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Dehydroascorbate Reductase cDNA from Sweet Potato (*Ipomoea batatas* [L.] Lam): Expression, Enzyme Properties, and Kinetic Studies

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A cDNA encoding a putative dehydroascorbate reductase (DHAR) was cloned from sweet potato. The deduced protein showed a high level of sequence homology with DHARs from other plants (67 to ~81%). Functional sweet potato DHAR was overexpressed and purified. The purified enzyme showed an active monomeric form on a 12% native PAGE. The protein's half-life of deactivation at 50 °C was 10.1 min, and its thermal inactivation rate constant K_d was 6.4×10^{-2} min^{-1}. The enzyme was stable in a broad pH range from 6.0−11.0 and in the presence of 0.8 M imidazole. The K_m values for DHA and GSH were 0.19 and 2.38 mM, respectively.

KEYWORDS: Sweet potato (*Ipomoea batatas* [L.] Lam); expression; dehydroascorbate reductase

INTRODUCTION

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) catalyzes the reduction of dehydroascorbate (DHA) to ascorbate (AsA) during redox processes and plays an important role in maintaining AsA in its reduced form. Humans are unable to synthesize AsA and depend on dietary intake to meet nutritional needs. In both plant and animal metabolism, a major area of research focus on AsA is its antioxidant properties. Evidence has shown that AsA is important in protecting plants and mammals from oxidative stress. An important source of AsA is by its recycling from its oxidative form, in which DHAR catalyzes the conversion of DHA to AsA using GSH as a reducing agent.

It has been reported that the overexpression of DHAR in tobacco leaves and in maize resulted in an increased AsA content. The results demonstrated that DHAR plays important roles in determining the pool size of AsA (*1*). Ozone is a serious air pollutant that leads to extensive damages to plants by generating reactive oxygen species (ROS). Recently, Yoshida et al. showed that ozone exposure increased the expression of the cytosolic DHAR gene, which is important for ozone tolerance in *Arabidopsis thaliana* (*2*). Lee et al. showed that the expression of multiple antioxidant enzymes, such as DHAR, superoxide dismutase, and ascorbate peroxidase in chloroplasts, is more effective than single or double expression for developing transgenic plants with an enhanced tolerance to environmental stresses (*3*).

Sweet potato is widely distributed in Taiwan. In Asia, it is considered to be one of the cash crops sustaining the agricultural economy. Therefore, this local crop is especially highly regarded in Taiwan. Several components from sweet potato have been shown to exhibit antioxidative effects (*4*−*9*). Although sweet potato shows physiological activities with potential medical applications (*10*), only a handful of scientific studies have been reported. This motivated us to search for active components from the tuberous root of sweet potato for use. Here, we report the cloning of a putative DHAR cDNA from sweet potato. The coding region of the cDNA was introduced into an *Escherichia coli* expression system. The target protein was overexpressed and showed high levels of activity in catalyzing DHA to AsA. The recombinant enzyme was purified, and its properties were investigated.

MATERIALS AND METHODS

Sweet Potato (*Ipomoea batatas* [L.] Lam). Sweet potato was naturally grown in a field from the northern part of Taiwan.

Total RNA Preparation and cDNA Synthesis. The tuberous roots of sweet potato (wet wt 3 g) were frozen in liquid nitrogen and ground to a powder in a ceramic mortar. PolyA mRNA (24 μg) was prepared using a Straight A's mRNA Isolation System (Novagen). Three micrograms of the mRNA was used for 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA synthesis using Clontech's SMART RACE cDNA Amplification Kit.

Isolation of DHAR cDNA. Using 3′-RACE-Ready cDNA of sweet potato as a template and a UPM (universal primer A mix, purchased from BD Biosciences) primer and a degenerate primer (5′ GYR ACA AGC CCR ART GGT TC 3′), a 0.774 kb fragment was amplified by PCR. The degenerate primer was designed based on the conserved

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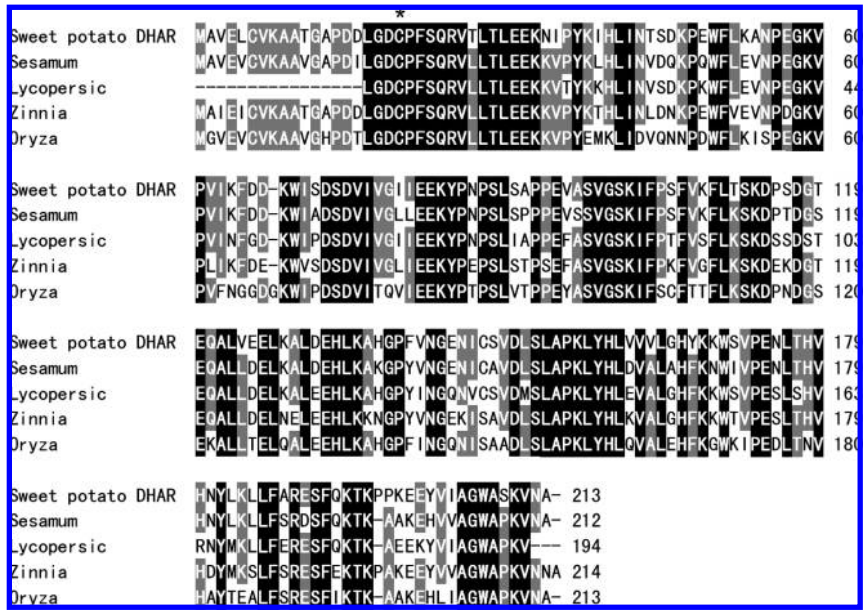

Figure 1. Alignment of amino acid sequences of DHAR. Sweet potato (this study), *S. indicum*, *L. esculentum*, *Z. violacea*, and *O. sativa*. Identical amino acids in all sequences are in black, and conservative replacements are in gray. The asterisk denotes the portion that may be involved in the formation of the disulfide bond with GSH.

sequences of DHAR from *Lycopersicon esculentum* (accession no. ABF59820), *Zinnia violacea* (accession no. BAD27392), and *A. thaliana* (accession no. AAG24946). The 0.774 kb fragment was subcloned and sequenced. On the basis of this DNA sequence, a reverse primer (5′ GCC AAC AGA GGC AAC TTC 3′) near the 5′end of the 0.774 kb fragment was synthesized. The reverse primer allowed sequence extension from the 5′end of the 0.774 kb fragment. Using 5′-RACE-Ready cDNA of sweet potato as a template, a 0.47 kb fragment between this reverse primer and the UPM primer was amplified by PCR. This DNA fragment was subcloned and sequenced. Sequence analysis revealed that the combined sequence of the 0.774 and 0.47 kb fragments covered an open reading frame of DHAR cDNA (948 bp, EMBL accession no. EF065676). The identity of the DHAR cDNA clone was assigned by comparing the DNA sequence and the inferred amino acid sequence in various databanks using the basic local alignment search tool (BLAST).

Recombinant DNA Preparation. The coding region of DHAR cDNA was amplified using gene specific flanking primers. The 5′ upstream primer contained the *Nde*I recognition site (5′ CAT ATG GCG GTG GAG CTC TGT G 3′) and the 3′ downstream primer contained the *Xho*I recognition site (5′CTC GAG TGC ATT AAC CTT TGA AGC CC 3′). Using 0.2 μg of sweet potato cDNA as a template, and 10 pmol each of 5′ upstream and 3′ downstream primers, a 0.65 kb fragment was amplified by PCR. The fragment was ligated into pCR4-Topo and transformed into *E. coli*. Plasmid DNA was isolated from a positive clone and digested with *Nde*I and *Xho*I. The digestion products were separated on a 1% agarose gel. The 0.65 kb insert was gel purified and subcloned into *Nde*I and *Xho*I sites of pET-20b(+) vector (Novagen). The recombinant DNA was then transformed into *E. coli* BL21(DE3). The recombinant protein was overexpressed and purified, and its function was checked by an enzyme activity assay.

Expression and Purification of Recombinant Sweet Potato DHAR. The transformed *E. coli* BL21(DE3) cells containing the putative DHAR cDNA were grown at 32 °C in 20 mL of Luria−Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.9. Protein expression was induced by the addition of isopropyl β-D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated at 32 °C for an additional 4 h under 80 rpm, and then the bacterial cells were harvested by centrifugation. Soluble proteins were extracted from the cell pellet with glass beads as described before (*11*). The His-tagged DHAR protein was purified by Ni-NTA affinity chromatography as per the manufacturer's instruction (Qiagen). The purified enzyme (1.2 mL) was dialyzed against 50 mL of 1/3 PBS containing 5% glycerol at 4 °C for 4 h. Fresh 1/3 PBS containing 5% glycerol was changed once during dialysis. The dialyzed sample was used directly for analysis.

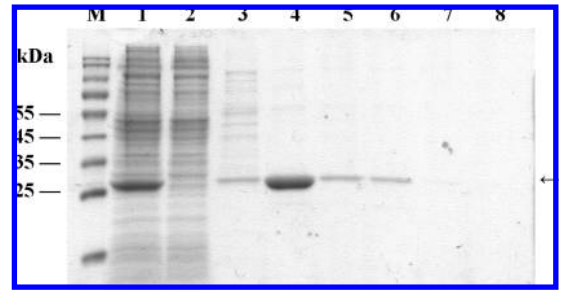

Figure 2. Purification of recombinant DHAR. Twelve milliliters of crude extract was obtained from 250 mL of culture. Each sample (15 μL) was analyzed on a 12% SDS-PAGE followed by Coomassie blue stain. Lane M, size marker; lane 1, crude extract; lane 2, flowthrough; lane 3, low concentration of washed imidazole; and lanes 4−6, eluted DHAR fractions.

Molecular Mass Determination by ESI Q-TOF. The protein sample (20 μg/50 μL) in 0.03 × PBS containing 0.05 mM imidazole and 2% glycerol was shipped to Gu-Yuan Biotech Services Corp. for molecular mass determination using an ESI Q-TOF mass spectrometer (Micromass).

Protein Concentration Measurement. The protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as the standard.

DHAR Activity Assay. The activity of DHAR was determined by monitoring the glutathione-dependent production of AsA as described elsewhere (*12, 13*) with some modifications. The assay mixture (100 μL) contained 50 mM potassium phosphate buffer (pH 8.0), 0.2 mM EDTA, 0.25 mM DHA, 0.2 mM GSH, and 0.05 μg of enzyme. The absorbance at 265 nm was recorded for 1.5 min. Nonenzymatic reduction of DHA by GSH was measured in a separate cell as a control, and the absorbance increment over that of the control activity was taken for DHAR activity. The absorbance coefficient of AsA at 265 nm was $\epsilon = 12.59\ \text{mM}^{-1}\ \text{cm}^{-1}$.

Enzyme Characterization. The enzyme sample was tested for stability under various conditions. The DHAR sample was tested for (1) thermal stability: The purified enzyme sample was heated to 50 °C for 2, 4, 8, or 16 min. After each treatment, triplicate sets of sample were electrophoresed onto a 12% SDS-PAGE, a 12% native gel, or

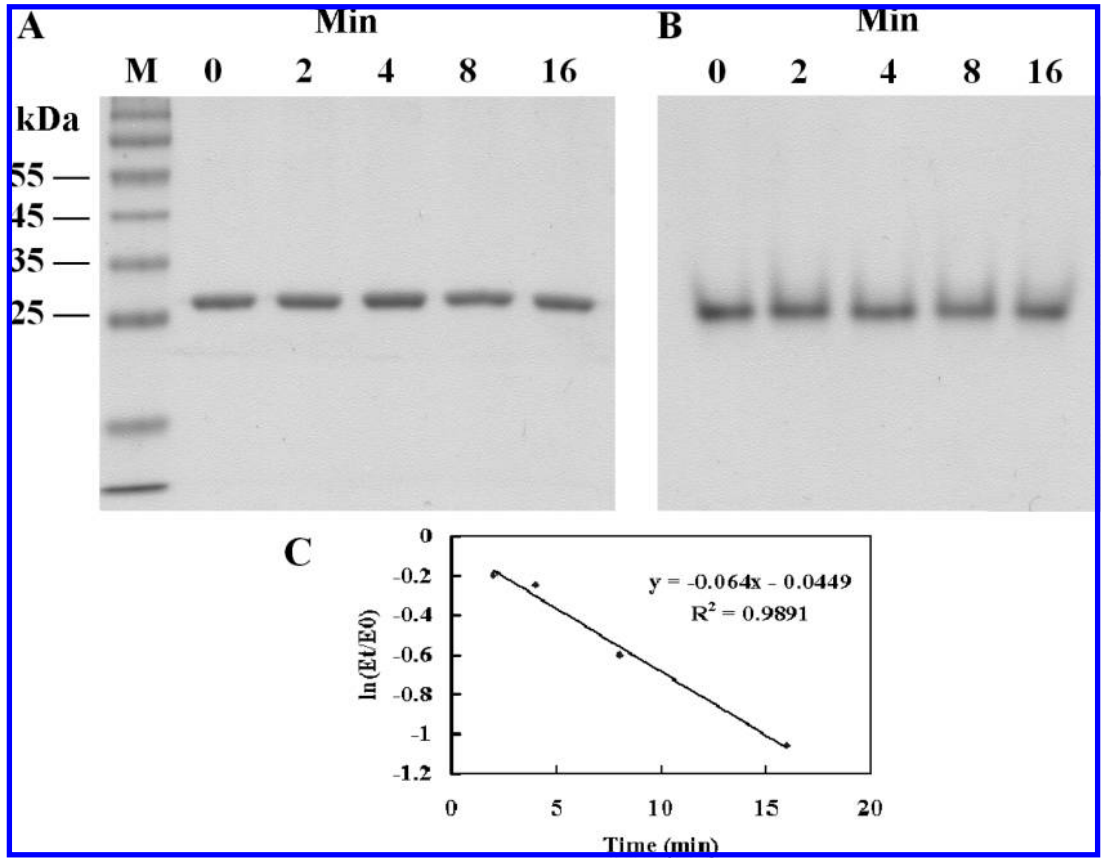

Figure 3. Effect of temperature on purified DHAR. The enzyme samples heated at 50 °C for various time intervals were analyzed by (**A**) 12% SDS-PAGE and (**B**) 12% native PAGE. Both staining for protein (1.6 μg/lane). Lanes 1−5 (0, 2, 4, 8, and 16 min). (**C**) Plot of thermal inactivation kinetics. The effect of temperature was determined by an activity assay. E_0 and E_t are original activity and residual activity after being heated for different time intervals. The data are the mean of three independent experiments.

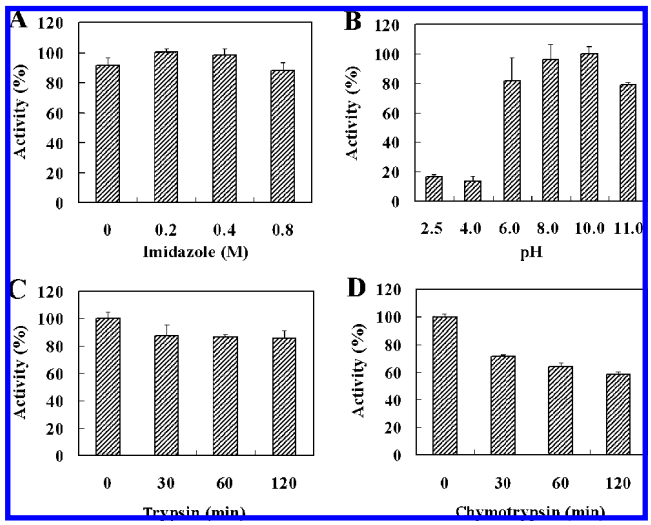

Figure 4. Effect of imidazole, pH, chymotrypsin, and trypsin on the stability of purified DHAR. (**A**) Enzyme samples were incubated with imidazole of different concentrations at 37 °C for 0.5 h and then subjected to activity assays. (**B**) Enzyme samples were incubated in buffers with different pH values at 37 °C for 0.5 h and then analyzed by activity assay (1.6 μg/lane). (**C**) Enzyme samples were incubated with trypsin (1:10 DHAR) at 37 °C for different time intervals and then subjected to activity assays. (**D**) Enzyme activities after treatment with chymotrypsin (1:10 DHAR) measured by activity assays. The data are the mean of three independent experiments.

were used to determine the changes in DHAR activity. (2) Imidazole effect: During protein purification, the DHAR enzyme was eluted with imidazole. Therefore, the effect of imidazole on the protein activity was examined. Imidazole was added to the enzyme sample to the levels of 0.2, 0.4, or 0.8 M and incubated at 37 °C for 0.5 h. (3) pH stability: Purified DHAR was adjusted to the desired pH by adding a half-volume of buffer with different pH values: 0.2 M citrate buffer (pH 2.5 or 4.0), 0.2 M potassium phosphate buffer (pH 6.0 or 8.0), or 0.2 M CAPS buffer (pH 10.0 or 11.0). (4) Proteolytic susceptibility: The enzyme was incubated with one-tenth its weight of trypsin or chymotrypsin at pH 8.0, 37 °C for a period of 0.5, 1, or 2 h. An aliquot of the treated sample was removed at various time intervals for activity analysis.

Kinetic Studies. The recombinant sweet potato DHAR activities were tested at 25 °C by monitoring the production of ascorbate at A_{265} for DHA reduction. The kinetic properties of DHAR (0.05 μg) in a total volume of 100 μL were determined using different concentrations of GSH (0.4−3.2 mM) with either a fixed amount of 0.1 mM DHA as the substrate or using different concentrations of DHA (0.025−0.75 mM) with a fixed amount of 0.2 mM GSH. The K_m, V_{max}, and K_{cat} values were calculated from Lineweaver−Burk plots.

RESULTS

Cloning and Characterization of a cDNA Encoding DHAR. A putative DHAR cDNA clone was identified as DHAR with a consensus pattern and sequence homology to the published DHARs. The DHAR cDNA (948 bp, EMBL accession no. EF065676) contains an open reading frame that encodes a protein of 213 amino acid residues with a predicted molecular mass of 25 kDa. **Figure 1** shows the amino acid sequence alignment of the putative sweet potato DHAR with DHAR from several sources. The DHAR shared 81, 79, 76, and 67% sequence identity with DHAR from *Sesamum indicum* (accession no. ABB89210), *L. esculentum* (accession no. ABF59820),

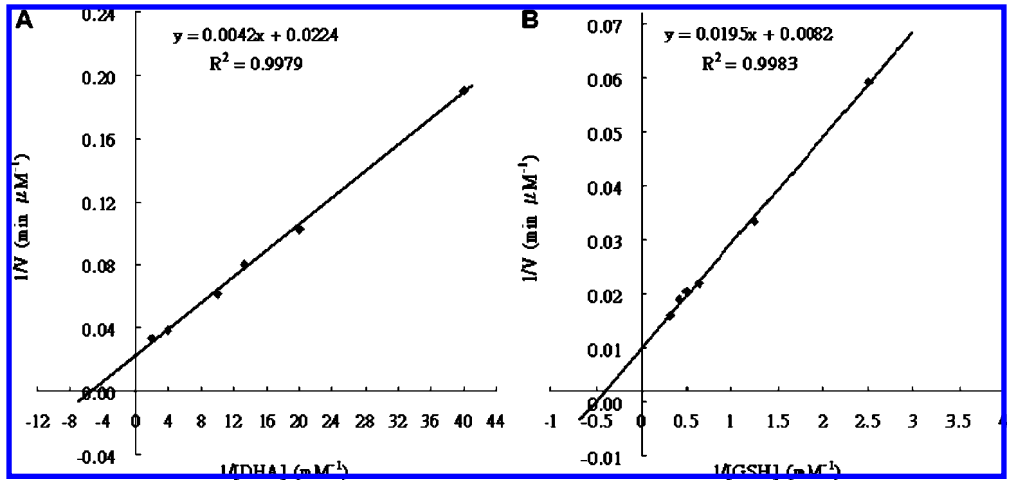

Figure 5. Double reciprocal plot of varying DHA and GSH on DHAR activity. The initial rate of the enzymatic reaction was measured at 0.2 mM GSH with the DHA concentration varying from 0.025 to 0.75 mM (**A**) and at 0.1 mM DHA with the GSH concentration varying from 0.4 to 3.2 mM (**B**). K_m, V_{max}, and K_{cat} were calculated from Lineweaver−Burk plots.

Z. violacea (accession no. BAD27392), and *Oryza sativa* (accession no. BAA90672), respectively. Using the InterProScan Sequence Search, we found three conserved domains: glutathione *S*-transferase N-terminal domain located at positions 20−82, thioredoxin fold at positions 20−89, and glutathione *S*-transferase C-terminal domain at positions 90−212. The most distinctive functional motif of this DHAR is the CxxS motif located at C^{20}PFS. CxxS and CxxC often are conserved in redox enzymes (*14*). This CPFS/CPFC domain shows itself to be highly conserved in all known DHARs. The homologous motif C ^{75}PFC was found in spinach chloroplast DHAR in which C^{75} was reported to play a key role in the formation of a disulfide bond with GSH. The disulfide bond formation with GSH is essential for the conversion reaction of DHA to AsA (*12, 13, 15, 16*). In addition, sweet potato DHAR may be classified into the cytosolic DHAR isozyme family group because it has a higher sequence homology with the cytosolic DHAR group (from the sequence alignment with *S. indicum* and *O. sativa*, this cDNA belongs to cytosolic DHAR) (*17*).

Expression and Purification of Sweet Potato Recombinant DHAR. cDNA was introduced into the *E. coli* expression system as described in the Materials and Methods. The recombinant DHAR protein was overexpressed, and the total cellular proteins were analyzed by SDS-PAGE (**Figure 2**). The DHAR fusion protein was purified by Ni-NTA affinity chromatography. The progress of purification was monitored by SDS-PAGE as shown in **Figure 2**. The results showed that the DHAR protein was purified to homogeneity in a single step. Only monomeric DHAR was detected in these fractions, suggesting that sweet potato DHAR is composed of monomer with an apparent molecular mass of 25 kDa. An ESI Q-TOF of DHAR confirms the presence of only one protein band (monomer) under the conditions of 0.03 × PBS containing 0.05 mM imidazole and 2% glycerol. When using native PAGE to analyze the DHAR, only one protein band was seen in **Figure 4B**. This indicates that the enzyme is monomeric in nature. The yield of the purified DHAR was 2.25 mg per 250 mL of culture. The specific activity was 52 units mg^{-1} (one unit is 1 μmol min^{-1} of AsA produced).

Characterization of Purified DHAR. Thermal stability of the recombinant sweet potato DHAR was examined as described in the Materials and Methods. The enzyme's inactivation kinetics at 50 °C fitted the first-order inactivation rate equation ln(Et/Eo) = $-K_d$ t, where Eo and Et represented the original activity and the residual activity after heating for time t, respectively. The thermal inactivation rate constant (K_d) calculated for the enzyme at 50 °C was 6.4 × 10^{-2} min^{-1}, and the half-life of inactivation was 10.1 min (**Figure 3C**). The DHAR showed a slight decrease in activity with increasing concentration of imidazole to 0.8 M (**Figure 4A**). The DHAR was stable in a broad pH range from 6.0 to 11.0 (**Figure 4B**). The enzyme was somewhat resistant to digestion by trypsin (**Figure 4C**) and chymotrypsin (**Figure 4D**) even at a high enzyme/substrate (w/w) ratio of 1:10. The results suggest that this DHAR has a rigid structure and that the potential cleavage sites were not accessible by the enzymes under the reaction conditions. 1-Cys peroxiredoxin was used as a positive control in the protease digestion. The peroxiredoxin was degraded within 20 min treatment with either trypsin or chymotrypsin (results not shown).

Kinetic Studies of Purified DHAR. As shown in **Figure 5A**,**B**, the Lineweaver−Burk plot of the velocity (1/*V*) against 1/DHA gave K_m = 0.19 mM, V_{max} = 44.64 μM/min, and K_{cat} = 2209.4 min^{-1}. The plot of the velocity (1/*V*) against 1/GSH gave K_m = 2.38 mM, V_{max} = 121.95 μM/min, and K_{cat} = 6035.4 min^{-1} for DHAR, respectively.

DISCUSSION

This study reported the first cloning and expression of an important reduction enzyme, DHAR, from sweet potato. The biological active form of the DHAR was successfully expressed in *E. coli*. We scanned the DHAR sequence against Swiss-Prot Prosite to locate potential post-translational modification sites. Although several potential phosphorylation sites, N-glycosylation sites, and a N-myristoylation site were found, it appears that eukaryotic specific post-translational modifications may not be required for the function of DHAR.

Biochemists have always been interested in enzyme's stabilities. It was evident that the recombinant sweet potato DHAR was stable in a broad pH range from 6 to 11. This pH value was different from those of other plant species. For instance, the sesame hairy roots DHAR had an optimal pH of 6 (*18*), and spinach leaves (*12, 15*) and rice (*19*) had optimal pH values between 7.8 and 8.2. Sweet protato DHAR is stable at 50 °C, which is consistent with the report that sesame hairy roots DHAR is active in between 30 and 60 °C (*18*). The sweet potato DHAR protein contains potential trypsin cleavage sites and potential chymotrypsin high specificity (C-term to [FYW], not

before P) cleavage sites or chymotrypsin low specificity (C-term to [FYWML], not before P) cleavage sites. However, the enzyme appeared to be resistant to digestion by trypsin and chymotrypsin even at a high enzyme/substrate (w/w) ratio of 1:10 (**Figure 4C**,**D**). This may be due to its rigid or compact structure and limits the accessibility of the enzymes to the potential cleavage sites under the reaction conditions.

Shimaoka et al. (*15*) reported that chloroplast DHAR had K_m values of 70 μM and 1.1 mM for DHA and reduced GSH, respectively. Sweet potato DHAR had a higher K_m value for DHA (0.19 mM) and reduced GSH (2.38 mM). A possible explanation for the discrepancy in K_m values of the two sources is that plant leaves are (root is underground) exposed to ozone, UV light, etc., causing ROS generation, which would require more efficient DHAR to produce AsA for protection (*16*).

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