzymes and of electron donors/acceptors had to be determined. These

data will not be presented in detail here. It should be mentioned that all enzyme activities could be improved – sometimes by more than 100% – over those obtained with literature assays developed for different living materials.

*Citrate synthase* (Citrate oxaloacetate-lyase [CoA-acetylating], EC 4.1.3.7) (after Bergmeyer, 1974)

Absorbance changes at 232 nm resulting from decrease in acetyl-CoA by oxaloacetate-dependent formation of citrate by the enzyme were determined.

Assay: 100 mM TRIS-NaOH-buffer pH 8.0, 0.2 mM acetyl-CoA, 25 µg crude cell extract protein/ml test volume, 0.2 mM oxaloacetate (start).

*Aconitase* (Aconitate hydratase; Citrate [isocitrate] hydrolyase, EC 4.2.1.3) (after Anfinsen, 1955, Fansler and Lowenstein, 1969)

Activity of the enzyme was determined in both directions. Conversions of citrate or of isocitrate were determined by absorbance changes at 240 nm resulting from production of aconitate from both substrates by the enzyme.

Assay 1: 100 mM phosphate buffer pH 7.5, 50 µg crude cell extract protein/ml test volume, 40 mM citrate (start).

Assay 2: 100 mM phosphate buffer pH 7.5, 50 µg crude cell extract protein/ml test volume, 10 mM isocitrate (start).

*Isocitrate dehydrogenase* (threo-D<sub>5</sub>-Isocitrate: NAD(P)<sup>+</sup> oxidoreductase [decarboxylating], EC 1.1.1.42) (after Bergmeyer, 1974)

Absorbance changes at 334 nm resulting from NADP<sup>+</sup> reduction by oxidation of isocitrate to 2-oxoglutarate by the enzyme were determined.

Assay: 100 mM phosphate buffer pH 7.0, 3.5 mM MgCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup>, 255 µg crude cell extract protein/ml test volume, 1.1 mM isocitrate (start).

*2-Oxoglutarate dehydrogenase (complex of 3 enzymes)*

E1 = 2-Oxoglutarate dehydrogenase, EC 1.2.4.2,  
E2 = Dihydrolipoyl transsuccinylase EC 2.3.1.61,  
E3 = Dihydrolipoyl dehydrogenase EC 1.8.1.4)  
(after Stein-Ludolph and Kowallik, 1985)

Absorbance changes at 334 nm resulting from NAD<sup>+</sup> reduction by oxidation of 2-oxoglutarate to succinyl-CoA by the enzyme complex were determined.

Assay: 40 mM glycylglycine-buffer pH 7.8, 1.0 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, 0.125 mM coenzyme A, 3.0 mM NAD<sup>+</sup>, 110 µg crude cell extract protein/ml test volume, 10 mM 2-oxoglutarate (start).

*Succinyl-CoA synthetase* (Succinate:)(CoA ligase [GDP], EC 6.2.1.4) (after Cha, 1969)

Absorbance changes at 334 nm resulting from NADH oxidation by reduction of pyruvate to lactate by added lactate dehydrogenase (LDH) were determined. Pyruvate was produced by pyruvate kinase (PK) from added phosphoenolpyruvate and from GDP produced by formation of succinyl-CoA from succinate, coenzyme A and GTP by the enzyme.

Assay: 110 mM TRIS-buffer pH 7.4, 0.1 mM coenzyme A, 0.1 mM GTP, 10 mM MgCl<sub>2</sub>, 40 units PK/ml test volume, 110 units LDH/ml test volume, 110 µg crude cell extract protein/ml test volume, 50 mM succinate (start).

*Succinate dehydrogenase* (Succinate: [acceptor] oxidoreductase, EC 1.3.99.1) (after Hatefi, 1978)

Reduction of the artificial electron acceptor DCPIP coupled to oxidation of succinate to fumarate by the enzyme was used. Decrease in absorbance at 600 nm was determined.

Assay: 33 mM phosphate buffer pH 7.25, 0.1 mM DCPIP, 0.1 mM PMS, 3.3 mM KCN, 530 µg crude cell extract protein/ml test volume, 3.3 mM succinate (start). Measurements had to be performed under anaerobic conditions. For this, the assay mixture was gassed with purified nitrogen before and after addition of succinate for 2 × 2 min in closed cuvettes with gas inlet. PMS had to be prepared every day and was kept ice-cooled in the dark.

*Fumarase* (L-malate hydro-lyase, EC 4.2.1.2) (after Bergmeyer, 1974)

Activity of the enzyme was determined in both directions. Conversion of fumarate to malate was determined by absorbance changes at 334 nm due

to reduction of  $\text{NAD}^+$  by oxidation of the produced malate to oxaloacetate. Product inhibition was prevented by amination of oxaloacetate to aspartate by added aspartate transaminase (GOT) and glutamate. Conversion of malate was determined by absorbance changes at 240 nm resulting from fumarate production by malate dehydration by the enzyme.

Assay 1: 300 mM glycylglycine + 50 mM glutamate-buffer pH 9.5, 2 mM  $\text{NAD}^+$ , 4 units malate dehydrogenase/ml test volume, 1 unit GOT/ml test volume, 120  $\mu\text{g}$  crude cell extract protein/ml test volume, 30 mM fumarate (start).

Assay 2: 70 mM phosphate buffer pH 7.40, 20  $\mu\text{g}$  crude cell extract protein/ml test volume, 50 mM malate (start).

*Malate dehydrogenase* (L-malate: $\text{NAD}^+$  oxidoreductase, EC 1.1.1.37) (after Bergmeyer, 1974)

Activity of the enzyme was determined in both directions. Malate conversion was followed by absorbance changes at 334 nm resulting from  $\text{NAD}^+$  reduction due to oxidation of malate to oxaloacetate by the enzyme. Product inhibition was prevented by removal via transamination to aspartate of the reaction product oxaloacetate by added glutamate and aspartate transaminase (GOT). Oxaloacetate conversion was followed by absorbance changes at 334 nm resulting from NADH oxidation by reduction of oxaloacetate to malate by the enzyme.

Assay 1: 300 mM glycylglycine + 50 mM glutamate-buffer pH 9.5, 2.0 mM  $\text{NAD}^+$ , 1.0 units GOT/ml test volume, 60  $\mu\text{g}$  crude cell extract protein/ml test volume, 16 mM malate (start).

Assay 2: 80 mM phosphate buffer pH 7.50, 0.2 mM NADH, 25  $\mu\text{g}$  crude cell extract protein/ml test volume, 0.5 mM oxaloacetate (start).

#### *Soluble protein*

Soluble protein in crude extracts was determined according to Lowry *et al.* (1951) with bovine serum albumin as reference.

#### *Fast performance liquid chromatography (FPLC)*

0.25 ml crude cell extract, filtered through a 0.2  $\mu\text{m}$  sterile filter (Sartorius GmbH, Göttingen Germany), were applied to a Superose 12 column

(HR 10/30, Pharmacia, Uppsala Sweden). The column was equilibrated and eluted with 100 mM phosphate buffer pH 7.5. Fractions of 250  $\mu\text{l}$  were collected at a flow rate of  $25 \text{ ml} \times \text{min}^{-1}$  at room temperature. For molecular mass determination, the column was calibrated with following standards: thyroglobulin dimer 1338 kDa, thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, aldolase 158 kDa, albumin 67 kDa, hemoglobin 64.5 kDa, chymotrypsin 25 kDa, myoglobin 17.2 kDa, and cytochrome c 12.2 kDa.

#### *Ion exchange chromatography*

Crude cell extracts were separated on Servacel, cellulose ion exchanger SE 23, (column  $2.2 \times 8 \text{ cm}$ ) using phosphate buffer pH 6.0 for elution. The column was washed with 2 bed volumes of 10 mM phosphate buffer and developed with a linear phosphate gradient (10–100 mM). 1.5 ml fractions were collected at a flow rate of  $90 \text{ ml} \times \text{h}^{-1}$ . Phosphate concentration in fractions was calculated from the refraction index (Abbe universal refractometer, Schmid and Haensch, Berlin Germany).

#### *Polyacrylamide gel electrophoresis (PAGE)*

Malate dehydrogenase containing samples were separated by native PAGE using 6% stacking gel (0.125 mM TRIS-buffer pH 6.8) and 10% separation gel (0.125 mM TRIS-buffer pH 8.8). Electrophoresis was performed at constant current (60 mA and 155 V in the beginning) for 150 min in a cold room ( $4^\circ\text{C}$ ). Gels were tested for enzyme activity with nitroblue tetrazolium (0.5 mM) plus PMS (75  $\mu\text{M}$ ) coupled to the above described assay 1. Resulting  $\text{NAD}^+$  led to formation of deep blue formazane bands. Dependent on total enzyme activity, the reaction was terminated after 5–15 min by addition of stop reagent (50% methanol, 12% acetic acid, 0.05% formaldehyde).

#### *Chemicals*

All biochemicals were purchased from Roche Diagnostics GmbH, Mannheim Germany, or from Sigma-Aldrich Chemie GmbH, Deisenhofen Germany.



## Results

**Citrate synthase.** Affinities for oxaloacetate ( $K_m = 0.018$  mM) and for acetyl-Co A ( $K_m = 0.014$  mM) are relatively high, and there is positive cooperativity for both substrates as well. No influence of  $Mg^{2+}$  ions is detected.

**Aconitase.** Maximum activity of the enzyme is found similar to that of citrate synthase, while affinities for its substrates, citrate and isocitrate, are much lower ( $K_m = 3.20$  mM and  $K_m = 0.62$  mM). Addition of  $Fe^{2+}$  ions (see: Fansler and Lowenstein, 1969) does not improve enzyme activity or substrate affinity, neither for citrate nor isocitrate.

**Isocitrate dehydrogenase.** In *Suillus bovinus*, only the  $NADP^+$ -dependent species can be detected. Affinity for this electron acceptor is about 6 times higher ( $K_m = 0.03$  mM) than that for the substrate, isocitrate ( $K_m = 0.19$  mM). There is no

isocitrate homotropy detectable ( $n = 0.98$ ). Enzyme activity is strictly dependent on divalent ions; added  $MgCl_2$  yields half maximum effect at 0.65 mM in the assay mixture. There is no indication for any cooperative function of these ions ( $n = 0.99$ ). Maximum *in vitro* activity of the enzyme is comparatively low ( $60 \pm 2$  nmol  $\times$  mg protein $^{-1}$   $\times$  min $^{-1}$ ). Additional extraction with buffer containing 0.1% Triton TX 100 of the sediments resulting during preparation of crude cell extracts does not yield any further activity. It also does not result in activity of an  $NAD^+$ -dependent enzyme species. Most – if not all – isocitrate dehydrogenase activity resides in the mitochondrion. Cell extracts without broken organelles exhibit only traces of respective activity. Whether this is due to a cytosolic species of the enzyme or to a few damaged mitochondria cannot be decided as yet.

Table I. Apparent  $K_m$ - or  $S_{0.5}$ -values, Hill coefficients and maximum activities of enzymes of the tricarboxylic acid cycle in crude extracts of *Suillus bovinus*. Growth conditions: liquid inorganic medium with glucose as sole carbon source and ammonia as sole nitrogen source, initial pH 5.0, continuous aeration with compressed air, 25 °C, darkness. For preparation of crude cell extracts and of mitochondria and for enzyme assays see Material and Methods.

| Enzyme  | Substrate              | $K_m$ or $S_{0.5}$<br>[mM]          | Hill coefficient<br>[n]            | Maximum activity<br>[nmol $\times$ mg protein $^{-1}\times$ min $^{-1}$ ] |
|---|------------------------|-------------------------------------|------------------------------------|---|
| Citrate synthase                                | oxaloacetate           | $0.018 \pm 0.002$                   | $1.84 \pm 0.10$                    | $256 \pm 6$   |
|   | acetyl-CoA             | $0.014 \pm 0.001$                   | $1.97 \pm 0.05$                    |   |
| Aconitase                                       | citrate                | $3.20 \pm 0.21$                     | $1.00 \pm 0.04$                    | $110 \pm 4$   |
|   | isocitrate             | $0.62 \pm 0.03$                     | $1.04 \pm 0.05$                    | $258 \pm 14$  |
| Isocitrate dehydrogenase<br>$NADP^+$ -dependent | isocitrate<br>$NADP^+$ | $0.19 \pm 0.02$<br>$0.03 \pm 0.002$ | $0.98 \pm 0.10$<br>$1.00 \pm 0.07$ | $60 \pm 2$  |
| Isocitrate dehydrogenase<br>$NAD^+$ -dependent  |                        | no activity<br>detected             |                                    |   |
| 2-Oxoglutarate<br>dehydrogenase                 |                        | no activity detected                |                                    |   |
| Succinyl-Co-A synthetase                        |                        | no activity detected                |                                    |   |
| Succinate dehydrogenase                         | succinate              | $0.38 \pm 0.03$                     | $0.96 \pm 0.06$                    | $15 \pm 1$  |
| Fumarase  | fumarate               | $2.09 \pm 0.11$                     | $1.00 \pm 0.05$                    | $94 \pm 3$  |
|   | malate                 | $1.19 \pm 0.04$                     | $1.06 \pm 0.04$                    | $222 \pm 8$   |
| Malate dehydrogenase<br>(crude extract = total) | oxaloacetate           | n.d.                                | n.d.                               | $29282 \pm 1285$  |
|   | malate                 | n.d.                                | n.d.                               | $854 \pm 21$  |
| (mitochondria)                                  | oxaloacetate           | $0.22 \pm 0.02$                     | $1.00 \pm 0.09$                    | n.d.  |
|   | $NADH$                 | $0.068 \pm 0.004$                   | $1.00 \pm 0.05$                    |   |
|   | oxaloacetate           | $0.24 \pm 0.01$                     | $1.02 \pm 0.01$                    | n.d.  |
|   | $NADH$                 | $0.070 \pm 0.004$                   | $0.99 \pm 0.10$                    |   |
|   | malate                 | $5.77 \pm 0.24$                     | $1.03 \pm 0.04$                    | n.d.  |
|   | $NAD^+$                | $0.46 \pm 0.02$                     | $1.04 \pm 0.02$                    |   |

n.d. = not determined

Up to now, we were unable to detect activity of 2-oxoglutarate dehydrogenase and we also failed to find succinyl-Co synthetase activity.

**Succinate dehydrogenase.** Enzyme activity is found extremely low and also substrate affinity is comparatively poor ( $K_m = 0.38$  mM). It remains open, whether the artificial assay system used does not reflect comparable optimum activities or whether the enzyme is a severe bottle neck within a metabolic sequence.

**Fumarase** turned out to be independent of added  $\text{Fe}^{2+}$ , i.e. class II type of the enzyme (= fumarase C). It exhibits more than two times higher maximum activity for malate conversion than for fumarate hydration, both maximum activities being comparatively high and thus giving no indication for regulatory significance of the enzyme. Affinity for malate ( $K_m = 1.19$  mM) is also better than that for fumarate ( $K_m = 2.09$  mM). There are no indications for cooperative conversion of either substance.

**Malate dehydrogenase.** Corresponding to other sources, very high activity in crude cell extracts results from different species of the enzyme also in *Suillus*. Native gel electrophoresis of crude cell extracts reveals two bands of malate dehydrogenase activity (Fig. 1, lane 1). Examination of extracts of isolated broken mitochondria (verified by an immunodiffusion assay with rabbit antiserum against a spinach preparation) reveal the faster running band (designated A) of mitochondrial and the slowly running much heavier band (designated B) of (mainly?) cytosolic origin (Fig. 1, lanes 1 and 2). Existence of some activity of band B in electroph-

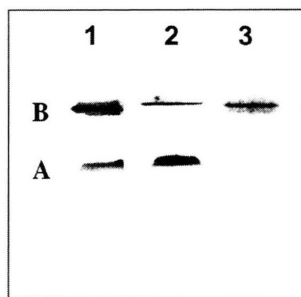


Fig. 1. Native polyacrylamide gel electrophoresis of malate dehydrogenase in crude cell extracts (lane 1), isolated mitochondria (lane 2) and crude cell extracts after exposure to 60 °C for 10 min (lane 3) of isolated mycelia of the ectomycorrhizal fungus, *Suillus bovinus*. For technical details see Material and Methods.

erograms of extracts of broken isolated mitochondria might very well be due to impurities of the preparation with cytosolic malate dehydrogenase attached to the organelles during preparation and not removed completely by the washing procedures applied. Separation of the crude cell extract proteins on the basis of their surface charges results in one rather narrow peak with a pronounced shoulder towards higher ion concentrations of malate dehydrogenase activity (Fig. 2). Native gel electrophoresis of the combined fractions of the main peak and of those containing the pronounced shoulder reveals only one, the slowly moving band (= B) in both cases (Fig. 2, inset). Since all activity was bound to and subsequently completely eluted from the column with the phosphate gradient used (subsequent treatment with 0–600 mM NaCl yielded no additional malate dehydrogenase activity containing fraction) the proposed mitochondrial enzyme species A appears to be less stable than the cytosolic enzyme species B. This is proved by exposure to 60 °C for 10 min of crude cell extracts. They reveal only the slowly running band B in native PAGE (Fig. 1, lane 3). It is also indicated by FPLC on Superose 12. It results in a single peak of malate dehydrogenase activity revealing only little activity of the fast running band A in subsequent native gel electrophoresis (Fig. 3). From these FPLC data,

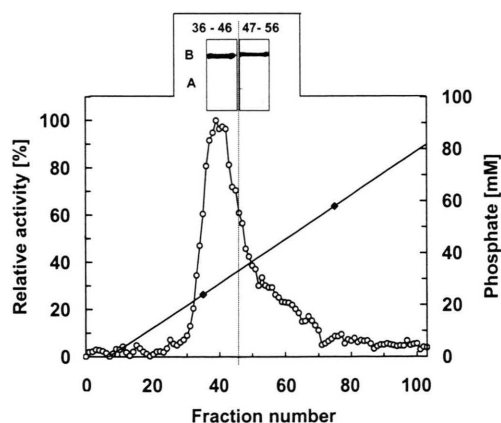


Fig. 2. Separation by ion exchange chromatography on Servacel (cellulose ion exchanger SE 23) of malate dehydrogenase in crude cell extracts of isolated mycelia of the ectomycorrhizal fungus, *Suillus bovinus* (relative activity ○—○, phosphate gradient ■—■). Inset: Native polyacrylamide gel electrophoresis of indicated fractions. For technical details see Material and Methods.

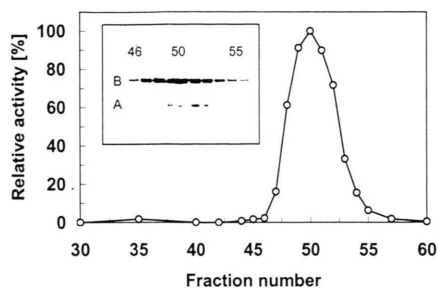


Fig. 3. Separation by FPLC on Superose 12 of malate dehydrogenase in crude cell extracts of isolated mycelia of the ectomycorrhizal fungus, *Suillus bovinus*. Inset: Native polyacrylamide gel electrophoresis of indicated fractions. For technical details see Material and Methods.

the enzyme exhibits a molecular mass of  $100 \pm 15$  kDa, the shifted maxima in subsequent PAGE pointing to a slightly smaller molecular mass of the proposed mitochondrial enzyme than that of

the cytosolic enzyme within the range of deviation.  $K_m$ -values for oxaloacetate and for NADH of the enriched cytosolic enzyme species B (FPLC or heat treatment) and of the mitochondrial preparation are identical within limits of significance (0.24 mM versus 0.22 mM and 0.070 mM versus 0.068 mM).

Affinities for malate ( $K_m = 5.77$  mM) and for  $\text{NAD}^+$  ( $K_m = 0.46$  mM) are remarkably lower for the cytosolic enzyme. Correspondingly, also maximum activity of the enzyme is lower for malate oxidation than for oxaloacetate reduction. Affinities for malate and for  $\text{NAD}^+$  have not been determined for the mitochondrial preparation because of very low activity. In no case any cooperativity could be detected.

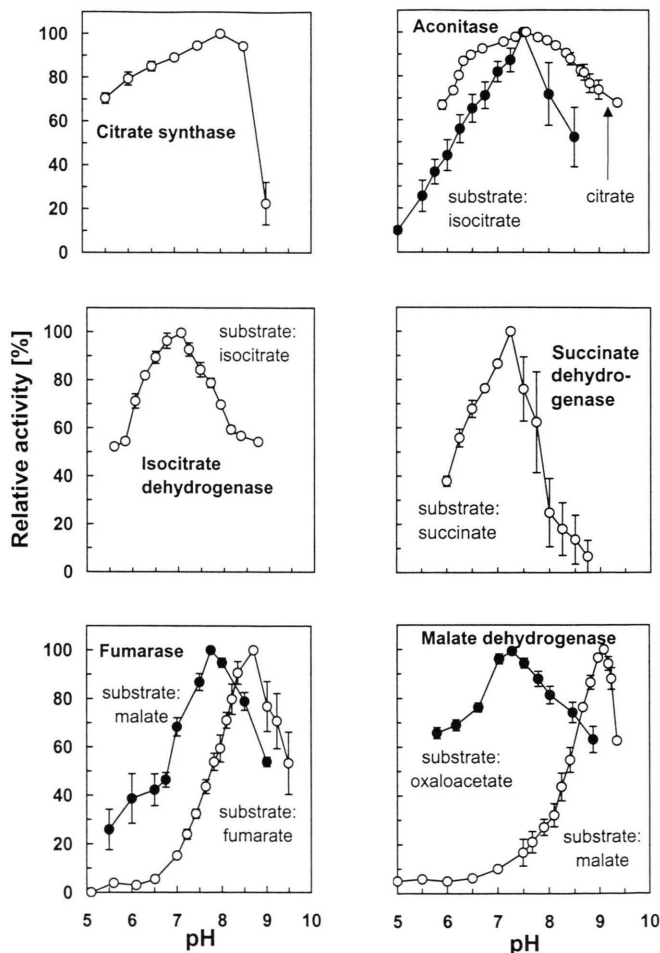


Fig. 4. pH-dependences of tricarboxylic acid cycle enzymes in crude cell extracts of isolated mycelia of the ectomycorrhizal fungus, *Suillus bovinus*. Buffers used: TRIS-buffer = citrate synthase; phosphate buffer = aconitase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase (malate converting); glycylglycine-buffer = fumarase (fumarate converting), malate dehydrogenase (malate converting); HEPES-buffer = malate dehydrogenase (oxaloacetate converting). For enzyme assays see Material and Methods.

All enzymes analyzed exhibit optimum activities between pH 7 and 8, fumarase and malate dehydrogenase, however only when functioning reductively with malate or oxaloacetate as their respective substrates (Fig. 4). Catalyzing hydration or oxidation, these two enzymes show pH-optima around 9.0.

## Discussion

Kinetic data of all enzymes examined fit into the frame of literature data for various heterotrophic organisms (Polakis and Bartley, 1965; van Etten *et al.*, 1966; Flavell and Fincham, 1968; Entian and Zimmermann, 1980; Saito 1995). This holds true also for the strict dependence on NADP<sup>+</sup> of isocitrate dehydrogenase which, in general, exists as separate, either NADP<sup>+</sup>- or NAD<sup>+</sup>-dependent species. Their ratio, however, has been reported to vary largely (from e. g. 2 : 1 [rat liver] to 0.1 : 1 [locust flight muscle], Pette, 1966) and there was no NAD<sup>+</sup>-dependent activity found in *Aspergillus niger* (Mattey, 1977). The NADP<sup>+</sup>-dependent enzyme is generally separated into a cytosolic and a mitochondrial species. But exclusive existence in mitochondria of the enzyme has been reported for 7-day old cultures of *Rhizopus arrhizus* by Osmani and Scrutton (1985).

Failure in demonstrating activities of 2-oxoglutarate dehydrogenase and of succinyl-CoA synthetase brings up the question whether or not a complete tricarboxylic acid cycle is functioning in *Suillus*. For some other mycorrhizal fungi, existence has been assumed mainly from incorporation of carbon isotopes such as <sup>14</sup>C (France and Reid, 1983) and <sup>13</sup>C (Martin and Canet, 1986; Martin *et al.* 1987, 1988) in some of its metabolites (see also Niederpruem, 1965; Jakobsen, 1991). To our knowledge comprehensive pulse chase experiments and proof of all enzymes involved are still missing. It may be that we did not succeed in stabilizing the appropriate molecular structure of these two complex enzymes. Nevertheless, with the method applied activity of 2-oxoglutarate dehydrogenase (formerly =  $\alpha$ -ketoglutarate dehydrogenase) could reliably be measured in the unicellular green alga *Chlorella* (Stein-Ludolph and Kowallik, 1985), an organism requiring even harder procedures for protein extraction than the fungal

cells. Various techniques to break the hyphae as soft and carefully as possible did not lead to positive results and also separation and purification of the *Suillus* proteins by gradual precipitation and by column chromatography did not yield respective enzyme activities. Attempts to prove the existence of the enzyme protein turned out impractical: Specific antibodies against 2-oxoglutarate dehydrogenase were unavailable to us, impurities in commercial enzyme preparations did not allow to produce them by ourselves, furthermore there are no respective fungal DNA base sequences in data banks accessible.

Should both enzymes be absent in *Suillus*, indeed, malate dehydrogenase, fumarase, and succinate dehydrogenase may be functioning reductively instead of oxidatively, *in vivo*.  $K_m$ -values, maximum activities and also *in vitro* pH-dependences, indicating only very low activities for oxidative reactions at generally assumed intracellular values of pH 7.0 - 7.5, do not exclude, but rather favour this possibility. In this case, oxaloacetate needed as a substrate for citrate synthase as well as for malate dehydrogenase could result from carboxylations of pyruvate or phosphoenolpyruvate. Activities of respective enzymes, however, could not yet be shown reliably for *Suillus*. Incorporation of C-isotopes from labelled CO<sub>2</sub> in components of the tricarboxylic acid cycle and in derived substances, such as malate and glutamate, has been reported repeatedly (Harley, 1964; France and Reid, 1983; Martin and Canet, 1986).

Finally, 2-oxoglutarate, produced by isocitrate dehydrogenase action, must not be oxidized to succinyl-CoA. It could also be aminated to glutamate. Activity of the respective enzyme, glutamate dehydrogenase, was shown to be high in *Suillus* (Grotjohann *et al.*, 2000).

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