Malic enzyme: its purification and characterization from _Mucor circinelloides_ and occurrence in other oleaginous fungi

J. Savitha, J.P. Wynn* and C. Ratledge

Malic enzyme was purified 43-fold from _Mucor circinelloides_. The enzyme was dependent on Mg$^{2+}$ or Mn$^{2+}$ for activity, was not active with D-malate and had a pH optimum at 7.8. The apparent $K_m$ values for malate and NADP$^+$ were 488 $\mu$M and 41 $\mu$M respectively. The $M_r$ of the native enzyme was 160 kDa. Five metabolic analogues of malate: oxaloacetate, tartronic acid, 1-methylenecyclopropane trans-2,3-dicarboxylic acid, malonic acid and glutaric acid, were found to inhibit malic enzyme activity at 10 mM. Four oleaginous fungi, _Mucor circinelloides_, _Mortierella alpina_, _Mortierella elongata_ and _Pythium ultimum_, were also examined, all possessed a soluble malic enzyme, two also possessed a microsomal malic enzyme.

Key words: Fungi, malic enzyme, _Mucor_, oleaginicity.

Malic enzyme [malate dehydrogenase (decarboxylating) (NADP$^+$) (EC 1.1.1.40)] catalyses the reaction

$$\text{L-malate + NADP}^+ \rightarrow \text{pyruvate + NADPH + CO}_2.$$  

Although malic enzyme has been detected in a number of fungi, its metabolic function remains somewhat unclear. In some fungi, malic enzyme has been reported to be involved primarily in pyruvate metabolism (Zink 1972; Zink & Katz 1973; McCullough & Roberts 1974); whereas in others it has also been implicated in the provision of NADPH for lipid biosynthesis and fatty acid desaturation (Evans & Ratledge 1985; Kendrick & Ratledge 1992).

In the oleaginous fungus, _Mucor circinelloides_, two isoenzymes of malic enzyme were detected, one soluble and the other membrane bound (Kendrick & Ratledge 1992). A survey was then initiated to examine if the possession of malic enzyme, and in particular the microsomal form of this enzyme, was a common feature of oleaginous fungi. The results of this survey are presented in this communication.

Due to the uncertainty concerning the metabolic function of this enzyme in fungi, malic enzyme has been studied further. This communication also outlines the purification and characterization of malic enzyme from _Mucor circinelloides_.

Materials and Methods

**Cultivation of Fungi**

Fungi were cultivated in 11 vortex-aerated bottles at 30°C. _Mucor circinelloides_ (CBS 108.16), _Mortierella alpina_ (CBS 210.32) and _Mortierella elongata_ (NRRL 5513) were cultivated on the medium of Kendrick & Ratledge (1992). _Pythium ultimum_ (IMI 342645) was grown on the medium of Vogel (1964) with sucrose as a carbon source. Mycelia were harvested by filtration after 56 h, except _Mucor circinelloides_ which was harvested after 24 h and washed with distilled water.

**Fractionation of Fungal Mycelia**

Washed fungal mycelia were suspended in 50 mM Tris/HCl buffer (pH 7.4) containing 20 mM magnesium acetate, 2 mM benzamidine...
Table 1. Typical purification of malic enzyme from *Mucor circinelloides*.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity (µmol/min)</th>
<th>Specific Activity (µmol/min mg protein)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>51.2</td>
<td>0.043</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(1) (NH₄)₂SO₄ precipitation</td>
<td>27.3</td>
<td>0.105</td>
<td>2.4</td>
<td>53</td>
</tr>
<tr>
<td>(2) Gel filtration (Sephacryl S-200)</td>
<td>8.4</td>
<td>0.272</td>
<td>6.3</td>
<td>16</td>
</tr>
<tr>
<td>(3) Ion exchange (DEAE sephadex A-50)</td>
<td>7.1</td>
<td>0.882</td>
<td>21.0</td>
<td>14</td>
</tr>
<tr>
<td>(4) Affinity chromatography (Mimetic Green 1A6XL)</td>
<td>1.1</td>
<td>1.880</td>
<td>43.0</td>
<td>2</td>
</tr>
</tbody>
</table>

All steps were carried out at 4°C. Phosphate buffers were used throughout, for steps (1) and (2) the buffer used was 20 mM (pH 7.0) for step (3), 100 mM (pH 7.0) and for step (4), 10 mM (pH 6.0). Between steps 2, 3 and 4 the active fractions were concentrated by ultrafiltration using an Amicon Diaflow PM30 membrane.

and 1 mM dithiothreitol and disrupted by passage twice through a French press at 35 MPa. The homogenate was centrifuged at 12,000 × g for 10 min at 4°C and the resultant extract was ultracentrifuged at 100,000 × g for 1 h at 4°C. This supernatant was retained as the soluble fraction and the resuspended pellet was termed the microsomal fraction.

**Determination of Malic Enzyme Activity**

The activity of malic enzyme was assayed using the method of Hsu & Lardy (1969).

**Purification of Malic Enzyme from Mucor circinelloides**

Purification of the soluble malic enzyme from *Mucor circinelloides* was carried out in four stages: (1) ammonium sulphate precipitation between 50 and 60% saturation. (2) gel filtration using a Sephacryl S-200 column (3 × 20 cm). (3) ion exchange chromatography using a DEAE Sephadex A-50 column (1.5 × 30 cm), protein was eluted with a NaCl gradient (50 mM to 250 mM). (4) affinity chromatography using a Mimetic Green 1A6XL column (Affinity Chromatography Ltd. Ballasalla, Isle of Man, UK), malic enzyme activity was eluted with 1 M NaCl.

**Characterization of the Purified Enzyme**

PAGE Electrophoresis. SDS-PAGE was carried out according to Hames (1985) using 7.5% (w/v) acrylamide. Non-denaturing PAGE was carried out using the same protocol but with the omission of SDS. On the non-denaturing gels malic enzyme activity was visualized using the activity stain of Zink & Katz (1973).

**Determination of Molecular Mass.** The molecular mass of the native enzyme was established using a Sephacryl S-200 column, calibrated using Blue Dextran, carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

**Inhibitor Studies.** The effects of various potential inhibitors were determined by their inclusion in the standard assay mixture.

**Kinetic Studies.** The apparent $K_m$ values for NADP⁺ and malate were calculated using a standard Lineweaver-Burk plot. The concentration of each substrate was varied in the assay mixture whilst the other was maintained in excess.

**Reproducibility of Results**

Unless otherwise indicated all values are average values calculated from three independently derived sets of data.

**Results and Discussion**

**Malic Enzyme**

Malic enzyme from *Mucor circinelloides* was purified 43-fold. A typical purification profile is given in Table 1. As judged by SDS-PAGE, the final preparation was not homogeneous. The preparation contained four protein bands, two of which were minor bands of low molecular weight that could not be removed by subsequent isoelectric focusing. The two major bands on the SDS-PAGE represented two isozymes of malic enzyme: both bands reacted strongly with the malic enzyme activity stain on the non-denaturing gels. These two major bands of malic enzyme did not appear to be the result of proteolytic degradation of a single native protein during purification as the inclusion of protease inhibitors, benzamidine, PMSF and EDTA, did not prevent the appearance of the two bands.

The purified malic enzyme preparation was not completely homogeneous but it was devoid of contaminating enzymes: malate dehydrogenase, NADP⁺-dependent isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, pyruvate carboxylase, citrate synthase and pyruvate kinase.

Although the purification of malic enzyme was successful, the recovery of the enzyme was poor (only 2%). This was due to apparent inactivation of the enzyme during the purification process. Although attempts were made to stabi-
Table 2. Malic enzyme activity in oleaginous fungi

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Malic enzyme activity (μmol/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Soluble fraction</td>
</tr>
<tr>
<td>Mucor circinelloides</td>
<td>0.052</td>
</tr>
<tr>
<td>Mortierella alpina</td>
<td>0.040</td>
</tr>
<tr>
<td>Mortierella elongata</td>
<td>0.064</td>
</tr>
<tr>
<td>Pythium ultimum</td>
<td>0.120</td>
</tr>
</tbody>
</table>

Malic enzyme whereas only two were found to possess a microsomal isoenzyme (see Table 2). The possession of a soluble malic enzyme has previously been confirmed for Entomophthora exitalis, Conidiobolus nanodes and Aspergillus nidulans although none of these possessed a microsomal form of the enzyme. Thraustochytrium aureum was found to be devoid of malic enzyme activity (Kendrick 1991).

Acknowledgement

JS gratefully acknowledges The Jawaharlal Nehru Memorial Trust for its financial help. JW is supported by a research grant (21.27.67) from the Biotechnology and Biological Sciences Research Council, UK.

References


(Received in revised form 18 December 1995; accepted 29 January 1996)