

# The putative coenzyme B<sub>12</sub>-dependent methylmalonyl-CoA mutase from potatoes is a phosphatase

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## ARTICLE INFO

### Article history:

Received 1 February 2008

Available online 29 July 2008

### Keywords:

Coenzyme B<sub>12</sub> in plants?

Methylmalonyl-CoA

Phosphatase

## ABSTRACT

The reported presence of a coenzyme B<sub>12</sub>-dependent methylmalonyl-CoA mutase in potatoes has been reexamined. The enzyme converting methylmalonyl-CoA was purified to electrophoretic homogeneity. Examination of the reaction product by <sup>1</sup>H, <sup>31</sup>P NMR and mass spectrometry revealed that it was methylmalonyl-3'-dephospho-CoA. The phosphatase enzyme needs neither coenzyme B<sub>12</sub> nor S-adenosylmethionine as a cofactor.

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## 1. Introduction

During the last decades of the past century a number of publications appeared claiming the presence of coenzyme B<sub>12</sub>-dependent enzymes in plants [1–5]. On the other hand, there is no evidence for the occurrence of cobalamin in these organisms. This contradiction and our continuing interest in B<sub>12</sub>-dependent enzymic reactions prompted us to reinvestigate the report on the presence of the coenzyme B<sub>12</sub>-dependent methylmalonyl-CoA mutase in potatoes [3].

In the latter paper it was claimed that the product of the reaction was succinyl-CoA “identified” only by the similar retention time in a certain chromatographic system. Obviously, such an identification does not meet serious standards. Though the authors also claimed that the conversion was dependent on coenzyme B<sub>12</sub>, these results appeared to us rather unlikely.

In the following we describe the results we reached in our endeavors.

## 2. Materials and methods

The potatoes were commercially available, methylmalonyl-CoA was supplied from Sigma. Methylmalonyl-CoA mutase and oxalacetate-transcarboxylase for the spectrophotometric activity assays are isolated as described [6]. Malate dehydrogenase was supplied from Sigma.

### 2.1. Enzyme assay 1

The spectroscopic assay was carried out using a Cary 3 UV/VIS-spectrophotometer as described [6–9].

### 2.2. Enzyme assay 2

The activity of the enzyme was quantified by HPLC (Agilent 1200) chromatography (RP-18 column, 125 × 4 mm) using a method described previously [10]. Forty-five microliters of enzyme solution was added to 150 µl 0.05 M K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.5, 5 µl methylmalonyl-CoA solution (4 mM) was added to start the reaction which was stopped by 10 µl TFA after 30 min incubation at 37 °C. The protein was removed by centrifugation (5 min, 13,000 rpm). Hundred and fifty microliters of supernatant was diluted 1:1 with buffer (90% buffer A 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, pH 4.5 and 10% buffer B (0.2 M NaH<sub>2</sub>PO<sub>4</sub> and 20% acetonitrile) was applied to the RP-18 column which was equilibrated with 10% buffer B/90% buffer A. Product and substrate of the enzymatic reaction were separated with a 12 min continuous gradient run (0.75 ml/min, starting with 10% of buffer B, 63% buffer B after 5 min, 60% buffer B after 7 min, 100% buffer B after 11 min), product and substrate of the reaction are eluted as resolved peaks and can be detected at 254 nm.

### 2.3. Purification of the enzyme

Washed and peeled potatoes were cut into pieces and homogenized in a mixer 1:1 (w/w) with 100 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.0 (4 °C). The suspension was centrifuged for 30 min at 15,000 rpm (4 °C). The supernatant was decanted, filtered through a 22 µm

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**Table 1**

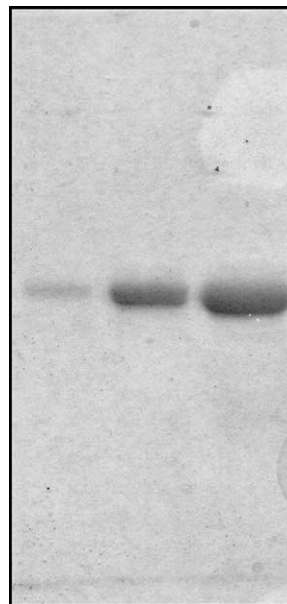
Isolation of putative methylmalonyl-CoA mutase out of 450 g potatoes

Purification step	Volume (ml)	Protein (mg)	Activity (U)	Specific act. (U/mg)
Cell disruption	150	1000		
DEAE column	100	80		
Gel filtration	30	30		
Anion exchange	8	28	0.004	0.001
Chromatofocussing	7.5	10.8	0.0008	0.0006
Anion exchange II	1	0.4		

The enzyme activities were determined by the HPLC assay [10]. After the last purification step (ion exchange column II) the amount of the protein was too small for the assay and was used for SDS-electrophoresis (see Fig. 2).

sterile filter and applied to a Fractogel-DEAE (100 ml) anion exchanger purchased from Merck Chemical Company which was previously equilibrated with buffer C (20 mM  $K_2HPO_4/KH_2PO_4$ , pH 7.0). The separation was carried out at 4 °C, the column was washed with 200 ml buffer C (4–5 ml/min), then with 200 ml 20% buffer B. The active fraction was eluted with 200 ml 30% buffer B and was concentrated with centricons to a volume of 15 ml which was applied to a Superdex HiLoad (Pharmacia, Sweden) gel filtration column equilibrated with buffer (100 mM  $K_2HPO_4/KH_2PO_4$ , pH 7.2). The enzyme solution from this purification step was loaded to a Resource Q anion exchanger after equilibrating the enzyme with buffer B (BB, 50 mM  $K_2HPO_4/KH_2PO_4$ , pH 7.0). The separation was carried out at a flow rate of 3 ml/min. The elution profile starts with 15 min BB, a continuous buffer gradient ends after 75 min with 100% elution buffer (EB, 50 mM  $K_2HPO_4/KH_2PO_4$ , pH 7.0), proteins were detected at 280 nm. After this (and all following) purification step fractions were assayed and the active enzyme fractions were pooled, protein content was measured at 280 nm with a in 1 ml quartz cuvettes using a Cary 3 UV/VIS-spectrophotometer.

The active enzyme was equilibrated with buffer 1 and applied to Mono P 5/20 (4 ml) chromatofocussing column. The column was equilibrated with buffer 1 (25 mM L-histidine, 35 mM NaCl, pH 6.2) at a flow rate of 0.75 ml/min. 40 mg of protein were loaded and eluted with buffer 2 (Polybuffer 74-HCl from Pharmacia).

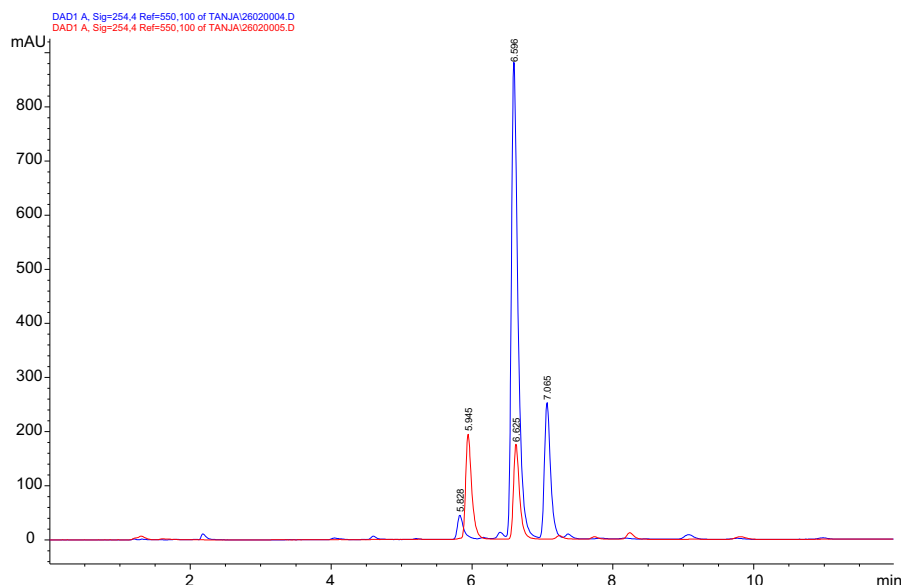
**Fig. 2.** SDS-electrophoresis of the active enzyme after the last purification step.

As a last purification step separation on a second Resource Q column was carried out in the same manner as described for the first column. For all protein purification steps a Pharmacia LCC-501 PLUS liquid chromatograph was used. The progress of the purification is shown in Table 1.

### 3. Results and discussion

#### 3.1. Enzyme assays

A fresh extract of potatoes, prepared as previously described [3], was used as enzyme source. The putative methylmalonyl-CoA mutase was assayed by two different methods. The spectrophotometric assay starts with the substrate succinyl-CoA and is

**Fig. 1.** Conversion of methylmalonyl-CoA with the putative mutase from potatoes and synthetic succinyl-CoA (retention time: 6.625 min), which is contaminated with some CoA (retention time: 5.945 min, red trace) and the reaction product mixture (retention times: 6.596 and 7.065 min, blue trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

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