Isolation of a cDNA Coding for L-Galactono-γ-Lactone Dehydrogenase, an Enzyme involved in the Biosynthesis of Ascorbic Acid in Plants

PURIFICATION, CHARACTERIZATION, cDNA CLONING, AND EXPRESSION IN YEAST

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L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3; GLDase), an enzyme that catalyzes the final step in the biosynthesis of L-ascorbic acid was purified 1693-fold from a mitochondrial extract of cauliflower (Brassica oleracea, var. botrytis) to apparent homogeneity with an overall yield of 1.1%. The purification procedure consisted of anion exchange, hydrophobic interaction, gel filtration, and fast protein liquid chromatography. The enzyme had a molecular mass of 56 kDa estimated by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis and showed a pH optimum for activity between pH 8.0 and 8.5, with an apparent \( K_m \) of 3.3 mM for L-galactono-γ-lactone. Based on partial peptide sequence information, polymerase chain reaction fragments were isolated and used to screen a cauliflower cDNA library from which a cDNA encoding GLDase was isolated. The deduced mature GLDase contained 509 amino acid residues with a predicted molecular mass of 57,837 Da. Expression of the cDNA in yeast produced a biologically active protein displaying GLDase activity. Furthermore, we identified a substrate for the enzyme in cauliflower extract, which co-eluted with L-galactono-γ-lactone by high-performance liquid chromatography, suggesting that this compound is a naturally occurring precursor of L-ascorbic acid biosynthesis in vivo.

Vitamin C or ascorbic acid (L-AA) is an important metabolite for most living organisms present in millimolar concentrations and is well known for its antioxidant properties. Its precise functions in plants is still poorly understood, although it is known to play an important role in the antioxidant system that protects plants from oxidative damage resulting from biotic and abiotic stresses as well as being a cofactor for a number of dehydrogenase enzymes.

L-AA is synthesized by all higher plants and by nearly all higher animals except humans, other primates, guinea pigs, bats, and some birds (1–3). L-AA has also been reported to be present in a number of yeasts (4), but several reports suggest that L-AA analogues, rather than L-AA, are present in microorganisms (5–7).

The biosynthesis of L-AA follows different pathways in the animal and the plant kingdom. In animals, D-glucose serves as the first committed precursor in the biosynthesis of L-AA and the last step in the pathway is catalyzed by a microsomal L-gulono-γ-lactone oxidase (EC 1.1.3.8), which oxidizes L-gulono-γ-lactone (L-GuL) to L-AA. This enzyme has been isolated and characterized from rat, goat, and chicken (8, 9).

Despite the importance of L-AA in plants, the biosynthetic pathway has still not been established, although current evidence suggests the existence of two discrete routes. A biosynthetic pathway from D-galactose proceeding via L-galactono-γ-lactone (L-GL) has been proposed as long ago as 1954 by Isherwood et al. (10) and Mapson et al. (11), based on initial studies of the oxidation of L-Gl to L-AA by the enzyme L-galactono-γ-lactone dehydrogenase (GLDase). GLDase activity has been described (11–13) in plants such as pea, cabbage, cauliflower florets, and potato, and recently Öba et al. (14) reported a purification of this enzyme from sweet potato roots. Loewus (15) has proposed an alternative pathway in which L-AA is synthesized from D-glucose via L-sorbose. The presence of an enzyme able to convert L-sorbose to L-AA with concomitant reduction of NADP was demonstrated in bean and spinach leaves (16, 17). Conceivably, these distinct routes might be present in different subcellular compartments or in different plant species.

Here, we report the purification and characterization of GLDase from cauliflower florets, followed by isolation and sequencing of the corresponding cDNA. This is the first description of a gene coding for an enzyme involved in the biosynthesis of L-AA in plants. The GLDase cDNA has furthermore been expressed in an active form in yeast, and we have strong indications that the substrate for GLDase, L-Gl, is naturally present in plant extracts. These findings emphasize for the first time the physiological relevance of the biosynthetic pathway proposed by Isherwood et al. and Mapson et al. (10, 11).

EXPERIMENTAL PROCEDURES

Materials—Sephacryl SF-200, DEAE-Sepharose, and phenyl-Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). L-Galactono-γ-lactone, L-galactono-γ-lactone, N-galactono-γ-lactone, L-gulono-γ-lactone, L-mannono-γ-lactone, N-galactonic acid, N-glucuronic acid,
d-glucuronic acid, and d-hydroxymercuribenzoic acid were from Sigma.

Extraction—Cauliflower florets (7.5 kg) were cut into small pieces and homogenized in a pre-cooled blender in ice-cold buffer A (400 mM sucrose, 100 mM sodium phosphate buffer, pH 7.4) at 1 liter/kg fresh weight. The homogenate was passed through four layers of Miracloth tissue (Calbiochem-Novabiochem, La Jolla, CA), and centrifuged at 13,500 g for 45 min in a GSA rotor. The pellet was resuspended in Buffer B (40 mM Tris-HCl, pH 9.0) and kept at 4 °C until further use.

2 ml were collected. Two peaks of activity eluted: peak I at 210 mM and peak II at 225 mM NaCl. Peak II was dialyzed against 10 mM sodium phosphate, pH 7.4. Fractions of 4 ml were collected. The activity of the main peak, which eluted at 120 mM NaCl, was collected and adjusted to pH 6.0 with glacial acetic acid.

GLDase Assay—GLDase activity was measured spectrophotometrically by following the l-GL-dependent reduction of cytochrome c at 550 nm and 22 °C. The reaction mixture (1 ml) consisted of enzyme extract, cytochrome c (1.5 mg/ml), and l-GL (4.2 mM) in 0.05 M Tris-HCl buffer (pH 8.4). Under these conditions the reaction rate was linear with respect to time for an initial period of at least 15 min. One unit of enzyme activity was defined as the amount that oxidized 1 μmol of l-α-A/min. This corresponds to the reduction of 2 μmol of cytochrome c as described by Oba et al. (13). Substrate specificity assays were carried out as described above using 4.2 mM of the different substrates to be tested.

Enzyme Purification—The protein extract (from 250 g of mitochondrial pellet) was loaded onto a DEAE-Sepharose column (5 × 12 cm) equilibrated with buffer B. After washing with 4 column volumes of buffer B at 60 ml/h, elution was carried out with 0.5 M NaCl in the same buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h, and fractions containing GLDase activity were pooled and ammonium sulfate was added to a concentration of 1 ml. The extract was then loaded on a p-beadose CL-4B column (2.2 × 15.0 cm) equilibrated in buffer C (1 mM ammonium sulfate, 25 mM sodium phosphate, pH 7.0). After washing with 2 column volumes of buffer C, elution was carried out at 30 ml/h by mixing buffer C with a 600-ml linear gradient of 80% ethylene glycol in 25 mM sodium phosphate (pH 7.0).

Fractions containing GLDase activity were again pooled, concentrated to 10 ml by ultrafiltration using a PM-10 membrane (Amicon, Beverly, MA), and then applied onto a Sephadryl SP-2000 gel filtration column (2.6 × 94 cm) equilibrated in buffer D (20% ethylene glycol, 40 mM NaCl, 80 mM sodium phosphate, pH 7.4). The enzyme was eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 5 ml were collected and fractions with activity pooled. The preparation could be stored at 4 °C for several weeks without any detectable loss of activity.

Two gel filtration preparations were combined and concentrated with buffer exchange to buffer E (20% ethylene glycol, 20 mM Tris-HCl, pH 8.0) by ultrafiltration (PM-10 membrane). The resulting solution was applied to a strong anion exchange column (Resource Q, 6 ml; Pharmacia Biotech Inc.) equilibrated in buffer E and connected to an FPLC system (Pharmacia). The column was eluted at 1 ml/min with a gradient of 0–450 mM NaCl in buffer E as follows: 0–85 mM in 18 min, 85–110 mM in 10 min, 110–130 mM in 14 min, and 130–450 mM in 10 min. Fractions of 1 ml were collected. The activity of the main peak, which eluted at 120 mM NaCl, was collected and adjusted to pH 6.0 with 50 mM sodium phosphate.

The pooled fractions were loaded onto a Poros 20 SP strong cation exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, 20% ethylene glycol and eluted using the FPLC at a flow rate of 1 ml/min. Elution was carried out with a gradient of 0–500 mM NaCl in buffer F as follows: 125–225 mM in 40 min and 225–500 mM in 37 min. Fractions of 2 ml were collected. Two peaks of activity eluted: peak I at 210 mM and peak II at 225 mM NaCl. Peak II was dialyzed against 10 mM sodium phosphate, pH 7.2, containing 1 mM l-AA and the volume was reduced to 200 μl by lyophilization (Heto Lab Equipment, Lyngby, Denmark).

As a final step, the pooled fractions of peak II were separated by HPLC using a Zorbax gel filtration column GF-250 (9.4 × 250 mm) (Rockland Technologies Inc., Newport, DE) equilibrated in 750 mM NaCl, 50 mM sodium phosphate (pH 7.2). Fractions of 1 ml were collected at a flow rate of 1 ml/min.

Protein Determination—The protein concentration of extracts was determined according to Bradford (18) using bovine serum albumin as standard.

Determination of Molecular Mass—The molecular mass of the native GLDase was estimated by gel filtration on a Sephacryl SF-200 column (2.5 × 94 cm) equilibrated in 40 mM NaCl, 80 mM sodium phosphate (pH 7.4). Fractions of 4 ml were collected at a flow rate of 20 ml/h and fractions of 4 ml were collected. The molecular mass was estimated by comparing the elution of GLDase with that of the standard proteins: ferritin (450 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa).

SDS-PAGE—Analytical SDS-PAGE was performed in slab gels of 10% polyacrylamide as according to Chua (19). Proteins were visualized either by Coomasie Brilliant Blue R-250 staining (19) or silver nitrate staining (20).

Affinity Retardation Chromatography—Cytochrome c was covalently bound to thiol-activated Sepharose 4B as described by Azzi et al. (21) and packed into a column (1.0 × 20 cm) that eluted at flow rates of 8 ml/h in 10 mM sodium phosphate buffer (pH 7.4). Fractions of 2 ml were collected and tested for activity.

Lycorine Extraction—Lycorine was purified from non-flowering, whole plants of Crinum jagus or Crinum asiaticum as described by Davey et al. (22).

Partial Amino Acid Sequence Determination—Purified GLDase from the Poros 20 SP purification step was applied to SDS-PAGE. The separated polypeptides were blotted onto polyvinylidene difluoride membranes (Millipore) as described by Bauw et al. (23). NH-terminal and internal amino acid sequence analyses of the polyvinylidene difluoride-bound proteins were performed as described by Bauw et al. (24). Trypsin was used for the in situ digests and the resulting peptides were separated by reversed-phase HPLC. Amino acid sequencing was performed on a 473 protein sequencer (Applied Biosystems, Foster City, CA).

Isolation of Total RNA and First-strand cDNA Synthesis—Cauliflower floret tissue (300 mg) was ground to a powder in liquid nitrogen with a mortar and pestle and RNA was extracted using a method based on LiCl precipitation as described by Goormachtig et al. (25). The RNA isolated from cauliflower florets (4 μg) was used to synthesize first-strand cDNA according to the instruction manual for Superscript™ Reverse Transcriptase System for first-strand cDNA synthesis (Life Technologies, Inc., Gaithersburg, MD).

Polymerase Chain Reaction—Degenerate oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems) and used as primers in polymerase chain reactions. The peptide sequences used for synthesizing the corresponding coding and complementary oligonucleotides were designed according to the partial amino acid sequence obtained earlier, and designated 3, 6, and 8 (underlined in Fig. 5).

First-strand cDNA synthesized from cauliflower florets was used as a template. The amplification mixture consisted of template, polymerase chain reaction buffer, 200–300 ng of each primer, 2.5 mM cNTP, and 1 unit of Taq polymerase in a total volume of 50 μl. The amplification program consisted of 32 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 1 min), and primer extension (72 °C, 2 min). Products of the reaction were separated on 1% agarose gels, excised, and then purified according to the QIAEX Handbook (Diagen GmbH, Hilden, Germany). The purified products were cloned into a pGEM-T vector (Promega, Madison, WI).

Sequencing of cDNA Library—A cauliflower cDNA library constructed in ZAP II (Stratagene, La Jolla, CA) was used. Aliquots of the cDNA library were plated out using Escherichia coli XL-1 Blue cells on 23 × 23-cm baking plates (Nunc, Roskilde, Denmark) containing NZY agar. Approximately 600,000 plaques of the library were transferred onto duplicate nylon membranes (Hybond N²; Amersham). The membranes were treated in accordance with the manufacturer’s instructions for plaque lifting to membranes with irradiation with ultraviolet light (UV Stratalinker; Stratagene). A 250-bp polymerase chain reaction-amplified fragment was labeled with [α-32P]dCTP using a random primed DNA labeling kit (Boehringer, Mannheim, Germany)

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and subsequently used as probe for screening the cDNA library. The membranes were washed for 4 h at 65 °C in hybridization buffer (1% (w/v) bovine serum albumin, 7% (w/v) SDS, 1 mM EDTA, and 0.25 M sodium phosphate, pH 7.2), before 20 h incubation with the 32P-labeled probe in hybridization buffer at 65 °C. The membranes were then rinsed twice for 15 min with 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na2-citrate, pH 7.0) and 1% SDS at room temperature and exposed to X-Omat AR film (Kodak, Rochester, NY) with an enhancer screen for autoradiography. Plaque-purified phage clones were converted into phagemids (Bluescript SK+; Stratagene) by in vitro excision using the ExAssist™ System.

DNA Sequence Determinations—DNA sequence determinations were carried out in accordance with protocols obtained from Applied Biosystems. Initial sequences were obtained by use of T7 and T3 vector primers. To complete the sequences on both strands, cDNA-specific primers were used. The sequence analyses were carried out using software of the Genetics Computer Group (Madison, WI).

Expression in Yeast—To express the GLDase cDNA in yeast (Saccharomyces cerevisiae), the Bluescript vector containing the full-length cDNA was digested with ApaI and KpnI and a 27-bp adapter containing a NotI restriction site subsequently ligated into the ApaI-KpnI-linearized vector. The resulting construct containing two NotI restriction sites was cloned into the NotI restriction sites of the pFL61 vector (26). Yeast cells of the strain W303B (Matα, ade2, ura3, his3, trp1, leu2, can1-100) (27) were transformed by the method of Dohmen (28) and plated on selective media containing ade2, ura3, his3, and tryrntophan, leucine and adenine at 30 °C. The cells were collected by centrifugation (8,000 × g, 15 min) from the initial mitochondrial fraction with a recovery of 1.1% (Table I). The purity of the final enzyme preparation was confirmed by SDS-PAGE, where we consistently obtained three polypeptide bands corresponding to approximately 56, 30, and 26 kDa (Fig. 1). Further purification of the enzyme by a high resolution gel filtration on a Zorbax GF 250 column did not confirm the partial amino acid sequence of the GLDase cDNA. The native molecular mass of the enzyme was estimated to be approximately 56 kDa by Sephacyr SF-200 (Fig. 3) and Zorbax GF 250 high resolution gel filtration. After passage through the strong cation exchange column (Poros 20 SP), GLDase activity was resolved into two peaks designated I and II (Fig. 1). The activity forming the latter peak was used for further analysis. At this stage GLDase was purified 1693-fold from the initial mitochondrial fraction with a recovery of 1.1% (Table I). The purity of the final enzyme preparation was confirmed by SDS-PAGE, where we consistently obtained three polypeptide bands corresponding to approximately 56, 30, and 26 kDa (Fig. 2). Further purification of the enzyme by a high resolution gel filtration on a Zorbax GF 250 column did not result in elimination of the 30- and 26-kDa polypeptide bands; and subsequent amino acid sequence analyses revealed them to be breakdown products of the 56-kDa band. The native molecular mass of the enzyme was estimated to be approximately 56 kDa by Sephacyr SF-200 (Fig. 3) and Zorbax GF 250 high resolution gel filtration.

Partial Amino Acid Sequence Determination of Purified GLDase Polypeptides

NH2-terminal sequence analysis of the complete 56- and 30-kDa polypeptide bands were found to be identical, and the partially determined sequence of the 26-kDa band was located within the deduced amino acid sequence of the GLDase cDNA (Asp-273 to Leu-289). Trypsin digestions of the 56-kDa protein yielded a series of peptides which were separated by reversed-phase HPLC. A number of the peptides were subjected to partial sequence analysis and could again be located in the GLDase cDNA, as indicated in Fig. 5.
Characterization

Substrate Specificity and pH Dependence—Various isomeric compounds were tested as possible substrates for the purified GLDase using cytochrome c as electron acceptor. These were L-GL, D-galactono-\(\gamma\)-lactone, D-gulono-\(\gamma\)-lactone, D-erythronic-\(\gamma\)-lactone, D-xylonic-\(\gamma\)-lactone, L-mannono-\(\gamma\)-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. Apart from L-GL, none of the compounds tested could serve as a substrate for GLDase because no reduction of cytochrome c was observed.

GLDase obeyed Michaelis-Menten-type kinetics using L-GL as substrate. With the method of Lineweaver and Burk (Fig. 4), the \(K_m\) value was determined to be 3.3 mM with a \(V_{max}\) of 7.1 units/min. Concentrations of L-GL used were from 1.0 to 32.6 mM. Substrate inhibition was observed at 32.6 mM.

The pH dependence of the enzyme activity was examined using 50 mM sodium phosphate buffer in the pH range from 6.0 to 7.6 and 50 and 100 mM Tris-HCl in the range between 7.4 and 8.8 at 22°C with 4.2 mM L-GL. A broad maximum of activity between pH 8.0 and 8.5 was observed (results not shown).

Electron Acceptors—The enzyme assay is based on the reduction of cytochrome c by GLDase, in which for each micromole of oxidized L-GL, 2 \(\mu\)mol of cytochrome c are reduced, because the L-AA formed is spontaneously oxidized by cytochrome c to dehydroascorbic acid. The purified GLDase showed strict specificity for cytochrome c, and neither FAD, NAD, NADP, nor molecular oxygen were able to serve as electron acceptors for the enzyme.

Inhibitors/Stimulators—The effect of various substrate analogues, organic inhibitors, and some divalent metal ions were examined for their influence on the enzyme activity. The oxidation of L-GL by GLDase was tested in the presence of equimolar concentrations of each of the following compounds: D-galactono-\(\gamma\)-lactone, D-gulono-\(\gamma\)-lactone, L-gulono-\(\gamma\)-lactone, D-erythronic-\(\gamma\)-lactone, D-xylonic-\(\gamma\)-lactone, L-mannono-\(\gamma\)-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. None of these had any influence on the reaction rate.

Of the divalent metal salts we tested, MgCl\(_2\), CaCl\(_2\), and SrCl\(_2\) had no effect on the GLDase activity at concentrations up to 15 mM. The chelating agent EDTA had no significant effect on the enzyme activity supporting the conclusion that there was no metal requirement for the enzymatic activity.

Sulfhydryl-modifying agents, however, were able to partially inhibit GLDase: \(N\)-ethylmaleimide, monoiodoacetic acid, and \(p\)-hydroxymercuribenzoic acid inhibited the enzyme activity by 18% at 12.5 mM, 42% at 26.9 mM, and 81% at 0.4 mM, respectively. These observations indicate that cysteine residues play an important role in the enzyme catalysis. We did not observe any inhibition of the GLDase-dependent reduction of cytochrome c in the presence of 5.2 mM riboflavin, a well known flavoprotein inhibitor (29–31).

Lycorine, an alkaloid isolated from members of the Amaryllidaceae has been reported to be a specific inhibitor of ascorbic acid biosynthesis in plants and animals at concentrations as
low as 1 μM (32–34); once again, however, no influence of lycorine on GLDase activity could be found at concentrations of up to 100 μM.

Cytochrome c Affinity Chromatography—Partly purified enzyme extract was observed to be slightly retarded compared with other proteins (measured as the absorption at 280 nm) when eluted from a cytochrome c affinity column. This indicated interaction between GLDase and cytochrome c.

Isolation and Sequencing of GLDase cDNA Clone

DNA fragments were obtained by polymerase chain reaction amplification of oligo(dT)-primed cDNA using degenerate oligonucleotides (based on the peptide sequences) as primers. These DNA fragments were subcloned into a pGEM-T vector and sequenced. One 400-bp fragment contained a nucleotide sequence which corresponded to the amino acid sequence of one of the sequenced internal peptides in addition to the sequences corresponding to the primers. Therefore, this fragment was radiolabeled and used as a probe to screen a cDNA library from cauliflower. We screened 2 × 10^6 plaques resulting in isolation of several positive clones. After in vitro excision of the Bluescript plasmid followed by digestion with EcoRI and KpnI, the two longest cDNA inserts were found to be approximately 2,000 bp. Subsequent subcloning and sequencing revealed an uninterrupted open reading frame of 1803 nucleotides, containing all of the partially sequenced tryptic peptides, the NH2-terminal amino acid sequence, the first ATG codon (position 56) representing the consensus sequence of an initiator codon (35), and a TAA terminator codon. The presence of these elements showed that the full-length cDNA corresponding to the purified protein had been isolated. Fig. 5 shows the deduced amino acid sequences of the 1803-bp open reading frame coding for 600 amino acids, a 55-bp putative 5′-noncoding region, and a 206-bp 3′-noncoding region including a poly(A) tail. A hexanucleotide AATAAA consensus signal for polyadenylation is found 20 nucleotides before the poly(A) tract. Interestingly, nucleotides coding for the determined NH2-terminal amino acid sequence were found 270 bp downstream from the initiator codon, indicating that the protein is synthesized as a preprotein (600 amino acids with a predicted molecular mass of

![Figure 5. Nucleotide sequence and predicted amino acid sequence of GLDase.](image)
Expression in Yeast

The GLDase cDNA was cloned into a pFL61 yeast vector (26) in both the sense and antisense orientations relative to the phosphoglycerate kinase promoter and terminator. Untransformed and transformed yeasts were grown and extracts were prepared and tested for GLDase activity. Extracts from yeast transformed with a sense-oriented GLDase cDNA showed a specific GLDase activity of 3.0 units/min/mg protein compared with those made from extracts from untransformed yeast and yeast transformed with antisense orientated GLDase cDNA in which no GLDase activity could be measured with L-GL as substrate (Fig. 6).

HPLC Analysis of L-GL

We used several different systems for the analysis of L-GL by HPLC. These included ion suppression reversed-phase HPLC, weak anion exchange HPLC, and strong anion exchange HPLC. In no case was it possible to obtain unequivocal resolution of L-GL from all other sugar-lactone analogues, but semi-preparative separations using weak anion exchange and reversed-phase HPLC in combination with spectrophotometric assays for GLDase activity, allowed us to consistently identify a fraction that co-migrated with L-GL standard and which served as a substrate for the GLDase-based reduction of cytochrome c. Peaks co-eluting in all three systems with L-GL standard were found to be able to serve as a substrate for GLDase (results not shown). This indirect evidence strongly suggests the presence of a natural substrate for GLDase in plant tissue extracts. In addition to this observation, acid extracts of plant tissues were resolved using pulsed amperometric detection and strong anion exchange on a Dionex PA-100 column. Under conditions of high pH (pH 11-12), it is possible to ionize neutral carbohydrates at the C-2 OH position, allowing the separation on appropriate ion exchange columns. Analysis of acid extracts from cauliflower and parsley by strong anion exchange HPLC with pulsed-amperometric detection at a gold electrode showed the presence of small amounts of a peak that co-migrated with L-GL (data not shown). However, in this system, L-GL also co-migrates with DL-GuL and with n-GL, so that it is not possible to unequivocally demonstrate the presence of this compound as a natural substrate.

DISCUSSION

GLDase was purified 1693-fold from cauliflower florets by a 5-step method with 1.1% recovery. The loss in recovery was approximately 20% in each purification step. This compares favorably with the results of Oba et al. (14) who recently published results from which they concluded that the alkaloid lycorine acts by inhibiting the conversion of L-GL to L-AA. Consequently, the enzyme we have purified from cauliflower is different to the homologous enzyme which Arrigoni et al. (37) recently published results from which they concluded that the partial amino acid sequences of tryptic peptides, the cDNA for GLDase was cloned and characterized. The complete amino acid sequence deduced from the cDNA and the localization of the NH₂-terminal amino acid sequence suggest that the mature GLDase protein is preceded by a 91-amino acid pre-peptide. We consider GLDase from cauliflower to be a mitochondrial enzyme as it was purified from a mitochondrially enriched extract from cauliflower florets. This corresponds well with the fact that the deduced pre-protein contains a relatively high number of Ala, Leu, Arg, and Ser residues (11, 10, 8, and 10, respectively); and relatively few Asp, Glu, Ile, and Val residues (0, 3, 2, and 0, respectively), which is characteristic for polypeptides targeted to the mitochondria (38, 39). In addition, the GLDase pre-protein cleavage site FR \( \downarrow \) YA resembles a
cleavage site motif (RXY↓S/A) which is relatively common in a number of higher and lower eukaryotes (36). These data are in accordance with results obtained by Oba et al. (14) who by unequivocally rule out the presence of GLDase from cauliflower by riboflavin or obtain the typical flavin protein absorption spectrum from the purified GLDase. In addition, there was no significant homology between the deduced amino acid sequence of cauliflower GLDase and the sequences of flavin-binding regions where the sequences have been extended to allow optimal sequence alignment. Identical residues are indicated by asterisks.

It has been suggested that GLDase contains a covalently bound flavin moiety as prosthetic group, because inhibition of GLDase activity by flavoprotein inhibitors was reported by Mapson and Breslow (12) and Oba et al. (14). However, we could not observe any inhibition of GLDase from cauliflower by riboflavin or obtain the typical flavin protein absorption spectrum from the purified GLDase. In addition, there was no significant homology between the deduced amino acid sequence of cauliflower GLDase and the sequences of flavin-binding regions where the sequences have been extended to allow optimal sequence alignment. Identical residues are indicated by asterisks.

Interestingly, an homology search with cauliflower GLDase in protein data bases revealed that the first 230 NH2-terminal amino acid of GLDase had 28% identity with an NH2-terminal stretch of L-gulono-lactone oxidase from rat (48), a sequence of L-gulono-1,4-lactone oxidase from rat (48), with the amino acid domain is involved in similar functional roles.

The results presented here are the first important step in the rationalization of the biosynthetic pathway of L-AA in plants. Furthermore, in the near future the isolation of the GLDase cDNA may give us the possibility to engineer crops containing stably increased levels of vitamin C.

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