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Purification and some properties of malate synthase from the methylotrophic yeast *Hansenula polymorpha*

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1. SUMMARY

Malate synthase, one of the key enzymes in the glyoxylate cycle, was purified 122-fold to homogeneity from ethanol-grown *Hansenula polymorpha*. SDS-polyacrylamide gel electrophoresis showed that the enzyme has a subunit size of 62 000 daltons. The molecular mass of native malate synthase was determined to be 250 000 daltons by gel filtration, indicating that the enzyme is a tetramer. Cell fractionation studies and immunogold staining, carried out on ultrathin sections of ethanol-grown *H. polymorpha*, using malate synthase-specific antibodies, showed that malate synthase was localized in the matrix of peroxisomes.

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2. INTRODUCTION

In yeast, the proliferation and metabolic function of microbodies (peroxisomes, glyoxysomes) is largely prescribed by environmental conditions. Dependent on the composition of the growth medium, organelles develop that are involved in the metabolism of either the carbon source, the nitrogen source or both [1,2]. For instance, in *Hansenula polymorpha* peroxisomes are massively induced during growth on methanol as sole carbon source [1]; under these conditions the organelles contain mainly alcohol oxidase, dihydroxyacetone synthase and catalase, key enzymes in methanol metabolism [3]. However, yeast microbodies may also play a role in C2-intermediary metabolism. During growth of *Candida utilis* or *Trichosporon cutaneum* on ethanol [2] or ethylamine [4], and also in fatty acid-grown yeast [5], microbodies are present which contain the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase. Here we report the purification of malate synthase from the methylotrophic yeast *H. polymorpha* and show that it is in the peroxisomes. The properties of the enzyme are compared with malate synthase from other fungal sources.

3. MATERIALS AND METHODS

3.1. Microorganism and cultivation

Hansenula polymorpha de Morais et Maia CBS 4732 was grown in batch fermenters at 37°C in a mineral according to [6]. After extensive precultivation on 0.25% (w/v) glucose [7], cells were transferred into media containing 0.25% (v/v) ethanol. Cells were harvested when in the mid-exponential growth phase ($OD_{633} = 1.5$).

3.2. Cell fractionation

Protoplasts were prepared as previously described [3]. Microbodies were purified by differential and sucrose density centrifugation [8]. Sucrose concentrations were determined by measuring the refractive index.

3.3. Analytical procedures

Cytochrome C oxidase [4], malate synthase, isocitrate lyase [9] and catalase [10] were assayed as described. Protein concentrations were determined by the method of Bradford [11] using bovine serum albumin as a standard.

3.4. Purification of enzyme and preparation of antisera

All steps were performed at 4°C. Cells were disrupted in 10 mM potassium phosphate buffer pH 7.2. containing 1 mM MgK_2EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethane sulphonyl fluoride, 0.02% (w/v) NaN_3. To avoid hydrophobic protein interactions, 0.1% (v/v) Lubrol was added to the cell-free extract and to all buffers used in the purification procedure. Subsequent steps for enzyme purification are described under RESULTS AND DISCUSSION. Polypeptides in protein fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis [12] and silver stained [13]. The molecular mass of the native enzyme was determined by gel chromatography on a Sepharose 6B column. Antibodies against malate synthase were raised in rabbits; the specificity of the antiserum was tested in Ouchterlony double diffusion tests [14] and by Western blotting using the protoblot immunoblotting system (Promega Biotec).

3.5. Electron microscopy

Whole cells and subcellular fractions were fixed and prepared for electron microscopy [3]. Immunocytochemical demonstration of malate synthase protein was performed on ultrathin sections of Lowicryl-embedded cells as described previously [3].

4. RESULTS AND DISCUSSION

4.1. Cell fractionation

The location of malate synthase activity in ethanol-grown *H. polymorpha* (Fig. 1A) was studied by cell fractionation. Catalase and cytochrome C oxidase were taken as marker enzymes for microbodies and mitochondria, respectively. Table 1 shows the enzyme activities in the marker enzyme peak fractions after sucrose gradient centrifugation of the $30000 \times g$ pellet, obtained by differential centrifugation of homogenates of ethanol-grown cells. A peak of malate synthase and isocitrate lyase activities cosedimented in a narrow band at 47% sucrose with the catalase

Table 1

Enzyme distribution in catalase and cytochrome C oxidase peak fractions after discontinuous sucrose gradient centrifugation of a fraction enriched in microbodies. This fraction ($30000 \times g$ pellet) was obtained by differential centrifugation of homogenized *H. polymorpha* protoplasts

Fraction	Catalase	Malate synthase	Isocitrate lyase	Cytochrome C oxidase	Protein (mg/ml)	Sucrose (%)
12	0.26	13	15	1.81	0.32	41
26	0.68	53	58	0.33	0.08	49

Malate synthase is expressed as nmol acetyl-CoA consumed $\times min^{-1} \cdot ml^{-1}$, isocitrate lyase as nmol isocitrate consumed $\times min^{-1} \cdot ml^{-1}$, catalase as $\Delta E240 \times in^{-1} \cdot ml^{-1}$ and cytochrome C oxidase as μmol reduced cytochrome oxidized $\times min^{-1} \cdot ml^{-1}$.

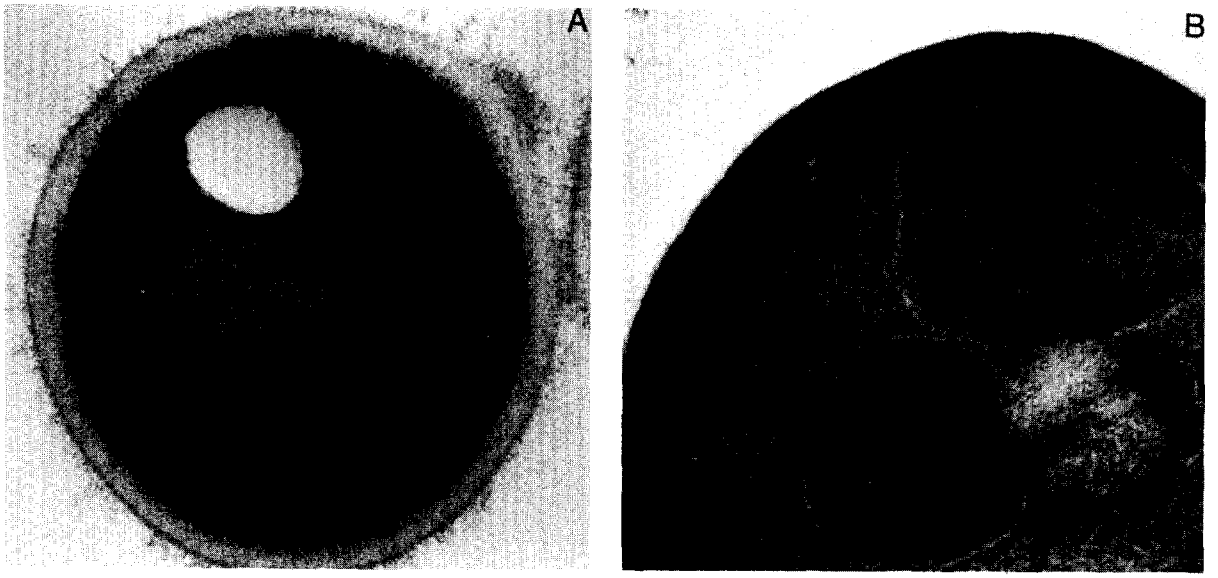

Fig. 1. Intracellular localization of malate synthase by immunocytochemistry. Fig. 1A: A thin section of a $KMnO_4$-fixed ethanol-grown *H. polymorpha* showing the overall cell morphology and microbody proliferation (42000×). Fig. 1B: Immunocytochemical localization of malate synthase protein in the microbody matrix (anti-malate synthase-protein A/gold; 51000×).

marker. In the electron microscope, these fractions were found to be highly enriched in microbodies (data not shown). The above enzymes were clearly separated from cytochrome C oxidase which was localized in a broad band at approximately 51% sucrose. These enzyme distribution patterns are essentially similar to those described for ethanol-grown *C. utilis* [8], ethylamine-grown *T. cutaneum* [4] and alkane-grown *C. tropicalis* [15] and suggest that malate synthase and isocitrate lyase are localized in the microbody matrix. The microbody-borne nature of malate synthase was confirmed immunocytochemically; after incubation of ultrathin sections of Lowicryl-embedded cells with an-

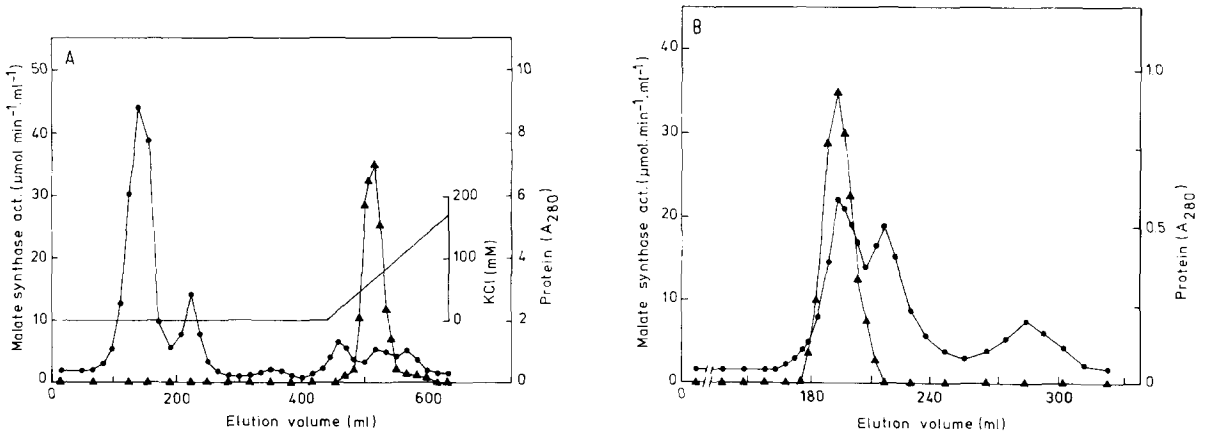

Fig. 2. Elution profile of protein and malate synthase activity upon DEAE-Sephacel column chromatography (Fig. 2A) and Sepharose 6B column chromatography (Fig. 2B). Symbols: (●) protein; (▲) malate synthase activity; (———) KCl concentration.

tibodies specific for malate synthase and protein A/gold, the labelling was largely confined to the microbody matrix (Fig. 1B).

4.2. Purification of malate synthase

A crude extract prepared from ethanol-grown *H. polymorpha* was applied to a DEAE-sephacel ion exchange column. Proteins were eluted by a linear gradient of 0-180 mM KCl in 10 mM KPi buffer pH 7.2. The major peak of malate synthase activity was eluted at 65 mM KCl, independent of the presence of Lubrol (Fig. 2A). Contrary to *H. polymorpha* malate synthase, the *C. tropicalis* enzyme does not absorb to DEAE-Sephacell column [15], indicating that the two enzymes differ in their net charge. The malate synthase activity-containing fractions were pooled, concentrated by ultrafiltration and loaded on a Sepharose 6B gel filtration column. The protein profile after elution with 50 mM KPi buffer pH 7.2 is shown in Fig. 2B. Fractions containing high enzyme activity were pooled, concentrated and further purified on an affinity column of Blue Sepharose CL-6B. A summary of the purification 122-fold with a yield of 40%. The purification was monitored by analysing aliquots of protein fractions from the chromatographic steps on sodium dodecyl sulphate-polyacrylamide gels. The resulting malate synthase preparation contained a single polypeptide band of approximately 62 kDa (Fig. 3, lane 4), indicating the homogeneity and subunit size of the enzyme. The purified protein remained stable for at least 3 months when stored at $-70\,^{\circ}C$. Antisera raised in rabbits against this preparation were shown to be specific by means of Ouchterlony double diffusion tests and Western blot analysis. Antibodies reacted with one single band (Fig. 3) of approximately 62 kDa in total cell-free extracts from ethanol-grown cells.

Table 2

Purification scheme of malate synthase from *H. polymorpha*. Cells grown on ethanol/ammonium sulphate (22 g wet weight) were used

Fraction	Total protein (mg)	Spec. act. (U·mg protein^{-1})	Yield (%)	Purification (fold)
Cell free extract	612	0.65	100	1
DEAE-Sephacel	18	13.4	62	21
Sepharose 6B	4	46	46	71
Blue Sepharose	2	79	40	122

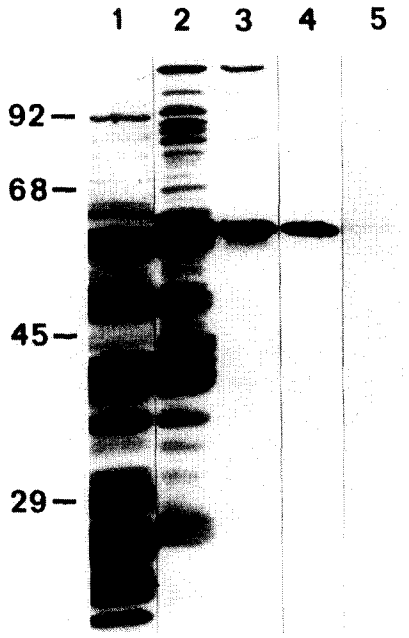

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of crude extracts of ethanol-grown *H. polymorpha* and peak fractions of malate synthase activity after the different chromatographic steps. Lane 1, crude extract; lane 2, DEAE-sephacel; lane 3, Sepharose 6B; lane 4, Blue Sepharose CL-6B; lane 5, crude extract showing malate synthase visualized by immunochemistry of a Western blot.

4.3. Properties of malate synthase

The molecular mass of the native form of malate synthase, as determined by gel filtration on Sepharose 6B column, is approximately 250 kDa. Given a subunit size of 62 kDa (Fig. 3), this implies that the native form of malate synthase consists of four identical subunits. Some properties of malate synthases of *H. polymorpha*, *C. tropicalis* [15] and *S. cerevisiae* [16] are summarized in Table 3. The molecular mass of the native enzyme and subunit of *H. polymorpha* is similar to the values determined for the *C. tropicalis* enzyme, suggesting that both enzymes are related. Malate synthase of *S. cerevisiae* has been reported to be trimeric [16]; however, the

Table 3

Properties of malate synthase from different yeasts

	H. polymorpha	*C. tropicalis*	*S. cerevisiae*
Molecular mass (kDa)			
Subunit	62	61	66
Native enzyme	250	250	180
Number of subunits	4	4	3
Specific activity ($U \cdot mg\ protein^{-1}$)	79	18.9	36
pH optimum	7.5	8.0	8.1
K_m value for			
Acetyl-CoA (μM)	11	80	83
Glyoxylate (mM)	0.06	1	0.1

subcellular localization of this enzyme has not yet been unequivocally established. The specific activity of the purified *H. polymorpha* malate synthase was two to four-fold higher than the homologous enzymes of *S. cerevisiae* and *C. tropicalis*, respectively. The enzyme showed the highest activity at pH 7.5, and K_m values measured at this pH were: 0.3 mM for Mg^{2+}, 11 μM for acetyl-CoA and 0.06 mM for glyoxylate, showing significant differences from the other yeast enzymes (Table 3). As discussed previously [8], the localization of the key enzymes of the glyoxylate cycle in the microbodies implies that an exchange of metabolites must exist between microbodies and mitochondria during growth of *H. polymorpha* on ethanol as the sole carbon source. The observed high affinity for substrates of *H. polymorpha* malate synthase may contribute to the driving force, facilitating the exchange of metabolites and interlocking the metabolic activities of the organelles. The substrate specificity of malate synthase of *H. polymorpha* and *C. tropicalis* are essentially identical. The enzyme activity of the *H. polymorpha* enzyme was strictly dependent on the presence of Mg^{2+}, which could not be substituted by Ca^{2+}, Ba^{2+} or Mn^{2+}. Furthermore, glycolate, oxalic acid, pyruvate, oxaloacetate and propionyl-CoA could not serve as substrates.

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