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Functional Characterization of D-Galacturonic Acid Reductase, a Key Enzyme of the Ascorbate Biosynthesis Pathway, from *Euglena gracilis*

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D-Galacturonic acid reductase, a key enzyme in ascorbate biosynthesis, was purified to homogeneity from *Euglena gracilis*. The enzyme was a monomer with a molecular mass of 38–39 kDa, as judged by SDS–PAGE and gel filtration. Apparently it utilized NADPH with a *K*m value of 62.5 ± 4.5 μM and uronic acids, such as D-galacturonic acid (*K*m = 3.79 ± 0.5 mM) and D-glucuronic acid (*K*m = 4.67 ± 0.6 mM). It failed to catalyze the reverse reaction with L-galactonic acid and $NADP^+$. The optimal pH for the reduction of D-galacturonic acid was 7.2. The enzyme was activated 45.6% by 0.1 mM H_2O_2, suggesting that enzyme activity is regulated by cellular redox status. No feedback regulation of the enzyme activity by L-galactono-1,4-lactone or ascorbate was observed. N-terminal amino acid sequence analysis revealed that the enzyme is closely related to the malate dehydrogenase families.

Key words: D-galacturonic acid reductase; ascorbate biosynthesis; *Euglena*; aldo-keto reductase

The biosynthetic pathways of ascorbic acid (vitamin C: AsA) between animals and plants are different. In animals, AsA is synthesized mainly through D-glucuronic acid, L-gulonic acid, and L-gulono-1,4-lactone as intermediates.[1)] On the other hand, several types of biosynthetic pathway of AsA have been proposed in plants. The D-Mannose/L-galactose (D-Man/L-Gal) pathway proceeds *via* GDP-D-Man, GDP-L-Gal, L-Gal, and L-galactono-1,4-lactone (L-GalL), and most of the enzymes related to the pathway have been identified and analyzed in detail.[2)] The D-Man/L-Gal pathway appears to be the predominant pathway according to diverse analysis of its constituent enzymes using transgenic and mutant plants. In addition to the D-Man/L-Gal pathway, some researchers have reported that other alternative pathways *via* uronic acid intermediates, such as D-galacturonic acid and D-glucuronic acid, contribute to AsA biosynthesis,[3,4)] but the contribution of these alternative pathways *via* uronic acid intermediates to the AsA biosynthesis is still largely unknown.

Unlike animals and plants, the unicellular alga *Euglena gracilis* has developed a unique pathway for AsA biosynthesis. Shigeoka *et al.*[5)] have reported that *Euglena* cells accumulate AsA at a high level, and that its biosynthesis pathway comprises D-galacturonic acid, L-galactonic acid, and L-GalL, judging from the results of a radio-tracer experiment (Fig. 1). These facts suggest that *Euglena* cells are eukaryotic algae having the great advantage of elucidating the undefined uronic acid pathway in plants. D-Galacturonic acid reductase (EC 1.1.1.19) catalyzes the reaction of D-galacturonic acid to L-galactonic acid, and the gene was first identified from ripening strawberry fruit, suggesting that the protein belongs to a novel aldo-keto reductase (AKR) family.[3)] Recently, Kuorelahti *et al.*[6)] reported enzymatic characterizations of the recombinant D-galacturonic acid reductase from a mold, *Hypocrea jecorina*. In the mold, the enzyme appears to contribute to D-galacturonic acid catabolism, converting pectin to pyruvate and D-glyceraldehyde 3-phosphate, but not to AsA biosynthesis. Overall, the available information on D-galacturonic acid reductase is quite limited. Moreover, it is an interesting problem to elucidate clearly the

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Abbreviations: ARK, aldo-keto reductase; AsA, ascorbic acid; BCS, bathocuproine disulfonate; DTT, dithiothreitol; L-Gal, L-galactose; L-GalL, L-galactono-1,4-lactone; D-Man, D-mannose; MDH, malate dehydrogenase; NEM, *N*-ethylmaleimide; *p*CMB, *p*-chloromercuribenzoate

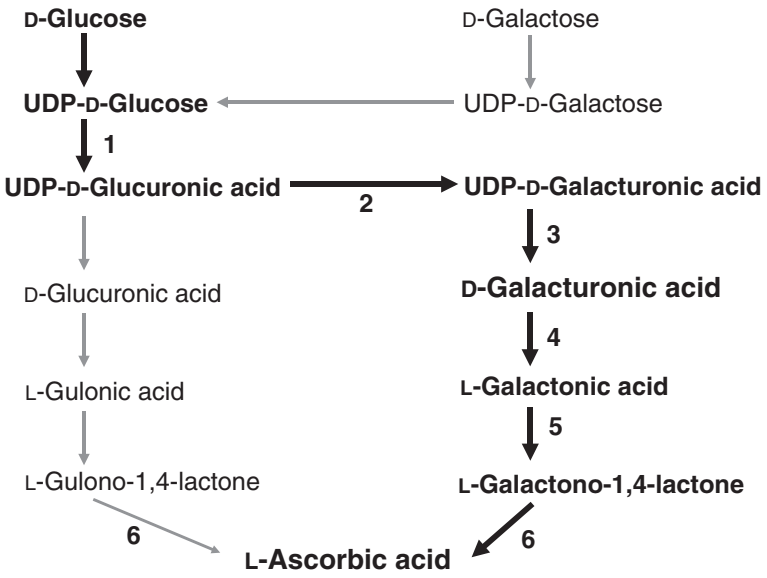

Fig. 1. The Ascorbate Biosynthesis Pathway in *Euglena gracilis*.
1, UDP-glucose dehydrogenase; 2, UDP-glucose-4-epimerase; 3, UDP-D-galacturonic acid pyrophosphatase; 4, D-galacturonic acid reductase; 5, lactonase (lactone hydroxylase); 6, L-gulono-1,4-lactone(L-galactono-1,4-lactone)dehydrogenase.

relationship between D-galacturonic acid reductase and a novel AKR family enzyme. In this study, we purified and characterized D-galacturonic acid reductase from *Euglena*. We discuss the physiological role of D-galacturonic acid reductase in AsA biosynthesis of *Euglena* cells which are photosynthetic organisms with AsA at a high level.

Materials and Methods

Strain and culture. Euglena gracilis strain Z was grown in Koren-Hutner medium under continuous light conditions at a photosynthetic photon flux density of 24 μmol m^{-2} s^{-1} at 26 °C for 6 d, by which time the stationary phase was reached.[7)]

Assay of D-galacturonic acid reductase. The assay mixture (1 ml) contained 50 mM Tris–HCl buffer (pH 7.2), 5 mM D-galacturonic acid, 0.2 mM NADPH and the enzyme. The reaction was monitored by the decrease in absorbance at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) at 25 °C. The specificity of the enzyme with alternate substrates was also assayed using the same reaction mixture. The protein concentration was measured with the Bio-Rad Protein Assay Reagent (Bio-Rad, Los Angeles, CA) using BSA as a standard. To check the reverse reaction, L-galactonic acid and $NADP^+$ were used as substitutes for the assay mixture. L-Galactonic acid was obtained by hydrolysis of L-GalL under basic conditions. Twenty μl of 0.3 M NaOH was added to 100 μl of 10 mM L-GalL and the mixture was vigorously agitated by vortexing for 20 s. Then 0.3 M hydrochloric acid (20 μl) was added to the mixture to neutralize the solution.

Purification of D-galacturonic acid reductase. Euglena cells were collected by brief centrifugation, suspended in two volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.2) containing 1 mM EDTA, and disrupted by sonication. The cell lysate was centrifuged at 100,000 × *g* at 4 °C for 30 min, and the supernatant was used as a crude extract for enzyme purification. It was loaded onto a CM-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden) column (25 × 40 mm) equilibrated previously with 50 mM Tris–HCl (pH 7.2). The column was washed with at least three column volumes of the buffer and then eluted with 100 ml of a 0–500 mM linear gradient of KCl in the buffer. The active fractions were pooled and applied onto a Butyl-Toyopearl (Tosoh, Tokyo) column (30 × 150 mm) equilibrated with Tris–HCl buffer (pH 7.2), containing 30% (W/V) ammonium sulphate. The column was developed with a linear gradient of 30–0% (W/V) ammonium sulphate in Tris–HCl buffer (pH 7.2). The active fractions were pooled and dialyzed in an ample volume of Tris–HCl buffer (pH 7.2) with two changes of the buffer. The fraction was further purified by affinity chromatography using a HiTrap Blue HP (Amersham-Pharmacia Biotech) column (10 × 50 mm) equilibrated with Tris–HCl buffer (pH 7.2). The column was washed with 20 ml of Tris–HCl buffer (pH 7.2) containing 0.8 M KCl, and then eluted with 10 ml of Tris–HCl buffer (pH 7.2) containing 1.5 M KCl. The active fractions were pooled, concentrated, and stored at −20 °C until use.

SDS–PAGE. SDS–PAGE was performed in a 12.5% polyacrylamide slab gel using a Tris/glycine buffer system, as described by King and Laemmli.[8)] The gels were stained with Coomassie Blue G-250.

Table 1. Purification of D-Galacturonic Acid Reductase from *Euglena gracilis* Z

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg protein)	Fold	Yield (%)
Crude extract	354	1498	4.22	1	100
CM Sepharose	334	1797	5.38	1.2	119.9
30% $(NH_4)_2SO_4$	294	1040	3.53	0.8	69.5
Butyl Toyopearl	7.78	248.4	31.9	7.6	16.6
HiTrap Blue HP	0.16	104.2	671.0	159.0	7.0

HPLC analysis of the enzyme reactant. D-Galacturonic acid and the corresponding reactant, L-galactonic acid, were assayed by HPLC on a Fusion-RP C18 column (150 × 4.6 mm; Phenomenex, Torrance, CA) at 210 nm with an eluent of dilute phosphoric acid (pH 2.5) at a flow rate of 1 ml/min.

Analysis of N-terminal amino acid sequence. Analysis of the N-terminal amino acid sequence was carried out with an automated pulse-liquid phase sequencer (Model 492; Applied Biosystems, Foster City, CA).

Results and Discussion

Purification of D-galacturonic acid reductase from Euglena

A typical result of purification of the enzyme from *Euglena gracilis* Z is summarized in Table 1. The crude extract showed a specific activity of 4.22 nmol/min/mg protein, which was approximately 40-fold higher than that of *Arabidopsis* leaves.[3] The elution patterns of all columns showed only one peak of the enzyme activity (data not shown). The purified enzyme was obtained in an overall yield of 7% and exhibited an increase of approximately 160-fold in specific activity. SDS–PAGE showed a single protein band with a molecular mass of 38 kDa (Fig. 2). The molecular mass of the *Euglena* enzyme was almost the same as those of the enzymes from the strawberry and the mold *H. jecorina*.[3,6] It was also approximately 39 kDa based on gel filtration on a calibrated Superose-200 column (data not shown). These results indicate that D-galacturonic acid reductase from *Euglena* cells exists in a monomer in its native state, corresponding to the known AKR families.[9]

Identification of the reaction product of Euglena *D-galacturonic acid reductase*

As shown in Fig. 3, L-galactonic acid was detected by the HPLC separation as a product of D-galacturonic acid reduction after a 10-min incubation of the reaction mixture at 25 °C, clearly indicating that the enzyme catalyzed D-galacturonic acid to L-galactonic acid in the forward direction.

Substrate specificity and kinetic study of Euglena *D-galacturonic acid reductase*

Next we studied the substrate specificity and kinetics

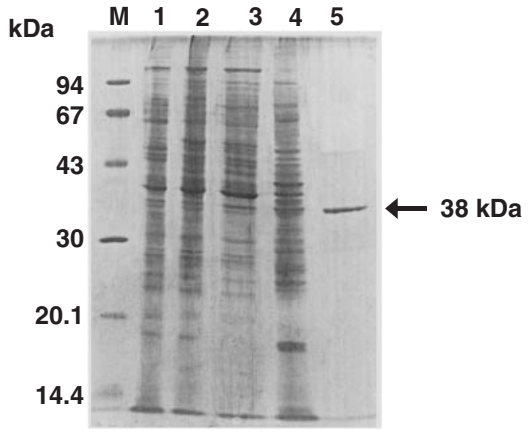

Fig. 2. SDS–PAGE Documenting the Progress of Purification of *Euglena* D-Galacturonic Acid Reductase.

D-Galacturonic acid reductase was purified as described in "Materials and Methods." The samples were loaded onto a 12.5% polyacrylamide gel and stained with Coomassie brilliant blue. Lane M, molecular marker; lane 1, crude extract; lane 2, CM sepharose chromatography; lane 3, 30% $(NH_4)_2SO_4$ fraction; lane 4, butyl Toyopearl chromatography; lane 5, HiTrap Blue HP chromatography. The positions of molecular markers are indicated on the left in kDa.

of the enzyme. The enzyme utilized both NADPH and NADH in a ratio of 10 to 1.8, indicating that it prefers NADPH to NADH as an electron donor. The apparent *K*m value of the enzyme for NADPH was 62.5 ± 4.5 μM, similar to that of the recombinant enzyme from *H. jecorina*,[6] and the *V*max value was 266.7 ± 19.2 μmol/min/mg protein. It has been reported that the recombinant enzymes from strawberry and *H. jecorina* are highly specific for NADPH, but not for NADH.[3,6] As shown in Table 2, the *Euglena* enzyme exhibited high activity with both D-galacturonic acid and D-glucuronic acid, and also catalyzed the reduction of D-xylose, D/L-galactose, D-glucose, L-arabinose, and DL-glyceraldehyde at appropriate rates, indicating that the enzyme possesses broad substrate specificity. No activity of the *Euglena* enzyme was observed with *p*-nitrobenzaldehyde or menadione, which are substrates for carbonyl reductase, one of the known AKR families. As shown in Table 3, the *K*m values for D-galacturonic acid, D-glucuronic acid and D-xylose were 3.79 ± 0.5, 4.67 ± 0.6 and 8.48 ± 0.8 mM, respectively, but the enzyme showed higher catalytic efficiency (*K*cat/*K*m)

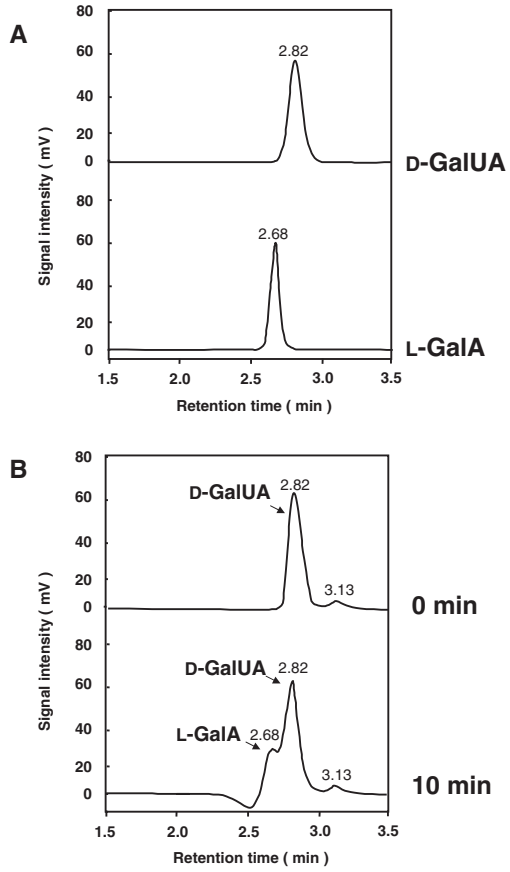

Fig. 3. Formation of L-Galactonic Acid from D-Galacturonic Acid by D-Galacturonic Acid Reductase Purified from *Euglena*.

The purified enzyme was incubated with 5 mM D-galacturonic acid and 0.2 mM NADPH at 25 °C for 10 min. The experimental conditions for HPLC analysis are described in "Materials and Methods." A, standard compounds; B, analysis of a reaction mixture. Arrows indicate the compounds. D-GalUA, D-galacturonic acid; L-GalA, L-galactonic acid.

Table 2. Substrate Specificity for Purified D-Galacturonic Acid Reductase from *Euglena*

Substrate	Relative activity (%)
Electron donor	
NADPH	100.0
NADH	17.7
Electron acceptor	
D-Galacturonic acid	100.0
D-Glucuronic acid	82.4
D-Xylose	29.9
L-Galactose	18.8
D-Galactose	14.9
D-Glucose	14.6
L-Arabinose	14.2
DL-Glyceraldehyde	14.2
p-Nitrobenzaldehyde	0.0
Menadione	0.0

for D-galacturonic acid and D-glucuronic acid than for D-xylose, indicating that it should dominantly reduce uronic acids *in vivo*. The purified recombinant D-galacturonic acid reductase of the strawberry exhibited much higher activity with D-galacturonic acid than with D-glucuronic acid.[3] On the other hand, the recombinant enzyme from *H. jecorina* showed similar Michaelis-Menten constants for both D-galacturonic acid (Km = 6 mM) and D-glucuronic acid (Km = 11 mM). These findings indicate that the affinity against uronic acids of the *Euglena* enzyme tends to be similar to that of the mold enzyme, but the mold enzyme did not show any significant activities with other aldoses, such as D-glucose, D-xylose, D-galactose, and L-arabinose.[6] It is worth noting that there is an alternative ascorbate biosynthesis pathway in *Euglena* that takes D-glucuronic acid and L-gulono-1,4-lactone, although the flux of the alternative pathway is much lower than that of the D-galacturonic acid pathway.[5] Therefore, it might be reasonable to consider that the *Euglena* enzyme has high catalytic efficiency with both D-galacturonic acid and D-glucuronic acid.

Table 3. Michaelis Constants and Catalytic Efficiency for Substrates of *Euglena* D-Galacturonic Acid Reductase

Substrate	$k$cat (s^{-1})	Km (mM)	$k$cat/Km ($\mu M^{-1}\ s^{-1}$)
D-Galacturonic acid	0.19 ± 0.01	3.79 ± 0.5	50.95
D-Glucuronic acid	0.19 ± 0.01	4.67 ± 0.6	41.35
D-Xylose	0.06 ± 0.004	8.48 ± 0.8	6.97

With respect to substrate specificity, AKR families are classified into three distinct groups.[9] The first group is aldehyde reductase, which catalyzes the reduction of various types of aldehyde, including uronic acids and some ketones. The second is aldose reductase, which catalyzes the reduction of aldehydes, such as glycolaldehydes, but is less active against uronic acids. The third group is carbonyl reductase, which catalyzes the reduction of quinines, other ketones, and short-chain aldehydes. On the basis of the present data, it appears likely that *Euglena* D-galacturonic acid reductase belongs to the first group of AKR families.

Kuorelahti *et al.*[6] have reported that fungal recombinant D-galacturonic acid reductase exhibits activity in the reverse direction. When we tested the reverse reaction of the enzyme, no reaction was observed with L-galactonic acid and $NADP^+$ as substrates (data not shown).

Effects of pH and various compounds on D-galacturonic acid reductase activities

The optimal pH for the reduction of D-galacturonic acid in *Euglena* D-galacturonic acid reductase was 7.2, with 75 and 18.5% of maximum activity at pH 6.5 and 9.0 respectively (Fig. 4).

Metal ions, except for Cu^{2+}, reducing and oxidizing reagents such as dithiothreitol (DTT), *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (*p*CMB), and chelators had no effect on enzyme activities (Table 4).

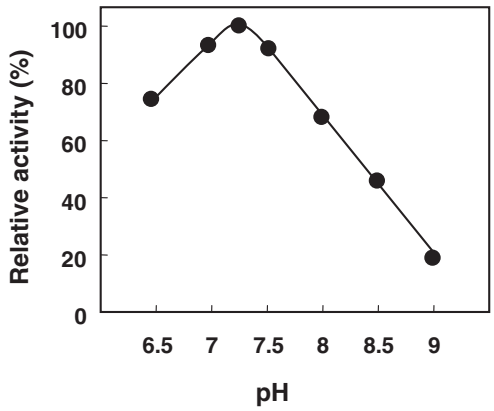

Fig. 4. Effect of pH on the Activity of the Purified D-Galacturonic Acid Reductase.

The activity of the purified enzyme was measured as described in "Materials and Methods."

Table 4. Effects of Various Compounds on Purified D-Galacturonic Acid Reductase from *Euglena*

Compound	Concentration (mM)	Relative activity (%)
None	—	100
$CaCl_2$	5	93.8
$CoCl_2$	5	102.9
$MnCl_2$	5	98.4
$NiSO_4$	5	94.8
$ZnSO_4$	5	100.7
$FeSO_4$	1	104.8
	5	110.2
$CuSO_4$	1	115.0
	5	135.3
EDTA	1	96.7
BCS	0.5	93.9
DTT	5	98.0
NEM	0.5	99.7
	1	84.0
*p*CMB	0.05	107.2
H_2O_2	0.1	145.6
L-GalL	2	99.8
AsA	2	100.8

Interestingly, 5 mM Cu^{2+} and 0.1 mM H_2O_2 activated the enzyme activity to 135.3% and 145.6% respectively. The ratio of direct oxidation of NADPH by H_2O_2 was at a negligible level. Treatment of the enzyme with a Cu chelator, bathocuproine disulfonate (BCS), had no effect on the activity (Table 4), and the enzyme exhibited no absorbance spectrum bands for a typical Cu-containing protein, except for a strong 280 nm peak (data not shown), indicating that it is not a typical Cu-containing protein, as reported in other known AKR families.[9] These findings suggest that activation of the enzyme in the presence of Cu^{2+} is due to generation of H_2O_2 caused by auto-oxidation of metal ions, and that the enzyme activity might be regulated by the redox status of the *Euglena* cells. Oxidative stress causes an increase in the levels of AsA, a functional antioxidant, in *Euglena cells*.[10,11] In addition, we have reported that H_2O_2 is generated under high light conditions in chloroplasts of *Euglena*.[12] Accordingly, it appears likely that activation of D-galacturonic acid reductase in the presence of H_2O_2 is a key regulation mechanism for the acceleration of AsA biosynthesis in *Euglena* cells under oxidative stress conditions. Treatment of the enzyme with thiol-modification reagents, such as DTT, *p*CMB, and NEM, had no effect on activity, suggesting that the enzyme does not include the SH base in the reaction center, and that the target site for the redox regulation of the enzyme activity is another amino acid residue, besides the cysteine residue. It has been reported that phenylalanine hydroxylase and dihydropteridine reductase from humans were activated by oxidation of Trp and Met residues respectively by H_2O_2 treatment.[13,14] These observations might fit in with the case of the activation mechanism of D-galacturonic acid reductase by H_2O_2.

Feedback inhibition of L-Gal dehydrogenase, the penultimate enzyme in the D-Man/L-Gal pathway in higher plants, and GDP-D-Man-3,5-epimerase by AsA, is known to be an important regulation mechanism in AsA biosynthesis.[15,16] To clarify the possibility of feedback regulation of *Euglena* D-galacturonic acid reductase, we studied the effect of two downstream compounds of the biosynthesis pathway, AsA and L-GalL, on enzyme activity. Neither AsA nor L-GalL had an effect on the activity up to 2 mM (Table 4), indicating no feedback regulation of the enzyme activity in the AsA biosynthesis pathway of *Euglena*.

N-terminal amino acid sequence

The N-terminal amino acid sequence of *Euglena* D-galacturonic acid reductase was determined by Edman degradation. The first 16 residues of the N-terminal sequence were FKVAV?GAAAGIGQPL. Surprisingly, homology of the N-terminal sequence apparently exists among malate dehydrogenases (MDHs) from various organisms, but not among known D-galacturonic acid reductases from strawberry or mold (Fig. 5). Next, we determined the MDH activity of the purified *Euglena* D-galacturonic acid reductase, no dehydrogenase activity was detected, even at high protein concentrations. Conversely, MDHs from pigs and yeast were not determined to have any D-galacturonic acid reducing activity (data not shown). These results indicate that the *Euglena* enzyme is clearly distinct from MDHs from other organisms. The conserved N-terminal region among MDH families is known to form an α-helix and to play an important role in the subunit–subunit interface to constitute its dimeric form together with another set of α-helix domain.[17,18] As described above, *Euglena* D-galacturonic acid reductase exists in monomeric form, indicating that the primary or the secondary structure should be distinct from that of MDH families although it has a similar N-terminal region with them. It is an interesting problem to define the evolutionary relation-

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Strawberry D-GalUAR          1 MAKVPSVTLSSCGDDI 16
H.jecorina D-GalUAR          1 MVATSFKLNNGLEIPA 16

Euglena D-GalUAR             1 FKVAV?GAAAGIGQPL 16
Trypanosoma mMDH             9 FKVAVLGAAGGIGQPL 24
Oryza gMDH                  43 FKVAVLGAAGGIGQPL 58
Arabidopsis gMDH            43 FKVAILGAAGGIGQPL 58
Stevia MDH                  41 FKVAILGAAGGIGQPL 56
Pig heart MDH                1 AKVAVLGASGGIGQPL 16
Yeast mMDH                  18 YKVTVLGAGGGIGQPL 33
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Fig. 5. Alignment of N-Terminal Amino Acid Sequences of D-Galacturonic Acid Reductase (D-GalUAR) and Malate Dehydrogenase (MDH).
The accession numbers for the sequences are as follows: strawberry D-GalUAR, AF039182; *H. jecorina* D-GalUAR, AY862503; *Trypanosoma* mitochondrial MDH (mMDH), AF027739; *Oryza* glyoxysomal MDH (gMDH), D85763; *Arabidopsis* gMDH, NM001036783; stevia MDH, DQ269456; pig heart MDH, M16427; yeast mMDH, J02841.

ship between D-galacturonic acid reductase and MDH in *Euglena* cells.

In the present study, we purified and characterized a D-galacturonic acid reductase from *Euglena*, the first enzyme identified from the AsA biosynthesis pathway proposed in *Euglena*. The present results clearly indicate that the substrate specificity and primary structure of the *Euglena* enzyme are distinct from those of strawberry and mold. We also found that the activity of D-galacturonic acid reductase is activated by the occurrence of H_2O_2, which is involved in the regulation of redox homeostasis. More experiments will be necessary to elucidate the relationship between redox regulation of the enzyme and AsA levels in *Euglena* cells under diverse environmental conditions.

Acknowledgments

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