

Rapid purification of malate synthase from cotyledons of *Brassica napus* L.

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Abstract A rapid and efficient method for the purification of malate synthase, an enzyme uniquely confined to glyoxysomes, from cotyledons of *Brassica napus* L. has been developed. The two step purification procedure is based on the consequent utilization of the tendency of malate synthase to form high molecular weight aggregates. Malate synthase was purified 75-fold to apparent homogeneity with a specific activity of 180 nkat/mg protein. The estimated molecular weight of malate synthase subunits was 63 kDa. Polyclonal antibodies raised against malate synthase in rabbits detect on Western blots only one single polypeptide with an identical molecular weight.

Key words: Malate synthase; Glyoxysome; Rape; *Brassica napus* L.

1. Introduction

During the heterotrophic growth period of oil seeds lipid breakdown is accompanied by the conversion of fatty acids to sugars for the production of cellular components [1]. In this process specialized organelles called glyoxysomes are responsible for both the β -oxidation of fatty acids and the formation of succinate from the resulting acetyl-CoA via the glyoxylate cycle. Malate synthase, a key enzyme for the glyoxylate cycle, catalyzes the condensation of acetyl-CoA and glyoxylate forming L-malate and CoA [2,3]. The enzyme was purified from several plant species [4–8]. Its activity is strictly dependent on divalent cations, such as Mg^{2+} and it is comprised of subunits with a molecular weight of around 63 kDa which readily aggregate to oligomeric complexes [6–9]. The aggregation behavior of malate synthase can be used for enzyme purification. Based on this property we developed a simple and rapid purification protocol for malate synthase from cotyledons of rape seedlings, which includes two steps only: selective precipitation and sedimentation centrifugation of the oligomeric complex.

2. Materials and methods

2.1. Purification of malate synthase

All steps were performed at 4°C. Cotyledons (11–19 g) of 4-day-old etiolated rape seedlings were homogenized in 150 mM MES-NaOH, pH 6.5, containing 500 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT, filtered through cheese cloth and centrifuged to clearness (last step 160,000 \times g, 90 min). The clear supernatant solution (soluble fraction) was mixed with PEG-400 (15% w/v), incubated for 1 h and centrifuged at 25,000 \times g for 20 min. Precipitated material was resuspended in 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$

and 1 mM EDTA. After incubation (30 min) insoluble proteins were precipitated (11,000 \times g, 10 min) and resuspended in a small volume of 50 mM Tris-HCl, pH 7.5, 4 mM glyoxylate and 1 mM EDTA (glyoxylate buffer). The resuspended precipitate was cleared by centrifugation (11,000 \times g, 10 min) and subsequently loaded onto a linear sucrose density gradient (34 ml, 5–50% w/w) buffered with 25 mM Tricine-NaOH, pH 7.5, 5 mM $MgCl_2$ and 1 mM EDTA for sedimentation centrifugation (116,000 \times g, 4 h). After sedimentation centrifugation the protein pellet and the fraction with the highest density were pooled and diluted with glyoxylate buffer.

Malate synthase activity was assayed according to [10] using acetyl-CoA prepared by the procedure of Simon and Shermin [11]. All malate synthase activities were corrected against unspecific deacylating activities. Protein contents were measured using the Coomassie dye binding method (Bio-Rad Inc.) with BSA as the standard.

2.2. SDS-PAGE and antibody preparation

SDS-PAGE was performed by the method of [12] with an acrylamide gradient of 7.5–18% (w/v) in the separating gel. Gel slices containing purified malate synthase were cut out and used for antibody production in rabbits (performed by Eurogentec s.a., Seraing, Belgium). Gel strips were pulverized and mixed with Freund's adjuvant. A initial injection of approximately 100 μ g of antigen with complete adjuvant was followed by two injections of antigen with incomplete adjuvant, lacking immunogenic bacteria. Globulin fractions from preimmune serum or malate synthase-antiserum were purified on a CM-affi-gel blue column according to the manufacturer's instructions (Bio-Rad Inc.) and finally concentrated by $(NH_4)_2SO_4$ -precipitation.

2.3. Immunochemical analysis

Following electrophoresis, polypeptides were transferred onto a nitrocellulose membrane (0.2 μ m; Schleicher and Schuell Inc.) by a semi-dry blotting method. The transfer buffer contained 48 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) EtOH. Electrotransfer was carried out at 10°C for 35 min using a constant voltage of 45 V. Western blots were blocked with BSA (5%, w/v) and immunoreactive peptides were localized using goat anti-rabbit IgG conjugated with alkaline phosphatase as described by [13].

3. Results and discussion

A common property of malate synthase in higher plants is its tendency to assemble to high molecular weight aggregates resulting in an exceptionally high mass of the enzyme preparation. This property may be utilized for the rapid purification of malate synthase. In the presence of Mg^{2+} highly aggregated forms of malate synthase are induced and as a consequence a protocol for swift purification of the enzyme without involvement of column chromatography steps could be developed.

In crude homogenates obtained from 4-day-old cotyledons of rape seedlings the total malate synthase activity was found to be 0.5 nkat/pair of cotyledons (60.3 nkat/g fresh weight). After differential centrifugation of the crude extracts a soluble supernatant fraction was obtained (specific activity of malate synthase 2.37 nkat/mg protein) and used for the purification of malate synthase. The purification scheme is given in Table 1.

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Purification of malate synthase to apparent homogeneity was achieved by only two steps yielding a protein fraction with a specific activity of 180 nkat/mg protein and 19% recovery of the total enzyme activity. These data are in good agreement with previous reports on malate synthase purifications from *Brassica napus* L. [7] or *Glycine max* L. [8]; however, these reports describe procedures that involve at least three different chromatographic steps. The purification of malate synthase from homogenates of cotton seedlings involved even six steps with three different columns and resulted in a apparent homogeneity on silver stained SDS-PAGE [5] yielding 2.6% of the total enzyme activity and a specific activity of 133 nkat/mg protein for the purified malate synthase [5].

Crucial for our easy and fast procedure is the homogenization of the rape seed cotyledons in MES-buffered sucrose at pH 6.5. Only MES-buffered homogenates were found to produce high molecular weight malate synthase aggregates in the presence of both PEG-400 and Mg^{2+} in the precipitating buffer. Homogenization in extraction media buffered with Tricine, HEPES or Bicine did not yield comparable results. The selective precipitation resulted in a specific malate synthase activity

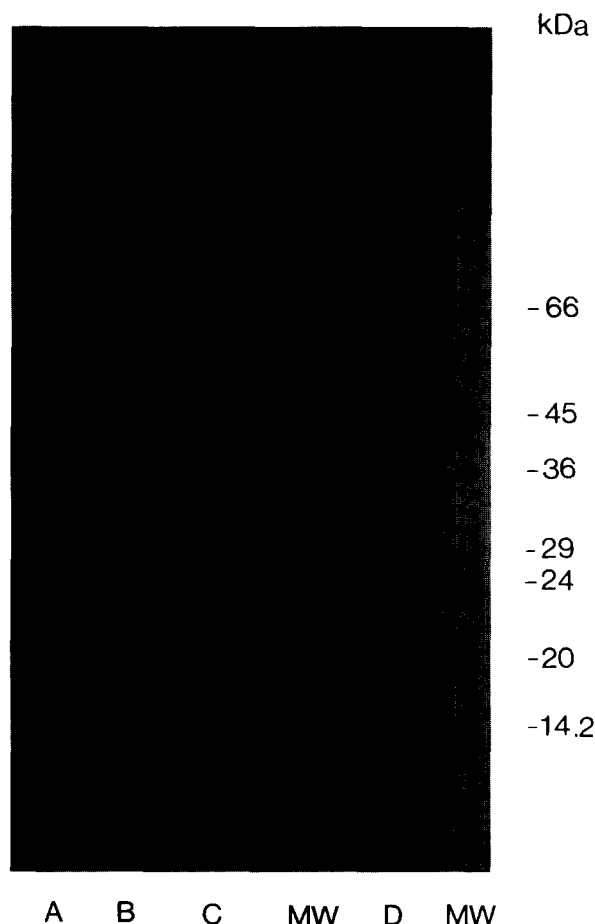


Fig. 1. Coomassie blue-R 250 stained SDS-PAGE of fractions in the course of malate synthase purification. Lane A = soluble supernatant fraction; lane B = enzyme preparation after selective precipitation; lanes C,D = purified malate synthase after sedimentation centrifugation from two different experiments (9.5 μ g protein). MW, molecular mass markers (kDa, as indicated on the right).

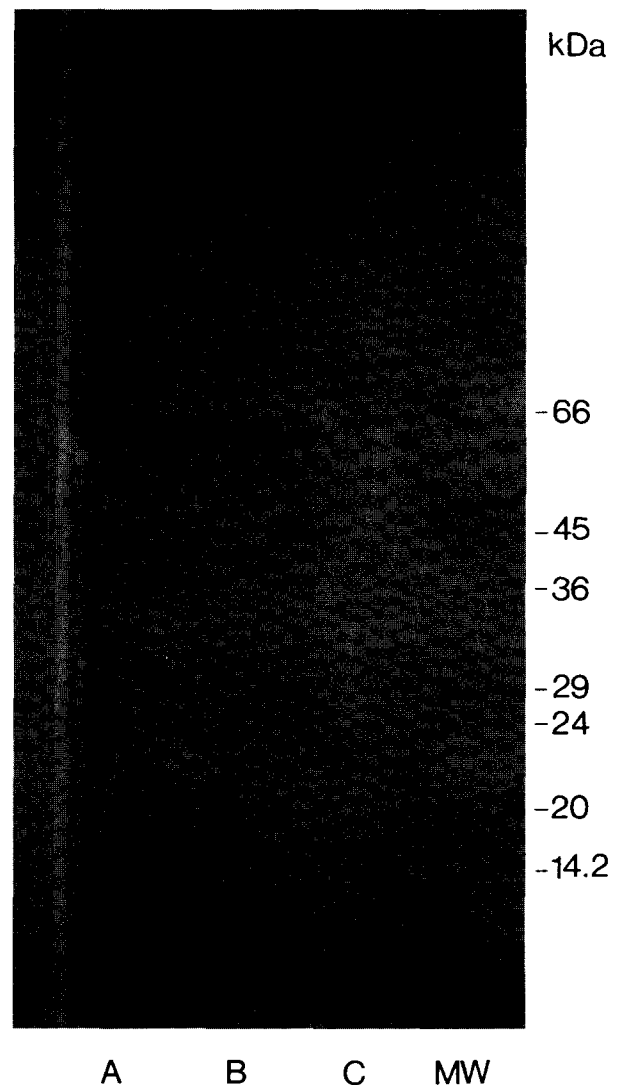


Fig. 2. Western blot of fractions in the course of malate synthase purification incubated with malate synthase antiserum. Lane A = purified malate synthase; lane B = enzyme preparation after selective precipitation; lane C = crude homogenate of cotyledons of rape seedlings. MW, molecular mass markers (kDa, as indicated on the right).

of 23 nkat/mg protein and a yield of 50%. In comparison, precipitation with $(NH_4)_2SO_4$ generally used in the purification protocols results in a specific activity of only 5.1 nkat/mg protein for preparations from rape seed cotyledons [7], 7.3 nkat/mg protein for soybean cotyledons [8] and 10.8 nkat/mg protein for cotton cotyledons. The final purification step of our procedure is a sedimentation centrifugation step which provides a good yield and high specific activity of malate synthase. In the presence of 5 mM Mg^{2+} in the gradient solutions a highly aggregated form of malate synthase may be precipitated through the sucrose gradient to the bottom of the tubes. In this regard our results confirm data about the aggregating effect of Mg^{2+} on malate synthase previously reported for the soybean system [8].

In addition, aggregated forms of malate synthase were also detected after sedimentation centrifugation in the absence of Mg^{2+} at high concentrations of sucrose, buffered with Tricine at pH 7.5 [6,9].

Table 1
Purification of malate synthase from etiolated cotyledons (16 g) of rape seeds

Step	Total protein (mg)	Total activity of ms (nkat)	Specific activity (nkat/mg protein)	Recovery (%)	Purification-fold
Soluble fraction	242.000	570.0	2.37	100.0	1.0
Selective precipitation	12.00	284.2	23.57	50.0	9.9
Gradient centrifugation	0.61	110.0	180.30	19.3	75.9

In Fig. 1 the polypeptide pattern of the different protein fractions obtained during the course of purification is shown. After the selective precipitation step of malate synthase activity a polypeptide of 63 kDa becomes predominant and it is this major peptide that remains detectable after sedimentation centrifugation. The estimated subunit size of malate synthase is in good agreement with previous reports using different oil storage tissues [4–8] and with the deduced amino acid sequence of malate synthase from rape seeds [14].

On Western blots, antibodies raised against malate synthase detected only one single polypeptide with a molecular weight of 63 kDa (Fig. 2). Also, on Western blots of crude homogenates of 4-day-old rape seed cotyledons only one single polypeptide with an identical molecular weight is stained demonstrating the monospecificity of the purified antibody fraction. Controls with the preimmune globulin fraction gave no immunoreactive staining.

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