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### Purification and Characterization of Two Isoforms of Glucose 6-Phosphate Dehydrogenase (G6PDH) from *Chlorella vulgaris* C-27

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## Purification and Characterization of Two Isoforms of Glucose 6-Phosphate Dehydrogenase (G6PDH) from *Chlorella vulgaris* C-27

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Two kinds of isoforms of glucose 6-phosphate dehydrogenase (G6PDH) were purified from cells of a freezing-tolerant strain, *Chlorella vulgaris* C-27, by sequential steps of chromatography on five kinds of columns, including a HiTrap Blue column which showed excellent separation of the isoforms from each other. The two isoforms (G6PDH<sub>1</sub> and G6PDH<sub>2</sub>) were purified up to 109-fold and 197-fold with specific activity of 14.4 and 26.0 U/mg-protein, respectively. G6PDH<sub>1</sub> showed an apparent  $M_r$  of 200,000 with a subunit  $M_r$  of about 58,000, whereas G6PDH<sub>2</sub> showed an apparent  $M_r$  of 450,000 with a subunit  $M_r$  of about 52,000. The kinetic parameters were measured and several enzymatic features of the isoforms, such as effects of metal ions on the enzyme activity, were clarified, which showed that the two isoforms were different from each other in many respects. Among the effective ions, Cd<sup>2+</sup> showed marked stimulating effects on both isoforms. G6PDH<sub>1</sub> and G6PDH<sub>2</sub> seem to be a cytosolic and a chloroplastic type, respectively, as judged by their sensitivity to DTT, and also from the results of sequence similarity searches using their N-terminal and internal amino acid sequences.

**Key words:** *Chlorella vulgaris* C-27; freezing tolerance; glucose 6-phosphate dehydrogenase (G6PDH); purification

Freezing injury is one of the most severe constraints limiting crop productivity.<sup>1)</sup> Investigating mechanisms of freezing injury and developing

methods to prevent the injury are very important to meet a worldwide food crisis. It is also quite interesting from the point of view of plant biochemistry and physiology to understand such a phenomenon as cold acclimation of plants. Freezing injury leads to severe dehydration within the cells, denaturation of proteins, and precipitation of various molecules,<sup>2,3)</sup> further leading to oxidative damage to the cells and finally killing them. Cold hardening, *i.e.* exposure of plants to non-freezing low temperatures for a few hours or days, results in alteration in gene expression, increased synthesis of certain proteins, changes in activity behaviors of many enzymes, and accumulation of soluble sugars.<sup>4-7)</sup> These events together could potentially contribute to freezing tolerance.

Glucose 6-phosphate dehydrogenase (G6PDH) has prime importance in carbon metabolism of plants and it catalyzes the first reaction in the oxidative pentose phosphate pathway.<sup>8)</sup> The main function of the enzyme is to provide NADPH, which is a critical modulator of the redox potential of the cells and is the principal intracellular reductant for various biosynthetic reactions.<sup>9,10)</sup> It has been observed by various workers that physiological processes associated with low temperature treatment of plants and animals lead to an increase in activity of G6PDH and increased capacity of the pentose phosphate pathway.<sup>4,11-15)</sup> Desiccation or oxidative stress, which are often associated with chilling, also tend to increase G6PDH activity in yeast, plants, and human cells.<sup>16-18)</sup> Under a condition of cold hardening, to

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Abbreviations: CBB, Coomassie brilliant blue; G6PDH, glucose 6-phosphate dehydrogenase; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride

which glycolysis is sensitive,<sup>12)</sup> increased activity of G6PDH may provide sustainable levels of NADPH to meet the biosynthetic events within the cells.<sup>15,19,20)</sup> An increased NADPH level is also essential to maintain sufficient reduced glutathione, which serves as antioxidant to detoxify H<sub>2</sub>O<sub>2</sub> within cells under oxidative stress.<sup>21)</sup>

From bacteria, yeast, and animal tissues, G6PDHs have been purified to homogeneity, as a homodimer or tetramer with a subunit molecular weight between 50,000 to 57,000.<sup>22–24)</sup> From plant cells, however, G6PDH isoforms have been difficult to purify to homogeneity,<sup>25,26)</sup> as they are very unstable during the purification process. The enzymes appear to be tetramers with molecular weights of from 210,000 to 240,000. Owing to the difficulties in purifying G6PDH from plant sources, there is little information available on its characteristics and kinetic properties.<sup>8)</sup>

Our previous studies indicated possible involvement of the pentose phosphate pathway (PPP) in the process of hardening in *Chlorella* cells, marked by increased activity of the PPP enzymes, *i.e.*, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.<sup>4)</sup> However, detailed studies regarding involvement of G6PDH in the development of freezing tolerance are still under way. Although G6PDH seems to be involved in the development of freezing tolerance of plants, the exact analysis of the enzymes have not been tried. There are at least two isoforms of G6PDH in higher plants; one is in the cytosol and the other is in the chloroplast.<sup>9,27,28)</sup> However, there are no reports on which isoform is involved in the development of freezing tolerance. Thus, as a part of the study to make clear the involvement of G6PDH and further, that of the isoforms in the development of freezing tolerance on a firm molecular basis, we tried first to purify two kinds of isoforms of G6PDH from hardened cells of *C. vulgaris* and second, to characterize their structural, kinetic, and other enzymatic properties.

## Materials and Methods

**Plant materials.** Cells of *Chlorella vulgaris* Beijerinck IAM C-27 were grown synchronously in MC medium<sup>29)</sup> at 25°C, under a photosynthetic photon flux density of about 250 μmol/m<sup>2</sup>s, with 1.3% CO<sub>2</sub> in air, to a concentration of about 1.0 × 10<sup>10</sup> cells/l, under a 16-h light/8-h dark regime, as described previously.<sup>30)</sup>

**Hardening.** Cold treatment was done using *Chlorella* cells grown up to an intermediate stage in the ripening phase of the cell cycle, the L<sub>2</sub> stage.<sup>30)</sup> The synchronized cells (1.0 × 10<sup>10</sup> cells/l) in the MC medium were treated at 3°C for 24 h. During the treatment, cultures were aerated with 1% CO<sub>2</sub>-air

and kept in the light (250 μmol/m<sup>2</sup>s). The hardened cells were used for purification of G6PDH.

**Purification of G6PDH.** The hardened cells of a five-liter culture were harvested by centrifugation and suspended in 40 ml of 50 mM potassium phosphate buffer (pH 7.5) that contained 1 mM PMSF. All the subsequent steps were done at 4°C. The cells were broken with a Vibrogen-Zellmühle (Vibrogen-Zellmühle; Edmund Bühler Co., Tübingen, Germany) for 12 min at 4°C. The homogenate was filtered using a sintered-glass funnel and then centrifuged at 168,000 × *g* for 30 min. The supernatant was concentrated to about 15 ml by ultrafiltration using a 20-kDa ultrafilter membrane (Advantec Co., Tokyo, Japan).

The concentrated sample was put directly on a column (2.5 × 66 cm) of Sepharose-4B (Amersham Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with buffer A (50 mM potassium phosphate buffer (pH 7.5), 10% glycerol). Proteins were eluted with the same buffer. Active fractions were mixed and used for a subsequent step.

The enzyme solution was put directly on a HiTrap Blue column (5 ml; Amersham Pharmacia), which had been equilibrated with buffer A. The non-adsorbed solution, which included one of the isoforms of G6PDH, was used for another affinity chromatography on a Blue Cellulofine column. The HiTrap Blue column was washed with 20 ml of buffer A and then adsorbed proteins were eluted by three kinds of the following buffers; first step: 30 ml of buffer A plus 1 mM NAD, second step: 30 ml of buffer A plus 1 mM NADP, third step: 30 ml of buffer A plus 1 M KCl. Active fractions eluted with 1 M KCl were mixed and dialyzed with buffer A. We designated an isoform, which was eluted with 1 M KCl, as G6PDH<sub>2</sub>.

The non-adsorbed fraction, which contained another isoform of G6PDH that did not bind to the HiTrap Blue column, was put on a Blue-Cellulofine (Seikagaku Co., Tokyo, Japan) column (1.6 × 8 cm), which had been equilibrated with buffer A. The column was washed with the same buffer and then washed with buffer A plus 1 mM NAD. Then the adsorbed enzyme was eluted by an increasing gradient of NADP in the buffer (0–1 mM, total volume 60 ml). Active fractions were mixed. The isoform separated on a Blue-Cellulofine column was designated G6PDH<sub>1</sub>.

The two enzyme solutions (G6PDH<sub>1</sub> and G6PDH<sub>2</sub>) were, separately, applied to a HiTrap Q-Sepharose-XL (Amersham Pharmacia Biotech) column which had been equilibrated with buffer A. The column was washed initially with 20 ml of the same buffer A and then proteins were eluted by an increasing gradient of KCl in the buffer (0.15–0.25 M; the total volume was 70 ml in the case of G6PDH<sub>1</sub>, 0.25–0.45 M; the total

volume was 70 ml in the case of G6PDH<sub>2</sub>). Active fractions were mixed and dialyzed against buffer A.

The two enzyme solutions were respectively put on a HiLoad 16/60 Superdex 200 pg (Amersham Pharmacia Biotech) column which had been equilibrated with buffer A. The column was eluted with the same buffer. Active fractions were mixed and used for further experiments.

**Molecular mass measurement.** The molecular masses of the purified G6PDHs were measured on a HiLoad 16/60 Superdex 200 pg column equilibrated with buffer A containing 0.15 M KCl. The column was calibrated with a mixture of proteins prepared in the same buffer: ferritin,  $M_r=450,000$ ; bovine liver catalase,  $M_r=240,000$ ; rabbit muscle aldolase,  $M_r=160,000$ ; bovine serum albumin,  $M_r=67,000$ ; and egg albumin,  $M_r=45,000$ . The purified enzyme samples were put on the column. The column was eluted with the above buffer at a flow rate of 1.0 ml/min and the absorbance was monitored at 220 nm. The molecular masses of the isoforms were then calculated from the plot of the molecular masses of the known proteins vs. the retention times.

The molecular masses of subunits of G6PDH isoforms were measured by SDS-PAGE on a 7.5% (w/v) polyacrylamide gel, by the procedure of Laemmli.<sup>31</sup> After separation, the gels were stained with Coomassie Brilliant Blue (CBB) R-250.

**Assay of G6PDH activity.** The activity of G6PDH was assayed by the method of Sagisaka.<sup>32</sup> An assay mixture in a total volume of 3 ml contained 55 mM Tris-HCl buffer (pH 7.8), 3.3 mM MgCl<sub>2</sub>, 0.2 mM NADP, 3.3 mM glucose 6-phosphate (G6P), and enzyme. The reaction was started by adding 0.1 ml of an enzyme solution to the reaction mixture. The reduction of NADP was followed by monitoring the increase in absorbance at 340 nm in a double-beam recording spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). One unit of the activity was defined as the amount of enzyme forming 1.0  $\mu$ mol of NADPH per minute at 25°C.

**Estimation of protein concentration.** Protein concentrations were measured by the method of Bradford<sup>33</sup> using bovine serum albumin as a standard.

**Treatment with DTT.** In order to examine the effects of DTT on G6PDH activity, purified enzyme solutions were diluted with an equal volume of buffer A containing 200 mM DTT. After incubation of the samples at 0°C for different times, portions were withdrawn and used for measurement of G6PDH activity. The corresponding controls were diluted with buffer alone.

**Temperature-, pH-dependence, and kinetic parameters.** The effects of temperature on the activity of G6PDH were measured by the enzyme activity at different temperatures from 20 to 70°C. For investigating the effects of temperature on the stability of the enzymes, the purified enzyme was incubated in buffer A at various temperatures for 30 min, and then the remaining activity was assayed at 25°C. The pH-dependence was investigated by doing assays in 55 mM of different buffers ranging from pH 3 to 11: citric acid-di-sodium hydrogenphosphate (pH 3–7), Tris-HCl (pH 7–9), and glycine-NaOH (pH 9–11). For investigating the effects of pH on the stability of the enzymes, the purified enzyme was incubated in the above buffers (55 mM) at 4°C overnight, and then a portion of the mixture was transferred into the assay mixture at pH 7.8 to measure the remaining activity.

The  $K_m$  for G6P was measured in 0.2 mM NADP. The  $K_m$  for NADP was measured in 3.3 mM G6P. The maximum velocity ( $V_{max}$ ) of G6PDH and Michaelis constant ( $K_m$ ) values were calculated by constructing Lineweaver-Burk plots.

**Amino acid sequencing of the N-terminus and digested peptides, and homology search.** For analysis of the N-terminal amino acid sequences, the purified G6PDHs were separated by SDS-PAGE, blotted electrophoretically onto a PVDF membrane (Bio-Rad, Richmond, CA, U.S.A.) in an electroblot apparatus (AE-6675P; ATTO Co., Tokyo, Japan), and detected by staining with CBB R-250. The amino acid sequences of the isoforms were analyzed with a gas-phase protein sequence analyzer (model PPSQ-21; Shimadzu, Kyoto, Japan).

For analysis of internal amino acid sequences of the G6PDHs, purified G6PDH<sub>1</sub> was cleaved by cyanogen bromide,<sup>34</sup> and purified G6PDH<sub>2</sub> was digested by V8 protease in a gel according to the method of Kennedy *et al.*<sup>35</sup> The digested peptide fragments were separated by SDS-PAGE on a 15% polyacrylamide gel and blotted electrophoretically onto a PVDF membrane. The peptides were stained with CBB R-250 and sequenced as described above.

The sequences obtained were compared with those of proteins in the databases, using the BLAST program<sup>36</sup> on the NCBI homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## Results and Discussion

### *Purification of two isoforms of G6PDH from Chlorella*

Two different enzymes showing activity of G6PDH have been purified from *Chlorella vulgaris* C-27. A typical purification protocol is described in Table 1. Although the activity profile from the Sepharose 4B column showed only one peak with G6PDH activity

**Table 1.** Purification of G6PDHs from *Chlorella vulgaris* C-27

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude extracts	133	17.55	0.132	1.00	100
Sepharose 4B	117	13.53	0.115	0.87	77.1
HiTrap Blue					
G6PDH <sub>2</sub>	8.61	5.76	0.669	5.08	32.8
Blue Cellulofine					
G6PDH <sub>1</sub>	0.85	2.51	2.95	14.3	22.4
HiTrap Q-Sepharose XL					
G6PDH <sub>1</sub>	0.10	1.04	10.2	77.1	5.50
G6PDH <sub>2</sub>	0.52	3.24	6.21	47.1	18.5
HiLoad 16/60 Superdex 200 pg					
G6PDH <sub>1</sub>	0.03	0.40	14.4	109	2.29
G6PDH <sub>2</sub>	0.08	1.99	26.0	197	11.3

(data not shown), the second chromatography using a HiTrap Blue column showed G6DPH activity both in the adsorbed and non-adsorbed fractions. The non-adsorbed isoform was designated as G6PDH<sub>1</sub> and the adsorbed isoform as G6PDH<sub>2</sub>. The G6PDH<sub>1</sub> solution was put on the third chromatography using a Blue Cellulofine column. After following ion exchange and gel filtration chromatography, both isoforms were respectively purified to homogeneity.

Various workers have attempted to purify G6PDH from plant sources. However, because of its high instability and its unspecific aggregation during purification, little success has resulted so far.<sup>9,26)</sup> Only two cytosolic G6PDHs from plant species have been purified in homogeneous states.<sup>9,26)</sup> Therefore, many experiments related to characterization of plant G6PDH have been done with partially purified preparations or crude extracts.<sup>9)</sup> In this study, as Fig. 1B shows, two kinds of isoforms of G6PDH have been purified, especially owing to the use of a HiTrap Blue column, which was very helpful and effective for purification of G6PDH<sub>2</sub>. According to the manufacturer's instructions, HiTrap Blue contains Cibacron Blue F3G-A as a ligand, to which the two isoforms showed different affinity, leading to their effective separation from each other. Thus, we succeeded in purification of the two isoforms without the problem of the unspecific aggregation of the enzymes. Another point for the successful purification of the isoforms in this study lies in our use of hardened *Chlorella* cells, because the total activity of G6PDH<sub>2</sub> of unhardened cells was low and thus it was difficult to purify the isoform.

The molecular mass of each purified G6PDH isoform was measured by gel-filtration chromatography using a HiLoad 16/60 Superdex 200 pg column. The retention time of G6PDH<sub>1</sub> in relation to the protein molecular mass standards indicated an apparent  $M_r$  of 200,000 for the native isoform (Fig. 1A). When the isoform was put through SDS-PAGE, a single band was observed, corresponding to a subunit  $M_r$  of 58,000 compared to the protein stan-

dards (Fig. 1B). The isoform thus seems to be a tetramer. On the other hand, the retention time of G6PDH<sub>2</sub> showed an apparent  $M_r$  of 450,000 for the native isoform. SDS-PAGE showed a subunit  $M_r$  of 52,000. These results suggested that G6PDH<sub>2</sub> formed an octamer.

These oligomeric properties and the subunit  $M_r$  are very similar to characteristics of G6PDHs from other species (bacteria, yeast, animal tissues, and plants), which have been purified as a homodimer or tetramer with a subunit  $M_r$  of 50,000 to 57,000.<sup>9,16-18)</sup> Cyanobacterial G6PDHs seems to form oligomers (from dimer to tetramer, finally to hexamer), leading to higher activity.<sup>37)</sup> Likewise, in *Chlorella* the formation of the octamer might occur during hardening, leading to the activation of G6PDH<sub>2</sub>, although the reason for the activation of G6PDH<sub>2</sub> during hardening remains unclear. Therefore, in order to make clear the involvement of G6PDH<sub>2</sub> in the development of freezing tolerance, the expression of its corresponding gene and the formation of the octamer of G6PDH<sub>2</sub> will be studied in the future.

#### *Properties of the purified G6PDH isoforms*

The effects of pH on activity and stability of the purified isoforms were measured. As Fig. 2A shows, both isoforms were most active at pH 8.0. The activities of both isoforms, G6PDH<sub>1</sub> and G6PDH<sub>2</sub>, were reduced to 50% above pH 9.0 and below pH 6.0. The stability of G6PDH<sub>1</sub> was reduced to 50% below pH 5.0 and above pH 10.0 (Fig. 2B). On the other hand, G6PDH<sub>2</sub> was stable between pH 4.0 and 11.0.

The effects of temperature on activity and stability of both isoforms were also examined. G6PDH<sub>1</sub> was most active at 50°C (Fig. 3A), while G6PDH<sub>2</sub> was most active at 40°C. The two isoforms were stable up to 20°C, but the stability of G6PDH<sub>1</sub> began to decrease at 30°C and that of G6PDH<sub>2</sub> at 50°C (Fig. 3B).

The results of kinetic studies are shown in Table 2; the purified G6PDH<sub>1</sub> of *Chlorella* has structural and kinetic properties comparable with those reported

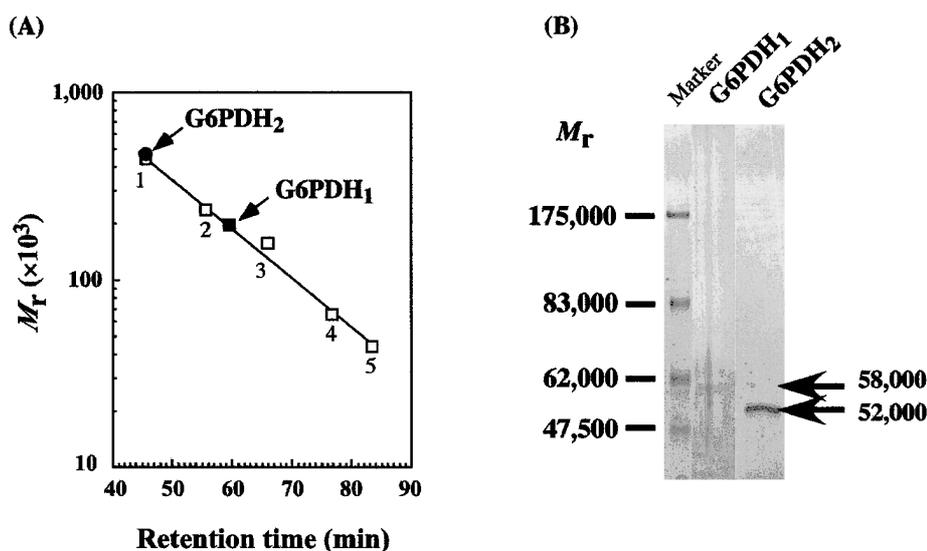


Fig. 1. Molecular Mass Determination of G6PDHs from *C. vulgaris*.

(A) Determination of molecular masses of G6PDH isoforms, G6PDH<sub>1</sub> and G6PDH<sub>2</sub> from *C. vulgaris* by chromatography on HiLoad 16/60 Superdex 200 pg. Marker proteins: ferritin from horse spleen ( $M_r = 450,000$ ) (1), catalase from bovine liver ( $M_r = 240,000$ ) (2), aldolase from rabbit muscles ( $M_r = 160,000$ ) (3), bovine serum albumin ( $M_r = 67,000$ ) (4), and ovalbumin ( $M_r = 45,000$ ) (5). (B) SDS-PAGE of the purified G6PDHs. The gel was stained with CBB R-250.

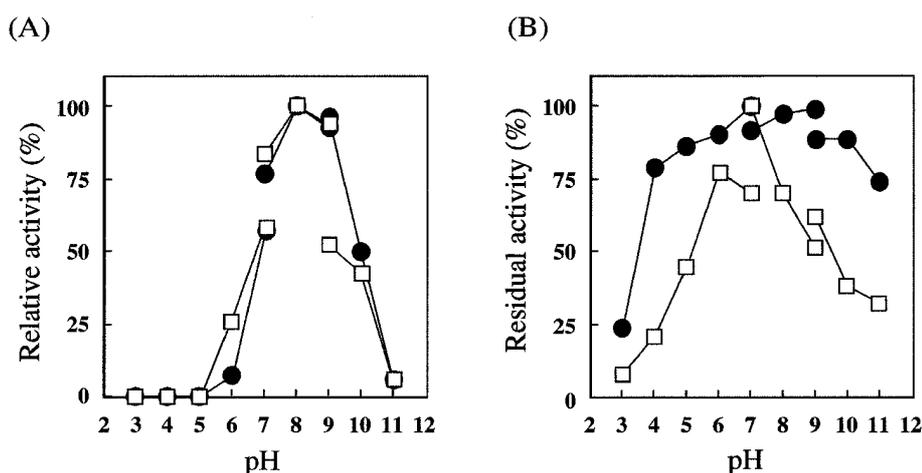


Fig. 2. Effects of pH on Activity and Stability of G6PDHs.

(A) Effects of pH on activity. The activity of the enzyme was assayed as described in Materials and Methods, except for the buffer. The activity at pH 8.5 in Tris-HCl buffer was taken as 100%. (B) Effects of pH on stability. The remaining activity at pH 7.0 in Tris-HCl buffer was taken as 100%. The open square symbols represent the activity of G6PDH<sub>1</sub> and the closed circular symbols represent the activity of G6PDH<sub>2</sub>.

from other sources<sup>38,39</sup> including a potato cytosolic enzyme.<sup>9</sup> For example, *Chlorella* G6PDH<sub>1</sub> has similar parameters to cytosolic G6PDH from potato ( $K_m$  values of  $260 \mu\text{M}$  for G6P and  $6 \mu\text{M}$  for NADP).<sup>9</sup> This shows the low  $K_m$  value of *Chlorella* G6PDH<sub>1</sub> for its substrate G6P compared to the potato enzyme. The  $K_m$  values of G6PDH<sub>2</sub> for both substrates (G6P and NADP) were higher than those of G6PDH<sub>1</sub>. As Esposito *et al.*<sup>40</sup> shows, the  $K_m$  values of a plastid G6PDH isoform for G6P and NADP are higher than those of the cytosol isoform. These results also supported the idea that G6PDH<sub>2</sub> of *Chlorella* is a chloroplast type, as will be discussed later.

#### Effects of DTT on G6PDH isoforms

Purification of chloroplast G6PDH had not been successful, partly because this isoform is inactivated by light because of redox modulation by thioredoxin, and partly because it easily aggregates unspecifically during purification.<sup>41</sup> Anderson *et al.*<sup>42</sup> reported an isoform of G6PDH as the cytosolic one because of its insensitivity to DTT. Incubation with DTT *in vitro* has been widely used to mimic the redox modulation of plastidic G6PDH to distinguish between the two plant isoforms, cytosolic and chloroplast types.<sup>41</sup> A disulfide bond between cysteine residues of the potato chloroplast G6PDH seems to be reduced by DTT,

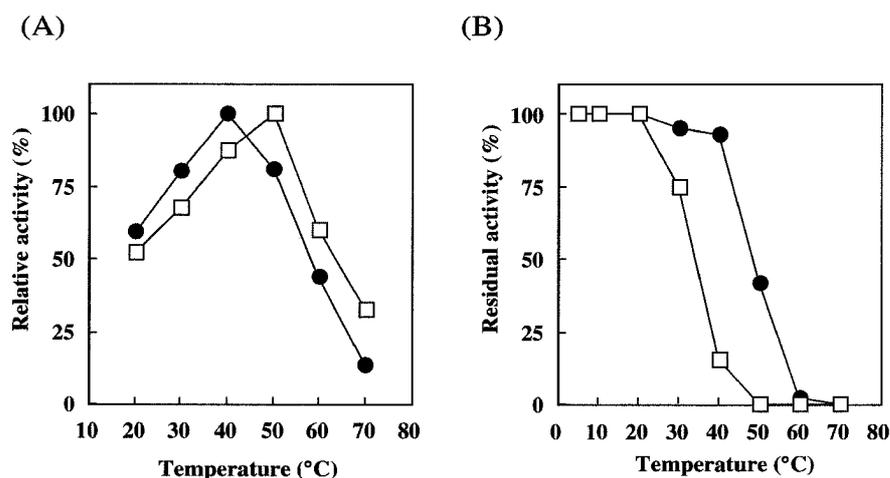


Fig. 3. Effects of Temperature on Activity and Stability of G6PDHs.

(A) Effects of temperature on activity. The activity was assayed as described in Materials and Methods at various temperatures as indicated in the figure. The maximal activity of each isoform was taken as 100%. (B) Effects of temperature on stability. The residual activity at 5°C was taken as 100%. The open square symbols represent the activity of G6PDH<sub>1</sub> and the closed circular symbols represent the activity of G6PDH<sub>2</sub>.

Table 2. Kinetic Parameters for Purified G6PDHs from *C. vulgaris*

	$K_m$ ( $\mu\text{M}$ )		$V_{\text{max}}$ (U/mg protein)	$V_{\text{max}}/K_m$ for G6P
	G6P	NADP		
G6PDH <sub>1</sub>	154	12.4	4.73	$3.07 \times 10^{-2}$
G6PDH <sub>2</sub>	335	15.6	13.9	$4.15 \times 10^{-2}$

leading to inactivation of the enzyme.<sup>41</sup> As shown in Fig. 4, the G6PDH<sub>1</sub> preparation probably represents the cytosolic isoform; but G6PDH<sub>2</sub>, which was inactivated by DTT, seems to have the characteristics of the chloroplast isoform.<sup>9,26</sup> The insensitivity of G6PDH<sub>1</sub> towards DTT suggests that this isoform is not under the control of redox modulation, similarly to potato cytosolic G6PDH<sup>9</sup> and contrarily to cyanobacterial G6PDH (chloroplast G6PDH).<sup>8</sup> Although it is necessary to investigate the localization of G6PDH<sub>2</sub>, this isoform is likely to be a chloroplast type as judged by its sensitivity to DTT.

#### Effect of metal ions on G6PDH activity

In order to compare other characteristics of the purified isoforms, the effects of metal ions on the activity were examined (Table 3). Several ions showed quite different effects on the two isoforms; for example, Mn<sup>2+</sup> ion had a slight effect on G6PDH<sub>1</sub>, but it stimulated the activity of G6PDH<sub>2</sub>. Ca<sup>2+</sup> ion had almost no effect on G6PDH<sub>1</sub>, while it inhibited the activity of the other isoform. These results, in addition to the different behavior in affinity chromatography and in electrophoresis, indicated that the two isoforms are different kinds of polypeptides, which was definitely clarified from amino acid sequence analysis as described later.

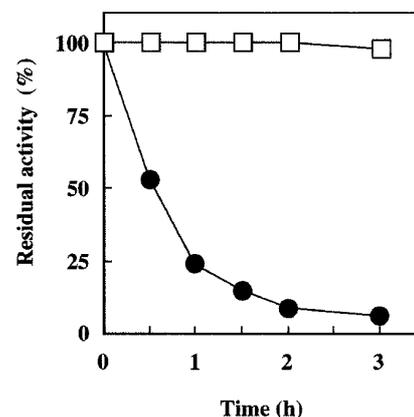


Fig. 4. Effects of DTT on the Activity of G6PDHs.

The open square symbols represent the activity of G6PDH<sub>1</sub> and the closed circular symbols represent the activity of G6PDH<sub>2</sub>.

Among the effective ions, Cd<sup>2+</sup> showed marked stimulating effects on both isoforms. Similar stimulation (up to 10-fold) by Cd<sup>2+</sup> was also reported with *Escherichia coli* threonine dehydrogenase,<sup>43</sup> which is supposed to have a zinc-binding site in which a cysteine residue is located.<sup>44</sup> In addition, Cd<sup>2+</sup> is known to bind to the sulfhydryl groups of several proteins.<sup>45</sup> An analysis of cDNA clones corresponding to genes encoding cytosolic and chloroplast G6PDHs from potato showed that six cysteine residues exist on the deduced amino acid sequence.<sup>41</sup> Out of them, two residues are suggested to be involved in the reductive inactivation of the chloroplast isoform by DTT.<sup>41</sup> At present, we suppose that the Cd<sup>2+</sup> ion activated both isoforms through binding to free sulfhydryl groups, although we do not know the exact site(s) of the Cd<sup>2+</sup> binding. The fact that EDTA did not dramatically affect the activities of both

isoforms suggests a possibility that they are not metalloenzymes, although whether the isoforms are metalloenzymes remains to be elucidated.

#### Amino acid sequences of peptide fragments of the two isoforms

For identification of the purified proteins as G6PDHs, analysis of the N-terminal amino acid sequence was done and that of G6PDH<sub>2</sub> was identified as NH<sub>2</sub>-GLQEENWEKAALSIV-, while that of G6PDH<sub>1</sub> was found to be blocked. In order to analyze the internal amino acid sequences of G6PDH<sub>1</sub>, the isoform was cleaved with cyanogen bromide and put through amino acid sequencing. Two peptide fragments derived from G6PDH<sub>1</sub> showed the sequences -NDDKLREKLK- (fragment 1) and -IVKKPGLEFD- (fragment 2). Homology search of the sequence of fragment 2 showed similarities to those of cytosolic G6PDHs from several higher plants (Fig. 5A). The two internal amino acid

sequences derived from G6PDH<sub>1</sub> were found in the deduced amino acid sequence of an isolated cDNA clone, which showed similarity to cytosolic types of G6PDH from several plants, in our laboratory (unpublished data). Thus, the purified G6PDH<sub>1</sub> was thought to be localized in the cytosol.

For identification of the internal amino acid sequences of G6PDH<sub>2</sub>, the isoform was digested by V8 protease. Three fragments obtained were sequenced. Fragment 1 was found to be as -LVIRIQ-PNEGIYLVNNKVP-, but fragments 2 and 3 could not be identified so far. Homology search of the sequence showed similarity to the sequences of plastid G6PDHs from several higher plants (Fig. 5B). This result also suggested that G6PDH<sub>2</sub> was localized in the chloroplast. Based on the sequences of G6PDH<sub>2</sub> obtained, the cDNA corresponding to G6PDH<sub>2</sub> will be isolated and characterized.

This study shows the purification and characteristics of two G6PDHs from hardened *Chlorella vulgaris* C-27. The obtained results suggested that G6PDH<sub>1</sub> was a cytosolic type as a tetramer and G6PDH<sub>2</sub> was a chloroplast type as an octamer in hardened *Chlorella* cells. A cDNA clone corresponding to G6PDH<sub>1</sub> has been isolated and characterization of the clone is in progress. Furthermore, based on the obtained amino acid sequences, isolation of a cDNA clone corresponding to a gene encoding G6PDH<sub>2</sub>, a chloroplast isoform, is also in progress. By investigating the expression patterns of the corresponding genes and producing transgenic plants, the relationship of G6PDHs with the development of freezing tolerance will become clearer.

**Table 3.** Effects of Metal Ions and EDTA on Activity of G6PDHs from *C. vulgaris*

Reagent	Conc. (mM)	Relative activity (%)	
		G6PDH <sub>1</sub>	G6PDH <sub>2</sub>
Control	—	100	100
ZnCl <sub>2</sub>	1	202	109
MnCl <sub>2</sub>	1	102	574
FeCl <sub>3</sub>	1	209	470
CuCl <sub>2</sub>	1	87.2	80.9
BaCl <sub>2</sub>	1	99.1	93.0
CdCl <sub>2</sub>	1	529	435
CaCl <sub>2</sub>	1	102	37.4
MgCl <sub>2</sub>	1	99.1	99.1
EDTA	1	90.0	100

The purified enzymes were dialyzed against 50 mM potassium phosphate buffer (pH 7.5) and used to measure the effects of metal ions on the enzyme activity. The enzyme activity was assayed in the presence of each metal ion tested under the conditions described in Materials and Methods without MgCl<sub>2</sub>.

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#### (A)

Fragment 2 of G6PDH <sub>1</sub>	1	<b>IVKKPGLEFD</b>	10
Cytosolic G6PDH from Rice (AAL79959)	409	<b>IVKKPGLEFD</b>	418
Cytosolic G6PDH from Tobacco (CAA04992)	401	t <b>VKKPGLE</b> ms	410
Cytosolic G6PDH from Parsley (AAB69319)	426	t <b>VKKPGLE</b> ms	435
Cytosolic G6PDH from Wheat (BAA97663)	403	g <b>VKKPGLE</b> ma	412

#### (B)

Fragment 1 of G6PDH <sub>2</sub>	1	<b>LVIRIQPNEGIYLVNNKVE</b>	20
Chloroplastic G6PDH from Tobacco (Q43793)	477	<b>LVIRVQPNEaIYLKiNNKVE</b>	496
Chloroplastic G6PDH from <i>Arabidopsis</i> (Q43727)	458	<b>LVIRVQPdEGIYLriNNKVE</b>	477
Plastidic G6PDH from Potato (BAC23041)	465	<b>LVIRVQPdEaIYLKiNNKVE</b>	484
Chloroplastic G6PDH from Parsley (T14890)	485	<b>LVIRVQPdEaIYLKiNNKVE</b>	504

**Fig. 5.** Alignment of the Amino Acid Sequences of Fragment 2 of G6PDH<sub>1</sub> (A) and of Fragment 1 of G6PDH<sub>2</sub> (B) with Those of the Proteins Identified by a Computer Search.

Accession numbers of databases (DDBJ/GenBank/EMBL) are in parentheses. Identical amino acids are shown in capital letters and shaded, and similar amino acids are in lower case and shaded.

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