Plant Cell Physiol. 39(12): 1350–1358 (1998)

L-Galactono-γ-lactone Dehydrogenase from Sweet Potato: Purification and cDNA Sequence Analysis

Tsuyoshi Imai [1,4], Shuichi Karita [2], Gen-ichi Shiratori [1], Miyo Hattori [3], Tsukasa Nunome [1], Kazuko Ôba [3] and Masashi Hirai [1]

[1] *Department of Vegetable Breeding, National Research Institute of Vegetables, Ornamental Plants and Tea, Ano, Mie, 514-2392 Japan*
[2] *Center for Molecular Biology and Genetics, Mie University, Tsu, Mie, 514-8507 Japan*
[3] *Department of Food Science and Nutrition, Nagoya Women's University, Mizuho, Nagoya, 467-8610 Japan*

L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3, GLDHase) was partially purified from mitochondria of sweet potato tuberous roots over 600-fold on a specific activity basis, followed by purification of the enzyme protein of 56 kDa by a preparative SDS-PAGE. The absorption spectrum of the hydroxylapatite column-purified GLDHase showed peaks at 448 and 373 nm, suggesting the presence of flavin as a prosthetic group. The activity of GLDHase was inhibited by lycorine, an alkaloid which inhibits ascorbic acid biosynthesis in vivo. N-terminal partial sequences of four internal polypeptides generated by partial digestion of GLDHase with V8 protease were determined. The deduced nucleotide sequences were used to amplify a cDNA fragment of the GLDHase gene. The clone encoded a polypeptide of 581 amino acid residues with a molecular mass of 66 kDa. The deduced amino acid sequence showed 77% identity with that of cauliflower GLDHase, and significant homology to those of L-gulono-γ-lactone oxidase (22% identity) from rat and L-galactono-γ-lactone oxidase from yeast (17% identity), which are enzymes involved in L-ascorbic acid biosynthesis in these organisms. The absorption spectrum and cDNA sequence suggested that the flavin group bound noncovalently. We conclude that GLDHase, L-gulono-γ-lactone oxidase and L-galactono-γ-lactone oxidase are homologous in spite of the difference in substrates and electron acceptors. Genomic Southern analysis suggested that GLDHase gene exists as a single copy in the genome of sweet potato.

Key words: L-Ascorbic acid — cDNA sequence — L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3) — Sweet potato (*Ipomoea batatas*).

With the exception of dry seeds and other dormant organs, plants generally contain substantial amounts of L-ascorbic acid (AsA) (Arrigoni et al. 1992, Foyer 1993, Loewus 1980). The most important role of AsA in plants is thought to be its action as a reductant (Foyer 1993). AsA functions not only as an antioxidant that scavenges radicals and active oxygen species but also as a cofactor in certain enzymatic reactions where it maintains the component metal ions, which are essential for the reaction, in the reduced state. A physiological role for AsA in plant cell development has also been proposed (Arrigoni 1994).

In spite of its importance as a plant constituent, the biosynthetic pathway for AsA in higher plants was not fully understood until recently (Foyer 1993, Nishikimi and Yagi 1996). The enzymatic activity of oxidation of L-galactono-γ-lactone to AsA in plant mitochondria was demonstrated many decades ago (Mapson et al. 1954). In animals, L-gulono-γ-lactone, a stereoisomer of L-galactono-γ-lactone, is a preferential precursor for AsA biosynthesis, which was confirmed by using purified enzyme L-gulono-γ-lactone oxidase (EC 1.1.3.8, GLO) (Kiuchi et al. 1982, Nishikimi et al. 1976). The biosynthetic pathway of L-galactono-γ-lactone has not been evidenced experimentally in plants, but the presence of a pathway similar to the well characterised one in animals has been proposed: D-galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone in plants vs. D-glucose → D-glucuronic acid → L-gulonic acid → L-gulono-γ-lactone in animals (Mapson et al. 1954, Nishikimi and Yagi 1996). However, tracer studies using specifically labelled [^{14}C]D-glucose at position 1 or 6 indicated that the biosynthetic pathway of AsA in plants was different from that in animals: the carbon skeleton was preserved in plants while it was inverted in animals (Horowitz et al. 1952, Horowitz and King 1953, Loewus 1963, 1980). An alternative pathway in agreement with this observation was proposed: D-glucose → D-glucosone → L-sorbosone → AsA (Loewus 1987, Saito et al. 1990). Recently, another pathway has been proposed: GDP-D-mannose → GDP-L-galactose → L-galactose → L-galactono-γ-lactone → AsA (Wheeler et al. 1998). Formation of AsA from these intermediates by plant extracts were demonstrated. The pathway harmonise with both of

Abbreviations: ALO, D-arabinono-γ-lactone oxidase; AsA, L-ascorbic acid; CSPD, disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]-decan}-4-yl)phenyl phosphate; GLDHase, L-galactono-γ-lactone dehydrogenase; GLO, L-gulono-γ-lactone oxidase; RACE, rapid amplification of cDNA ends.

The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL, Genbank under accession number AB017357.

[4] Corresponding author: E-mail, imagic@nivot.affrc.go.jp; FAX, +81-59-268-1339.

the previous findings: preservation of carbon skeleton and immediate precursor for AsA is L-galactono-γ-lactone.

L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3, GLDHase) catalyses the final step of AsA biosynthesis in plants: oxidation of L-galactono-γ-lactone to AsA. The enzyme has recently been highly purified from sweet potato roots (Ôba et al. 1995) and cauliflower florets (Østergaard et al. 1997). Among the enzymes involved in AsA biosynthesis in plants, GLDHase is the only enzyme which has been highly purified. To elucidate the biosynthesis of AsA molecular biologically, we have isolated a cDNA clone encoding GLDHase from sweet potato. The homology in the deduced amino acid sequences of plant GLDHases, rat GLO and yeast L-galactono-γ-lactone oxidase is discussed.

Materials and Methods

Purification of GLDHase from sweet potato tuberous roots—Tuberous roots of sweet potato (*Ipomoea batatas* L., cv. Beniazuma) were homogenised in an extraction medium consisting of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 M sucrose and 10 mM 2-mercaptoethanol, at a ratio of 0.75 ml per g tissue. Isolation of mitochondria, solubilisation of enzymes by sonication, fractionation by centrifugation, Sephacryl gel filtration and DE-52 anion exchange column steps were performed essentially as described previously (Ôba et al. 1995). In the present purification procedure, hydroxylapatite chromatography (Econopack-HTP, 5 ml, Nippon Bio-Rad Laboratories, Tokyo, Japan) was used instead of chromatofocusing. The hydroxylapatite column was equilibrated with a buffer consisting of 5 mM potassium phosphate (pH 7.4), 5 mM GSH and 10% (w/v) glycerol. Fractions from the DE-52 column with GLDHase activity more than 0.25 units ml^{-1} were pooled and concentrated by ultrafiltration. The concentrate was then diluted 8-fold with 1 mM potassium phosphate buffer (pH 7.4) containing 5 mM GSH and 10% glycerol, and applied to the hydroxylapatite column. After washing with 8 ml of equilibration buffer, we performed linear gradient elution of potassium phosphate buffer (5 to 800 mM, 40 ml, pH 7.4). Fractions with GLDHase activity of more than 0.6 units ml^{-1} were combined, concentrated by ultrafiltration, and stored at −30°C.

GLDHase activity assay—The activity was measured at 25°C according to the method of Ôba et al. (1995) after slight modification. The reaction mixture contained 1.05 mg ml^{-1} (85 μM) Cyt *c*, 10 mM potassium phosphate buffer (pH 7.8), 5.6 mM L-galactono-γ-lactone and 2.5 μl of enzyme preparation in a total volume of 50 μl. The increase in the absorbance at 550 nm was followed immediately after the addition of L-galactono-γ-lactone. One unit of activity is defined as the amount of extract required to oxidise 1 μmol of L-galactono-γ-lactone (equivalent to the formation of 2 μmol of reduced Cyt *c*) per min. A molar coefficient value of 17.3 mM^{-1} cm^{-1} for the difference between oxidised and reduced form of Cyt *c* was used.

For the inhibition experiments, mixtures containing inhibitors in which the substrate was omitted were incubated for 4 min at 25°C prior to the addition of L-galactono-γ-lactone. Lycorine was donated by Dr. Takagi, Mukogawa Women's University, and dissolved in 0.1 M hydrochloric acid and added to the GLDHase assay mixture.

Protein assay—Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoretic purification of GLDHase—A preparative gel electrophoresis system (BIOFORESIS III, Atto, Tokyo, Japan) was used for obtaining a single band preparation of GLDHase. The hydroxylapatite column-purified sample was loaded onto a cylindrical gel, with a diameter of 16 mm, consisting of a 5 mm long stacking gel (4.4% acrylamide–0.12% bisacrylamide) and 24 mm long separating gel (15% acrylamide–0.067% bisacrylamide). Electrophoresis was performed at a constant current of 15 mA. Proteins which migrated to the bottom of the separating gel and diffused into recovery buffer were fractionated according to the manufacturer's instruction. A portion of each fraction was analysed by SDS-PAGE, and fractions containing 56 kDa protein were combined and concentrated by centrifuge-based ultrafiltration (Microcon 30, Amicon, MA, U.S.A.).

V8 protease digestion and amino acid sequencing—About 10 μg of purified GLDHase protein (56 kDa) was loaded onto a stacking gel (4.7% acrylamide–0.13% bisacrylamide) onto which 1.4 μg of V8 protease (Wako Pure Chemical, Osaka, Japan) solution was overlayed. A current (12 mA) was applied for 1 h, then electrophoresis was aborted for 25 min to allow the digestion of GLDHase protein in the stacking gel (Cleveland et al. 1977). Partially digested polypeptides were then separated in a gel (16% acrylamide–0.44% bisacrylamide), blotted onto a polyvinyliden difluoride membrane and stained with Coomassie Brilliant Blue R-250. Major bands were excised from the membrane, and amino acid sequences were determined by an automated sequencer (model 476A, PERKIN ELMER Japan, Chiba, Japan).

PCR-amplification and sequence analysis of cDNA clone for GLDHase—Based on the amino acid sequences obtained, degenerate oligonucleotide primers were designed and used for amplification of GLDHase-specific cDNA from tuberous roots of the same cultivar. Poly(A^+) RNA was isolated from frozen and powdered tuberous roots using FastTrack kit (Invitrogen, The Netherlands). cDNA was synthesised using a commercial kit (TimeSaver, Pharmacia Biotech, Tokyo, Japan). PCR amplification was performed: 41 cycles of denaturation at 94°C for 60 s, annealing at 49°C (53°C for cycles 1–3 and 51°C for cycles 4–6) for 60 s and elongation at 72°C for 120 s, followed by 72°C for 5 min. The reaction mixture contained 100 pmol of sense primer S3B (5′-AA(C/T)-GA(A/G)AA(A/G)AA(A/G)CA(A/G)AAIAT-3′, corresponding amino acid sequence: NEKKQK(I/M), see Fig. 1C) and 100 pmol of antisense primer A1D (5′-TC(A/G)AAICC(C/T)AAIAT(C/T)-TC(A/G)TC-3′, corresponding amino acid sequence: DEILGF(D/E), see Fig. 1C), 5 nmol of each dNTP, 1.5 units of Taq polymerase (Takara Shuzo, Kyoto, Japan) and 0.5 μl of cDNA in a total volume of 25 μl. A 0.8 kb fragment was amplified then cloned into pUC18 and sequenced. Based on the nucleotide sequence obtained, 3′- or 5′- extension of the cDNA clone was performed using the rapid amplification of cDNA ends (RACE) technique (Frohman et al. 1988). Amplified fragments were cloned into plasmid pCR2.1 (Invitrogen) and nucleotide sequences were determined for about 600 and 800 bases for 5′- and 3′-extended fragments, respectively. A set of primers were designed from the sequences of the 5′- or 3′-noncoding regions to amplify a full length cDNA encoding GLDHase: sense primer, 5′-CTTTAATCCCTC-CATAGCCAC-3′; antisense primer, 5′-TTGCATAATCATG-TGAATCC-3′. PCR was performed using cDNA from tuberous roots as template. The reaction condition was 42 cycles of denaturation at 94°C for 45 s, annealing at 43°C for 45 s and elongation at 72°C for 200 s, followed by 72°C for 7 min. The mixture contained 5 pmol of each primer, 5 nmol of each dNTP, 1.5 units of Taq polymerase, 0.38 μl of cDNA in a total volume of 25 μl.

An amplified fragment of about 2.0 kb was cloned into pCR2.1 plasmid. Four independent plasmid clones were sequenced to eliminate any missequence formed during PCR amplification. DNA sequencing was done using a DNA sequencing kit and model 373S DNA sequencer (PERKIN ELMER Japan). Nucleotide sequences were analysed with DNASIS (Hitachi Software Engineering, Yokohama, Japan) and GeneWorks (IntelliGenetics, CA, U.S.A.) software.

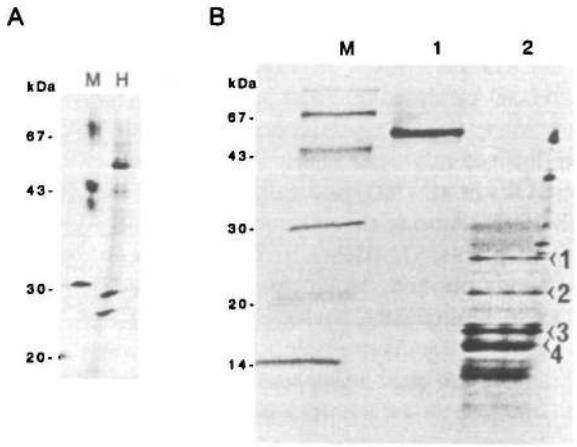
Genomic Southern analysis—Genomic DNA was purified from young leaves of sweet potato (*I. batatas* L. cv. Tsukubakomachi). Five micrograms of DNA was digested with *Hin*dIII or *Eco*RI, size fractionated in a 0.7% agarose gel (128 × 135 mm) and blotted onto a Hybond N^+ membrane (Amersham, Tokyo, Japan). The cloned cDNA fragment (2.0 kb) was labeled with digoxigenin, and was used as a probe. Hybridisation (at 68°C)/detection (using CSPD as a phosphatase substrate) was done according to the manufacturer's protocol (Boehringer Mannheim, Tokyo, Japan).

Results

Purification of GLDHase—About 100 μg of GLDHase was partially purified from 10 kg of tuberous roots, with a recovery of 23% on a total activity basis and an increase in specific activity of more than 600-fold (Table 1). The activity reported previously (Ôba et al. 1995) was a miscalculation, and should be divided by 10^3.

SDS-PAGE analysis of the hydroxylapatite column-purified GLDHase preparation revealed the existence of 3 polypeptides: GLDHase protein of 56 kDa (Ôba et al. 1995) and two other polypeptides of 29 and 26 kDa (Fig. 1A, lane H). Due to the absence of an appropriate method for further purification after the step of hydroxylapatite chromatography, we employed preparative SDS-PAGE to obtain a GLDHase preparation for amino acid sequencing. Proteins fractionated by BIOFORESIS III from 135 to 160 min (fractions 22-26) contained only the 56 kDa protein (data not shown). The pooled fractions were concentrated and separated by SDS-PAGE. A single band with a M_r of 56 kDa was observed (Fig. 1B, lane 1).

Spectral properties—Hydroxylapatite column-purified preparation of GLDHase showed absorption maxima at 276 nm (not shown), 373 and 448 nm (Fig. 2). The ratio of A_{276} to A_{448} was 7.9. The absorption spectrum between 340 nm and 540 nm was similar to that of riboflavin (Fig. 2) and



C

Band 1	GYRVQ ?SDRI LGPD?
Band 2	T?FPA GTLSK PS(I+M)KD
Band 3	AAVKD SRKKK Q(V+K)IRP VGSG
Band 4	(S+M)(I+V)(P+E)(A+H)(P+V) APIEQ R?TA?S

Fig. 1 (A) and (B) Electrophoretic analysis of GLDHase preparation and of polypeptides generated after V8 protease digestion. Lane M; Marker proteins. The size of markers is shown. Lane H; GLDHase purified with hydroxylapatite column. Lane 1; GLDHase purified by BIOFORESIS fractionation. Lane 2; GLDHase treated with V8 protease. Polypeptide bands indicated by arrowheads with numbers were analysed by an amino acid sequencer. (C) N-terminal amino acid sequences of V8 protease-digested polypeptides shown in panel B. "?" denotes no amino acid was detected. If more than one amino acid was detected, the alternatives are shown in brackets. The amino acid sequence used for designing degenerate oligonucleotide primers S3B and A1D are underlined.

Table 1 Purification of GLDHase from mitochondria of sweet potato tuberous roots

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units (mg protein)$^{-1}$)	Purification (-fold)
Mitochondria	105	557	39.1	100	0.070	1
12,000 × *g* sup[a]	240	309	23.4	60	0.076	1.1
250,000 × *g* sup[a]	220	231	25.6	65	0.11	1.6
Sephacryl S-200	36	34	19.1	49	0.56	8.0
DE52	20	6.6	16.5	42	2.5	36
Hydroxylapatite[b]	1.0	0.20	8.9	23	44.5	640

[a] Supernatant fractions obtained after centrifugation of sonicated mitochondria suspensions.
[b] After ultrafiltration.

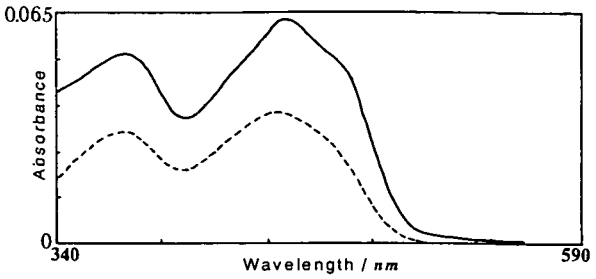


Fig. 2 The absorption spectra of the hydroxylapatite column-purified GLDHase (0.46 $\mu g\ \mu l^{-1}$ (solid line)) and a riboflavin solution in potassium phosphate buffer (3.5 μM, dashed line) in visible wavelength range.

FAD (not shown). The absorbance ratio at absorption maxima (A_{373}/A_{448}) was 0.85 which was the same as that of riboflavin.

Inhibitors—Results of inhibition experiments are summarised in Table 2. The GLDHase activity was inhibited by a sulfhydryl-modifying agent, *N*-ethylmaleimide. The activity was partially restored by the addition of GSH. However, the addition of GSH itself inhibited the activity to some extent. The activity was inhibited by divalent cations such as Cu^{2+} (10^{-5} M) and Zn^{2+} (10^{-4} M) and by lycorine (10^{-5} M), a specific inhibitor of AsA biosynthesis.

Amino acid sequencing—Polypeptides generated after V8 protease digestion of a single band preparation of GLDHase were separated by SDS-PAGE (Fig. 1B, lane 2). Partially digested polypeptides with a M_r smaller than 26 kDa were detected. Four bands were analysed by an automatic protein sequencer. The amino acid sequences thus determined are shown in Fig. 1C.

PCR amplification of cDNA encoding GLDHase—A 0.8 kb fragment was amplified with a set of primers S3B (a degenerate primer coding for NEKKQKI of band 3 in sense orientation) and A1D (a degenerate primer coding for DEILGFD of band 1 in antisense orientation) (Fig. 1C). This fragment was cloned into plasmid pUC18 and the nucleotide sequence was analysed. The insert encodes a 289-residue polypeptide (from Asn-125 to Asp-413, Fig. 3), which had significant similarity to rat GLO protein: 83 residues (28.8%) were identical and 27 (9.4%) were conservative substitutions (Fig. 3). Two stretches of 6 amino acid-long residues were perfectly matched (Fig. 3): VDKEKK (residue 78 to 83 of rat GLO) and VTLQCV (residue 180 to 185). Furthermore, since the deduced amino acid sequences flanking to both ends of the amplified fragment were completely matched with the determined ones (Fig. 1C and 3), we conclude that the clone encodes part of GLDHase pro-

Table 2 Inhibitory effect of *N*-ethylmaleimide, Cu^{2+}, Zn^{2+} and lycorine on GLDHase activity

	Additive	Relative activity (%)
Exp. 1	0.064 mM *N*-ethylmaleimide (NEM)	40
	0.64 mM NEM	4
	0.64 mM NEM+2 mM GSH[a]	59
	2 mM GSH	66
Exp. 2	0.05 mM $CuSO_4$	1
	0.05 mM $CuSO_4$+1 mM EDTA[a]	71
	0.5 mM $ZnSO_4$	31
	0.5 mM $ZnSO_4$+1 mM EDTA[a]	90
	1 mM EDTA	97
Exp. 3	2 mM HCl	90
	68 μM Lycorine with 2 mM HCl	57
	170 μM Lycorine with 2 mM HCl	14
	340 μM Lycorine with 2 mM HCl	0

GLDHase activity was measured in a 50 μl (Exp. 1 and 2) or 200 μl (Exp. 3) reaction mixture at 25°C, pH 7.8. Activity was expressed as relative percentage to that measured with no additive. 1.8×10^{-3} (Exp. 1), 0.49×10^{-3} (Exp. 2) and 3.8×10^{-3} (Exp. 3) units of GLDHase (specific activity: 10 units per mg protein) was used. Final concentrations of additives in the reaction mixture are shown. In Exp. 3, 22 mM potassium phosphate buffer (pH 7.8) was used. Lycorine was added as a hydrochloric acid solution and incubated for 4 min at 25°C prior to measurement. Mean values from duplicate measurements are shown.

[a] The reaction mixture was incubated for 4 min with inhibitors, then GSH or EDTA was added. L-Galactono-γ-lactone was added after incubation at 25°C for another 4 min.

```
Sweet potato        MFRAHHFRRS LRSLLAHSHS HPHSNPHINP -RLLC----S LSSQPP---- -----SSDA-    45
Cauliflower         ---------M LRSLLLRRSN ARSLRPPFPP LRTLCTSGQT LTPAPPPPPP PPPPISSSAS    51

Sweet potato        --EVRKYIGY TVLVLGCAAA TYYSFPFPAD AKHKKAQLFR YAPLPDDLHT VTNWSGTHEV   103
Cauliflower         EKEFRKYAGY AALALFSGAA TYFSFPFPEN AKHKKAQIFR YAPLPEDLHT VSNWSGTHEV   111
Rat GLO             -----MVHGY KGVQFQ---- ---------- ---------- ---------- --NWAKTYGC    19

Sweet potato        QTRTFLQPES LQELERAVKD SNEKKQKIRP VGSGLSPNGI GLTRAGMVNL GLMDKVLEVD   163
Cauliflower         QTRNFNQPET LADLEALVKE AHEKKNRIRP VGSGLSPNGI GLSRSGMVNL ALMDKVLEVD   171
Rat GLO             SPEVYYQPTS VEEVREVLAL AREQKKKVKV VGGGHSPSDI ACTDGFMIHM GKMNRVLQVD    79
Yeast Gal Ox              34  EVVELVKS ARLAEKSLVT VGSGHSPSNM CVTD  65
A. oxidans 6-HDNO         49  DVAKSVRY ACDNGLEISV RSGGHNPNGY ATND  80
                                                   *

Sweet potato        KEKKRVTVQA GIRVQQLVDS IKEYGLTLQN FASIREQQVG GIVQVGAHGT GARLPPIDEQ   223
Cauliflower         KEKKRVRVQA GIRVQQLVDA IQEYGLTLQN FASIREQQIG GIIQVGAHGT GARLPPIDEQ   231
Rat GLO             KEKKQITVEA GILLADLHPQ LDEHGLAMSN LGAVSDVTVA GVIGSGTHNT GIKHGILATQ   139

Sweet potato        VISMKLVTPA KGTIEISKEK DPDLFYLARC GLGGLGVVAE VTLQCVERQE LVEHTYISNM   283
Cauliflower         VIGMKLVTPA KGTIELSKDN DPELFHLARC GLGGLGVVAE VTLQCVERQE LLEHTYVSTL   291
Rat GLO             VVALTLMTAD GEVLECSESR NADVFQAARV HLGCLGIILT VTLQCVPQFQ LQETSFPSTL   199

Sweet potato        KDIKKNHKKL LSENKHVKYL HIPYTDAVVV VTCNPISKWK GPPKYKPKYS PEEAVGHVQD   343
Cauliflower         EEIKKNHKKL LSTNKHVKYL YIPYTDTVVV VTCNPVSKWS GAPKDKPKYT TEEALKHVRD   351
Rat GLO             KEVLDNLDSH LKRSEYFRFL WFPHTENVSI IYQDHTNK-- -APSSASNWF WDYAIGFYL-   255

Sweet potato        LYRESLKKYR STEN------ --ESEINELS FTELRDKLLA LDPLNTDHVK KTNQAEAEFW   395
Cauliflower         LYRESIVKYR VQDSSKKTPD SREPDINELS FTELRDKLIA LDPLNDVHVG KVNQAEAEFW   411
Rat GLO             ---------- ---------- --------LE FL-LWTSTY- LPCL-VGWIN RFF-FWMLFN   283

Sweet potato        RKSEGYRVGW SDEILGFDCG GHQWVSETCF PAGTLSKPSM KDLEFIEQLM QLIEKESIPA   455
Cauliflower         KKSEGYRVGW SDEILGFDCG GQQWVSETCF PAGTLAKPSM KDLEYIEQLK ELIQKEAIPA   471
Rat GLO             CKKESSNL-- SHKIFTYECR FKQHVQDWAI PREK-TKEAL --LE-LKAML EAHPK--VVA   335

Sweet potato        PAPIEQRWTA CSKSLMSPAY SSVDDDIFSW VGIIMYLPTM DARERKHITE EFFHYRHLTQ   515
Cauliflower         PSPIEQRWTG RSKSPMSPAF STAEEDIFSW VGIIMYLPTA DPRQRKDITD EFFHYRHLTQ   531
Rat GLO             HYPVEVRFTR GDDILLSPCF QR--DSCY-- MNIIMYRPY- ----GKDVPR --LDYW-LAY   383

Sweet potato        AHLWDHYSAY EHWAKIEVPK DKEELQALQA RLRKKFPVDA YNRARQELDP NRILSNNMLE   575
Cauliflower         AKLWDQYSAY EHWAKIEIPK DKEELEALQE RLRKRFPVDA YNKARRELDP NRILSNNMVE   591
Rat GLO             ETIMKKFGGR PHWAKAHNCT QK-DFEEMYP TFHK------ FCDIREKLDP TGMFLNSYLE   436

Sweet potato        KLFPSS---                                                         581
Cauliflower         KLFPVSKTA                                                         600
Rat GLO             KVFY-----                                                         440
```

Fig. 3 Alignment of the deduced amino acid sequences of GLDHase from sweet potato (this study), GLDHase from cauliflower (Østergaard et al. 1997), GLO from rat (Koshizaka et al. 1988) and partial sequences of L-galactono-γ-lactone oxidase from yeast (Yeast Gal-L Ox; Nishikimi et al. EMBL accession number AB009401) and of 6-hydroxy-D-nicotine oxidase from *Arthrobacter oxidans* (*A. oxidans* 6-HDNO; Brandsch et al. 1987). Identical or similar (S/T, D/E, N/Q, R/K, V/L and V/I) residues are shaded. Gap "-" was introduced to maximise homology. The determined amino acid sequences of polypeptides generated after partial digestion with V8 protease are boxed (see also Fig. 1B, C). Note that W and C residues were not detected y the protein sequencer. A qutative cleavage site of signal peptide for cauliflower GLDHase (Østergaard et al. 1997) is indicated by an arrowhead. A His residue to which covalently-bound flavin group of 6-HDNO is indicated by an asterisk. Alignment was done using Gene Works software.

tein.

In order to obtain 5′- and 3′-flanking sequences of the 0.8 kb clone, we applied the RACE technique. Full length cDNA was then amplified using a set of primers corresponding to 5′- and 3′-untranslated regions (see Materials and Methods). A clone obtained contained a 2,023 bp-long cDNA fragment with an open reading frame of 1,747 bp, coding for 581 amino acid-long polypeptides with a molecular mass of 66 kDa (Fig. 3). The deduced amino acid sequence contained those of the four polypeptides generated by V8 protease digestion of GLDHase (Fig. 1B, C and 3). Accordingly, we conclude that the 2.0 kb clone encodes GLDHase protein.

Among the deduced amino acid residues of GLDHase, 130 residues (22.4%) were identical to rat GLO protein and another 31 (5.3%) were conservative substitutions (Fig. 3). In addition, 99 (17.0%) residues were identical to yeast L-galactono-γ-lactone oxidase (EC 1.1.3.24) (EMBL accession number AB009401, data not shown). The overall homology among GLDHase, GLO and L-galactono-γ-lactone oxidase was 51 residues (8.8%) (not shown).

Genomic Southern analysis—Genomic Southern analysis was performed with the 2.0 kb entire cloned cDNA fragment as a probe. Two fragments of 2.9 and 1.4 kb were detected in the *Eco*RI digest and three fragments of 10, 8.7 and 7.9 kb in the *Hin*dIII digest (Fig. 4), suggesting that several sequences homologous to the GLDHase gene exist in the genome of sweet potato. Considering the hexaploid nature of sweet potato cultivars and heterogeneous organisation of genomes due to self-incompatibility, it is likely that GLDHase gene exists as a single copy.

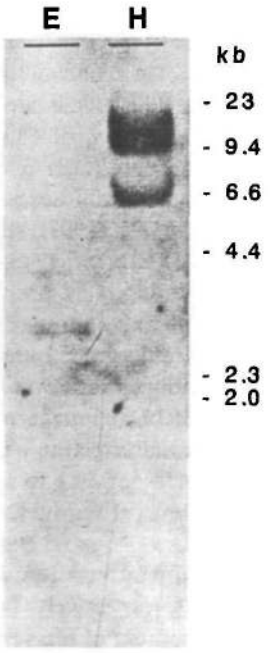


Fig. 4 Genomic Southern analysis of GLDHase gene. Five micrograms of genomic DNA from sweet potato (cv. Tsukubakomachi) leaves was digested with *Eco*RI (lane E) or *Hin*dIII (lane H), probed with 2.0 kb of digoxigenin-labeled cDNA fragment encoding GLDHase.

Discussion

GLDHase has recently been highly purified from sweet potato roots and some properties described (Ôba et al. 1995). By using the hydroxylapatite column described in this paper, the recovery of activity was greatly improved: about 23% of the initial activity in the mitochondrial fraction was present in the hydroxylapatite-purified fraction (Table 1). The specific activity of the hydroxylapatite column-purified GLDHase was almost 10-fold higher than that reported for GLO (Kiuchi et al. 1982, Nishikimi et al. 1976) and L-galactono-γ-lactone oxidase (Nishikimi et al. 1980), the enzymes involved in AsA biosynthesis in animals and yeast, respectively. The post-hydroxylapatite preparation contained in addition to GLDHase protein of 56 kDa, two other polypeptides of 29 and 26 kDa (Fig. 1A, lane H). These two small polypeptides are possibly degradation products of GLDHase protein. In cauliflower, two degradation products of 30 and 26 kDa were co-purified with GLDHase protein (Østergaard et al. 1997).

The hydroxylapatite column-purified GLDHase preparation showed a typical absorption spectrum of flavin between wavelengths of 340 nm and 540 nm (Fig. 2), suggesting that a flavin prosthetic group is associated with the enzyme. Inhibition of the enzyme activity by acriflavine previously reported by Ôba et al. (1995) also supports this hypothesis. Østergaard et al. (1997) found no typical flavin absorption spectrum in their preparation. This may be due to the noncovalent interaction of the flavin group in GLDHase. GLO (Kiuchi et al. 1982, Nishikimi et al. 1976) and L-galactono-γ-lactone oxidase (Nishikimi et al. 1980) have similar absorption spectra but the absorption maximum of these enzymes is around 350 nm and is hence distinct from the 373 nm maximum observed for GLDHase. Only a faint fluorescence was observed by excitation at 365 nm at the 56 kDa band after SDS-PAGE separation of hydroxylapatite column preparation of GLDHase (data not shown). The results suggest that the flavin prosthetic group is noncovalently bound to GLDHase in contrast to the covalently bound flavins in GLO (Kenny et al. 1976, Kiuchi et al. 1982) and L-galactono-γ-lactone oxidase (Kenny et al. 1979).

Covalently bound flavin groups have also been found in two other related enzymes of fungal and yeast origin which catalyse the synthesis of ascorbic acid analogues: D-glucono-γ-lactone oxidase (Harada et al. 1979, Takahashi et al. 1976; The enzyme was described as D-glucono-γ-lactone dehydrogenase but it should be D-glucono-γ-lactone oxidase because the enzyme uses molecular oxygen as the

Table 3 A comparison of properties of enzymes involved in the biosynthesis of AsA and its analogues

Enzyme	EC number	Product	O_2 as an electron acceptor	Binding form of flavin	Organism
GLDHase	1.3.2.3	AsA	No	Noncovalent[b]	Sweet potato[c], Cauliflower[d]
GLO	1.1.3.8	AsA	Yes	Covalent	Rat[e], Goat[e], Chicken[f]
L-galactono-γ-lactone oxidase[a]	1.1.3.24	AsA	Yes	Covalent	Bakers' yeast[g]
D-glucono-γ-lactone oxidase	1.1.3.-	D-erythrobic acid	Yes	Covalent	*Penicillium cyaneo-fulvum*[h]
ALO[a]	1.1.3.-	D-erythroascorbic acid	Yes	Covalent	*Candida albicans*[i]

[a] The nucleotide sequences encoding these enzymes from bakers' yeast were recently found to be the same (EMBL accession numbers AB009401 for L-galactono-γ-lactone oxidase and U40390 for ALO). Therefore, the enzyme has bifunctional catalytic activity.
[b] Preliminary result.
[c] Ôba et al. (1995) and this study.
[d] Østergaard et al. (1997).
[e] Nishikimi et al. (1976) and Kenny et al. (1976).
[f] Kiuchi et al. (1982).
[g] Nishikimi et al. (1980) and Kenny et al. (1979).
[h] Takahashi et al. (1976) and Harada et al. (1979).
[i] Huh et al. (1994).

best electron acceptor) and D-arabinono-γ-lactone oxidase (ALO, Huh et al. 1994). Recently, identity of the genes has revealed that L-galactono-γ-lactone oxidase and ALO from baker's yeast have the same amino acid sequences (EMBL accession numbers AB009401 for L-galactono-γ-lactone oxidase and U40390 for ALO). Some properties of these enzymes are summarised in Table 3. The presence of the noncovalently bound flavin prosthetic group of GLDHase is characteristic among the known AsA synthesising enzymes. The type of binding, the structure of the flavin prosthetic group and electron acceptor in vivo require further study.

GLDHase activity was inhibited by *N*-ethylmaleimide (Table 2), indicating involvement of a sulfhydryl group at the catalytic site. A similar inhibition has been reported for GLDHases from spinach leaf (Mutsuda et al. 1995) and *Euglena* (Shigeoka et al. 1979; The enzyme designated as L-gulono-γ-lactone dehydrogenase should be termed L-galactono-γ-lactone dehydrogenase because of its substrate preference). GLDHase activity was also inhibited by Cu^{2+} or Zn^{2+} as has been reported for the *Euglena* GLDHase (Shigeoka et al. 1979).

Lycorine has been found to have an inhibitory effect on AsA biosynthesis in higher plants (Arrigoni et al. 1975). De Gara et al. (1994) showed that the target reaction of inhibition was oxidation of L-galactono-γ-lactone to AsA, by using maize embryo system. Recently, the same group reconfirmed the in vivo inhibition on GLDHase activity but found no inhibitory effect on the activities of ascorbate peroxidase, ascorbate free radical reductase and dehydroascorbate reductase (Arrigoni et al. 1997). The results shown in Table 2 clearly demonstrate the inhibitory effect of lycorine on GLDHase activity in vitro, supporting these findings. However, Østergaard et al. (1997) found no inhibition of GLDHase activity with up to 100 μM of lycorine. The reason for this discrepancy is unclear.

The cDNA clone for GLDHase encodes a 66 kDa polypeptide while purified enzyme showed a M_r of 56 kDa (Ôba et al. 1995). Two explanations are possible for the discrepancy between these values: (i) A sonication process for solubilisation caused release of a truncated peptide from the mitochondrial membrane. This idea is supported by the observation of GLDHase with a M_r of 68 kDa from potato tubers solubilised by detergent (Ôba and Nakano 1995). (ii) Removal of a mitochondrial targeting peptide. In cauliflower, Østergaard et al. (1997) suggested that the mature form GLDHase (58 kDa) was generated by removing the N-terminal 91-amino acid-prepeptide from the preform (68 kDa). A similar value was observed in this study; the calculated molecular mass based on the deduced sequence of the open reading frame was 66 kDa while the purified GLDHase was 56 kDa. Furthermore, characteristic features of mitochondrial targeting peptide were found in the first 83 residues corresponding to a putative prepeptide of cauliflower GLDHase (91 residues, Fig. 3); a relatively high number of Ala, Leu, Arg and Ser residues (8, 9, 7 and 11, respectively), and relatively few Asp, Glu, Ile and Val residues (2, 1, 2 and 3, respectively) (von Heijne 1986). An extraordinarily high content of His residues (7 residues) in the first 27 residues is characteristic for the sweet potato GLDHase (Fig. 3).

We found that the deduced amino acid sequence of GLDHase showed homology to those of GLO (Fig. 3) and L-galactono-γ-lactone oxidase (not shown). These two en-

zymes catalyse reactions similar to GLDHase but not the same reaction in animals and yeast, respectively (Table 3). GLO also catalyses the oxidation of L-galactono-γ-lactone utilising molecular oxygen as an electron acceptor (Kiuchi et al. 1982), while GLDHase does not use O_2 (Østergaard et al. 1997, Table 3). On the other hand, GLDHase does not catalyse the oxidation of L-gulono-γ-lactone (Arrigoni et al. 1997, Ôba et al. 1995, Østergaard et al. 1997). In spite of the difference in the substrate specificity, electron acceptor and subcellular localisation, the deduced amino acid sequences showed that these three enzymes are homologous with each other. Almost no homologous sequences to those of GLO (Fig. 3) or L-galactono-γ-lactone oxidase (not shown) were found in the first 90 residues of GLDHase. A stretch of sequence ^{134}VGSGLSP^{140} of sweet potato GLDHase was well conserved among the three enzymes (Fig. 3). A corresponding position of 6-hydroxy-D-nicotine oxidase from *A. oxidans*, a flavoprotein having moderate homology to rat GLO (Nishikimi and Yagi 1996), is also compared in Fig. 3. The His residue of 6-hydroxy-D-nicotine oxidase indicated by an asterisk is known as the site to which FAD binds covalently (Brandsch et al. 1987, Mauch et al. 1989). The corresponding residue His-54 of GLO is thought to be a putative FAD-binding site (Nishikimi and Yagi 1996). The corresponding residue in plant GLDHases is Leu instead. The difference in the amino acid residue at this position supports the hypothesis that the binding of the flavin group in GLDHase is different from that in GLO. To evaluate the evolutionary relationships among plant GLDHase, animal GLO and yeast L-galactono-γ-lactone oxidase at the gene level, genomic DNA structure of plant GLDHase genes needs to be studied.

The authors thank Mr. K. Katayama and Mr. S. Tamiya, Agricultural Research Centre, MAFF, Japan, for generously providing sweet potato tuberous roots, Dr. S. Takagi, Mukogawa Women's University, for the generous gift of lycorine. We also thank Mr. H. Maeda for technical assistance, Dr. E.E. Conn, Dr. A. Kingston-Smith (IGER, U.K.) and Dr. C. Foyer (IGER, U.K.) for helpful discussion. This work was supported financially by the Biotechnology Plant Breeding Project, MAFF, Japan, given to T.I. and M.H.

References

Arrigoni, O. (1994) Ascorbate system in plant development. *J. Bioenerg. Biomem.* 26: 407–419.

Arrigoni, O., Arrigoni Liso, R. and Calabrese, G. (1975) Lycorine as an inhibitor of ascorbic acid biosynthesis. *Nature* 256: 513–514.

Arrigoni, O., De Gara, L., Paciolla, C., Evidente, A., De Pinto, M.C. and Liso, R. (1997) Lycorine: a powerful inhibitor of L-galactonolactone-γ-lactone dehydrogenase activity. *J. Plant Physiol.* 150: 362–364.

Arrigoni, O., De Gara, L., Tommasi, F. and Liso, R. (1992) Changes in the ascorbate system during seed development of *Vicia faba* L. *Plant Physiol.* 99: 235–238.

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Brandsch, R., Hinkkanen A.E., Mauch, L., Nagursky, H. and Decker, K. (1987) 6-Hydroxy-D-nicotine oxidase of *Arthrobacter oxidans*. Gene structure of flavoenzyme and its relationship to 6-hydroxy-L-nicotine oxidase. *Eur. J. Biochem.* 167: 315–320.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252: 1102–1106.

De Gara, L., Paciolla, C., Tommasi, F., Liso, R. and Arrigoni, O. (1994) In vivo inhibition of galactono-γ-lactone conversion to ascorbate by lycorine. *J. Plant Physiol.* 144: 649–653.

Foyer, C.H. (1993) Ascorbic acid. *In* Antioxidants in Higher Plants. Edited by Alscher, R.G. and Hess, J.L. pp. 31–58. CRC Press, Boca Raton.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 85: 8998–9002.

Harada, Y., Shimizu, M., Murakawa, S. and Takahashi, T. (1979) Identification of FAD of D-gluconolactone dehydrogenase: D-erythrobic acid producing enzyme of *Penicillium cyaneo-fulvum*. *Agric. Biol. Chem.* 43: 2635–2636.

Horowitz, H.H., Doerschuk, A.P. and King, C.G. (1952) The origin of L-ascorbic acid in the albino rat. *J. Biol. Chem.* 199: 193–198.

Horowitz, H.H. and King, C.G. (1953) The conversion of glucose-6-C^{14} to ascorbic acid by the albino rat. *J. Biol. Chem.* 200: 125–128.

Huh, W-K., Kim, S-T., Yang, K-S., Seok, Y-J., Hah, Y.C. and Kang, S-O. (1994) Characterisation of D-arabinono-1,4-lactone oxidase from *Candida albicans* ATCC 10231. *Eur. J. Biochem.* 225: 1073–1079.

Kenny, W.C., Edmondson, D.E., Singer, T.P., Nakagawa, H., Asano, A. and Sato, R. (1976) Identification of the covalently bound flavin of L-gulono-γ-lactone oxidase. *Biochem. Biophys. Res. Commun.* 71: 1194–1200.

Kenny, W.C., Edmondson, D.E., Singer, T.P., Nishikimi, M., Noguchi, E. and Yagi, K. (1979) Identification of the covalently-bound flavin of L-galactonolactone oxidase from yeast. *FEBS Lett.* 97: 40–42.

Kiuchi, K., Nishikimi, M. and Yagi, K. (1982) Purification and characterization of L-gulonolactone oxidase from chicken kidney microsomes. *Biochemistry* 21: 5076–5082.

Koshizaka, T., Nishikimi, M., Ozawa, T. and Yagi, K. (1988) Isolation and sequence analysis of a complementary DNA encoding rat liver L-gulono-γ-lactone oxidase, a key enzyme for L-ascorbic acid biosynthesis. *J. Biol. Chem.* 263: 1619–1621.

Loewus, F.A. (1963) Tracer studies on ascorbic acid formation in plants. *Phytochemistry* 2: 109–128.

Loewus, F.A. (1980) L-ascorbic acid: metabolism, biosynthesis, function. *In* The Biochemistry of Plants. Vol. 3. Edited by Preiss, J. pp. 77–99. Academic Press, New York.

Loewus, F.A. and Loewus, M.W. (1987) Biosynthesis and metabolism of ascorbic acid in plants. *CRC Crit. Rev. Plant Sci.* 5: 101–119.

Mapson, L.W., Isherwood, F.A. and Chen, Y.T. (1954) Biological Synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. *Biochem. J.* 56: 21–28.

Mauch, L., Bichler, V. and Brandsch, R. (1989) Site-directed mutagenesis of the FAD-binding histidine of 6-Hydroxy-D-nicotine oxidase. Consequences on flavinylation and enzyme activity. *FEBS Lett.* 257: 86–88.

Mutsuda, M., Ishikawa, T., Takeda, T. and Shigeoka, S. (1995) Subcellular localization and properties of L-galactonolactone-γ-lactone dehydrogenase in spinach leaves. *Biosci. Biotech. Biochem.* 59: 1983–1984.

Nishikimi, M., Noguchi, E. and Yagi, K. (1980) Redox properties of L-galactonolactone oxidase purified from Baker's yeast. *Biochem. Int.* 1: 155–161.

Nishikimi, M., Tolbert, B.M. and Udenfriend, S. (1976) Purification and characterization of L-gulono-γ-lactone oxidase from rat and goat liver. *Arch. Biochem. Biophys.* 175: 427–435.

Nishikimi, M. and Yagi, K. (1996) Biochemistry and molecular biology of ascorbic acid biosynthesis. *In* Subcellular Biochemistry. Vol. 25, Ascorbic acid: Biochemistry and Biomedical Cell Biology. Edited by Harris, J.R. pp. 17–39. Plenum Press, New York.

Ôba, K., Ishikawa, S., Nishikawa, M., Mizuno, H. and Yamamoto, T. (1995) Purification and properties of L-galactonolactono-γ-lactone dehy-

drogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *J. Biochem. (Tokyo)* 117: 120–124.

Ôba, K. and Nakano, A. (1995) Purification and characterization of L-galactono-γ-lactone dehydrogenase from potato tubers. (in Japanese) *Nippon Nogeikagaku Kaishi* 69(Suppl.): 101.

Østergaard, J., Persiau, G., Davey, M.W., Bauw, G. and Van Montagu, M. (1997) Isolation of a cDNA coding for L-galactonolactono-γ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. *J. Biol. Chem.* 272: 30009–30016.

Saito, K., Nick, J.A. and Loewus, F.A. (1990) D-glucosone and L-sorbosone, putative intermediates of L-ascorbic acid biosynthesis in detached bean and spinach leaves. *Plant Physiol.* 94: 1496–1500.

Shigeoka, S., Nakano, Y. and Kitaoka, S. (1979) Some properties and subcellular localization of L-gulono-γ-lactone dehydrogenase in *Euglena gracilis* Z. *Agric. Biol. Chem.* 43: 2187–2188.

Takahashi, T., Yamashita, H., Kato, E., Mitsumoto, M. and Murakawa, S. (1976) Purification and some properties of D-glucono-γ-lactone dehydrogenase. D-Erythrobic acid producing enzyme of *Penicillium cyaneofulvum*. *Agric. Biol. Chem.* 40: 121–129.

von Heijne, G. (1986) Mitochondrial targeting sequences may form amphiphilic helices. *EMBO J.* 5: 1335–1342.

Wheeler, G.L., Jones, M.A. and Smirnoff N. (1998) The biosynthetic pathway of vitamin C in higher plants. *Nature* 393: 365–369.

(Received May 18, 1998; Accepted October 5, 1998)