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Chloroplastic monodehydroascorbate radical reductase from spinach.

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Introduction

Ascorbate (AsA) plays a central function to protect plant cells from oxidative stress. Especially in the water-water cycle in chloroplasts, AsA works as a reductant of the hydrogen peroxide (H_2O_2) photogenerated via the AsA peroxidase reaction and protects the H_2O_2-sensitive enzymes of the Calvin-Benson cycle in the stroma (Asada 2000). In AsA peroxidase reaction, monodehydroascorabte (MDA) radical is the primary oxidation product of AsA (Hossai et al 1984). For effective operation of the scavenging system of H_2O_2 in chloroplasts, it is essential to regenerate AsA from oxidized AsA; MDA and dehydroascorbate. In chloroplasts MDA is reduced to AsA by photoreduced ferredoxin in PS I and by NAD(P)H catalyzed with MDA reductase (Asada 2000). MDA reductase is an FAD-enzyme and the only enzyme known to use an organic radical as an enzyme substrate (Hossain & Asada 1984). Its amino acid sequence has putative FAD and pyridine nucleotide binding domains of flavoenzymes (Sano & Asada 1994). MDA reductase has been purified and its cDNA has also been cloned from several plant sources, including cucumber (Hossain & Asada 1984, Sano & Asada 1994). Except for the enzyme from mitochondria of potato tuber (De Leonardis et al. 1995), MDA reductases purified so far are cytosolic isoforms. The cDNAs from *Arabidopsis* (DDBJ/EMBL/ Genbank databases D84417) and leaf mustard (DDBJ/EMBL/Genbank databases AF109695) which show high homologies to MDA reductase have the putative regions encoding the transit peptides for targeting to cell organelles but the localization of their translation products have not been confirmed. Namely, the chloroplastic MDA reductase and its cDNA have never been isolated.

In this presentation, we report the purification and characterization of chloroplastic MDA reductase from spinach. Then, the cDNA clone of this isozyme was isolated with the information of amino acid sequence of its amino terminus.

Materials and methods

MDA reductase was assayed as described at Hossain et al. (1984). Suspension of Percoll-purified intact chloroplasts (Asada et al. 1990) from 3 kg of spinach leaves was hypotonically disrupted in buffer A (50 mM potassium phosphate, pH7.5, 10 mM 2-mercaptoethanol, 0.1 mM EDTA) containing 1 mM PMSF. After centrifugation at 20,000 x *g* for 30 min, $(NH_4)_2SO_4$ was added to the stromal fraction up to 40% saturation, and the supernatant obtained by centrifugation was loaded on a column of Butyl-Toyopearl (Tosoh). The pool of peak fractions containing the activity of MDA reductase were dialyzed and applied onto columns of Q Sepharose (Amersham

Pharmacia) and then Blue Sepharose (Amersham Pharmacia). The former two chromatographies were performed with FPLC system (Amersham Pharmacia). For purification from whole leaves, spinach leaves (700 g) were homogenized with buffer A containing 1 mM PMSF. After removal cell debris by centrifugation, the supernatant was fractionated by $(NH_4)_2SO_4$ (40-80% saturation). The precipitated proteins suspended in buffer A containing 40% saturation $(NH_4)_2SO_4$ was loaded on columns of Butyl-Toyopearl, Q Sepharose and Blue Sepharose. All procedures for purification were carried out at 4°C. For immunoblotting analysis, the antibody raised against cytosolic MDA reductase from cucumber fruits (Sano & Asada 1984) was used as a primary antibody. The amino acid sequences of the amino terminus of enzymes were determined with a peptide sequencer (model 492; Applied Biosystems).

Total RNA was isolated from greening cotyledons of spinach seedlings (one-week growth) with RNeasy plant mini kit (Qiagen). A fragment containing 5'-end of cDNA encoding chloroplastic MDA reductase was amplified with 5'-RACE system (Invitrogen) using degenerate primers designed based upon the amino terminal sequence of the purified isozyme. Refering from the nucleotide sequence of the resultant fragment, a cDNA fragment containing 3'-end was obtained with 3'-RACE. The cDNAs having the complete reading frame of the chloroplastic MDA reductase from spinach were obtained with RT-PCR by KOD plus (Toyobo). The nucleotide sequences were determined with a DNA sequencer (model 373A; Applied Biosystems).

Results and discussion

MDA reductase was purified from the stroma fraction of the Percoll-purified intact chloroplasts. No activity of MDA reductase was found in the thylakoid fraction, in agreement with the previous observation (Hossain et al. 1984). Spinach chloroplasts seem to have a single isoform of MDA reductase as evidenced from a single activity peak observed in hydrophobic, ion exchange and affinity chromatographies. The Blue Sepharose fraction of chloroplastic MDA reductase after separation by SDS-PAGE showed several protein bands and an antibody-cross-reacted band at a position corresponding to a molecular mass of 51 kDa (Fig. 1). The cytosolic MDA reductase from cucumber showed a band at molecular mass of 47 kDa as expected (Hossain & Asada 1984, Sano & Asada 1994). The molecular mass of chloroplastic MDA reductase was about 50 kDa as determined by Superdex 200 column, indicating that chloroplastic isozyme is a monomer as the cytosolic (Hossain & Asada 1984,) and mitochondrial isozymes (De Leonardis 1995). We also tried to purify chloroplastic MDA reductase from whole leaves of spinach effectively. Two isozymes were separated from each other at the purification step with Butyl-Toyopearl. With comparison to the elution profile of stroma fraction, it was seemed that the former peak contained chloroplastic isozyme and the latter was another isozyme, perhaps cytosolic one. Therefore, we purified further the enzyme contained at the former peak and obtained the isozyme that showed the band corresponding to a molecular mass of 51 kDa on SDS-PAGE. (Fig. 1) The both of bands corresponded to MDA reductase from stroma fraction and crude extract of leaves were subjected to sequence analysis of amino acid residues. We could determine the sequences up to 8th and 25th residues of the amino termini, respectively (Fig. 2). These sequences were identical up to 8th residue. We succeeded to purify the chloroplastic MDA reductase from crude extract of spinach leaves.

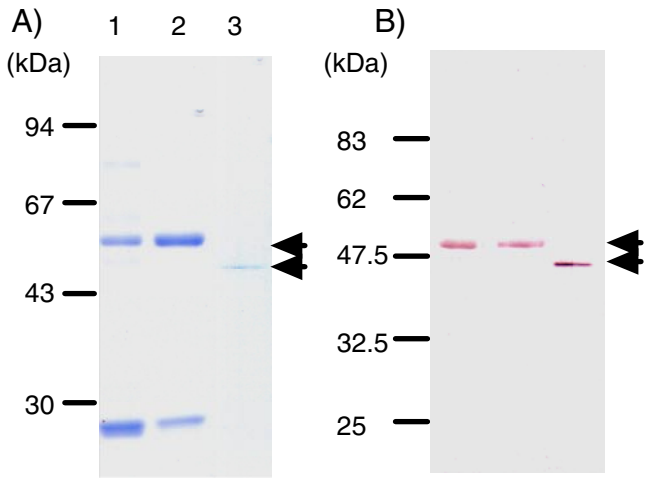

Fig. 1. Analysis of SDS-PAGE of purified MDA reductase from spinach. Purified MDA reductase was subjected to SDS-PAGE (A). Immunoblotting was detected by anti-MDA reductase from cucumber fruits (B). Lane 1, MDA reductase purified from stroma fraction ; Lane 2, MDA reductase isozyme from crude extract of spinach leaves, containing in the former peak at the column step of Butyl-Toyopearl; Lane 3, recombinant cytosolic MDAR of cucumber.

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                10         20         30         40         50        ▼60         70
Sp   MSTVGRFMAT MSNSLSLKHG VSLYSSTSSS FSLSKLHCKP SLVASRPFYN RRCFSISASS SFANDNREYV
Ar   MSAVRRVMAL ASTTLPTKSG LSLWCPSSPS LAR-RFPARF SPIG-----S RIASRSLVTA SFANENREFV
Cu   ---------- ---------- ---------- ---------- ---------- ---------- -MADETFKYV

                80          90        100        110        120        130        140
Sp   IVGGGNAAGY AARTFVEHGL ADGKLCIVTK EAYAPYERPA LTKGYLFPLD KKPARLPGFH TCVGGGGERQ
Ar   IVGGGNAAGY AARTFVENGM ADGRLCIVTK EAYAPYERPA LTKAYLFPPE KKPARLPGFH TCVGGGGERQ
Cu   ILGGGVAAGY AAREFVKQGL NPGELAIISK EAVAPYERPA LSKAYLFP-- ESPARLPGFH VCVGSGGERL

               150        160        170        180        190        198 ✖      208
Sp   TPEWYQEKGI EMIYEDAVTG VDIEKHTLQT QSGKSLKYGS LIVATGCTAT RFPEK--IGG NLPGVHYVRD
Ar   TPDWYKEKGI EVIYEDPVAG ADFEKQTLTT DAGKQLKYGS LIIATGCTAS RFPDK--IGG HLPGVHYIRE
Cu   LPDWYKEKGI ELILSTEIVE ADLPAKRLRS AHGKIYNYQT LIIATGSTVI KLSDFGVQGA DAKNIFYLRE

               218        225        235        245        255        265        275
Sp   VADADSLIES LKKAK---KV VIVGGGYIGM EVAAAAVGWN LDTTVIFPED HLLQRLFTPS LARKYEELYE
Ar   VADADSLIAS LGKAK---KI VIVGGGYIGM EVAAAAVAWN LDTTIVFPED QLLQRLFTPS LAQKYEELYR
Cu   IDDADQLVEA IKAKEN-GKV VVVGGGYIGL ELGAALRINN FDVSMVYPEP WCMPRLFTPE IAAFYEGYYA

               285        295        305        315        325        335        345
     QNGVKFVKGA MIKNLEAGSD GSVAAVNLEN GSTIEADTII IGIGAKPAVG PFENVGLDTT VGGIEVDGLF
     QNGVKFVKGA SINNLEAGSD GRVSAVKLAD GSTIEADTVV IGIGAKPAIG PFETLAMNKS IGGIQVDGLF
     QKGITIIKGT VAVGFTVDTN GEVKEVKLKD GRVLEADIVV VGVGARPLTS LFKGQIVEE- KGGIKTDEFF

               355        365        375        385        395        401        411
Sp   RSKVPGIFAI GDVAAFPLKM YDRVARVEHV DHARKSAQHC VSALLSARTH T----YDYLP YFYSRVFEYE
Ar   RTSTPGIFAI GDVAAFPLKI YDRMTRVEHV DHARRSAQHC VKSLLTAHTD T----YDYLP YFYSRVFEYE
Cu   KTSVPDVYAV GDVATFPLKL YNELRRVEHV DHSRKSAEQA VKAIKASEEG KAIEEYDYLP YFYSRSFDLS

               421        431       440        446        456        466        476
Sp   GSQRKVWWQF FGDNVGEAVE VGNFDPKIA- ----TFWIDS GRLKGVLVES GSPEEFQLLP KIAKAQPLVD
Ar   GSPRKVWWQF FGDNVGETVE VGNFDPKIA- ----TFWIES GRLKGVLVES GSPEEFQLLP KLARSQPLVD
Cu   -------WQF YGDNVGDAVL FGDNSPDSAT HKFGSYWIKD GKVVGAFLES GSPEENKAIA KVARIQPSVE

               486        496
Sp   KAKLQSASSV EEALEIIHQS M
Ar   KAKLASASSV EEALEIAQAA LQS
Cu   SSDLLLKEGI SFASKV
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Fig. 2. Comparison of the deduced amino acid sequence of chloroplastic MDA reductase from spinach with those of MDA reductases from various plants. Sp, chloroplastic isozyme from spinach; Ar, organelle isozyme from *Arabidopsis* (D84417); Cu, cytosolic isozyme from cucumber (Sano & Asada 1994). The determined sequence of amino terminus of chloroplastic MDA reductase from spinach is indicated by underlining. The position shown by closed triangle (▼) is the predicted cleavage site of the transit peptide of the spinach enzyme. The sequences were aligned using ClustalW program. The conserved Cys residues are indicated by the cross (✖) . This comparison located three finger prints (, for binding FAD; , for binding NADH).

We obtained the full-length sequence of the cDNA encoding chloroplastic MDA reductase from spinach with RT-PCR. The determined sequence was 1,990 bp length and revealed an ORF of 1,494 bp encoding 497 amino acids (Fig. 2). The amino acid sequence deduced from the ORF has the corresponding sequence with the purified chloroplastic MDA reductase from spinach and an extension region consisting of 58 residues at amino terminus, which is expected to function as a transit peptide. Cleavage of the transit peptide resulted in a mature enzyme of 439 amino acid residues with a calculated molecular mass of 47,675 Da. This does not agree with that the purified enzyme from spinach showed a molecular mass of 51 kDa on SDS-PAGE. The recombinant protein of the chloroplastic MDA reductase from spinach as its mature form produced in *E. coli* cells with pET expression system (Novagen) were showed a band at the same position as that of the chloroplastic isozyme purified from spinach on SDS-PAGE and immunoblotting. These results indicate that the molecular mass of the chloroplastic MDA reductase from spinach is actually 48 kDa but the isozyme shows a molecular mass of 51 kDa apparently on SDS-PAGE.

Amino acid sequence of this gene product showed homologies higher than 75% to those of MDA reductases from *Arabidopsis* (D84417) and leaf mustard (AF109695), that are thought to be organelle enzymes. In contrast, this sequence showed low (about 45%) homologies with those of cytosolic enzymes. The FAD- and NAD(P)H-binding domains of flavoproteins are also found in the chloroplastic MDA reductase from spinach (Fig. 1), as those from other sources (Sano & Asada 1994). It has been shown that thiol-modifying reagents suppress the reduction of MDA reductase-FAD by NAD(P)H (Sano et al. 1995), and inhibit its activity. Two Cys residues are conserved in aminoacid sequences of cytosolic MDA reductase. However, are the Cys residue at the conserved position is only one, corresponding to Cys132 in the chloroplastic enzyme from spinach and Cys69 in the cytosolic enzyme from cucumber (Fig. 2). This Cys residue must react with thiol reagents and participate in the transfer of electrons from NADH to the FAD.

Acknowledgments

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