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Expression, purification, and characterization of two NADP-malic enzymes of rice (*Oryza sativa* L.) in *Escherichia coli*

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Abstract

NADP-malic enzymes (NADP-ME) are isozymes in plants. To clarify the diversity and function of NADP-ME isozymes in rice, we produced two active GST-fused NADP-ME proteins, NADP-ME_2 and NADP-ME_3 in *Escherichia coli*, and the fusion proteins were purified by affinity chromatography using a glutathione–Sepharose 4B column. After enzymatic cleavage of the GST tag, final yields were 1.4 mg/g wet cell weight (wcw) for NADP-ME_2 and 3.5 mg/g wcw for NADP-ME_3, respectively, and the molecular weights of NADP-ME_2 and NADP-ME_3 were about 65 and 62 kDa, respectively. The optimum pH is 7.3 for NADP-ME_2 and 7.7 for NADP-ME_3. The K_m values for malate of NADP-ME_2 and NADP-ME_3 were 2.6 and 3.1 mM, whereas the K_m values for NADP were 79 and 93 μM, respectively. The K_{cat} values of NADP-ME_2 and NADP-ME_3 for malate were about 91.7 and 96.7 s^{-1}, respectively, and the K_{cat} values for NADP about 88.3 and 98.3 s^{-1}, respectively. These results suggest that the two rice isozymes of NADP-ME in vitro have similar kinetic parameter.

Keywords: Rice NADP-malic enzyme; Purification; Glutathione *S*-transferase fusion protein; Isozyme; Enzyme activity; *Escherichia coli*

NADP-malic enzyme (NADP-ME; EC 1.1.1.40),[1] an important malate metabolizing enzyme, catalyzes the oxidative decarboxylation of malate: L-malate + $NADP^+ \rightarrow$ pyruvate + CO_2 + NADPH + H^+ in the presence of a bivalent metal ion [1,2]. NADP-ME acts in a wide range of metabolic pathways in both plants and animals. In plants, NADP-MEs have been classified as photosynthetic and non-photosynthetic isoforms based on their function [3,4]. The photosynthetic isoform of NADP-ME is localized in the chloroplasts of bundle sheath cells of NADP-ME-type C_4 plants, where it plays a key role in photosynthesis by supplying CO_2 in the Calvin cycle [2,3,5]. Another photosynthetic isoform of NADP-ME is found in certain CAM plants, where it has a similar role [3,6,7]. Non-photosynthetic isoforms of NADP-ME are either cytosolic or plastidic, and are present in C_3, C_4, and CAM plants [3]. They may have several different roles, such as in regulating intracellular pH [2,8], providing carbon and NADPH for fatty acid biosynthesis [9,10] and participating in defense reactions/stress responses [11,12]. Individual plant species, such as maize and *Flaveria* species are reported to have two and four genes encoding NADP-MEs [3,13,14]. Recently, NADP-ME isozymes with molecular weights from 62 to 72 kDa (62, 64 or 65 or 67, 72 kDa) were detected in several plants by Western blot analysis or by NADP-ME activity staining on native PAGE gels [6,15–18].

Although plant isozymes perform the same function in a biochemical reaction, they are different in some characteristics. First, isozymes have different intracellular localizations such as cytosol, chloroplasts, mitochondria, and peroxisomes [19], where they function in different metabolic pathways. For example, vacuolar Na^+/H^+ antiporters transport Na^+ into vacuoles, while on the plasma membrane they export Na^+

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[1] *Abbreviations used:* NADP-ME, NADP-malic enzyme; IPTG, isopropyl-β-D-thiogalactopyranoside; CBB, Coomassie brilliant blue R-250; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; wcw, wet cell weight.

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from the cytosol back to the apoplastic spaces or into the growth medium [20–22]. Second, the genes encoding isozymes may be regulated differently at the transcription level in tissue- and development-dependent manners [13,14]. Additionally, the effects of certain environmental cues and hormones (e.g., light, salt, and ABA) are different among different isoenzyme genes [13,21], probably because of their different promoter activities. Third, isozymes may differ in their enzymatic properties. Little is known about the individual functions of the NADP-ME isozymes in rice, partly because it is difficult to obtain sufficient amounts of homogeneous and active isozymes for analysis. Here, we compared two rice NADP-ME isozymes by expressing them in *Escherichia coli* (*E. coli*).

Recently, we isolated a new gene encoding NADP-ME (GenBank Accession No. AB053295; named NADP-ME_2) from a rice root cDNA library that was constructed under sodium carbonate and sodium bicarbonate stress (unpublished work). Additionally, the cytosolic NADP-ME gene was reported on GenBank (GenBank Accession No. AY435404; named NADP-ME_3). In rice, four genes encoding NADP-ME exhibit distinct expression profiles [23]. In this study, the two cDNAs (NADP-ME_2 and NADP-ME_3) encoding rice NADP-ME isozymes were successfully expressed in *E. coli* cells as fusion proteins. The optimized purification procedures for obtaining milligram amounts of homogeneous active recombinant proteins are presented. Two obtained recombinant proteins were used to investigate their enzymatic properties.

Materials and methods

Construction of expression plasmids

The complete coding regions of two rice NADP-ME genes, NADP-ME_2 (1782 bp) and NADP-ME_3 (1713 bp), were amplified by polymerase chain reaction (PCR). The primers for NADP-ME_2 were 5′-CCCGGGTCATGGAGAGCACCATGAAG-3′ (*Sma*I site underlined) and 5′-GCGGCCGCTCACCGGTAGTTGCGGTA-3′ (*Not*I site underlined). The primers for NADP-ME_3 were 5′-GGATCCATGGCCGGCGGCGGCGT-3′ (*Bam*HI site underlined) and 5′-GTCGACTCAGCGGTAGCAGCGGT-3′ (*Sal*I site underlined). The PCR products were ligated into the vector pGEX-6p-3 after it had been treated with the appropriate restriction enzymes. The resulting plasmids were confirmed by DNA sequencing and named pGEX-6p-3-NADP-ME_2 and pGEX-6p-3-NADP-ME_3, respectively. Cells of *E. coli* BL21 (Amersham-Pharmacia) were transformed with pGEX-6p-3-NADP-ME_2 and pGEX-6p-3-NADP-ME_3, respectively, and cells containing transformed plasmids were used to produce the recombinant proteins with GST.

Expression and purification of two GST-NADP-ME fusion proteins from E. coli

Escherichia coli cells transformed with pGEX-6p-3-NADP-ME_2 or pGEX-6p-3-NADP-ME_3 were used for the expression of the fusion proteins (GST-NADP-ME_2 or GST-NADP-ME_3). The cells were cultured overnight in 2× YT medium (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) containing 100 μg/ml ampicillin at 37 °C, diluted 1:100 with fresh pre-warmed 2× YT medium containing 100 μg/ml ampicillin and incubated at 28 °C with shaking at 130 rpm. When OD_{600} had reached a value of 1.35, the expression of GST-NADP-ME_2 was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 10 μM for an additional 12 h, and GST-NADP-ME_3 expression was induced by addition of IPTG to 100 μM for an additional 5 h. The cells were pelleted by centrifugation at 6000*g* for 5 min. Cell pellets were resuspended in pre-cooled lysis buffer (100 mM NaC1, 100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, 5 mM DTT, and 1 mM PMSF), and then lysozyme was added to the suspension to a final concentration of 1 mg/ml. The suspension was incubated on ice for 30 min, and centrifuged at 15,000*g* for 30 min. The supernatant containing fusion proteins was filtered through a 0.45 μm Millipore membrane and the filtrate was loaded onto a glutathione–Sepharose 4B column (Amersham-Pharmacia) pre-equilibrated with buffer A (100 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF). Non-specifically bound proteins were removed by washing with buffer A, and the bound fusion proteins with GST tag were eluted with 50 mM Tris–HCl (pH 8.0) buffer containing 10 mM reduced glutathione.

Cleavage of the GST tag

The recombinant proteins of GST-NADP-ME bound to the column were digested by the addition of PreScission Protease (Amersham-Pharmacia) in elution buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.01% Triton X-100) for 16 h at 4 °C. The desired recombinant proteins (NADP-ME_2 and NADP-ME_3) were eluted from the column, and used for activity tests immediately or stored at −80 °C for later use.

SDS– and native PAGE

Protein samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [24], and stained with Coomassie brilliant blue R-250 (CBB). Native PAGE, for determining NADP-ME activity in the gels, was done with a discontinuous pH system under nondenaturing nonreducing conditions essentially as described by Laemmli, except that SDS and DTT were omitted. BCA Protein Aassay Kit (PIERCE, USA) was used to determine the protein concentration.

Assay of NADP-ME activity and activity staining

NADP-ME activity was determined spectrophotometrically with Ultrospec 4300 pro UV/visible spectrophotometer (Amersham-Pharmacia) at 30 °C by monitoring NADPH production at 340 nm as described by Ferreyra