Enhancement of Freezing Tolerance by Expression of a Late Embryogenesis Abundant Protein Gene in Transgenic Plants

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INTRODUCTION

The human population on earth was over 2.5 billion in 1950 and has reached about 5.8 billion, according to the World Population Prospects (1994). The rate of increase in populations will reach 900 million in 10 years and the population will reach about 8.5 billion in the year 2025. Such increases in human populations mean that an increase in food production is needed. After the Green Revolution of 1960's, food production has continued to grow slowly, however, prospects for increases of food production is not bright. Since about 1984, increase in world populations has outpaced cereal (staple food) production and destruction of the natural environment has played a role in this regard, e.g., soil erosion, salinization, flooding, and desertification of agricultural land (Dyson 1996). One strategy to increase food production is to develop new crop varieties which can tolerate environmental stress, if genetic engineering techniques are applied.

Some plant species develop freezing tolerance when exposed to low non-freezing temperatures (Graham and Patterson 1992, Guy 1990). If tolerance could be provided to cold-sensitive plants, the contribution to stable production of crops would be significant. Protection from cold and frost damage will be afforded and areas where crops can be grown will be more available. Such improvements will

also produce crops of good quality, even in case of storage at low temperatures.

Since Weiser (1970) first proposed that cold acclimation involves changes in gene expression, efforts have been made to identify specific proteins that confer freezing resistance on plants (Robertson et al. 1987, Mohapatra et al. 1988, Guy 1990, Thomashow 1990). With cold acclimation, physiological and biochemical changes occur in plants cells, for example, alterations in lipid composition, increases in levels of sugars and other compounds as well as changes in enzymatic activities (Graham and Patterson 1982, Uemura and Yoshida 1984, Calderon and Pontis 1985, Lynch and Steponkus 1987, Guy 1990). Changes specific to cold acclimation in mRNA and protein profiles have been identified in a wide variety of higher plants (Guy 1990, Thomashow 1990). Attempts have been made to isolate and characterize genes that are expressed in response to low temperature. These efforts led to identification of a number of novel genes, including *cor15a* (Lin and Thomashow 1992), *kin1* (Kurkela and Franck 1990), *lti78* (Nordin et al. 1993) genes of *Arabidopsis thaliana*; the *MsaciA* gene family of alfalfa (Laberg et al. 1993); the *pt59* and *pao86* genes of barley (Cattivelli and Bartels 1990); and the *hiC2*, *hiC6*, and *hiC12* genes of *Chlorella* (Joh 1993). Accumulated levels of transcripts corresponding to these genes closely corresponded to the development of freezing tolerance. Functions of cold-induced

proteins are less well understood.

Transgenic approaches have been used to clarify function and effects of stress-induced proteins related to the development of stress tolerance. These are reports that genetic manipulation of accumulated low-molecular-weight osmolytes resulted in increased stress tolerance to water or salt stress in transgenic plants (Tarczynski et al. 1993, Holmstorm et al. 1994, Rathinasabapathi et al. 1994, Saneoka et al. 1995). There are also reports of transgenic approaches to confer freezing tolerance by plants (Hightower et al. 1991, Artus et al. 1996). However, despite these efforts to enhance levels of freezing tolerance, much remains to be done. I chose to investigate *Chlorella vulgaris*, an unicellular green algae, because it has advantages over higher plants for purposes of experimentation.

In our laboratory, we found that hardened cells of *Chlorella vulgaris* C-27 survive slow freezing to –196℃ (Hatano et al. 1976) and that levels of sugars, soluble proteins, and certain enzymatic activities, as well as lipid composition, are altered during the hardening process (Sadakane et al. 1980, Hatano et al. 1982, Yoshimoto et al. 1991, Joh et al. 1993a). As the acquisition of frost hardiness of *Chlorella* is inhibited by cycloheximide (Sadakane et al. 1980), protein synthesis is apparently essential for the development of frost hardiness. These physiological and biochemical changes which occur during hardening are accompanied by the expression of specific genes, as is the case in

higher plants (Joh et al. 1993b). The protein encoded by the most abundant cDNA clone of hardening-induced genes in *Chlorella* is homologous to a late embryogenesis abundant (LEA) protein (Joh et al. 1995). However, the exact function of hardening-induced proteins has remained to be clarified. It is important to confirm if hardening-induced protein is involved in development of freezing tolerance and for this the corresponding gene can be introduced into the freezing sensitive organism.

The objective of my study was to produce new plants with a greater freezing tolerance. To identify the hardening induced proteins, soluble proteins from hardened *Chlorella* were analyzed by two-dimensional high-performance liquid chromatography (HPLC) and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The N-terminal amino acid sequences of the hardening-induced proteins were compared with those of deduced proteins corresponding to hardening-induced *Chlorella* (*hiC*) genes. Two proteins (HIC6 and HIC12) were confirmed to be induced at both transcriptional and translational levels and to be homologous to LEA proteins. Subsequently, two genes (*hiC6* and *hiC12*) encoding the proteins were expressed, respectively, as glutathione *S*-transferase (GST)-fusion proteins in *E. coli.* Two purified proteins, HIC6 and HIC12, were found to have cryoprotective activity for protecting freeze-labile enzymes, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH). Of the

two HIC proteins, the HIC6 protein was given particular attention. Involvement of the number of 11-mer amino acid motif, a characteristic of group 3 LEA proteins in cryoprotection was also examined, and for this polypeptides which are based on the sequence of the HIC6 protein and have different numbers of the 11-mer motif were used. Based on approaches described above, it was deduced that if the *hiC6* gene encodes a homologue or analog of the putative cryoprotective proteins, expression of the gene might increase the freezing tolerance of freeze-sensitive organisms. Thus, expression of the *hiC6* gene was attempted in order to enhance levels of freezing tolerance of yeast and chilling-sensitive tobacco leaves.

CHAPTER 1. Isolation and characterization of hardening-induced

Chlorella **proteins**

1-1. Introduction

Some plant species develop freezing tolerance when exposed to low non-freezing temperature (Graham and Patterson 1982, Guy 1990, Thomashow 1990).   Since Weiser (1970) first proposed that cold acclimation involves changes in gene expression, many efforts have been made to identify specific proteins that confer freezing resistance upon plants (Robertson et al. 1987, Mohapatra et al. 1988, Guy 1990, Thomashow 1990).   Some of the mRNAs induced during cold acclimation encode a thiol protease (Schaffer and Fischer 1988), sucrose synthase (Crespi et al. 1991), and an antifreeze protein (Kurkela and Frank 1990, Gilmour et al. 1992, Lin and Thomashow 1992).   However, the mechanism of freezing resistance is not sufficiently understood to allow us effective improvement of the freezing resistance of plants by gene manipulation.

A two-dimensional high performance liquid-chromatographic (2D-HPLC) separation system was used for analysis of changes in soluble proteins of *Chlorella* during hardening.   The 2D-HPLC separation system using a cation-exchange column as the first dimension and a reserved-phase column as the second dimension

(Bushey and Jorgenson 1990) has a higher resolving power and peak capacity. For example, there are reports of excellent resolution and reproducibility of results for the isolation of oat seed prolamins (Pernollet et al. 1989), proteins and neuropeptides from a single rat brain nucleus (Mobbs et al. 1989).

In the present chapter, the author isolated and purified a large number of hardening-induced proteins by a combination of 2D-HPLC and SDS-PAGE, and some of these proteins were characterized by analysis of their N-terminal amino acid sequences. Two hardening-specific proteins were identified to be homologous to late embryogenesis abundant proteins (LEAs).

1-2. Materials and methods

1-2-1. *Strain*

Chlorella vulgaris Beijerink IAM C-27 (formerly *Chlorella ellipsoidea* Gerneck IAM C-27) was obtained from the Algal Culture Collection of the Institute of Applied Microbiology, University of Tokyo, Tokyo.

1-2-2. *Synchronous culture*

Cells of *C. vulgaris* C-27 were grown synchronously in the MC medium of Watanabe (1960), at 25°C, under a photosynthetic photon

flux density of about 250 μmol m^{-2} s^{-1}, with 1.3% CO_2 in air, to a concentration of about 1.0–1.5×10^{10} cells per liter, under a 16-h light/8-h dark regime, as described previously (Hatano et al. 1976). Since such cells were hardened to the greatest extent at the L_2 stage (an intermediate stage during the ripening phase of the cell cycle; Hatano et al. 1976), L_2 cells were used for experiments.

1-2-3. *Hardening*

L_2 cells of strain C-27, whose growth had been synchronized at 25℃, were directly hardened at 3℃. During hardening, the culture was aerated with 1.3% CO_2 in air and kept in the light (ca. 250 μmol m^{-2} s^{-1}). At intervals, about 1.5×10^{10} cells were collected by centrifugation at $1,000 \times g$ for 5 min, washed with distilled water, and resuspended in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM phenylmethylsulfonylfluoride (PMSF). The suspension of cells was frozen and stored at $-80℃$.

1-2-4. *Preparation of soluble proteins*

All the procedures described below were carried out at 0–4℃. The frozen suspension of cells was homogenized by agitation with glass beads of 0.5 mm in diameter on a reciprocal shaker (Vibrogen-Zellmühle; Edmund Bühler Co., Tübingen, F.R.G.), operated at 4,500 rpm for 12 min. The homogenate was freed from the beads by passage

through a sintered-glass funnel and then centrifuged at 165,000 $\times g$ for 1 h. The supernatant was dialyzed overnight against 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA and 0.1 mM PMSF. The dialyzate was concentrated using polyethylene glycol #20,000 and used for subsequent studies.

1-2-5.   *2D-HPLC*

Soluble *Chlorella* proteins were separated by a manually operated 2D-HPLC system (Matsuoka et al. 1990): separation in the first dimension was performed with a TSKgel DEAE-5PW column (7.5 mm i.d. $\times$ 75 mm; particle size, 10 μm; pore diameter, 1,000 Å; Tosoh, Tokyo, Japan) and separation in the second dimension was performed with a TSKgel Phenyl-5PWRP column (4.6 mm i.d. $\times$ 75 mm; particle size, 10 μm; pore diameter, 1,000 Å; Tosoh, Tokyo, Japan). This system consisted of a Shimadzu LC-9A system (Shimadzu, Kyoto, Japan) and 2D-HPLC was performed as follows. Each sample (10 mg of protein) was injected through a 1-ml loop and eluted at room temperature (25℃). The separation in the first dimension was achieved by elution with the mixture of solvents A (25 mM Tris-HCl, pH 7.5) and B (solvent A with 1 M NaCl). The following elutions conditions were used: 0% B plus 100% A from 0 to 20 min, 2.5% B from 20 to 40 min, 5.0% B from 40 to 60 min, 7.5% B from 60 to 80 min, 10.0% B from 80 to 100 min, 12.5% B from 100 to 120 min, 15.0% B from 120

to 140 min, 17.5% B from 140 to 160 min, 20.0% B from 160 to 180 min, and finally 50.0% B from 180 to 200 min, at a constant flow rate of 1.0 ml min^{-1}. The eluent was collected in 20-ml fractions.

The solvents for separation in the second dimension were designated C (0.1% trifluoroacetic acid) and D (70% acetonitrile with 0.075% trifluoroacetic acid). Each fraction (20 ml) from the first dimension, after the column had been equilibrated with solvents C. After loading of the sample, the column was eluted with a gradient of 30 to 85.7% solvent D plus 70 to 14.3% solvent C over the course of 45 min at a constant flow rate of 1.0 ml min^{-1}. The absorbance of the eluate was monitored at 220 nm. Fractions of 1.5 ml were collected and each initial sample was finally separated into 300 fractions. All fractions were lyophilized and resolved by SDS-PAGE. The concentrations of proteins were measured by the method of Bradford (1976) with a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.).

1-2-6. *SDS-polyacrylamide gel electrophoresis*

All fractions after 2D-HPLC were characterized by SDS-PAGE on a 13.5% (w/v) polyacrylamide gel by the procedure of Laemmli (1970). To avoid artificial blocking of the N-terminal amino acid, 0.1 mM sodium thioglycolate was added to the cathode buffer. Equal volumes of sample were applied and gels were run at a constant current of 15 mA. Then proteins were stained with 0.1% Coomassie brilliant blue (R-250).

1-2-7. *Preparation of boiling-soluble proteins*

Boiling-soluble proteins (BSPs) were prepared from the soluble proteins of *Chlorella* as described by Lin et al. (1990).   In brief, the soluble proteins in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM EDTA and 1 mM PMSF were placed in a boiling water bath for 10 min and the insoluble materials were removed by a centrifugation at 15,000 $\times g$ for 15 min.   Polypeptides that remained soluble were precipitated with seven volumes of acetone and collected by centrifugation. Thereafter, 100 μg of BSPs were separated by 2D-PAGE.

1-2-8. *2D-PAGE*

For 2D-PAGE, the system described by O'Farrell (1975) was used. Proteins were separated in the first dimension by isoelectric focusing in cylindrial gels that contained Ampholine pH 3.5-10 (Pharmacia, Uppsala, Sweden) and in the second dimension by SDS-PAGE on a 15% (w/v) polyacrylamide gel.   Gels were stained with Coomassie brilliant blue (R-250).

1-2-9. *Determination of N-terminal amino acid sequences of hardening-induced Chlorella proteins*

For N-terminal sequencing, proteins that had been separated by SDS-PAGE or 2D-PAGE were blotted onto polyvinyl-difluoride (PVDF)

membranes (Bio-Rad, Richmond, CA, U.S.A.) in an electroblot apparatus (AE-6675P; ATTO Co., Tokyo, Japan) and detected by staining with Coomassie blue. The N-terminal amino acid sequences of the proteins were determined with gas-phase protein sequence analyzers (model PSQ-1; Shimadzu, Kyoto, Japan; and model 473A; Applied Biosystems, Inc., Foster City, CA, U.S.A.). Sequences were compared with those of proteins in the Protein Research Foundation (PRF; Release 95-03), Protein Information Resource (PIR; Release 43.0), and GenBank (Release 87.0) databases, using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990).

1-3. Results

1-3-1. *Isolation of hardening-specific proteins by 2D-HPLC and SDS-PAGE*

Soluble proteins prepared from unhardened cells or from cells that had been hardened for 6, 12, and 24 h were separated by 2D-HPLC. There were few changes during hardening in the elution profiles after HPLC in the first dimension (Fig. 1-1).   After chromatography in the second dimension, all the fractions were resolved by SDS-PAGE.   A number was assigned to new peaks and to peaks whose height increased clearly after hardening.   The results are summarized in Table 1-1.   For some peaks, no bands of protein were detected after SDS-PAGE (data not shown), so these are not included in the table.   After hardening for 24 h, there were 23 new peaks or peaks whose heights were enhanced. Among these, nos. 5, 13, 25, 27, and 29 contained several proteins (Table 1-1).   SDS-PAGE analysis of the materials in the other peaks listed in the table revealed that there was only one induced protein or only one protein whose amount was increased in each peak. Consequently, it appeared that 31 proteins had been induced or had increased in abundance during hardening for 24 h at 3℃.
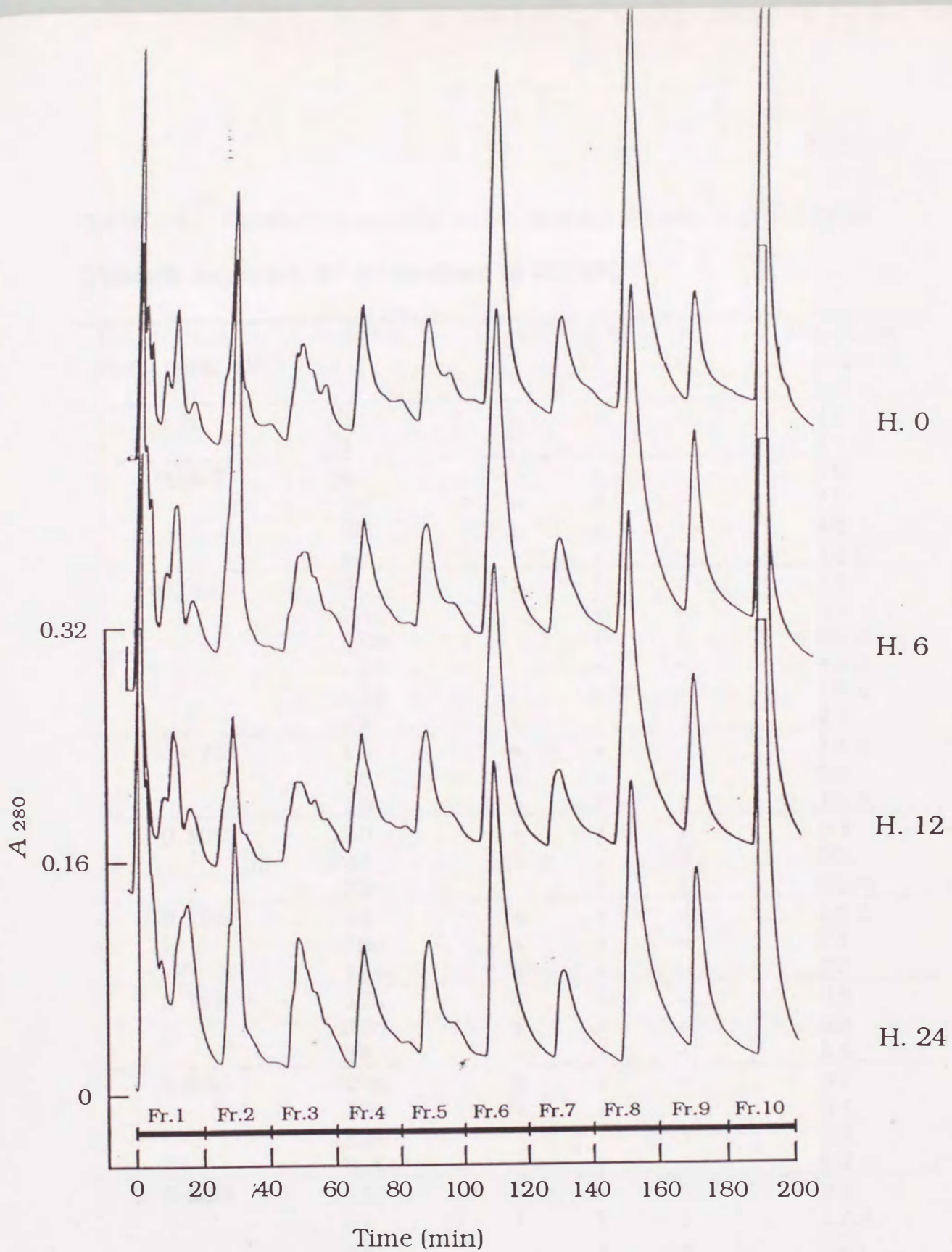
Fig. 1-1. Profiles after ion-exchanged HPLC of soluble proteins after hardening of *Chlorella vulgaris* C-27.

Numbers to the right of the graph indicate hardening times.

Table 1-1. **Hardening-specific or hardening-enhanced proteins in**

Chlorella vulgaris **C-27 determined by 2D-HPLC.**

DEAE fraction [NaCl conc. (M)]	Peak No.	Hardening time (h) 6	12	24	Molecular mass (kDa)
0	2	+	+	+	12.4
	3	I	−	−	14.5
0.025	4		I	I	10
	5a[a]	+	+	+	19
	5b	+	+	−	18
	5c	+	+	−	16.5
0.050	9	I	I	I	14
	13a		+	+	35
	13b		I	I	23.5
	13c		+	+	19.5
	13d	I	−	−	12.4
	15	I	−	−	44
0.075	16	+	+	+	15.5
	18	+	+	+	90
	19	I	I	I	12.4
0.100	20	+	+	+	24
	21	+	+	+	52
	22		I	I	22.5
0.125	24	+	+	+	16.5
	25a	+	+	+	71
	25b	+	+	−	57
0.175	27a	+	+	+	43
	27b	+	+	+	28
	28	+	+	+	14
0.200	29a	+	+	+	91
	29b	+	+	+	74
	29c			I	29
	N. D.[b]		+	+	83
0.500	30	+	+	+	36
	31	I	I	I	12.4
	32		+	+	22

[a] Lowercase letters are added for convenience.

[b] N.D., Not detectable.

I, induced; +, increased; −, decreased.

1-3-2.　*Characterization of hardening-specific proteins by N-terminal amino acid sequencing*

Seven proteins, which were either induced or whose levels increased during cold hardening, were chosen for determinations of N-terminal amino acid sequences (Table 1-2) and their sequences were compared with those of other previously reported proteins.

10-kDa protein (peak 4): This protein was not detectable during the first 6 h of hardening; it appeared only after 12 h and had accumulated at a high level after hardening for 24 h (Fig. 1-2A, B). The 10-kDa protein had the N-terminal amino acid sequence AGNKPITEQISDAVGAAGQKVG- (Table 1-2). A search of databases failed to reveal any significant homology to other known proteins.

18-kDa and 16.5-kDa proteins (peak 5): The levels of these proteins increased within 6 h of hardening (Fig. 1-2C). The 18-kDa and 16.5-kDa proteins had the N-terminal amino acid sequences VFAGNVTNKSGFVPY- and SGFVPYAGEGFAV-, respectively (Table 1-2). Both amino acid sequences include the sequence SGFVPY, and both are homologous to the N-terminal sequence of the oxygen-evolving enhancer protein 2 of PS2 of *Chlamydomonas reinhardtii* (Mayfield et al. 1987; Fig. 1-3).

14-kDa protein (peak 9): This protein was detectable after 6 h of hardening and the absorbance of peak 9 at 220 nm remained roughly constant between 6 h and 24 h (Fig. 1-4A, B). The 14-kDa protein had

the N-terminal amino acid sequence

ALGEESLGDKAKNAFEDAKDAVKDAAGNVKEAV- (Table 1-2). A homology search revealed considerable homology to the putative proteins encoded by pCC3-06 (a desiccation-induced transcript of *Craterostigma plantagineum*; Piatkowski et al. 1990), pT59 (a cold-induced transcript from barley, *Hordeum vulgare* L.; Cattivelli and Bartels 1990), and D-7 and D-29 (*lea* clones from cotton, *Gossypium hirsutum*; Baker et al. 1988) (Fig. 1-5).

19.5-kDa and 23.5-kDa proteins (peak 13): The N-terminal amino acid sequence of the 19.5-kDa protein in peak 13 was LSVQPHEALEASEPQ- and that of the 23.5-kDa protein in the same peak was AXFVLIVATXXTK- (Table 1-2). However, a homology search did not reveal any significant similarities to sequences in the databases.

22-kDa protein (peak 32): The N-terminal amino acid sequence of a 22-kDa protein whose level increased during hardening (Fig. 1-6A), was AAPLVGGPAPDFTAAAVFD- (Table 1-2), exhibiting significant homology to the sequence of a thiol-specific antioxidant enzyme, which is now called thioredoxin peroxidase, from rat brain (Chae et al. 1994a, 1994b) (Fig. 1-6B).

Fig. 1-2. 2D-HPLC analysis and SDS-PAGE of fractions No. 2.

(A) Profiles after reversed-phase HPLC of soluble proteins eluted by 25 mM NaCl from TSKgel DEAE-5PW column after hardening of *Chlorella vulgaris* C-27. (B) SDS-PAGE of fractions (fraction Nos. 2-6, 2-7, and 2-8) that correspond to peak 4 in (A). Numbers on the left indicate the molecular masses of marker proteins. Upper numbers show hardening periods and lower numbers are fraction numbers that correspond to peak 4. (C) SDS-PAGE of fractions 2-14, 2-15, and 2-16 that correspond to peak 5 in (A). The numbers are the same as the those in (B).

18-kDa protein	1	VFAGNVTNKSGFVPY	15
16.5-kDa protein	1	••••••AGEGFAAV	14
Oxygen-evolving enhancer protein 2 of PS2	7	Nvf•k•••••••••••••d•••ll	30

Fig. 1-3.   Alignment of the N-terminal amino acid sequences of the 18-kDa and 16.5-kDa proteins with that of oxygen-evolving enhancer protein 2 of PS2.

Identical amino acids are indicated with dots and similar amino acids with lowercase letters.   Residues are numbered with the N-terminal residues of the mature proteins taken as residue 1.
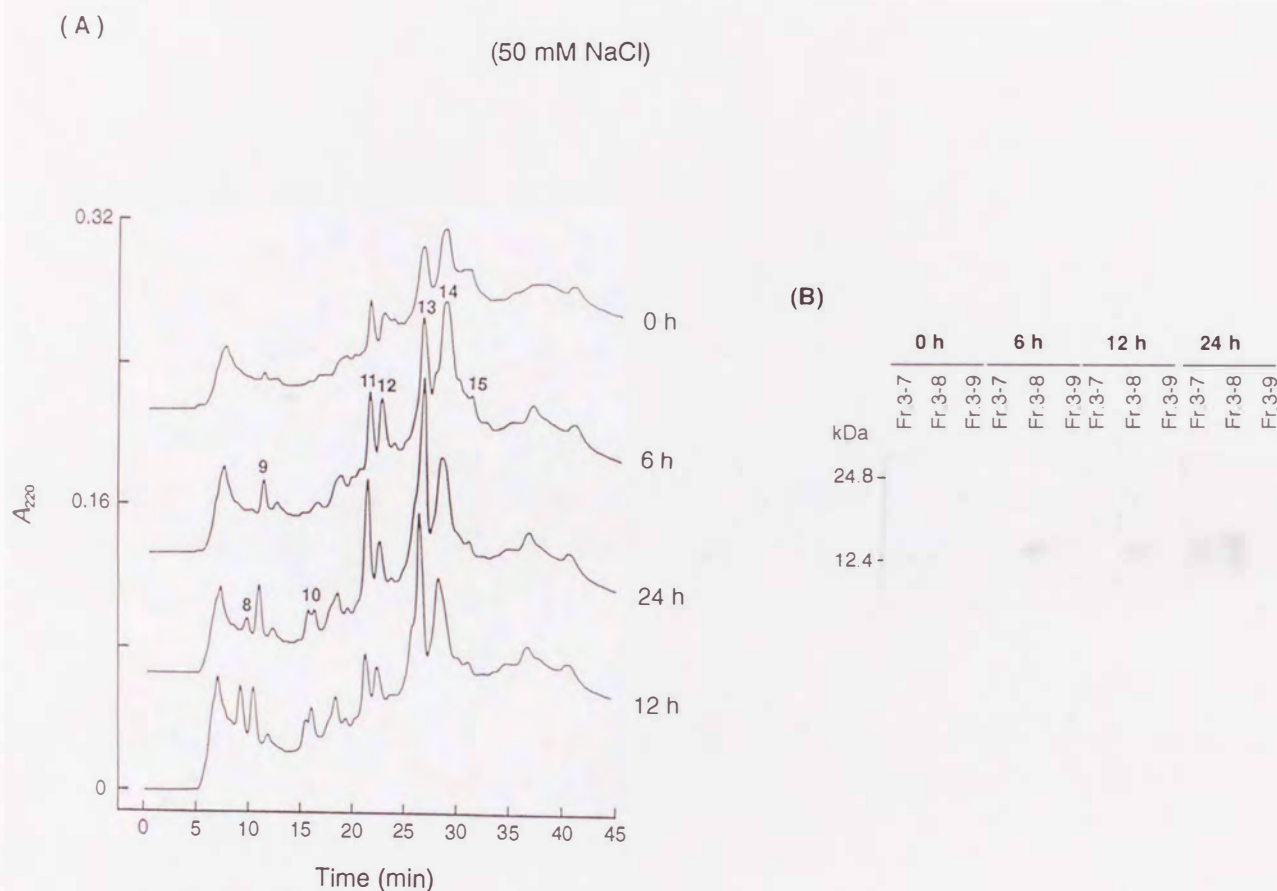
Fig. 1-4. 2D-HPLC analysis and SDS-PAGE of fraction No. 3.
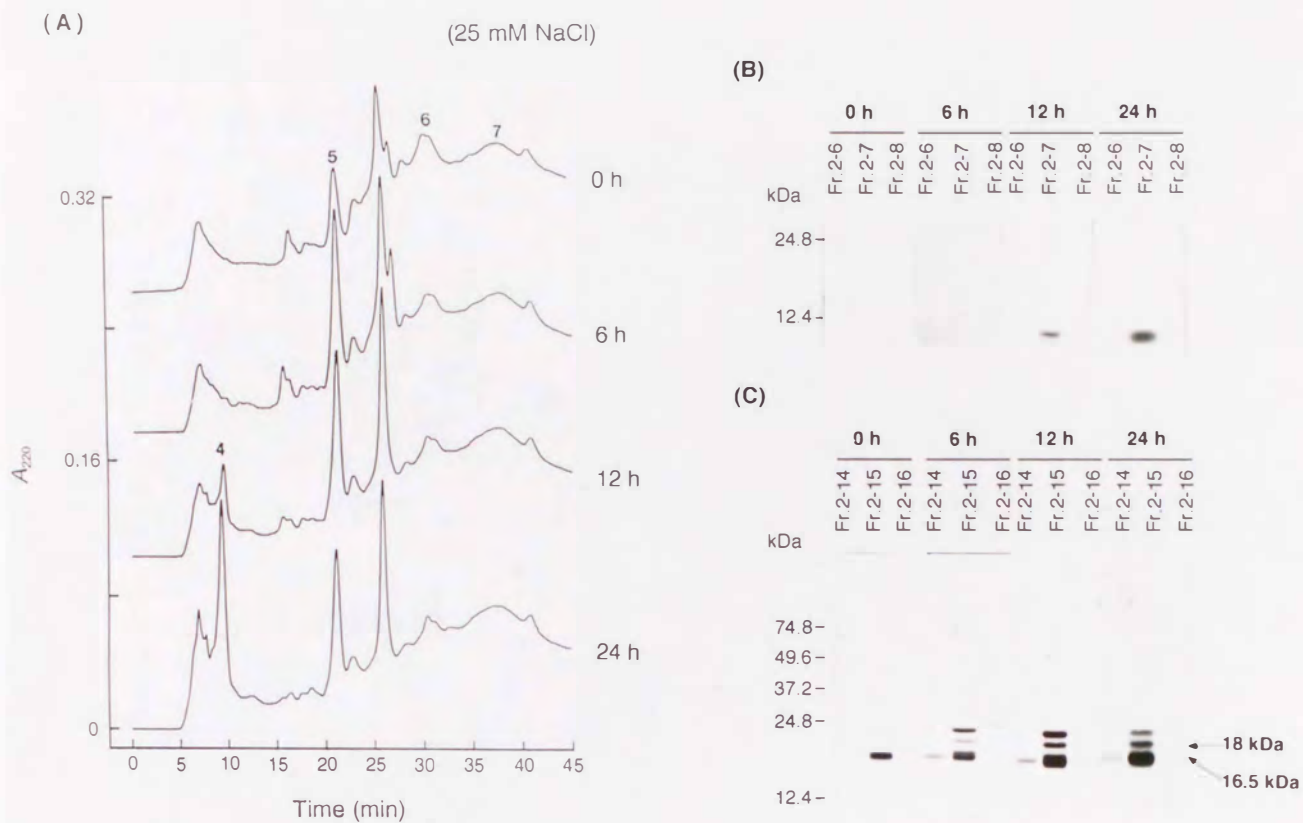
(A) Profiles after reversed-phase HPLC of soluble proteins eluted by 50 mM NaCl from the TSKgel DEAE-5PW column after hardening of *Chlorella vulgaris* C-27.   Numbers to the right of the graph indicate hardening times.   (B) SDS-PAGE of fractions (fraction Nos. 3-7, 3-8, and 3-9) that correspond to peak 9 in (A).   For other details, see legend to Figure 1-2B.

14-kDa protein	1	ALGEESLGDKAKNAFEDAKDAVKDAAGNVKEAV	33
Boiling-stable 14-kDa protein	1	••••••••••••••••••	18
HIC6 protein	37	•••••••••••••••••••••••••••••••••	69
pT59 (cold-regulated) protein	110	••s•G•SVV••A••g•sk•S•t	131
Dessication-related protein	117	•A•nvRe••Md•Gn••MeKTrn•gER•Adg•	147
D-7 protein	98	•e•Vr••AqG•T••••HtF•	117
D-29 protein	264	e•••QgydA••SKAEetIEsA•dti	288

Fig. 1-5.   Alignment of the amino acid sequences of the N-terminal region of the 14-kDa protein isolated by 2D-HPLC and of a boiling-soluble 14-kDa protein from hardened *Chlorella vulgaris* C-27 with those of the proteins identified by a computer search.

Identical amino acids are shown with dots and similar amino acids with lowercase letters.   The proteins compared with the 14-kDa protein were T59 protein from barley, a desiccation-related protein from *Craterostigma plantagineum*, and D-7 and D-29 proteins from cotton. Residues are numbered with the N-terminal residues of the proteins taken as residue 1.

(A)
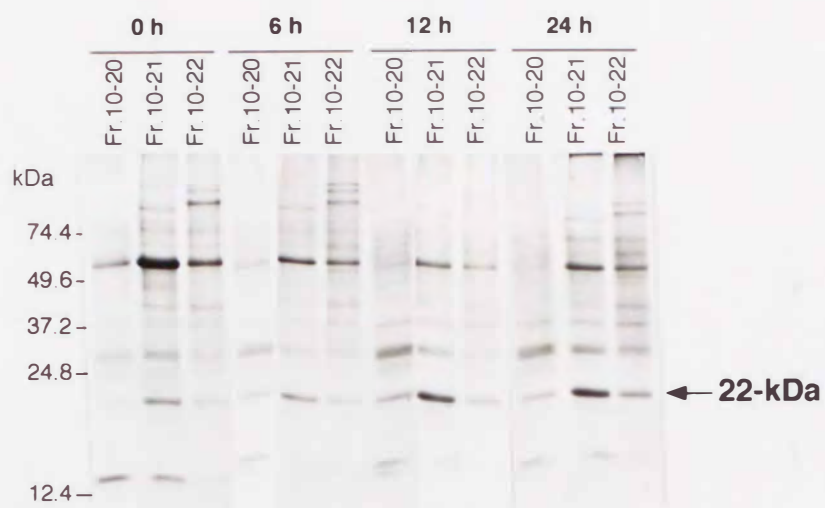
(B)

22-kDa protein:	1	**AAPLVGGPAPDFTAAAVFD**	19
Thiol-specific		**+· ······ ·· ·**	
antioxidant enzyme:	4	**GNARIGKPAPDFTGTAVVD**	22

Fig. 1-6 SDS-PAGE of fractions No. 10-20, 10-21, and 10-22.

(A) All fractions that correspond to peak 32.   (B) Alignment of the
N-terminal amino acid sequence of a 22-kDa protein with that of a
thiol-specific antioxidant enzyme.   Identical amino acids are
indicated with dots and a similar amino acid with +.   Residues are
numbered with the N-terminal residues of the mature proteins taken as
residue 1.

Table 1-2.   N-terminal amino acid sequences of soluble proteins separated by the 2D-HPLC system

Peak No.	Molecular mass (kDa)	Amino acid sequence	Homologous protein
4	10	AGNKPITEQISDAVGAAGQKVG-	LEA protein
5b	18	VFAGNVTNKSGFVPY-	Oxygen-evolving enhancer 2 of PS2
5c	16.5	SGFVPYAGEGFAAV-	Oxygen-evolving enhancer 2 of PS2
9	14	ALGEESLGDKAKNAFEDAKDAVKDAAGNVKEAV-	LEA protein
13b	23.5	AXFVLIVATXXTK-	Unknown
13c	19.5	LSVQPHEALEASEPQ-	Unknown
32	22	AAPLVGGPAPDFTAAAVFD-	Thiol-specific antioxidant protein

1-3-3. *Solubility of the 10-kDa and 14-kDa proteins even after boiling*

Boiling-soluble proteins (BSPs) prepared from unhardened or 24-h hardened cells were separated by 2D-PAGE (Fig. 1-7A). Several BSPs appeared after a 24-h hardening. In particular, levels of a 14-kDa protein (pI 5.3) and a 10-kDa protein (pI 7.7) were especially enhanced by hardening. These proteins were electroblotted onto PVDF membranes and sequenced. The N-terminal amino acid sequences of the 14- and 10-kDa proteins were ALGEESLGDKAKNAFEDA- and AGNKPITE-, respectively (Fig. 1-7B). The N-terminal amino acid sequences of these proteins coincided, respectively, with those of 14-kDa (peak 9) (Fig. 1-5) and the 10-kDa protein (peak 4) detected by 2D-HPLC. These results indicated that the 14- and 10-kDa proteins detected by 2D-HPLC were BSPs.

(A)

(B)

14-kDa protein (pI 5.3)

ALGEESLGDKAKNAFEDA–

10-kDa protein (pI 7.7)

AGNKPITE–
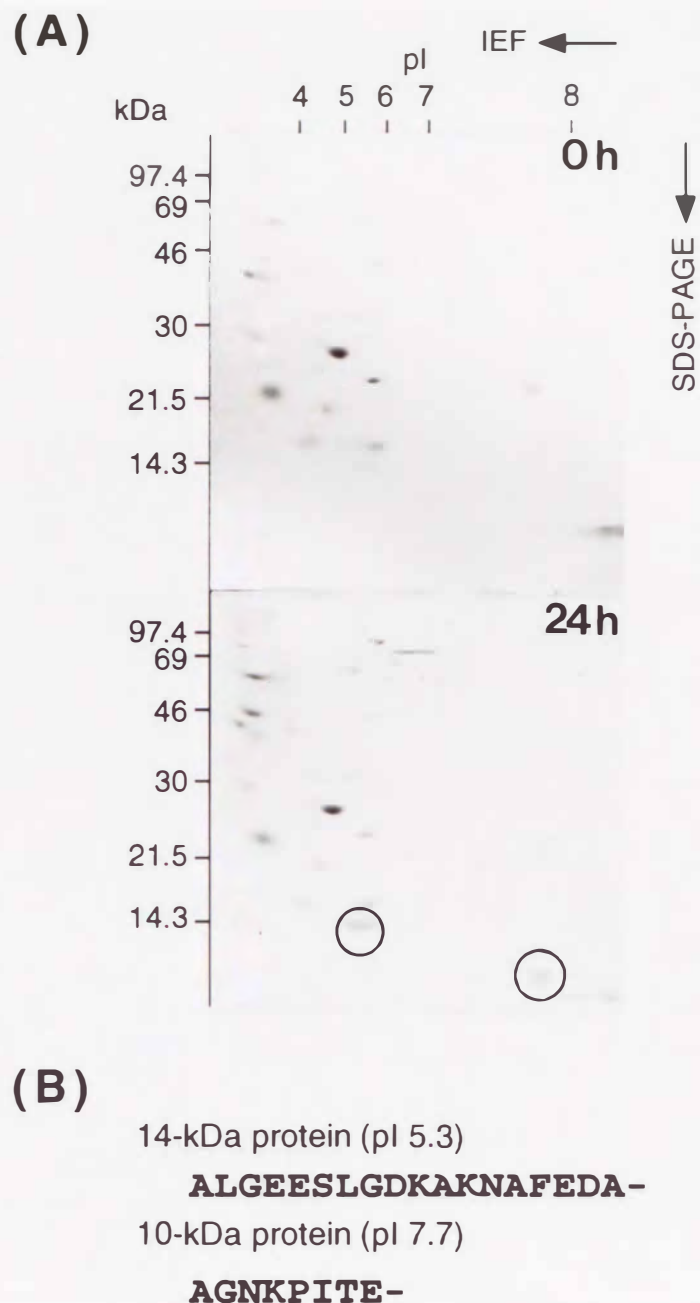
Fig. 1-7.   Analysis by 2D-PAGE of boiling-soluble proteins from unhardened (0 h) and 24-h hardened (24 h) *Chlorella vulgaris* **C-27.**

(A) Open circles indicate a 14-kDa protein (pI 5.3) and a 10-kDa protein (pI 7.7), respectively.   The pH values in the first-dimensional gel and the mobilities of markers, with molecular masses in kDa, are indicated at the top and on the right, respectively.   (B) The N-terminal amino acid sequences of the two proteins indicated in Figure 1-7A.

1-4. Discussion

Analysis 2D-PAGE of the soluble proteins of *C. vulgaris* C-27 revealed that 17 proteins were induced or increased in level during hardening (Yoshimoto et al. 1991). Using a 2D-HPLC system followed by SDS-PAGE, 31 proteins were identified. The result indicates that a combination of 2D-HPLC and SDS-PAGE is more effective than 2D-PAGE alone in terms of resolution and in the measurement of relative amounts of proteins.

In the author's laboratory, Joh et al. (1995) isolated 17 cDNA clones that corresponded to hardening-induced (*hiC*) genes in *Chlorella* by differential screening of a cDNA library from 6-h hardened C-27 cells. Their nucleotide sequences and deduced amino acid sequences were determined. The N-terminal amino acid sequence of the present 10-kDa protein was found in HIC12 polypeptide (Joh 1993) which was homologous to the LEA protein encoded by pMA2005 (Curry et al. 1991). However, the deduced molecular mass of the HIC12 protein was 8.6 kDa (Joh 1993). There was a discrepancy in molecular masses between the 10-kDa protein and the deduced HIC12 protein. The N-terminal amino acid sequence of the present 14-kDa protein was also found in the HIC6 polypeptide (Joh et al. 1995) which is homologous to the LEA proteins D29 and LEA76 (Baker et al. 1988, Harada et al. 1989). Although *hiC6* was the most abundant clone (Joh et al. 1995), the level

of expression of the corresponding 14-kDa protein in the present study was lower than expected (Fig. 1-4B). However, Western blotting analysis suggests that the level of expression of this protein might not be so low (Mori 1995). Expression was monitored in the present study by staining with Coomassie brilliant blue, which might be misleading in this case since the 14-kDa protein was only weakly sensitive to staining with Coomassie brilliant blue. At present, it is clear from the results of 2D-HPLC that both 10-kDa and 14-kDa proteins are LEA-like proteins, which are synthesized *de novo* during hardening (Fig. 1-2B, 1-4B).

LEA proteins are very hydrophilic (Baker et al. 1988) and some of them (LEA group 2 proteins) remain soluble even after boiling (Close et al. 1989, Gilmour et al. 1992). The N-terminal sequences of the 10- and 14-kDa BSPs of *Chlorella* were determined (Fig. 1-7). They were identical to the N-terminal amino acid sequences of the 10- and 14-kDa proteins isolated by 2D-HPLC and the deduced amino acid sequences of two HIC polypeptides (HIC12 and HIC6), respectively. The solubility of the proteins in boiling aqueous solution might be due to high hydrophilicity (Jacobsen and Shaw 1989). Thus, BSPs might modify the structure or propagation of intracellular ice crystals during freezing and, perhaps, trap enough water inside the cell to prevent local dehydration (Houde et al. 1992).

The fact that the induction of two LEA-like proteins (10-kDa and 14-kDa proteins) was confirmed by 2D-HPLC (Fig. 1-2, 1-4), 2D-PAGE of

BSPs (Fig. 1-7), and cloning of *hiC12* and *hiC6* (Joh et al. 1995) suggests that they function in the development of frost hardiness.

The N-terminal amino acid sequences of two proteins in peak 5 (18-kDa and 16.5-kDa proteins) were determined (Table 1-2). These sequences were homologous to that of the oxygen-evolving enhancer protein 2 of PS2 (Mayfield et al. 1987) but the amino-termini of the three proteins were not identical. From their amino acid sequences, the 18- and 16.5-kDa proteins seemed to be identical and appeared to be degraded at low temperature. Since the oxygen-evolving enhancer protein 2 of PS2 is liberated from the lumen of thylakoid membranes at 0℃ (Shen et al. 1990), these two proteins might similarly be liberated and degraded by proteinases in *Chlorella*. The liberation of the protein at low temperature supports the hypothesis that *Chlorella* cells decrease the rate of evolution of O_2 to reduce photooxidative damage at low temperatures (Hatano et al. 1978). Wang et al. (1992) suggested that the protein might not be absolutely essential for the evolution of O_2 and the liberation of the protein might decrease the evolution of O_2.

A homology search showed that the 22-kDa protein was homologous to a thiol-specific antioxidant enzyme which is induced by thiol compounds that are converted to reactive sulfur species (e.g., RS˙, RSSR˙, RSSO˙) under oxidative stress (Kim et al. 1988). The function this thiol-specific antioxidant enzyme seems to scavenge reactive sulfur species (Kim et al. 1988). According to Chae et al. (1994b), reactive

sulfur species cause damage that leads to protein oxidation, lipid peroxidation, DNA base modifications, and DNA strand breaks. Given that the level of this protein increased markedly during hardening in *Chlorella* (Fig. 1-6A), the author can postulate that it might be necessary to prevent damage by oxidative stress at low temperatures. The level in *Chlorella* of manganese-superoxide dismutase, which is H_2O_2-resistant, is much higher in chilling-resistant than in chilling-sensitive strains (Clare et al. 1984). Scavenging of reactive radicals may be important for survival at low temperatures. Thus, the relationship between the function of the thiol-specific antioxidant enzyme and low-temperature stress should be studied. To my knowledge, the involvement of this enzyme in the resistance of plants to stress has not previously been reported.

1-5. Summary

Hardening-induced soluble proteins of *Chlorella vulgaris* Beijerink IAM C-27 (formerly *Chlorella ellipsoidea* Gerneck IAM C-27) were isolated and purified by two-dimensional high-performance liquid chromatography (2D-HPLC) on an anion-exchange column, with subsequent reversed-phase chromatography. Some of the proteins were analyzed by SDS-PAGE, characterized by N-terminal sequencing and identified by searching for homologies in databases. Separation of the soluble proteins during the hardening of *Chlorella* by a combination of 2D-HPLC and SDS-PAGE revealed that at least 31 proteins were induced or increased in abundance. Of particular interest was the induction of a 10-kDa protein after 12-h hardening with the N-terminal amino acid sequence AGNKPITEQISDAVGAAGQKVG and the induction of a 14-kDa protein after 6 h with the N-terminal sequence ALGEESLGDKAKNAFEDAKDAVKDAAGNVKEAV. The N-terminal sequences of these proteins indicated that they were homologous to late embryogenesis abundant (LEA) proteins. Furthermore, the level of a 22-kDa protein also increased after 12 h. The N-terminal sequence of this protein, AAPLVGGPAPDFTAAAVFD, indicated that it was homologous to a thiol-specific antioxidant enzyme.

CHAPTER 2. Relationship between cryoprotection and 11-mer amino acid motif of LEA protein

2-1. Introduction

LEA proteins were first characterized in cotton as a set of proteins that accumulated highly in the embryos at the late stage of seed development (Dure et al. 1981). Based on their common amino acid sequence domains, LEA proteins are grouped into three major groups (Baker et al. 1988, Dure et al. 1989). The motif is characterized by apolar residues in positions 1, 2, 5, 9 and charged or amino residues in positions 3, 6, 7, 8 and 11 (Dure 1993). The regions containing repeats 11-mer amino acid motifs seem to form an amphiphilic α-helix structure (Baker et al. 1988, Dure et al. 1989, Dure 1993). Based on the correlation of *lea* gene expression with physiological and environmental stresses and on the predicted structure of the LEA proteins, it has been hypothesized that they play a protective role in plant cells under various stress conditions; and that the protective role may be essential for the survival of the plant under extreme stress conditions (Baker et al. 1988, Dure et al. 1989, Skriver and Mundy 1990, Chandler and Robertson 1994).

In the Chapter 1, 31 proteins were shown to be induced or have increased in abundance during hardening. Among them, 10-kDa and 14-kDa proteins were homologous to group 3 LEA proteins, identical

with the deduced HIC12 and HIC6 proteins (Joh 1993), respectively, and they seemed to be involved in the development of freezing tolerance of *Chlorella*. In order to investigate the roles of the two proteins, it is important to suppose the deduced function of the proteins.

In the present chapter, the author discussed the exact nucleotide sequences of *hiC6* and *hiC12* cDNA clones, cryoprotective activities of group 3 LEA proteins (HIC6 and HIC12) from *Chlorella vulgaris* C-27, and the involvement of an 11-mer amino acid motif of *Chlorella* group 3 LEA (HIC6) protein in cryoprotection.

2-2. Materials and methods

2-2-1. *Isolation and nucleotide sequence analysis of hiC6 and hiC12 cDNA clones*

Poly(A)$^+$ RNAs were prepared from the suspension of 6-h hardened *Chlorella* as described previously (Joh et al. 1995). A cDNA library corresponding to poly(A)$^+$ RNAs obtained from 6-h hardened cells was constructed in a λZAPII vector with a commercial cDNA synthesis and cloning kit (Stratagene, La Jolla, CA, U.S.A.). The library was screened with partial fragments of *hiC6* and *hiC12* clones (Joh 1993) as probes, which were labeled with digoxigenin-11-dUTP (DNA Labeling and Detection Kit Non-radioactive; Boehringer Mannheim

Gmbh, Germany). According to the instruction manual, the obtained positive clone was converted into a pBluescript vector, which included an objective clone, for further characterization. The nucleotide sequences of the *hiC6* and *hiC12* clones were determined by the method of Sanger et al. (1977) using a dyedeoxy terminator cycle sequencing kit and an automated DNA-sequencing system (model 373A; Applied Biosystem, Inc., Forester City, CA, U.S.A.). DNA sequences were determined in both orientations. The nucleotide and deduced amino acid sequences were analyzed by a GeneWorks computer software (IntelliGenetics, Mountain View, CA, U.S.A.). For homology search, the deduced amino acid sequence was compared with those of proteins in the PRF, PIR, and GenBank databases, using the BLAST program (Altschul et al. 1990).

2-2-2. *Construction of pGEX-1λT/hiC6 and pGEX-1λT/hiC12*

The *hiC6* and *hiC12* genes from *Chlorella* were mutated, using PCR, to introduce a *Bam*HI restriction site just before the codon, which corresponds to the N-terminus of the mature HIC6 and HIC12 proteins (Chapter 1), respectively, and an *Eco*RI restriction site just after the stop codon. For this purpose, four kinds of oligonucleotide fragments were synthesized as primers for amplification of coding regions of *hiC6* and *hiC12*. The first oligonucleotide, 5'-TG GTT G<u>GA TC</u>C GCC CTC GGG GAG GAG T-3', was homologous to the coding strand of *hiC6* with the exception of the 4 nucleotides (underlined) of the introduced

*Bam*HI site.   The second oligonucleotide, 5'-GC TGC <u>GAA</u> T<u>T</u>C TCA GAG CTT GTT GTC CTC-3', was complementary to the coding strand of it with the exception of the 4 nucleotides (underlined) of the introduced *Eco*RI site.   The third oligonucleotide, 5'-CAC A<u>GG ATC</u> <u>C</u>AT GGC CGG CAA C-3', was homologous to the coding strand of *hiC12* with the exception of the 5 nucleotides (underlined) of the introduced *Bam*HI site.   The fourth oligonucleotide, 5'-CTA GTG <u>AA</u>T T<u>C</u>G GGC AAA GGA GAG G-3', was complementary to the coding strand of it with the exception of the 3 nucleotides (underlined) of the introduced *Eco*RI site.   The open reading frame of *hiC6* and *hiC12* were amplified by PCR and the amplified fragment was subcloned into a pUC119 vector (Takara, Kyoto, Japan).   After confirming these sequences, the inserts were digested with *Bam*HI and *Eco*RI and then ligated into the *Bam*HI-*Eco*RI-digested pGEX-1λT plasmid vector (Pharmacia, Uppsala, Sweden).   The fragments of *hiC6* and *hiC12* were located downstream of the *tac* promoter to allow a high level of expression as GST fusion proteins.   The pGEX-1λT/*hiC6* and pGEX-1λT/*hiC12* plasmids were introduced into *E. coli* XL1-Blue by the method of Hanahan (1985).

2-2-3.   *Construction of pGEX-1λT/hiC6A and pGEX-1λT/hiC6B*

In order to study the involvement of the number of an 11-mer amino acid motif in cryoprotection, the two kinds of shortened HIC6 protein were expressed by gene engineering.   Two shortened polypeptide fragments were designated as HIC6A and HIC6B protein,

respectively.　The *hiC6* gene was mutated by PCR to make two genes coding for the HIC6A and HIC6B proteins (Fig. 2-5).　For this purpose, three oligonucleotides were synthesized as primers for amplification of coding regions of *hiC6A* and *hiC6B*.　The first oligonucleotide, 5'-TG GTT GGATCC GCC CTC GGG GAG GAG T-3', was the same primer (*Bam* HI site) as that for amplification of *hiC6*.　The second oligonucleotide, 5'-C TCC CTT GAA TTC GGC AAA TCA GTT GCT A-3', was complementary to the coding strand of *hiC6A* with the exception of the 6 nucleotides (underlined) of the introduced *Eco*RI and translational termination sites.　The third oligonucleotide, 5'-CTT GGG AAT TCC CAG TCA GAA GCA G-3', was homologous to the coding strand of *hiC6B* with the exception of the 6 nucleotides (underlined) of the introduced *Eco*RI and translational termination sites.　The amplified fragments were inserted into a pUC119 vector.　After confirming these sequences, the inserts were digested with *Bam*HI and *Eco*RI, and then ligated into the *Bam*HI-*Eco*RI-digested pGEX-1λT plasmid vector. These fragments were located downstream of *tac* promoter to allow a high level of expression as GST fusion proteins in *E. coli* and the pGEX-1λT/*hiC6A* and pGEX-1λT/*hiC6B* plasmids were introduced into *E. coli* XLI-Blue.

2-2-4.　*Expression and purification of LEA proteins*

　　　　Fresh overnight cultures of *E. coli* XL1-Blue carrying pGEX-1λT recombinants were diluted 1:10 in Luria-Bertani medium (LB)

containing ampicillin (100 μg/ml). *E. coli* carrying recombinant plasmids were grown to an A_{610} of 0.5, induced with a final concentration of 0.1 mM of isopropyl-ß-D-thiogalactopyranoside (IPTG) and further shaken for 3-4 h.

The bacterial cells were collected by centrifugation at 13,800 $\times g$ for 5 min and suspended in 5 to 10% of the culture volume of phosphate buffered saline (PBS; 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3)). The cells were disrupted by sonication on ice. After adding of Triton X-100 to a final concentration of 1%, the lysate was centrifuged at 13,800 $\times g$ for 20 min. Fusion proteins in the supernatant were purified by the use of a glutathione Sepharose 4B column (Pharmacia, Uppsala, Sweden) according to the manufacturer's instruction. Then, the fusion proteins were cleaved by thrombin (Sigma, St. Louis, MO, U.S.A.) and the objective proteins were purified. The purified proteins were analyzed by SDS-PAGE on a 15% (w/v) polyacrylamide gel (Laemmli 1970). The concentration of proteins were measured by the method of Bradford (1976) with a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.).

2-2-5. *Construction of 11-mer amino acid motifs*

Based on the sequences of the deduced HIC6 protein, three kinds of peptide fragments for the 11-mer amino acid motif were synthesized (Biologica Co., Nagoya, Japan) for measurement of cryoprotective activity. The amino acid sequences of the three

fragments were selected as follows (see Fig. 2-5):

(1) P-1, AFEDAKDAVKD; the first 11-mer amino acid motif of the HIC6 protein.

(2) P-2, AADDAEGAAKD; the seventh 11-mer amino acid motif of the HIC6 protein.

(3) P-3, AARDAKRSVKN; the tenth 11-mer amino acid motif of the HIC6 protein.

They were suspended in 10 mM Tris-HCl buffer (pH 7.5) that containing 1 mM EDTA and 1 mM PMSF and used for cryoprotective assay.

2-2-6. *Cryoprotective activities of LEA proteins and of various substances for freeze-labile enzymes against freezing*

The cryoprotective activities of LEA proteins and of various substances against freezing were assayed as described by Tamiya et al. (1985). Lactate dehydrogenase (LDH) from rabbit muscle (Boehringer Mannheim Gmbh, Germany) was dissolved in 10 mM sodium phosphate buffer (pH 7.5) at a concentration of 2 μg/ml. Malate dehydrogenase (MDH) from pig heart (Boehringer Mannheim Gmbh, Germany) was dissolved 0.01 M sodium phosphate buffer (pH 7.0) at a concentration of 1 μg/ml. Alcohol dehydrogenase (ADH) from baker's yeast (Oriental Yeast Co. Lt., Tokyo, Japan) was dissolved in 0.01 M sodium phosphate buffer (pH 7.5) at a concentration of 5 μg/ml. Freeze-labile enzyme solution (2.5 ml) was put into a test tube and 2.5

ml of the LEA proteins dissolved in 10 mM potassium phosphate buffer (pH 7.5) was added. Sample was frozen at –20℃ for 24 h, thawed at room temperature for 5–10 min, and submitted immediately to assay for the enzymatic activity. Among other substances recognized as being effective cryoprotectants and protein stabilizers, BSA (Nakalaitesuku, Japan), ovalbumin (Nakalaitesuku, Japan), trehalose (Ajinomoto, Japan) and sucrose (Nakalaitesuku, Japan) were used for comparison.

CP_{50} (50% cryoprotection) value was defined as the concentration of the additive required to give 50% residual enzyme activity after the freeze-thaw cycle, and then average values were compared. Activities of the enzymes were measured by a SHIMADZU UV-160 spectrophotometer at 25℃. All samples were assayed in triplicate as follows.

LDH: The reaction mixture consisted of 1 ml of 0.24 M Tris-HCl buffer (pH 7.5), 1 ml of 0.3 M KCl, 1 ml of 6 mM sodium pyruvate, and 1 ml of 15 mM NADH. The enzyme reaction was started by the addition of 60 μl of enzyme solution. Activity of the enzyme was determined by measuring the absorbance change at 340 nm depending on the oxidation of NADH.

MDH: The reaction mixture consisted of 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4), 0.2 ml of 3.75 mM NADH dissolved in 0.1 M Tris-HCl buffer (pH 7.4), and 0.1 ml of 6 mM oxaloacetate dissolved in 0.1M sodium phosphate buffer (pH 7.4). The enzyme reaction was

started by the addition of 0.1 ml of enzyme solution. Activity of the enzyme was determined by measuring the absorbance change at 340 nm depending on the oxidation of NADH.

ADH: The reaction mixture consisted of 1.3 ml of 50 mM sodium phosphate buffer (pH 8.8), 0.1 ml of 95% ethanol, and 1.5 ml of 15 mM NAD. The enzyme reaction was started by the addition of 0.1 ml of enzyme solution. Activity of the enzyme was determined by measuring the absorbance change at 340 nm depending on the reduction of NAD.

2-3. Results

2-3-1. *Nucleotide and deduced amino acid sequences of hiC6 and hiC12*

The DNA sequence of *hiC6*, which accounted for the largest percentage of *hiC* clones, was determined (Fig. 2-1A). The coding region starts at nucleotide 43 and ends at nucleotide 576. The deduced HIC6 protein is composed of 178 amino acid residues with a predicted molecular mass of about 18.6 kDa. No typical polyadenylation signal was found. The deduced HIC6 protein had high levels of alanine (22%), aspartic acid (13%) and lysine (12%) residues and was devoid of cysteine, histidine, and tryptophan residues. The hydropathy profile of HIC6 indicated that the putative polypeptide was hydrophilic (Fig. 2-1B).

A computer search of the PIR database revealed amino acid sequence homology between HIC6 protein and Group 3 late embryogenesis abundant (LEA) protein from *Brassica napus*, LEA76 (Harada et al. 1989; Fig. 2-2). The highest degree of conservation was found between residues 53 to 167 of HIC6 and residues 23 to 137 of LEA76 and an alignment of the sequences revealed 21% identical and 48% biochemically similar amino acids. An imperfect repeating motif of 11 amino acids, a predominant feature of Group 3 LEA proteins, was found in the amino acid sequence of HIC6 (Dure et al. 1989).

The nucleotide sequence of the full-length *hiC12* was 580 bp

long and contained an open reading frame coding for 95 amino acid residues with a molecular mass of about 9.7 kDa (Fig. 2-3A).   Sequence analysis revealed that the deduced HIC12 protein had high levels of alanine (22%) and glycine (13%) and was devoid of cystein, tryptophan, and tyrosine.   The hydropathy profile of the HIC12 protein indicated that the putative polypeptide was hydrophilic (Fig. 2-3B).

A computer search of the databases revealed amino acid sequence homology between the HIC12 protein and Group 3 LEA protein from *Hordeum vulgare*, HVA1 (Hong et al. 1988, Fig. 2-4). The highest degree of conservation was found between amino acid residues 9 to 87 of the HIC12 protein and residues 94 to 172 of the HVA1 protein.   Alignment of the sequences revealed 31% identical and 45% biochemically similar amino acids.   An imperfect repeating motif of 11-mer amino acids was also found in the amino acid sequence of the HIC12 protein (Fig. 2-3A).

(A)

```
  1 CTCTCCTACTCCCACCACCTACTCTCACCAAATACAGCCATCATGCAGGCCGCAGCCATG  60
                                              M  Q  A  A  A  M
 61 TCCGTCTCCTTCACTCGCCCCGCCCTGCGTGCGGCTCGCCCGGCAGTCAGCGCCCGCCGC 120
     S  V  S  F  T  R  P  A  L  R  A  A  R  P  A  V  S  A  R  R
121 ACGCTGACCCAGCACTCCATGGTTGTGCGCGCCCTCGGGGAGGAGTCCCTGGGCGACAAG 180
     T  L  T  Q  H  S  M  V  V  R▼ A  L  G  E  E  S  L  G  D  K
181 GCCAACAATGCCTTTGAGGATGCCAAGGACGCAGTCAAGGATGCGGCCGGTAACGTCAAG 240
     A  K  N  A  F  E  D  A  K  D  A  V  K  D  A  A  G  N  V  K
241 GAGGCTGTTGTGGGAGCTGCTGATGACGCAGAGGGTGCAGCCAAGGATGCTGGTCGCAAG 300
     E  A  V  V  G  A  A  D  D  A  E  G  A  A  K  D  A  G  R  K
301 GTTGACCGCAACACCGACAACCTGGCAGACAAGGCCAGCAACGCTCTGGGAGATGCCAAG 360
     V  D  R  N  T  D  N  L  A  D  K  A  S  N  A  L  G  D  A  K
361 GACGCAGCCAAGGATGCGTACAACACCGTGAAGGACAAGGTGTCCAACGCAGCTGACGAC 420
     D  A  A  K  D  A  Y  N  T  V  K  D  K  V  S  N  A  A  D  D
421 GTGGAGGGAGCAGCCAAGGACACTGGCCGCAAGATTGACCGCAACACGGATAACCTGGCA 480
     V  E  G  A  A  K  D  T  G  R  K  I  D  R  N  T  D  N  L  A
481 GACAAGGCTAGCAACAAGTTTGCCGAGGTGAAGGGAGATGTGAAGGACGCAGCACGTGAC 540
     D  K  A  S  N  K  F  A  E  V  K  G  D  V  K  D  A  A  R  D
541 GCAAAGCGCAGCGTGAAGAATGAGGCTAACAAGCTCTGAGCAGCAGCAGCACCGGCTCTT 600
     A  K  R  S  V  K  N  E  A  N  K  L  *
601 CTCCAAGCCTCAGCAGAGCAAGCGAGAAGATTTGTTTATCCTTTGCAGCCGGCGGCAAA 660
661 CTATTGCAGTTGCCTGCCACCCCACATGTATTAACTGTACACTGGTACTTGAACCATCTT 720
721 TTGCTGAAGGGCTTTGCTGAATTGTAAATGTGACGCATGAAAAAAAAAAAAAAAAAAAAA 779
```
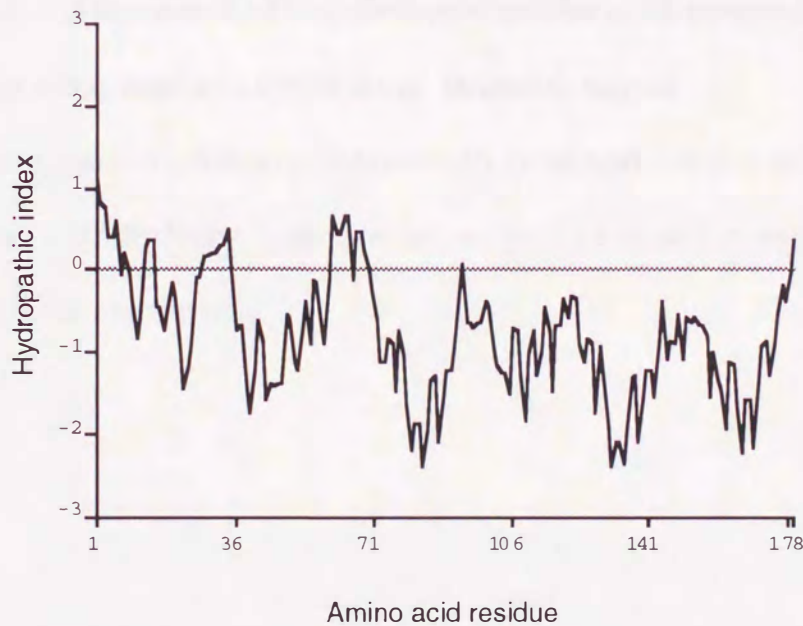
(B)

Fig. 2-1. Nucleic acid and deduced amino acid sequences of *hiC6* and hydropathy profile of the putative HIC6 protein.

(A) Underlines represent imperfect 11-residue repeats in the amino acid sequence. An arrow indicates the putative cleavage site. (B) The plot was constructed by the method of Kyte and Doolittle (1982). Negative values indicate hydrophilic regions.

```
HIC6     53  DAKDAVKD AAGNVKEAVVG AADDAEGAAKD AGRKVDRNTDN LADKASN 100
                 +•  +••+   •  +  •+     +•+ •+  •  +   +++   +•    •+ +
LEA76    23  QAMGAMRD KAEEGKDKTSQ TAQKAQQKAQE TAQAAKDKTSQ AAQTTQQ  70


HIC6    101  ALGDAKDAAKD AYNTVKDKVSN AADDVEGAAKD TGRKIDRNTDN LADKASN 151
                ++ +••••  +    +         •++    ••••      + •+   •  ++
LEA76    71  KAQETAQAAKD KTSQAAQTTQQ KAQETAQAAKD KTSQAAQTTQQ KAHETTQ 121


HIC6    154  KFAEVKGDVKD AARAK 167
                 •   ++  + +•+ •
LEA76   124  SSKEKTSQAAQ TAQEK 137
```

Fig. 2-2.   Alignment of the deduced amino acid sequence of the HIC6 protein with that of LEA76 from *Brassica napus*.

Identical amino acids are shown with dots and similar amino acids with crosses.   Underlines indicate imperfect 11-residue repeats in the amino acid sequence.

(A)

```
  1  CAACCACTCCAAAGCCCCCACACACTTACAATGGCCGGCAACAAGCCCATCACTGAGCAG  60
                                  M  A  G  N  K  P  I  T  E  Q
 61  ATCAGCGACGCCGTCGGCGCAGCAGGCCAGAAGGTCGGCGAGACCTTCGAGGCTGCCAAG 120
      I  S  D  A  V  G  A  A  G  Q  K  V  G  E  T  F  E  A  A  K
121  GCACAGGCCGCCAAGCCTGACCGGCACCGCCGAGCAGAAGGCCACTGAGGCCAAGCACGAT 180
      A  Q  A  A  S  L  T  G  T  A  E  Q  K  A  T  E  A  K  H  D
181  GCCAACCGCCAGGGCGGTGGTGTTG CGACGACATCAAGGGTGCTGCTGCTGAGGCCCAG 240
      A  N  R  Q  G  G  G  V  V  D  D  I  K  G  A  A  A  E  A  Q
241  CACCGCGCAGGCGAGACTGCGGAGAAGGCCAAGCACAACGTCAGGAGGGCTGGACCGAGA 300
      H  R  A  G  E  T  A  E  K  A  K  H  N  V  R  R  A  G  P  R
301  CCAAGCACAGGTCGA GAGGCGCGACCGAACGCGACCCGCTAAGTGTACAGCTTTAGCAC 360
      P  S  T  G  R  *
361  CACGGCCATATGTACTGTCTATCCTTTTAGCACAT CCAGAAGCCAGGCACAGCAGCATG 420
421  TTATTGGGCATACGGCATGCCGCTAATTTTAGCCGTTTATCGATGCATGTCCTTGACCTC 480
481  CCCTCCTCCCTCTCCTTTGCCCCAAGGCACTAGTGCCATTGCACTATGTACTTGCCTCCA 540
541  TCCATGTAAGCAGATGTAG AAAAAAAAAAAAAAAAAAAAA              580
```
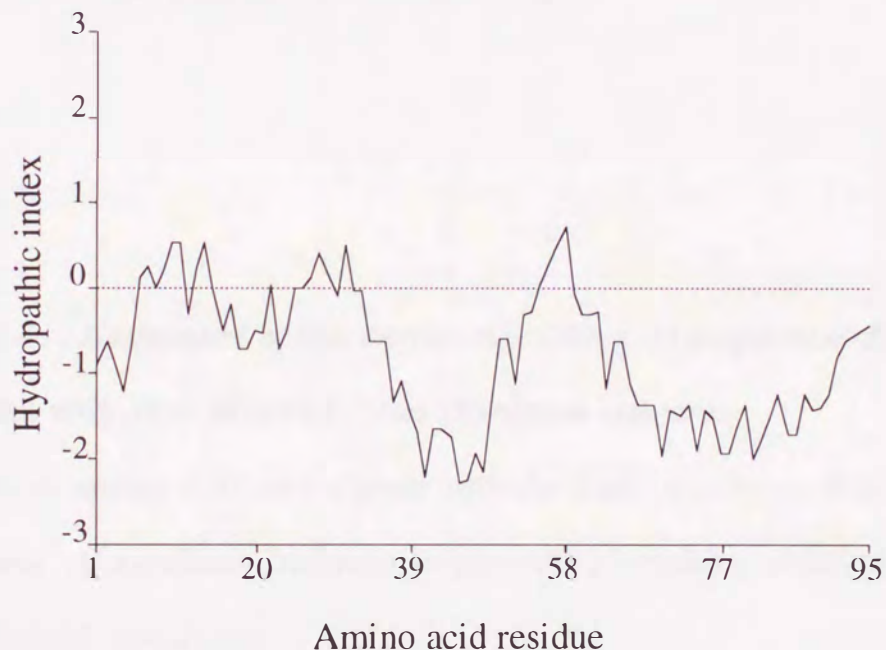
(B)

Fig. 2-3. **Nucleic acid and deduced amino acid sequences of *hiC12***

and hydropathy profile of the putative HIC12 protein. (A) Underlines

represent imperfect 11-residue repeats in the amino acid sequence.

(B) The plot was constructed by the method of Kyte and Doolittle

(1982). Negative values indicate hydrophilic regions.

```
HIC12:  9  EQISDA VGAAGQKVGET FEAAKAQAASL TGTAEQKATEA KHDANRQGGGV 58
           •+ +          •   +••   •••• +••   •   •+••• ••    • ++
HVA1:  94  ERAAQG KDQTGSALGEK TEAAKQKAAET TEAAKQKAAEA TEAAKQKASDT 143


HIC12: 59  VDDIKGAAAEA QHRAGETAEKA KHNVRRA                      87
              • +•    + + •   ++•    •  •
HVA1: 144  AQYTKESAVAG KDKTGSVLQQA GETVVNA                     172
```

Fig. 2-4. Alignment of the deduced amino acid sequence of the HIC12 protein with that of HVA1 from _Hordeum vulgare_.

Identical amino acids are shown with dots and similar amino acids with crosses. Underlines indicate imperfect 11-residue repeats in the amino acid sequence.

2-3-2. *Expression of HIC6, HIC12, HIC6A, and HIC6B proteins*

PCR fragments corresponding to the coding region of *hiC6* and *hiC12* were 461 bp and 488 bp, respectively, and the sequences of the fragments were confirmed to be coincident with those of the original clones.   They were introduced into pGEX-1λT vector separately. These plasmids (pGEX-1λT/*hiC6* and pGEX-1λT/*hiC12*) were introduced into *E. coli* and expression of the objective proteins was induced by the addition of IPTG.   This induction led to the accumulation of large amounts of 37-kDa and 33-kDa proteins (data not shown) consistent with the size of the GST-HIC6 and GST-HIC12 fusion proteins, respectively.   The GST fusion proteins were purified from *E. coli* and cleaved with thrombin.   Then, the objective proteins were purified by being passed though a glutathione Sepharose 4B column.   SDS-PAGE analysis showed that the molecular masses of the purified HIC6 and HIC12 proteins were approximately 14 kDa and 10 kDa, respectively (Fig. 2-6).

The amplified fragments of *hiC6A* and *hiC6B* were 377 bp and 220 bp, respectively.   They were introduced into pGEX-1λT vector separately.   These plasmids (pGEX-1λT/*hiC6A* and pGEX-1λT/*hiC6B*) were introduced into *E. coli* and expression of the objective proteins was induced by the addition of IPTG.   HIC6A and HIC6B proteins were also purified by the methods described above.   By SDS-PAGE analysis, it was confirmed that the molecular masses of the HIC6A and HIC6B proteins were approximately 12-kDa and 7-kDa, respectively (Fig. 2-6).
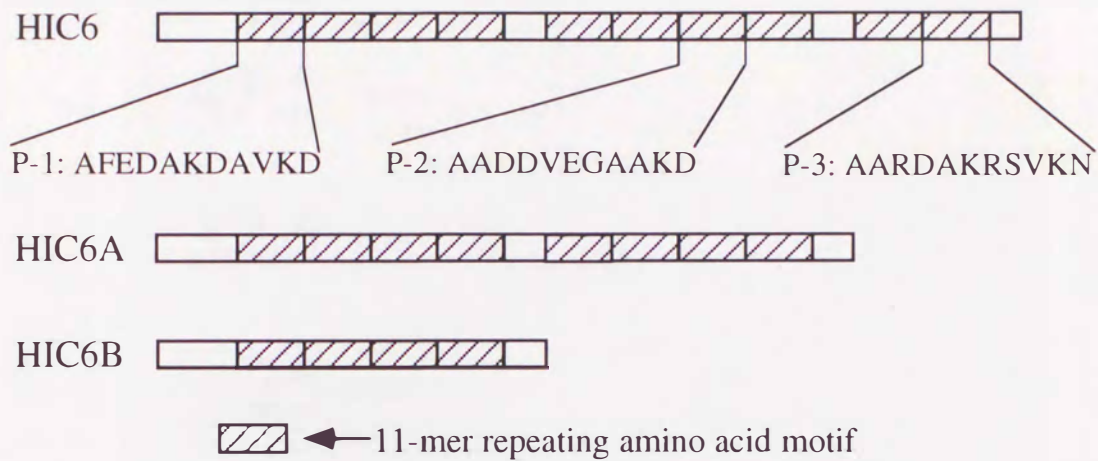
HIC6

P-1: AFEDAKDAVKD   P-2: AADDVEGAAKD   P-3: AARDAKRSVKN

HIC6A

HIC6B

◄——— 11-mer repeating amino acid motif

Fig. 2-5. Design of 11-mer amino acid motifs and artificially shortened polypeptide fragments derived from the HIC6 protein.

Two kinds of polypeptides designated as HIC6A and HIC6B were overexpressed in *Escherichia coli.* P-1, -2, and -3 indicate the selected and synthesized 11-mer amino acid motifs.
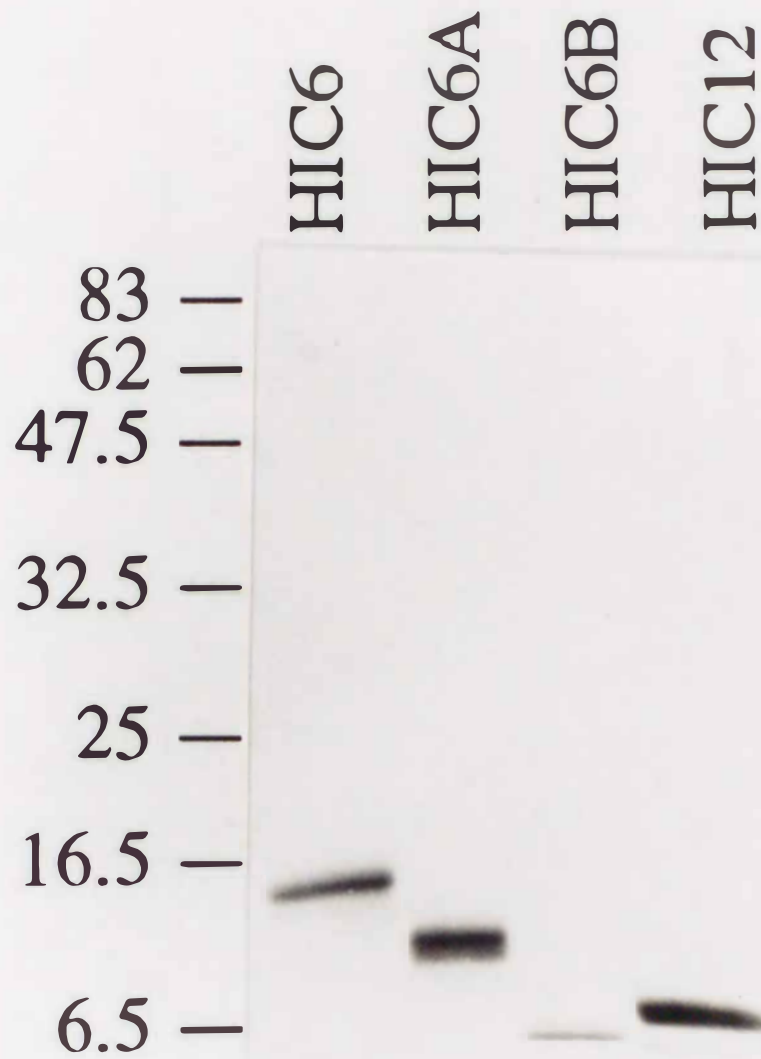
Fig. 2-6.   SDS-PAGE analysis of purified HIC proteins from *E. coli.*

The proteins were separated on a 15% polyacrylamide gel and

visualized by staining with Coomassie brilliant blue R-250.

2-3-3. *Activity of HIC6 and HIC12 proteins on cryoprotection for LDH*

Without the addition of cryoprotectants, a freeze-thaw cycle resulted in loosing more than 85% of LDH activity (data not shown). However, the addition of HIC6 or HIC12 protein at a concentration of 0.1 μM resulted in almost complete protection for LDH against freeze-inactivation (Fig. 2-7). The cryoprotective activity of the HIC6 protein was substantially the same as that of the HIC12 protein.

The cryoprotective activities of the HIC6 or HIC12 proteins were much higher than those of the other substances (Table 2-1). Whereas sucrose, which is commonly regarded as an effective cryoprotectant and protein stabilizer, had a CP_{50} value of about 1.4×10^8 nM, each CP_{50} value of the HIC6 and HIC12 proteins was about 1.86 and 3.16 nM, respectively (Fig. 2-7, Table 2-1). Thus, on a concentration basis, LEA proteins of *Chlorella vulgaris* C-27 was approximately 4.4 $\times 10^7$ times more effective than sucrose in protecting LDH from freeze-inactivation. Further, both HIC6 and HIC12 proteins were over 10 times more effective in protecting LDH than BSA, which had been shown to be effective in protecting LDH against freeze-inactivation.
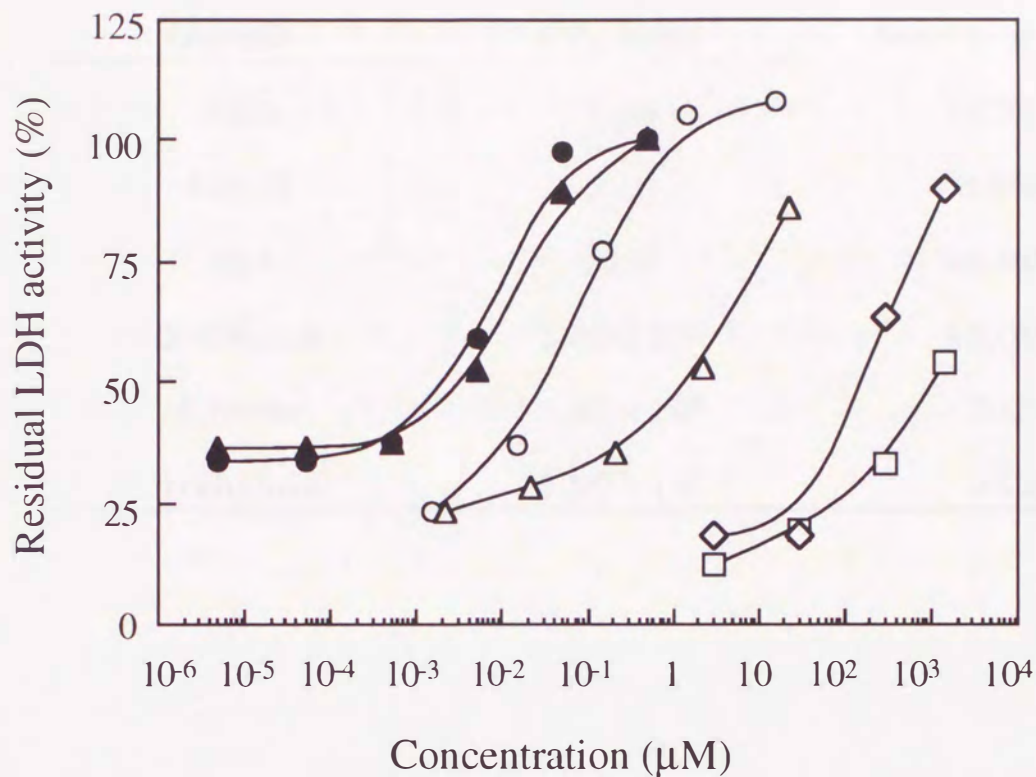
Fig. 2-7. Cryoprotective activities of various substances on L-lactate dehydrogenase.

The curve shows the percentage of LDH activity remaining after a freeze/thaw cycle in the presence of different concentrations of sucrose (◇), trehalose (□), ovalbumin (△), BSA (O), HIC6 (●), and HIC 12 (▲). The concentration of each substances is final concentration during freezing. Each experiment includes at least three replicated samples per point.

Table 2-1. CP_{50} values of the HIC proteins and other substances on LDH.

Agents	CP_{50} (nM)	Molecular mass
HIC6	1.86	14,720
HIC12	3.16	9,660
BSA	32.0	66,000
Ovalbumin	1.40×10^3	45,000
Sucrose	1.40×10^5	340
Trehalose	1.00×10^7	340

2-3-4. *Activity of HIC6 and HIC12 proteins on cryoprotection for two*

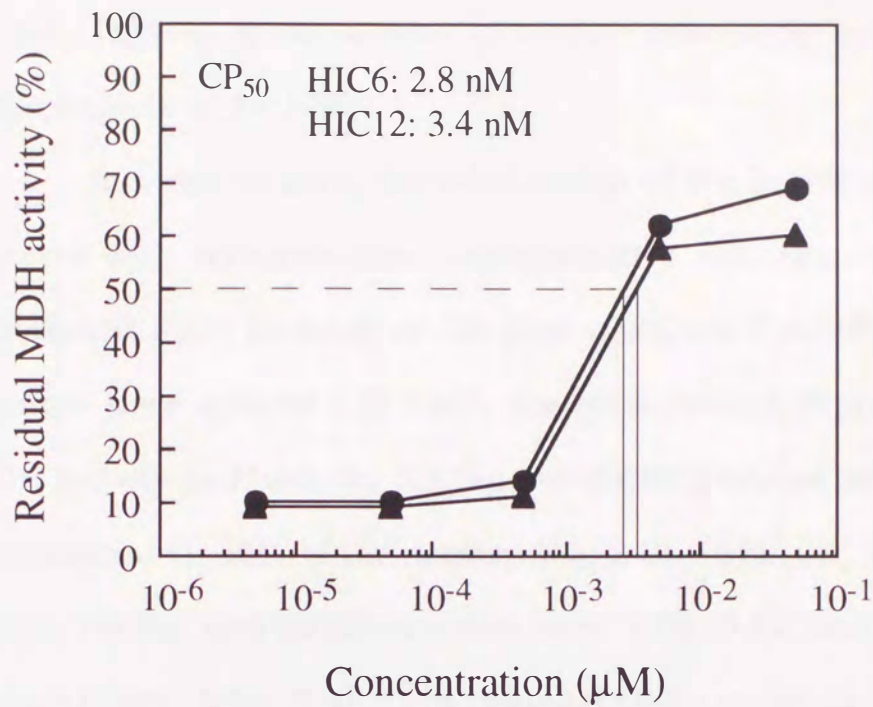*freeze-labile dehydrogenases, ADH and MDH*

The HIC6 and HIC12 proteins were also shown to be effective in

protecting MDH against freeze-inactivation.   A CP_{50} value of the HIC6

protein was about 2.8 nM and that of the HIC12 protein was about 3.4

nM on MDH (Fig. 2-8A).

However, both the HIC6 and HIC12 proteins were not effective

in protecting ADH (Fig. 2-8B).   Addition of HIC6 or HIC12 at a

concentration of approximately 0.5 μM resulted in under 20%
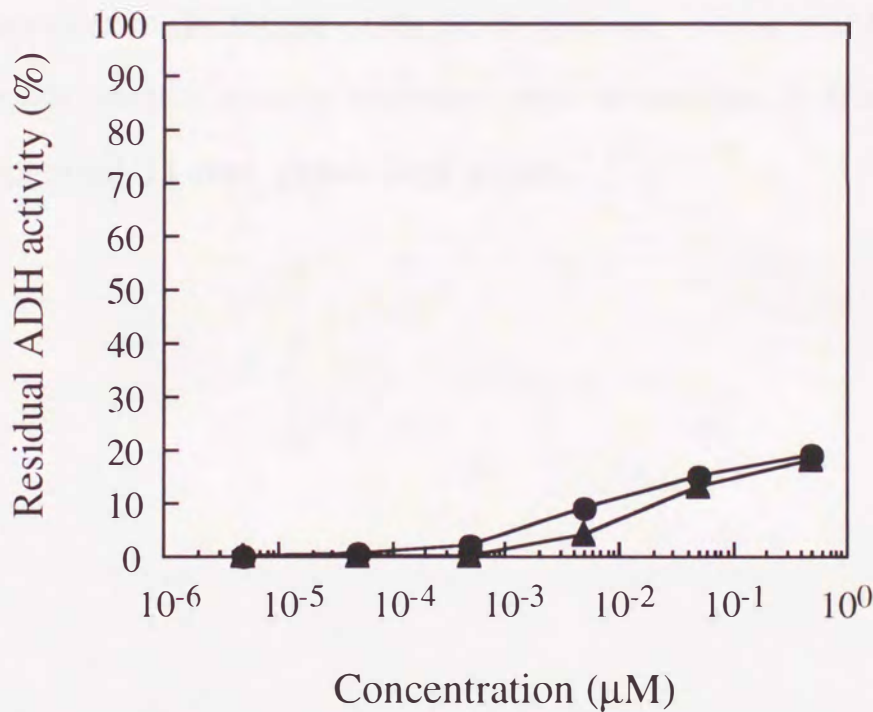
cryoprotection for ADH.

(A)

(B)

Fig. 2-8. Cryoprotective activities of HIC6 and HIC12 on MDH (A) and ADH (B).

The curve shows the percentage enzyme activity remaining after a freeze/thaw cycle in the present of different concentration of HIC6 (●) and HIC12 (▲). The concentration of each protein solution was final concentration during freezing. Each experiment includes at least three replicated samples per point.

2-3-5. *Effects of the number of 11-mer amino acid motif on cryoprotection for LDH*

In order to study the relationship of the length of the HIC6 protein with cryoprotection, cryoprotective activities of two kinds of shortened HIC6 proteins which were designated as HIC6A and HIC6B protein were assayed.   At 5 nM, the HIC6 protein kept about 60% of LDH activity and both the HIC6A and HIC6B proteins also did approximately 50% of the activity (Fig. 2-9).   The CP_{50} values of the HIC6, HIC6A, and HIC6B proteins were 1.86, 5.37, and 5.75, respectively (Table 2-2).   The residual LDH activity decreased with a decrease in the length of the HIC6 protein.   These results suggest that cryoprotective activity increases with an increase in the number of repeating 11-mer amino acid motifs.

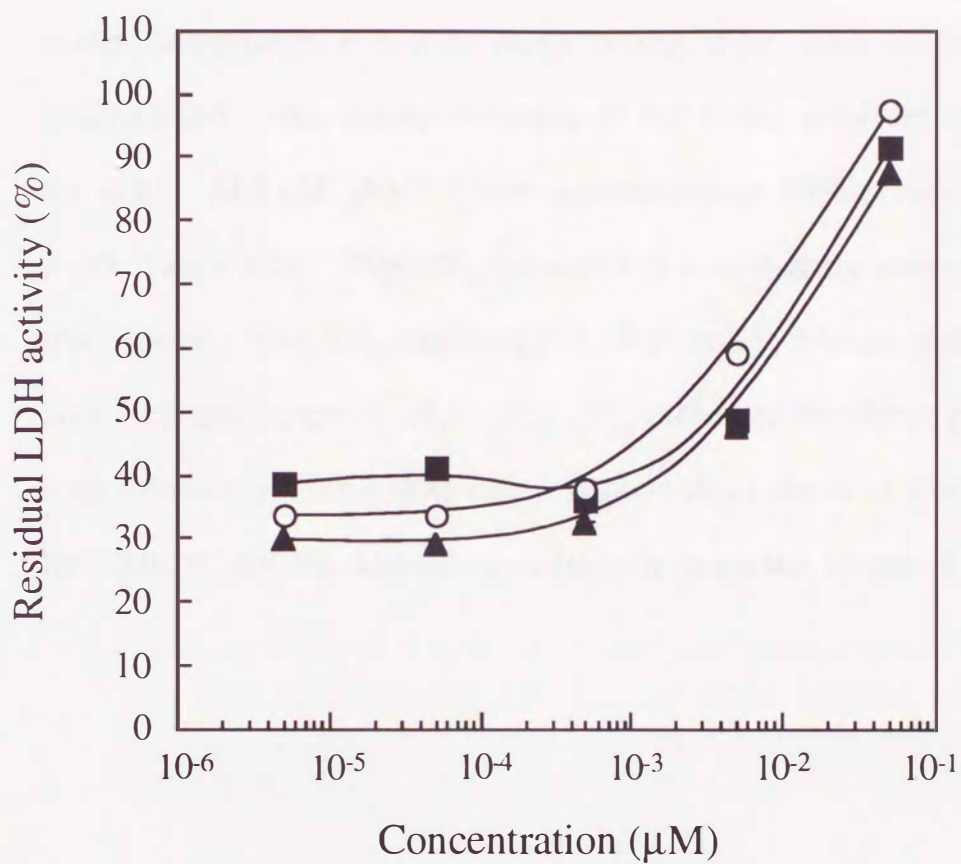**Fig. 2-9. Cryoprotective activities of HIC6, HIC6A, and HIC6B proteins on LDH.**

The curve shows the percentage of LDH activity remaining after a freeze/thaw cycle in the presence of different concentrations of HIC6 (O), HIC6A (■), and HIC6B (▲). The concentration of each protein solution is final concentration during freezing. Each experiment includes at least three replicated samples per point.

2-3-6. *Effects of the kinds of 11-mer amino acid motifs on cryoprotection for LDH*

In order to investigate the involvement of the 11-mer amino acid motif in cryoprotection, three kinds of the 11-mer amino acid motif, designated P-1, P-2, and P-3 (Fig. 2-5), were selected and synthesized. The cryoprotection of the three peptides were assayed for LDH. At 5 µM, the P-1 has approximately 55% cryoprotection (Fig. 2-10, Table 2-2). The CP_{50} value of P-1 was slightly lower than those of the others. The CP_{50} values of P-1, P-2, and P-3 were about 2.10, 9.05, and 13.5 µM, respectively. The CP_{50} values of the three peptides were approximately 350-7,000 times higher than those of the four kinds of HIC (HIC6, HIC6A, HIC6B, and HIC12) proteins (Table 2-1 and 2-2).

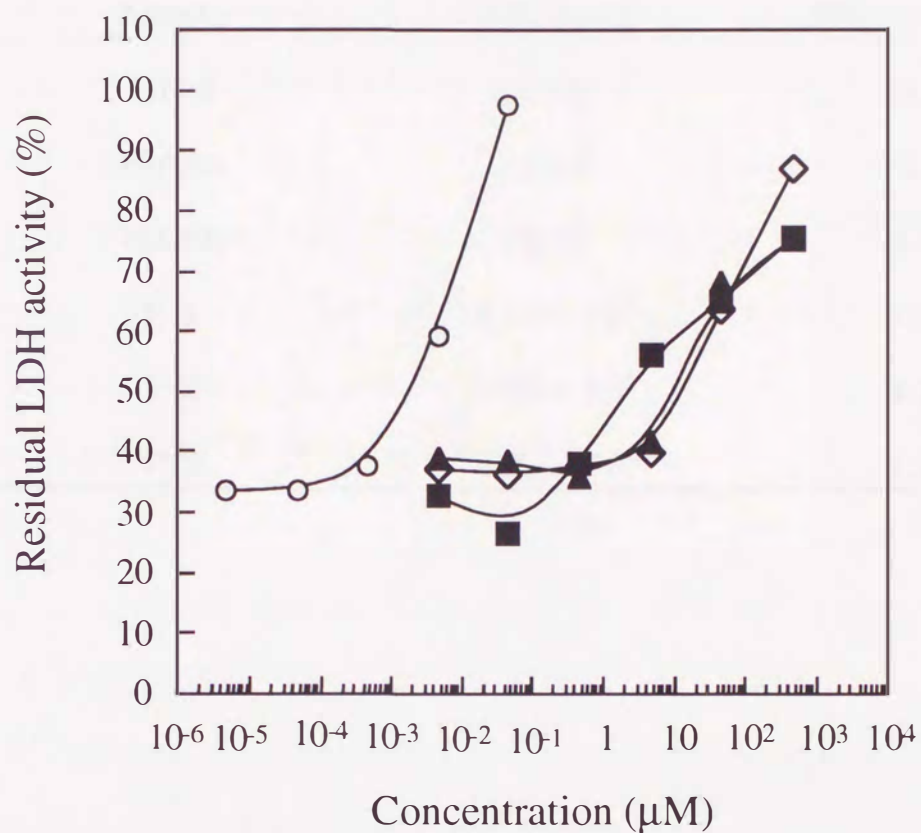**Fig. 2-10. Cryoprotective activities of three kinds of 11-mer amino acid motifs on LDH.**

The curve shows the percentage of LDH activity remaining after a freeze/thaw cycle in the presence of different concentrations of P-1 (■), P-2 (▲), P-3 (◇), and HIC6 (○). The concentration of each polypeptide solution is final concentration during freezing. Each experiment includes at least three replicated samples per point.

Table 2-2. CP_{50} values of the HIC proteins and 11-mer amino acid motifs on LDH.

Agents	CP_{50} (nM)	Molecular mass
HIC6	1.86	14,720
HIC6A	5.37	11,750
HIC6B	5.75	6,459
P-1	2.10×10^{3}	1,208
P-2	9.05×10^{3}	1,289
P-3	1.35×10^{4}	1,215

2-4. Discussion

The amino acid sequence of the most abundant clone (*hiC6*) was deduced from its nucleotide sequence (Fig. 2-1A). As shown in the Chapter 1, the amino-terminus of the mature HIC6 protein is located at amino acid position 37. Thus, it appears that the mature HIC6 protein is modified post-translationally and that the protein has a sequence that resembles a transit peptide. As do the chloroplast transit peptides reported by Gavel and Von Heijne (1990), the putative transit peptide of the HIC6 protein has a high level (19%) of hydroxylated Ser and Thr residues and contains positively charged amino acids (19%) but no acidic amino acids. In addition, the putative cleavage site (Val-Val-Arg↓Ala) of the HIC6 protein is very similar to the cleavage site (Val/Ile-X-Ala/Cys↓Ala) defined by Gavel and Von Heijne (1990). These observations suggest that the HIC6 protein might be transported into the organelles such as chloroplast.

The amino acid sequence of the *hiC12* clone was deduced from its nucleotide sequence (Fig. 2-3A). As shown in the Chapter 1, the N-terminal amino acid sequence of 10-kDa protein, which is a hardening-inducible protein, is AGNKPITEQISDAVGQKVG- and was identical with a part of the deduced amino acid sequence of HIC12 protein. The amino-terminus of the mature HIC12 protein is located at position 2 of the deduced amino acid sequence (Fig. 2-3A). Although the HIC6 protein had an amino acid sequence like a transit

peptide, the HIC12 protein seems not to have one and would be located in cytoplasm of *Chlorella*.

A computer search showed that the putative HIC6 and HIC12 proteins are, respectively, homologous to LEA76 (Harada et al. 1989) and HVA1 (Hong et al. 1988) proteins, group 3 LEA proteins (Fig. 2-2 and 2-4). The LEA76 and HVA1 proteins, respectively, have 13 and 10 repeating units of 11-mer amino acid sequence, a characteristic of group 3 LEA protein. The HIC6 and HIC12 proteins also seem to have ten and three of 11-mer amino acid motifs, respectively. However, the motifs of the HIC12 protein was not so clear as much as the 11-mer motifs of the HIC6 and the other Group 3 LEA proteins.

LEA proteins are very hydrophilic (Baker et al. 1988) and remain soluble even after boiling (Close et al. 1989, Gilmour et al. 1992). Both the 10-kDa and 14-kDa proteins, which are encoded by *hiC12* and *hiC6*, respectively, remain soluble even after boiling as shown in the Chapter 1. The solubility of the proteins in boiling aqueous solution might be due to high hydrophilicity. The polypeptides having three characteristics, cold-regulated expression, hydrophilicity, and heat-stability, might act as cryoprotectants by helping plant cells withstand the dehydration stress associated with freezing (Houde et al. 1992). Thus, boiling-soluble proteins might modify the structure or propagation of intracellular ice crystals during freezing and, perhaps, trap enough water inside the cell to prevent local dehydration. For further characterization of one function of the

HIC6 and HIC12 proteins, it was investigated whether the boiling-soluble polypeptides act as cryoprotectants.

Figure 2-7 shows that *Chlorella* LEA proteins (HIC6 and HIC12) have highly cryoprotective activities in standard *in vitro* cryoprotection assay. On a concentration basis, they were about 10 times higher in protecting LDH against freeze-inactivation than BSA which was recognized as an effective cryoprotectant and stabilizer (Tamiya et al. 1985, Greiff and Kelly 1966). Lin and Thomashow (1992) reported that COR15, a cold-regulated polypeptide, also has a potent cryoprotective activity. Since CP_{50} values of the HIC6 and HIC12 were 1.86 nM and 3.16 nM, respectively, they were slightly smaller than that of COR15 (5.6 nM). The results suggest that LEA proteins of *Chlorella* have a little stronger cryoprotective activity than COR15.

For measurement of cryoprotective activities, LDH from rabbit muscle was used. The molecular mass of the LDH was about 148 kDa. In the present study, the final concentration of the LDH is about 6.75 nM (1 µg ml^{-1}) during freezing. In order to keep about 100% of the LDH activity, it was necessary to add the HIC6 protein at a concentration of 25 nM. That is, the ratio of the amount of the HIC6 protein to that of LDH is approximately 4:1. Considering that LDH is a tetramer, the molar ratio of the HIC6 protein to monomer of LDH seems to be 1:1. There is a possibility that the protein protects LDH against freeze denaturation by affecting the enzyme directly.

Three kinds of 11-mer amino acid motifs were selected and

assayed for cryoprotection using LDH. There was only a little difference in cryoprotection among them and their cryoprotective activities were much lower than the activities of the HIC proteins (Fig. 2-10). It could not be concluded that the cryoprotective activities of the peptides were dependent on the components consisting 11-mer amino acid motif. The CP_{50} values of the three kinds of peptides were ca. 350 - 7,000 times higher than those of the HIC proteins. If only the existence of an 11-mer amino acid motif is involved in the cryoprotective function, the enough amounts of the motif would have cryoprotective activity. However, only single unit of the 11-mer amino acid motif could not function as a cryoprotectant even enough amounts of the motif existed with the freeze-labile enzyme during freezing. This result suggests that the conformation of the HIC6 protein would be very important for cryoprotection.

In order to investigate the cryoprotective activities of the HIC6 and HIC12 proteins in protecting other freeze-labile dehydrogenases, residual activities of ADH and MDH were assayed after freezing and thawing. Although their cryoprotection in protecting MDH were relatively high as well as LDH, their cryoprotection for ADH were quite low (Fig. 2-8). These results show that cryoprotection of the HIC proteins have different effects against freeze-labile enzymes even if the enzymes belong to dehydrogenase groups which are thought to have similar active sites. Thus, sites that HIC proteins act might be different from active sites.

Group 3 LEA proteins are suggested to be correlated with the development of tolerance to water-deficit stress (Curry et al. 1991, Dure 1993). Dure (1993) proposed that D-7 protein from cotton acts as an ion sequestrator. Imai et al. (1996) suggested that LE25, a group 4 LEA, protein acts as an ion scavenger. Sequestration or scavenging of ions seem to be one of the important functions of LEA proteins. However, the exact functions of LEA proteins have not become apparent. The present study showed that the *Chlorella* group 3 LEA protein have cryoprotective activities *in vitro* and suggested that the protein directly interacts with the freeze-labile enzymes.

To the author's knowledge, this is the first report that repeats of 11-mer amino acid motifs of group 3 LEA proteins is responsible for cryoprotective activity of LEA proteins. For further characterization of the development of frost hardness in *Chlorella*, it will be necessary to determine the mechanism of cryoprotection of LEA proteins.

2-5. Summary

Nucleotide sequences of *hiC6* and *hiC12* (hardening-induced *Chlorella*) cDNA clones was determined. Both clones encode late embryogenesis abundant (LEA) proteins. In order to overexpress the *hiC6* and *hiC12* genes, the coding regions of the two genes were mutated by PCR and the amplified fragments were subcloned into an expression vector, pGEX-1λT. The HIC6 and HIC12 proteins were expressed as glutathione S-transferase (GST) fusion proteins in *E. coli*. Molecular masses of the purified HIC6 and HIC12 proteins were 14-kDa and 10-kDa, respectively. Cryoprotective activities of the HIC6 and HIC12 proteins were assayed. Specifically, these proteins were very effective in protecting a freeze-labile enzyme, LDH, against freeze-inactivation. On a concentration basis, it was about 4.4×10^7 times more effective in protecting LDH against freeze-inactivation than sucrose and about over 10 times more effective than other proteins. Furthermore, HIC6A and HIC6B proteins, which were artificially shortened polypeptides based on the HIC6 protein, also have cryoprotective activities. However, CP_{50} values of the HIC6A and HIC6B proteins were about 2.9 and 3.1 times higher than that of the HIC6 protein. Three kinds of peptides for 11-mer amino acid motifs were synthesized and analyzed for cryoprotection. The cryoprotective activities of the peptides were very low. The CP_{50} of them were approximately 350–7,000 times higher than that of the HIC6 protein.

CHAPTER 3. Enhancement of freezing tolerance of yeast

3-1. Introduction

Some plants can acquire freezing tolerance during exposure to low, nonfreezing temperatures (Graham and Patterson 1982, Guy 1990). Polypeptides, which are encoded by low-temperature-inducible genes, are suggested to be involved in the development of freezing tolerance (Guy 1990, Thomashow 1990, Gilmour et al. 1992, Houde et al. 1992). The elucidation of the functions of these genes is expected to make it possible to confer freezing tolerance to plants. Since the functions of almost all of the genes is not well known, however, it is difficult to effectively improve the freezing tolerance of plants by genetic manipulation.

As one of the strategies to identify genes conferring stress tolerance, the author can utilize yeast, which has two advantages, high growth rates and ease of gene manipulation. As well as other plants, yeast is one of the organisms in which freezing tolerance is desirable, because frozen bread dough methods have been gaining acceptance in the baking industry (Hsu et al. 1979a, 1979b). However, because of the injury of yeast cells induced by freezing, frozen dough gives poorer quality bread than unfrozen dough (Wolt and D'Appolonia 1984). Conferring freezing tolerance to yeast by genetic manipulation has

already been tried (McKown and Warren 1991, Imai et al. 1996), but it was not satisfactory.

In the previous Chapters, the HIC6 protein was indicated to be important for the development of freezing tolerance of *Chlorella*. As a next step, clarification of the *in vivo* function of the protein is necessary. This chapter describes the expression of the *Chlorella lea* gene, *hiC6*, in a laboratory strain of *Saccharomyces cerevisiae* and the high survival rate of the transformed yeast after freezing and thawing.

3-2. Materials and methods

3-2-1. *Strains and culture*

The yeast strain used in this study was *Saccharomyces cerevisiae* INVSc2 (*MATα, his3-D200, ura3-167*; Invitrogen, CA, U.S.A.). Yeast cells were cultured in YPD medium containing 1% yeast extract, 2% peptone, and 2% dextrose (Sherman et al. 1986) or SC-uracil medium (synthetic complete medium lacking uracil) containing 2% raffinose, 0.67% yeast nitrogen base, and a supplement with all amino acids, adenine, inositol, and aminobenzoic acid. For the expression of the gene, *hiC6*, galactose was added to the media to a final concentration of 2%, in the control, glucose was added to the media instead of galactose.

3-2-2.   *Construction of pYES2/hiC6*

The *hiC6* gene from *Chlorella* (Joh et al. 1995) was mutated, by PCR, to introduce a *Bam*HI site just before the ATG start codon and an *Eco*RI site just after the stop codon.   For this purpose, two oligonucleotides were synthesized as primers.   The first oligonucleotide, 5'-CCA AAT GGA TCC ATC CAT CAT GCA GGC C-3', was homologous to the coding strand of *hiC6* with the exception of the 3 nucleotides (underlined) of the introduced *Bam*HI site.   The second oligonucleotide, 5'-GCT GCG AAT TCT CAG AGC TTG TTA GCC TC-3', was complementary to the coding strand with the exception of the 4 nucleotides (underlined) introduced to produce an *Eco*RI site.   The open reading frame of *hiC6* was amplified by PCR and the amplified fragment (563 bp) was subcloned into a pUC118 vector (TaKaRa, Kyoto, Japan).   After confirming its sequence, the insert was then digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI-*Eco*RI-digested multicopy yeast plasmid pYES2 (Invitrogen, CA, U.S.A.).   It was placed downstream of the *GAL1* promoter to allow a high level of expression in yeast.   *Escherichia coli* XL1-Blue (Stratagene, CA, U.S.A.) was used for the propagation of this plasmid.

3-2-3.   *Introduction of a lea(hiC6) gene into yeast*

S. cerevisiae INVSc2 was transformed with the plasmids pYES2/*hiC6* or pYES2 by the lithium acetate method (Ito et al. 1983).

Transformants were screened on SC-uracil agar plates and confirmed by the method of Hoffman and Winston (1987) as follows. The transformants were grown in 2 ml of SC-uracil liquid medium overnight. The cells of the culture were collected by centrifugation at 13,800 × g for 1 min and suspended in 50 μl of lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100). And 50 μl of a mixture of phenol, chloroform, and isoamyl alcohol (25 : 24 : 1, v/v/v) was added to the cell suspension. The mixture was vortexed with glass beads of 0.3 mm in diameter for 2 min. Then the mixture was centrifuged at 13,800 × g for 5 min. One μl of the aqueous phase was used to transform *E. coli* to confirm transformation of the yeast with pYES2/*hiC6* or pYES2.

3-2-4. *Western blot analysis of HIC6 protein in transformed yeast*

After confirming the transformation, individual transformed yeast cells were grown with shaking at 30°C overnight. The overnight cultures in SC-uracil medium were diluted into SC-uracil/galactose medium for expression of the HIC6 protein or into SC-uracil/glucose medium for the control. The yeast cells were harvested by centrifugation at 1,400 × g for 10 min and suspended in 10 mM Tris-HCl buffer (pH 7.5) that contained 1 mM EDTA and 1 mM PMSF. The cell suspension was homogenized with glass beads of 0.3 mm in diameter on a reciprocal shaker (Vibrogen-Zellmühle; Edmund Bühler

Co., Tübingen, F.R.G.), operated at 4,500 rpm at 4°C for 20 min. The homogenate was centrifuged at 13,800 × g at 4°C for 20 min. The soluble cell lysate was placed in a boiling water bath for 10 min and the insoluble materials were removed by centrifugation at 13,800 × g for 15 min. The polypeptides that remained soluble were lyophilized. The lyophilized samples (5 µg) were boiled for 5 min after the addition of 20 µl of 100 mM Tris-HCl buffer (pH 6.8) containing 1% (w/v) SDS, 10% (v/v) glycerol, and 2.5% (v/v) 2-mercaptoethanol, and subjected to SDS-PAGE (Laemmli 1970) on a 15% polyacrylamide gel. The polypeptides were electrophoretically transferred onto a nitrocellulose membrane (Towbin et al. 1979). The membrane was probed with rabbit antibodies against the HIC6 protein, which was expressed in *E. coli.* The antibodies against the protein were produced by established procedures (Harlowe and Lane 1988). The antibodies bound to the protein were detected by the immunoperoxidase method (Schneppenheim et al. 1991).

3-2-5. *Freezing tolerance of transformed yeast*

       S. cerevisiae carrying pYES2/*hiC6* or pYES2 were grown in SC-uracil medium with shaking at 30°C overnight. Aliquots were diluted into SC-uracil/galactose medium or SC-uracil/glucose medium. After 12-h or 24-h of incubation, the yeast cells were harvested by centrifugation at 560 × g for 10 min and resuspended in 5 ml of a

0.85% NaCl solution at a cell concentration of 5×10^6 cells

ml^{-1}. The sample in a sterilized test tube was cooled in an air-blast

freezer at $-20°C$ for 24 h. The frozen specimen was thawed in a bath

kept at 25°C. The cooling and thawing rates, represented by the time

required to change the temperature between 10 and $-10°C$, were about

41 min and 57 s, respectively. After thawing, the samples were

diluted with a 0.85% NaCl solution and spread on YPD agar plates.

After 2 days of growth at 30°C, the individual colonies were counted.

The relative survival rate was determined by comparing frozen-thawed

cells to unfrozen cells. The number of colonies that form on agar

plates after freezing-thawing is expressed as a percentage of the

number of colonies that form on agar plates without freezing. For the

growth curve, 5 ml of cell suspension was incubated with shaking at

30°C and changes in optical density were measured at 660 nm over a

10 h period.

3-3. Results

3-3-1.   *Changes in expression level of HIC6 protein*

    The PCR fragment corresponding to the coding region of *hiC6* was sequenced and the sequence of the fragment was confirmed to be coincident with that of the *hiC6* original clone (data not shown).   In order to investigate whether the *hiC6* gene is expressed in transformed yeast cells or not, immunoblot analysis was carried out. Figure 3-1 shows the result of immunoblot analysis probed with rabbit antibodies raised against the HIC6 protein.   The HIC6 protein was clearly expressed in yeast cells carrying pYES2/*hiC6* after induction (lanes 2 and 3) and the expression level of the protein after a 24-h induction was much higher than that after a 12-h induction.   The molecular mass of the protein was about 14-kDa.   In the absence of galactose, the level of expression of the protein was very low (lane 4).
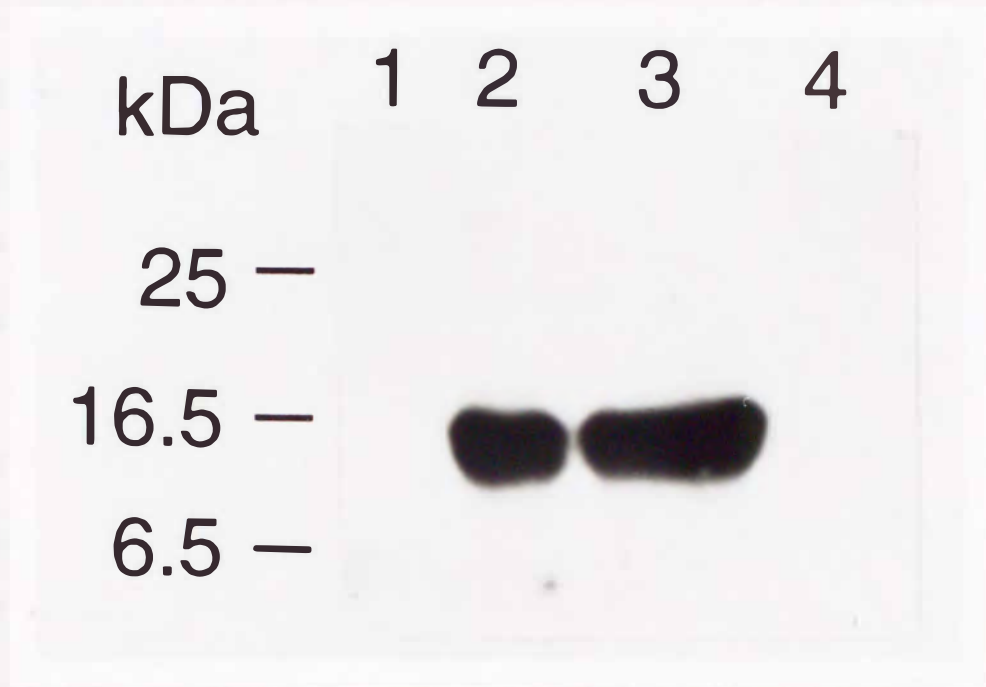
Fig. 3-1. Immunoblot analysis of HIC6 protein expression in transformed _Saccharomyces cerevisiae_.

Lane 1, pYES2 (+galactose, induction time: 24 h); lane 2, pYES2/_hiC6_ (+galactose, 12 h); lane 3, pYES2/_hiC6_ (+galactose, 24 h); lane 4, pYES2/_hiC6_ (-galactose, 24 h).

3-3-2. *Freezing tolerance of transformed yeast with hiC6 gene*

The author utilized two indicators to estimate the viability of yeast cells subjected to freezing-thawing.   One indicator of cell viability is the growth curves on the basis of OD_{660} after freezing-thawing (Fig. 3-2).   Yeast cells expressing the HIC6 protein after 12 and 24 h induction showed a faster increase in OD_{660} than non-expressing transformants.   An increase in OD_{660} of yeast after 24 h induction was faster than that of yeast after 12 h induction.

Another indicator is an ability to form colonies on agar plates. As shown in Table 1, the transformants carrying pYES2/*hiC6* had a survival rate approximately 3.3 times (from 20% to 66%) higher than the transformants carrying pYES2 after 24-h galactose induction.   The survival rate of the yeast expressing HIC6 protein increased with elongation of the induction time.   The results of the growth curves (Fig. 3-2) were confirmed by those of the colony counts (Table 3-1). These results indicate that freezing tolerance of yeast expressing the HIC6 protein was enhanced with an increase in the expression level of the HIC6 protein.
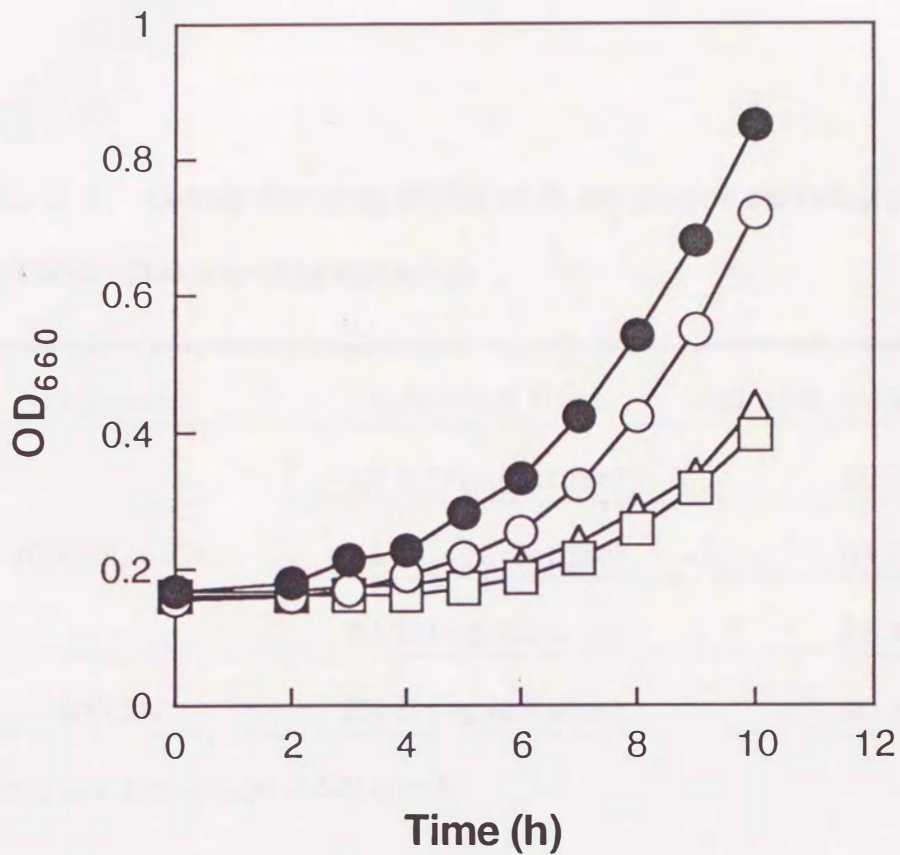
Fig. 3-2. Growth curves of S. *cerevisiae* carrying pYES2/*hiC6* or pYES2 after freezing-thawing.

Open circles, pYES2/*hiC6* (+galactose, induction time: 12 h); closed circles; pYES2/*hiC6* (+galactose, 24 h); triangles, pYES2/*hiC6* (−galactose, 24 h); squares, pYES2 (+galactose, 24 h).

Table 3-1.   Colony forming ability of S. *cerevisiae* carrying pYES2/*hiC6* or pYES2 after freezing-thawing.

Plasmids	Induction time	Relative colony number (%)
pYES2/*hiC6*	12 h (+galactose)	40.9 ± 2.7
	24 h (+galactose)	65.8 ± 3.6
	24 h (–galactose)	28.1 ± 1.4
pYES2	24 h (+galactose)	20.1 ± 6.0

Values are the mean ± SE (n=3).

3-4. Discussion

The expression of the HIC6 protein in yeast was confirmed by immunoblot analysis (Fig. 3-1). The expression level of the protein was increased with the galactose-induction time. This result was correlated with the results of the viability (Fig. 3-2 and Table 3-1), suggesting that the accumulation of the protein is important for acquisition of freezing tolerance of yeast.

The molecular mass of the expressed HIC6 protein in yeast cells was about 14-kDa (Fig. 3-1) and equal to that of the mature HIC6 protein in *Chlorella* (Honjoh et al. 1995). The molecular mass of the protein deduced from the nucleotide sequence of *hiC6* was about 18.6-kDa (Joh et al. 1995). Although Joh et al. (1995) reported that the predicted HIC6 protein has an N-terminal amino acid sequence that is similar to that of the transit peptides of chloroplast, the PSORT program (Nakai and Kanehisa 1992) indicated that the N-terminal amino acid sequence may be involved in transportation of the protein to mitochondria. Thus, the HIC6 protein might be transported to mitochondria in yeast. Future experiments will include determination of the localization of the HIC6 protein in yeast cells.

The survival rates of the yeast expressing the HIC6 protein are approximately 65.8% after 24-h induction and this was 3.3 times higher than control yeast cells which carry plasmid pYES2 (Table 3-1).

Previously, two groups reported the enhanced freezing tolerance of yeast by introducing an other exogenous gene. Introduction of an antifreeze gene analogue into yeast increased the survival rate up to approximately 2.5% (McKown and Warren 1991) and that of a *lea*-like gene increased survival by approximately 8.4% (Imai et al. 1996). Although there were differences between the strains used in each experiment, the survival rate obtained in the present study was much higher than that in others. This might be due to differences in both the accumulation level and amino acid sequences of the three proteins.

Group 3 LEA proteins are correlated with stress tolerance (Baker et al. 1988, Ried and Walker-Simmons 1993, Joh et al. 1995). Some functions of LEA proteins have been proposed. Dure (1993) proposed that the D-7 (group 3 LEA) protein from cotton acts as an ion sequestrator. Imai et al. (1996) suggested that the LE25 (LEA-like) protein functions as an ion scavenger. Sequestration or scavenging of ions seems to be one of the important functions of LEA proteins. Houde et al. (1992) suggested that hydrophilic proteins, such as LEA proteins, might modify the structure or propagation of intracellular ice crystals during freezing and trap enough water inside the cell to prevent local dehydration. Alternatively, these proteins might protect cells from freezing damage by preventing the removal of bound water from cellular substances. Although a clear account of the mechanism of the function of the HIC6 protein has not been given, the

present results indicate that this protein, which is a member of the group 3 LEA proteins, is involved in the development of freezing tolerance.

To the author's knowledge, it has not previously been shown that the introduction of only one gene conferred a considerable level of freezing tolerance to yeast. Manipulation of endogenous *lea* gene expression, in combination with the introduction of additional stress tolerance genes, may provide the genetic improvement of freezing tolerance in yeast.

3-5. Summary

The *hiC6* gene, encoding a homologue of a LEA protein, was introduced into *S. cerevisiae*. It was inserted on a multicopy plasmid under the transcriptional control of the yeast *GAL1* promoter. Expression of HIC6 protein was confirmed by immunochemical methods. Expression level of the protein was increased gradually with the elongation of induction period by galactose. With maximum induction time, the freeze-tolerance of yeast transformed with *hiC6* gene was approximately 3.3 times (from 20% to 66% survival rate) higher than that of the control yeast.

CHAPTER 4.   **Enhancement of freezing tolerance of transgenic tobacco leaves by introduction of *hiC6* gene**

4-1. Introduction

Some plants have to be able to acclimate to a variety of environmental stresses for optimal function and survival.   Many genes are thought to be involved in the development of freezing tolerance (Guy 1990, Thomashow 1990, Gilmour et al. 1992, Houde et al. 1992). They are expected to confer freezing tolerance to plants.   However, the improvement of freezing tolerance has never been done at a satisfactory level.

In the Chapters 1, 2, and 3, the involvement of the HIC6 protein in the development of freezing tolerance was discussed.   Until now, transgenic plants expressing group 3 LEA protein have not been confirmed to enhance the level of freezing tolerance.   In the present chapter, the author has taken a transgenic approach to determine whether the HIC6 protein, *Chlorella* group 3 LEA protein, confers freezing tolerance to a chilling-sensitive higher plant (*Nicotiana tabacum* var. Samsun).

4-2. Materials and methods

4-2-1. *Plant material and growth conditions*

Wild-type tobacco plants (*Nicotiana tabacum* var. Samsun), which were kindly provided by Professor N. Murata, were cultured on MS agar medium (Murashige and Skoog 1962) containing 0.8% agar and 3% sucrose, in a growth chamber (LX-3000; TAITEC, Tokyo, Japan) at 25℃, under a photosynthetic photon flux density of about 70 μmol m^{-2}s^{-1} with a 16 h photoperiod.

4-2-2. *Construction of pBI121/hiC6*

The *hiC6* gene from *Chlorella* (Joh et al. 1995) was mutated, using PCR, to introduce a *Bam*HI restriction site just before the ATG start codon and a *Sac*I site just after the stop codon.   For this purpose, two oligonucleotides were synthesized as primers.   The first oligonucleotide, 5'-CCA AAT GGA TCC ATC CAT CAT GCA GGC C-3', was homologous to the coding strand of *hiC6* with the exception of the 3 nucleotides (underlined) of the introduced *Bam*HI site.   The second oligonucleotide, 5'-GCT GCG AGC TCT CAG AGC TTG TTA GCC TC-3', was complementary to the coding strand with the exception of the 4 nucleotides (underlined) introduced to produce a *Sac*I site.   The open reading frame of *hiC6* was amplified by PCR, and the amplified fragment (563 bp) was subcloned into a pUC119 vector (TaKaRa, Kyoto,

Japan). The amplified fragment was sequenced using an AutoRead
sequencing kit (Pharmacia, Uppsala, Sweden), the possibility of the
misincorporation of nucleotide during PCR being eliminated. After
confirming its sequence, the recombinant plasmid pUC119/*hiC6* was
digested with *Bam*HI and *Sac*I and the insert was ligated into the
*Bam*HI-*Sac*I-digested pBI121 plasmid vector (Clontech, Palo Alto, CA,
U.S.A.). The insert was placed under the control of the cauliflower
mosaic virus (CaMV) 35S promoter to allow a high level of expression
in tobacco plants. For control, the *Bam*HI-*Sac*I-digested pBI121
plasmid, from which ß-glucuronidase gene was deleted and self-ligated,
was used. *Escherichia coli* HB101 was used for the propagation of
these plasmids.

4-2-3. *Transformation of tobacco plants*

The pBI121 and pBI121/*hiC6* expression vectors were
separately transferred from *E. coli* HB101 to *Agrobacterium*
tumefaciens LBA4404 via triparental mating with *E. coli* HB101
carrying a helper plasmid, pRK2013 (Bevan 1984). Cells of
Agrobacterium carrying the expression vectors were selected on
minimal medium containing 50 µg/ml kanamycin. Tobacco
(*Nicotiana tabacum* vr. Samsum) leaf disk was infected by the method
of Rogers et al. (1986). Leaf disks of the sterile tobacco were
incubated with *A. tumefaciens* carrying chimeric genes described

above for 1 min, and then placed on a MS1 medium (MS medium supplemented with 1.0 µg ml^{-1} 6-benzyladenin (BA) and 0.1 µg ml^{-1} 1-naphthaleneacetic acid (NAA)) after rinsing with a sterilized filter paper to remove excess bacteria. After 3 days, they were transferred onto a MS2 medium (MS1 medium supplemented with 100 µg ml^{-1} carbenicillin). After 1 week, they were transferred onto a MS3 medium (MS2 medium supplemented with 200 µg ml^{-1} kanamycin). After 10 days, calli were transferred onto a MS4 medium (MS medium supplemented with 1.0 µg ml^{-1} BA, 100 µg ml^{-1} carbenicillin, and 200 µg ml^{-1} kanamycin) and grown until shoot formation were recognized. Shoots were transferred onto a MS5 medium (MS medium supplemented with 200 µg ml^{-1} kanamycin) until root formation were recognized. Plants regenerated from the same resistant calli were regarded as clones of the same line. All transgenic plant materials were incubated at 25℃, under a photosynthetic photon flux density of about 70 µmol m^{-2}s^{-1} with continuous illumination. However, only regenerated plants were cultured in a mixture of vermiculites and perlites which was supplemented with 0.1% Hyponex (Hyponex Japan Co., Osaka, Japan) solution under a 16-h light/8-h dark regime.

4-2-4. *Preparation of genomic DNA from tobacco plants and detection of hiC6 gene*

Preparation of genomic DNA from tobacco leaves was

performed basically according to the CTAB (cetylmethylammonium bromide) method (Murray and Thompson 1980). Approximately 500 mg of the healthy green leaves of tobacco were lyophilized and then the tissues were ground with a glass rod in a Eppendorf tube to a fine powder prior to extraction. The dried powder was gently dispersed in 1 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 1%(w/v) CTAB, 0.7 M NaCl, 10 mM EDTA, 0.5%(w/v) polyvinylpyrolidone (PVP)). The mixture was incubated for 15-30 min at 60°C with occasional gentle mixing. Then, the extract was emulsified by gentle inversion with an equal volume of a mixture of chloroform and isoamyl alcohol (1:1, v/v) and centrifuged at 13,800 × g for 30 min. One tenth volume of 10% CTAB and 0.7 M NaCl solution was added to the upper aqueous phase containing total nucleic acids. The chloroform and isoamyl alcohol treatment was repeated for complete removal of cells debris, denatured proteins, and most polysaccharides. A CTAB-nucleic acid precipitate was formed when the NaCl concentration was reduced from 0.7 to 0.5 M by the addition of an equal volume of 50 mM Tris-HCl buffer (pH 8.0) that contained 1% CTAB and 10 mM EDTA. The precipitate was collected by centrifugation at 13,800 × g for 2 min. The precipitate was dissolved in 0.4 ml of 10 mM Tris-HCl buffer (pH 8.0) that contained 1 mM EDTA and 1 M NaCl by incubation at 65°C for 1 h. After cooling to room temperature, genomic DNA was precipitated by the addition of two volumes of cold ethanol and

centrifuged at $13,800 \times g$ for 5 min. After washing with 70% ethanol and then with 100% ethanol, the precipitate was dissolved in 250 μl of 1 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. The genomic DNA solution was extract with an equal volume of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v/v) and re-precipitated by addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitate was dissolved in 20 μl of 1 mM Tris-HCl buffer (pH 8.0) that contained 0.1 mM EDTA.

The genomic DNA was used to amplify the coding region of *hiC6* gene by PCR with the primers described above. The PCR products were separated on a 1.4% agarose gel by electrophoresis.

4-2-5. *Preparation of boiling soluble proteins*

Protein extracts were prepared by homogenizing healthy green leaves in 25 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 1 mM PMSF with a glass rod in Eppendorf centrifuge tubes. The homogenates were centrifuged at $13,800 \times g$ for 20 min at 4℃. The supernatants were boiled for 10 min at 100℃, and then centrifuged at $13,800 \times g$ for 20 min. Proteins that remained soluble were precipitated with seven volumes of acetone and collected by centrifugation. The pellet was resuspended in water and concentration was measured by the method of Bradford (1976) using a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.).

4-2-6. *Immunoblot analysis and quantitation of HIC6 protein in transgenic tobacco*

Proteins were directly dotted onto a nitrocellulose membrane, or separated by SDS-PAGE on a 15% (w/v) polyacrylamide gel (Laemmli 1970) and then transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 100 mM Tris-HCl buffer (pH 7.5) containing 0.9% (w/v) NaCl and 0.1% (v/v) Tween 20, incubated with rabbit anti-HIC6 antibody, and then incubated with goat anti-rabbit IgG horseradish phosphatase conjugate (Dainihonseiyaku, Osaka, Japan). The secondary antibody was detected using chemiluminescence (Schneppenheim et al. 1991).

Using the dotted membrane, the amount of the HIC6 protein was measured by scanning the x-ray film that had been exposed to immunoblots that reacted with chemiluminescent substrate. For measurement of the amounts of HIC6 protein, NIH Image Software 1.57 was used. Values from samples of transgenic leaves were compared with a standard curve generated from immunoblots of known concentrations of purified HIC6 protein.

4-2-7. *Measurements of freezing tolerance of leaves*

Two kinds of experiments were performed to assess the degree of freezing tolerance of tobacco leaves. One was measurement of

electrolyte leakage (EL) of leaves and the other was observation of browning of them.

(a) Measurement of EL: To assess the degree of freezing tolerance of tobacco leaves, EL of the leaves was measured by the method of Lång et al. (1989). The leaves (0.1 g fresh weight), which were almost the same size, were cut into small pieces (8 mm × 8 mm) and placed on the wet filter paper in petri dishes. Samples were cooled to $-2°C$ for 1 h, and then ice nucleation was achieved by touching the wet filter paper with a spatula precooled in liquid N_2. After 2-h equilibration period at $-2°C$, the samples were cooled to the indicated temperatures at a rate of $2°C$ per hour. The samples were withdrawn at $1°C$ intervals and thawed at $5°C$ overnight. Deionized water (5 ml) was added to each petri dishes and the samples were gently shaken at room temperature for 1 h. The conductivity of the resulting solution (EL_{frozen}) was measured. To obtain a value for 100% leakage (EL_{LN}), the samples were subsequently frozen by submerging them in liquid nitrogen followed by re-extraction with the original solution for 1 h and by measuring conductivity. EL from unfrozen leaves ($EL_{unfrozen}$) were taken as 0% EL. The percentage of EL from frozen leaves at the specified temperatures was calculated by the following equation (Webb et al. 1994): %EL = (EL_{frozen} – $EL_{unfrozen}$) / (EL_{LN} – $EL_{unfrozen}$) × 100.

Data were analyzed by inspection of all differences by Duncan's (1955) multiple range test. Differences were considered significant

at $P < 0.05$.

(b) Observation of browning: Fresh leaves were punched out into 1 cm in diameter and placed onto wet filter papers in petri dishes. The samples were frozen and thawed as described above, then they were returned to the normal growth conditions at 25°C. The browning of leaves was observed for 5 days.

4-3. Results

4-3-1. *PCR analysis of genomic DNA from transgenic tobacco*

The *hiC6* gene was introduced into tobacco plants under transcriptional control of CaMV 35S promoter by *Agrobacterium*-mediated transformation. By three transformation experiments with pBI121/*hiC6*, 26 regenerated plants were obtained from each of independent callus. However, out of them, the leaves of 11 lines turned yellow and died. Fifteen transgenic lines showed complete kanamycin-resistance and were further analyzed. In order to confirm introduction of the *hiC6* gene into transgenic tobacco plants, these 15 transgenic lines were analyzed by PCR using the primers described in "Materials and methods". Among 15 transgenic lines analyzed, all lines except for No. 26 tobacco plant contained the expected 563-bp fragment corresponding to the coding region of the *hiC6* gene (Fig. 4-1).
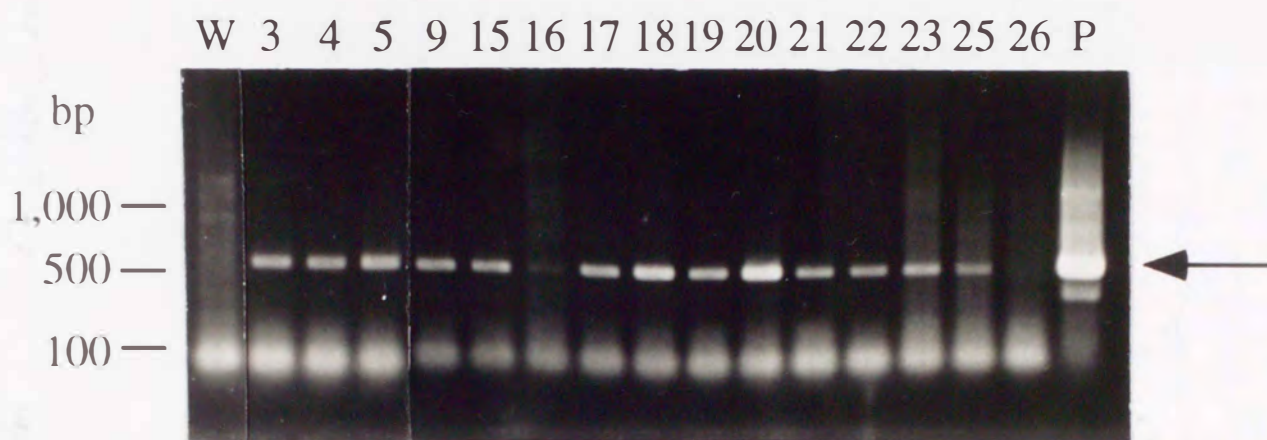
Fig. 4-1. PCR analysis of genomic DNA from transgenic tobacco plants.

Coding region of *hiC6* gene was amplified by PCR. An arrow indicates the 563-bp PCR products. The identification numbers of transgenic lines are indicated on the top. Lane W: Wild-type tobacco plants, lane P: Positive control.

4-3-2. *Detection of HIC6 protein expressed in transgenic tobacco*

The expression of HIC6 protein in the transgenic lines was investigated by immunoblot analysis. Because LEA proteins remain soluble even after boiling (Close et al. 1989, Gilmour et al. 1992), the protein extracts were used for immunoblot analysis after boiling at 100°C for 10 min. The expressed HIC6 protein in tobacco was detected by polyclonal antibodies raised against HIC6 protein overexpressed in *E. coli.* As shown in Fig. 4-2, a single band of 14 kDa, which corresponded to the molecular mass of the deduced HIC6 protein, was detected in 12 lines. The identification numbers of transgenic lines were 3, 4, 5, 9, 15, 17, 18, 19, 20, 21, 22, and 25. There was no additional bands of other sizes in the protein extracts of the transgenic tobacco plants.
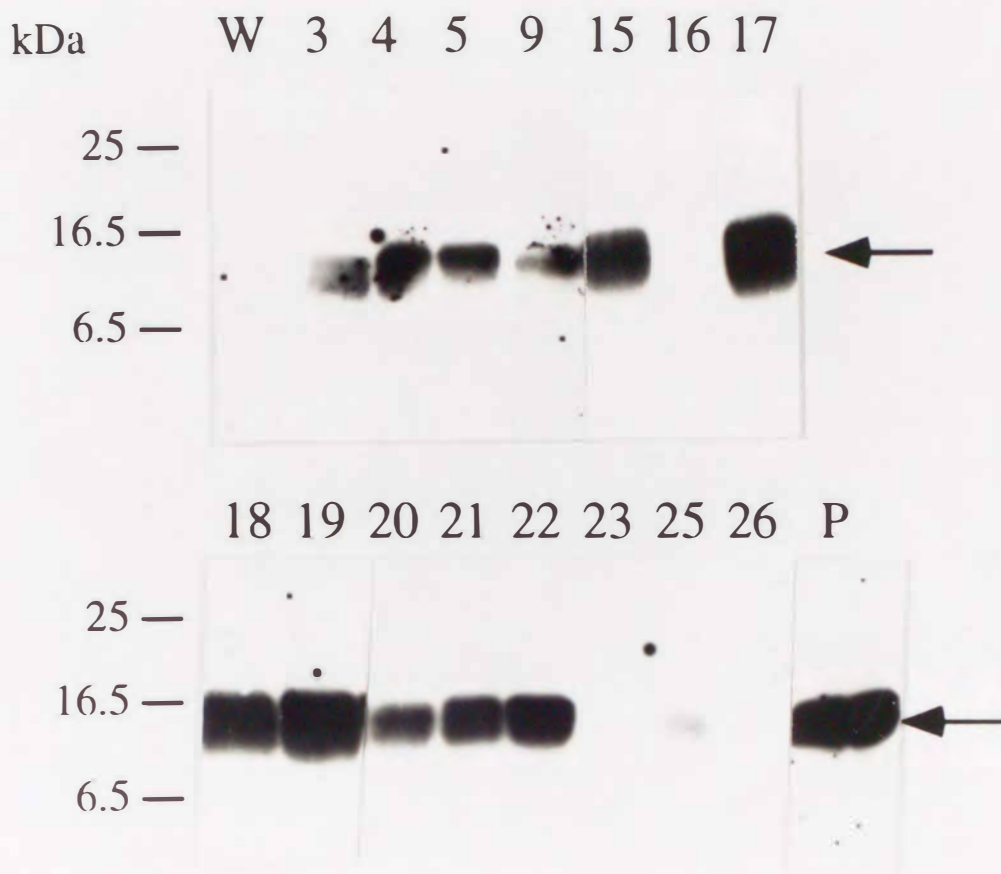
Fig. 4-2. Immunoblot detection of HIC6 protein accumulation in transgenic tobacco plants. Equal amounts of boiling-soluble proteins were separated on 15% SDS-PAGE and immunoblot detection was performed as described in "Materials and methods". Molecular mass markers are indicated in kDa on the left. The HIC6 protein shown as a 14-kDa band is indicated by an arrow at the right. The identification numbers of transgenic lines are indicated on the top. Lanes W: boiling soluble proteins from wild-type tobacco plants, P: purified HIC6 protein as the positive control.

4-3-3. *Accumulated levels of expressed HIC6 protein in transgenic tobacco*

Equal amounts of the boiling soluble proteins, which had been confirmed to include the expressed HIC6 protein, were dotted onto a nitrocellulose membrane and detected by immunological methods. Table 4-1 shows the levels of the expressed HIC6 protein in each tobacco plant expressing the protein. The accumulated level of the HIC6 protein in No. 3 tobacco leaves was the highest (6.5×10^{-2}% of the soluble proteins) and that in No. 25 leaves was the lowest (1.5×10^{-2}%). Therefore, No. 3 tobacco leaves were used for following experiments.

Table 4-1. Accumulated levels of expressed HIC6 protein in different transgenic lines.

Transgenic lines	Accumulated level	
	% of boiling soluble protein	% of soluble protein
3	0.30[a]	6.5×10^{-2}
4	0.19	6.0×10^{-2}
5	0.26	5.2×10^{-2}
9	0.12	3.3×10^{-2}
15	0.21	3.9×10^{-2}
17	0.26	5.2×10^{-2}
18	0.20	3.7×10^{-2}
19	0.18	2.9×10^{-2}
20	0.18	3.4×10^{-2}
21	0.20	4.6×10^{-2}
22	0.14	2.9×10^{-2}
25	0.08	1.5×10^{-2}

[a]Values from samples of transgenic leaves were compared with a standard curve generated from immunoblots of known concentrations of the purified HIC6 protein.

4-3-4. *Freezing tolerance of tobacco transformed with hiC6*

In order to examine the level of freezing tolerance of transgenic tobacco plants, EL values were measured after freezing.   The EL values from leaves of the transgenic tobacco plant, which expressed the HIC6 protein, were significantly lower than those of the control (wild-type and transgenic plant transformed with pBI121) plants ($p < 0.05$), when they were frozen to –2 or –3°C (Fig. 4-3).   After freezing to –4 or –6°C, any significant differences in the EL values were not observed between the transgenic and the control leaves.

4-3-5. *Browning of tobacco leaves after freeze/thaw treatment*

The results of observing browning of tobacco leaves which were frozen at -2°C, is shown in Fig. 4-4.   The transgenic plant (No. 3) browned more slowly than those of the control plants.   While control leaves wilted, the transgenic leaves kept green for 5 days after being transferred to the normal growth conditions (at 25°C).   When leaves were frozen to –3, –4, or –6°C, transgenic leaves browned as same as the control leaves (data not shown).
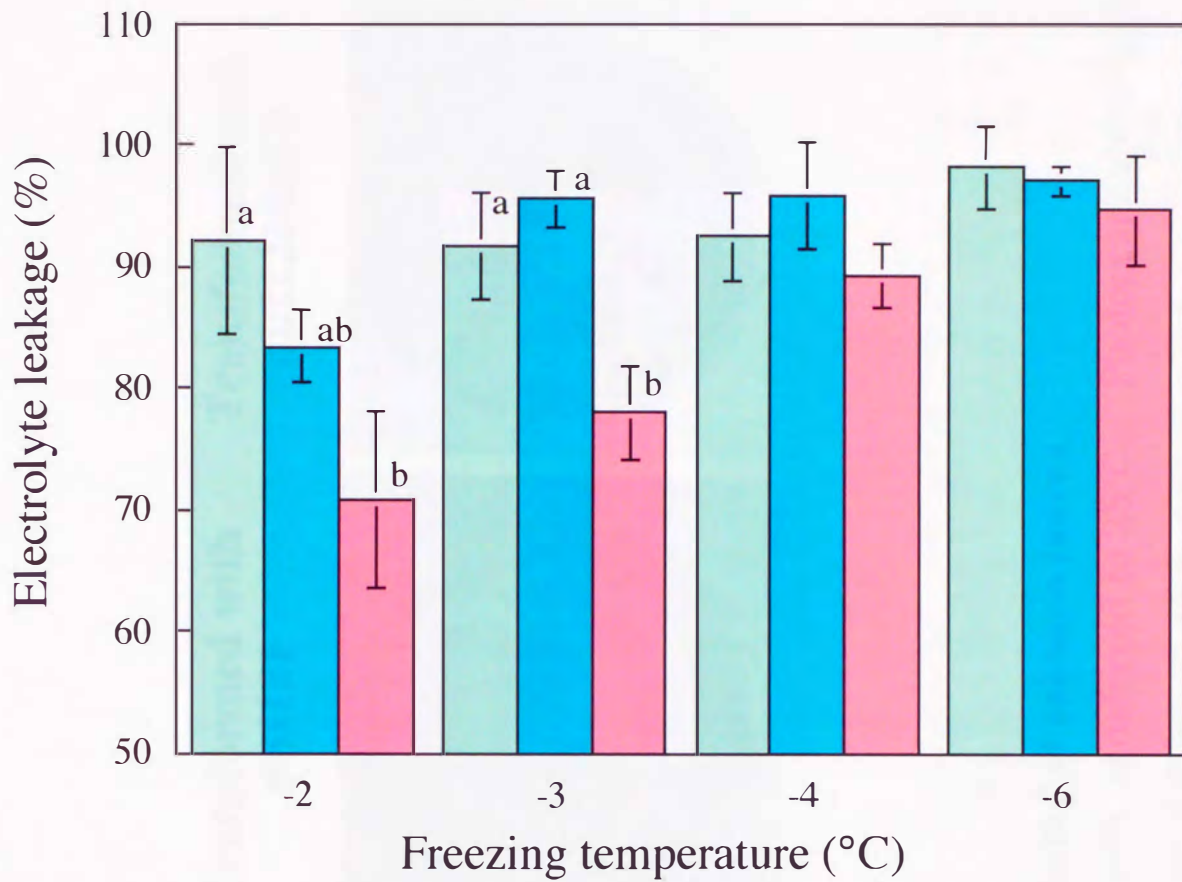
Fig. 4-3. Freezing tolerance of leaves of transgenic tobacco plants as determined by measurement of electrolyte leakage (EL) after freezing.
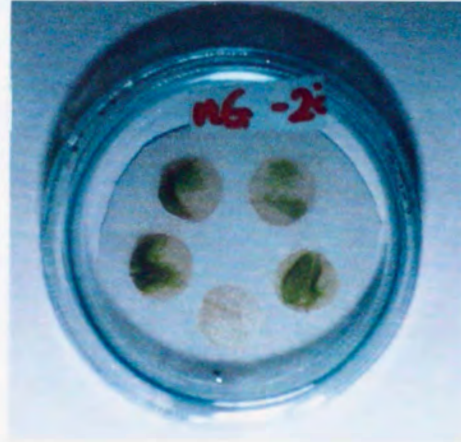
Results are the mean ± SE (n=3) and [ab]values not sharing a common letter are significantly different at $p < 0.05$.

☐ Wild type   ■ Transformed with pBI121   ■ Transformed with pBI121/*hiC6*
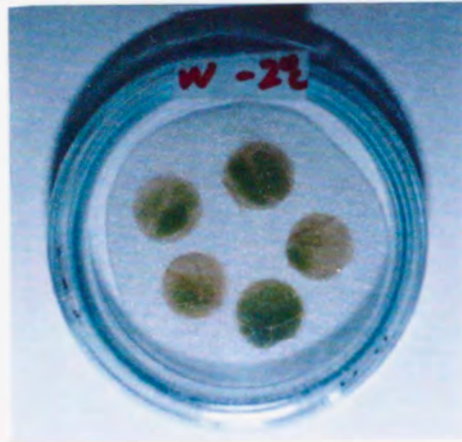
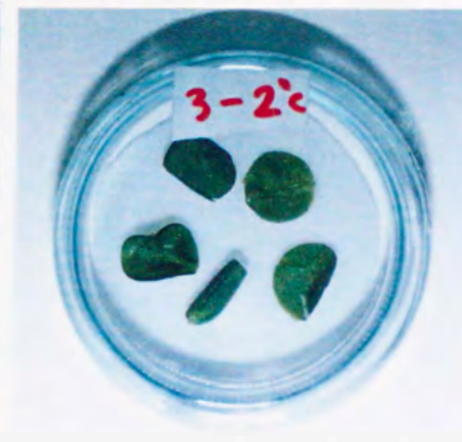**Wild type**   **Transformed with pBI121**   **Transformed with pBI121/*hiC6***

Fig. 4-4. Comparison of browning of tobacco leaves.
The leaves were frozen at −2°C and transferred to 25°C.   Photographs were taken after 5 days from beginning of incubation at 25°C.

4-4. Discussion

A hardening-induced *Chlorella* gene, *hiC6*, was introduced into tobacco plants. By the introduction of *hiC6* gene using the leaf-disk method, fourteen lines of transgenic tobacco plants which had the *hiC6* gene in the genome were obtained (Fig. 4-1). Out of these transgenic lines, twelve lines constantly expressed the HIC6 protein (Fig. 4-2). These transgenic tobacco plants appeared morphologically normal compared with wild-type tobacco plants. Thus, accumulation of the HIC6 protein appeared to have no detrimental effects on the growth and development of tobacco plants.

By using the 12 transgenic lines, dot blot analysis was carried out and the accumulated levels of the protein were determined in each transgenic line (Table 4-1). The levels of the HIC6 protein accumulated in the leaves of different transgenic lines were distributed in the range of 1.5×10^{-2} to 6.5×10^{-2}% of the soluble proteins (Table 4-1). On the other hand, the accumulated level of the HIC6 protein in *Chlorella vulgaris* IAM C-27 was approximately 2.7% of the soluble proteins (data not shown) and one of a group 3 LEA (D-7) protein in the embryos of cotton is approximately 2.6% (Roberts et al. 1993). Compared with these data, the amounts of the protein in the transgenic tobacco lines were quite low. These results suggest that the amounts of HIC6 protein may not be enough to confer a high level of freezing tolerance to the tobacco leaves (Fig. 4-3, -4).

By the measurement of EL from leaves frozen to -2 or -3℃, the transgenic leaves showed low EL values, 71 and 78%, respectively (Fig. 4-3). On the other hand, EL values of the wild type tobacco leaves were 92% at both –2 and –3℃. It is generally considered that plants showing 50% or more EL are dead (Lång et al. 1989). Thus, it seems that the low expression of the HIC6 protein could not lower the killing temperature of the tobacco plants. However, there were significant differences in the EL values between transgenic and control tobacco leaves. Furthermore, the degree of browning of the transgenic leaves were apparently reduced after freezing at –2℃ (Fig. 4-4). These results suggest that the accumulation of the HIC6 protein alleviated the damage caused by freezing and slightly conferred freezing tolerance to tobacco leaves.

Group 3 LEA proteins such as HIC6 protein are correlated with stress tolerance (Baker et al. 1988, Ried and Walker-Simmons 1993, Joh et al. 1995). The functions of some LEA proteins have been proposed. Dure (1993) proposed that D-7 (group 3 LEA) protein from cotton acts as an ion sequestraor. Imai et al. (1996) suggested that LE25 (group 4) protein acts as an ion scavenger. However, it remains unclear what roles group 3 LEA proteins play in the development of freezing tolerance. As Houde et al. (1992) suggested, hydrophilic proteins such as LEA proteins might modify the structure or propagation of intracellular ice crystals during freezing and trap

enough water inside the cell to prevent local dehydration. The proteins might protect cells from freezing damage by preventing removal of bound water from cellular substances. From the present study, it was shown that the HIC6 protein significantly reduced EL of tobacco leaves after freezing. Considering that EL values are based on the degree of injury of cell membranes, the result suggests that the protein reduced freezing injury of cell membranes by an unknown mechanism in which HIC6 protein involved.

Recently, Xu et al. (1996) reported that the transgenic rice plants with higher accumulated levels (0.5 to 2.5% of soluble proteins) of a group 3 LEA protein, HVA1 protein, showed more increased tolerance to water deficit and salt stress. They reported that the transgenic plants showed better recovery than did the control plants when the stress conditions were removed. For assay of stress tolerance, they used whole plants instead of leaves. In the author's future work, it will be necessary to use plants for observing appearance and development of the major damages (wilting and necrosis of leaves) caused by freezing.

To the author's knowledge, a group 3 LEA protein has not been shown to confer freezing tolerance to plants. This study showed that a *Chlorella* LEA protein (HIC6) slightly enhanced freezing tolerance of the transgenic tobacco leaves. However, the accumulated level of the HIC6 protein was quite low and not satisfactory. An increase in the

accumulated protein level will help the enhancement of freezing tolerance of transgenic tobacco plants. Furthermore, in addition to introduction of *hiC6* gene, it will be necessary to introduce other genes such as genes encoding ω-3 fatty acid desaturase (Kodama et al. 1994) or scavenging enzymes, superoxide dismutase (McKersie et al. 1993) and ascorbate peroxidase (Kuroda et al. 1991) etc., for highly enhancement of freezing tolerance of plants.

4-5. Summary

The *hiC6* gene of *Chlorella*, encoding a homologue of LEA protein, was introduced into a chilling sensitive tobacco plant (*Nicotiana tabacum* var. Samsun) by the leaf-disk method. For constitutive expression of the *hiC6* gene and high-level accumulation of the encoded protein, the gene was inserted under the transcriptional control of the cauliflower mosaic virus 35S promoter. Fifteen transgenic tobacco lines were acquired. PCR analysis showed that fourteen lines carried the *hiC6* gene. Out of fourteen lines, twelve lines expressed the HIC6 protein. The accumulated levels of the protein were from 1.5×10^{-2} to 6.5×10^{-2}% of the soluble protein. Compared to control tobacco leaves, EL values were statistically lowered in the transgenic tobacco leaves frozen at -2 and -3°C. The result was also supported by observation of browning. These results indicate that accumulation of HIC6 protein slightly alleviated the damage of tobacco leaves after freezing.

CONCLUSION

Now, a world-wide food shortage is foreseen because of the increase of human population, salinization and desertification of agricultural lands, and abnormal weather. In order to break through this subject, new crop varieties having tolerance to environmental stresses are desired to be developed by recombinant DNA technology.

Some plant species develop freezing tolerance when exposed to low nonfreezing temperature. The development of freezing tolerance has been shown to involve expression of specific genes. It is expected that the tolerance can be conferred to chilling-sensitive plants by the introduction of the specific genes expressed during hardening. However, it remains unclear what roles the hardening-induced proteins have and whether they can confer freezing tolerance to plants.

C. vulgaris IAM C-27 is able to survive slow freezing to $-196°C$ when hardened at 3°C for 24 h. Cycloheximide, an inhibitor of protein synthesis, prevents the development of frost hardiness in *Chlorella*, suggesting that specific proteins are directly involved in the frost hardiness. In the present study, the changes in soluble proteins of *C. vulgaris* C-27 during hardening were first analyzed by a 2D-HPLC system. The results revealed that 31 soluble proteins were induced or increased in abundance. Out of them, seven proteins were characterized by N-terminal sequencing and identified by searching

for homology in databases. Especially, a 10-kDa protein was induced after a 12-h hardening and a 14-kDa protein after a 6-h hardening. The N-terminal amino acid sequences of both 10-kDa and 14-kDa proteins were similar to those of group 3 LEA proteins. The N-terminal amino acid sequences of the proteins were identical to the partial amino acid sequences of the deduced proteins from *hiC6* and *hiC12*, which had been confirmed to be induced at a transcriptional level. These results reveal that the 10-kDa and 14-kDa proteins were induced also at a translational level and that the 14-kDa protein had a transit peptide which might involve in the transportation of the protein into organelles such as chloroplast and mitochondria.

Next, the author attempted the determination of the exact nucleotide sequence of *hiC6* and *hiC12* clones, overexpression of the *hiC6* and *hiC12* genes in *E. coli*, and the measurements of cryoprotective activities of the *Chlorella* LEA proteins (HIC6 and HIC12). The molecular mass of a deduced protein encoded by *hiC12* clone exhibited 9.7-kDa and it was almost close to that of the hardening-induced 10-kDa protein. The HIC6 and HIC12 protein was overexpressed as glutathione *S*-transferase-fusion proteins in *E. coli*. After purification of them, cryoprotective activity of both the proteins was assayed for protecting freeze-labile enzymes. Each CP_{50} value of HIC6 and HIC12 was 1.86 and 3.16 nM, respectively. The molar ratio of the proteins to a freeze-labile enzyme, LDH, is shown to be

approximately 4:1. Furthermore, two kinds of artificially shortened HIC6 proteins, HIC6A and HIC6B proteins, were also overexpressed. Each CP_{50} value of HIC6A and HIC6B was approximately 5.37 and 5.75 nM, respectively. However, single units of the motif had low cryoprotection (CP_{50} values: 2.10×10^3 to 1.35×10^4 nM) and the CP_{50} values were about 350 to 7,000 times higher than that of the HIC6 protein. The results show that the number of 11-mer motifs was important for cryoprotective function and suggest that *Chlorella* LEA proteins protect LDH directly against freezing by forming tertiary-structure.

In order to determine whether the HIC6 protein has the ability to confer freezing tolerance, the *hiC6* gene was introduced into *S. cerevisiae*. The gene was located under *GAL1* promoter of a pYES2 plasmid vector. Expression of the gene was induced by galactose. The accumulated level of the HIC6 protein was increased with an increase of induction period. Furthermore, freezing tolerance of the transformed yeast was also enhanced with the elongation of induction time. The survival rates of the yeast expressing the HIC6 protein are approximately 65.8% after 24-h induction and this was 3.3 times higher than control yeast cells which carry pYES2 plasmid. These results suggest the accumulation of the protein is important for acquisition of freezing tolerance of yeast.

In order to investigate whether the HIC6 protein will confer

freezing tolerance to higher plants, the coding gene was introduced into tobacco plant. Twelve lines of transgenic plants expressing the HIC6 protein were obtained. In each transgenic plant, the accumulated levels of the protein were from 1.5×10^{-2} to 6.5×10^{-2}% of the soluble proteins. Compared to wild-type and control tobacco leaves, electrolyte leakage values of the transgenic tobacco leaves frozen at -2 and -3°C were significantly lowered. However, the level of freezing tolerance of the transgenic tobacco leaves was relatively low and not satisfactory.

In summary, two kinds of group 3 LEA proteins, HIC6 and HIC12 proteins, were found to be significantly involved in the development of freezing tolerance in *Chlorella*. Especially, the author showed that the HIC6 protein has cryoprotective activity for protecting a freeze-labile enzyme, LDH, against freezing. The expressed HIC6 protein enhances the level of freezing tolerance in yeast. Although there were statistical differences between the levels of freezing tolerance of the transgenic and wild-type tobacco plants, the level of the tobacco leaves transformed with the *hiC6* gene was not satisfactory. Many kinds of the proteins encoded by low-temperature-induced genes are reported to be involved in the development of freezing tolerance of plants. Introduction of a single gene into chilling-sensitive plants may not be enough to highly increase the levels of the freezing tolerance of higher plants. Therefore, introduction of the

hiC6 gene, in combination with additional genes such as genes encoding ω-3 fatty acid desaturase or scavenging enzymes, superoxide dismutase and ascorbate peroxidase etc., will be necessary for a highly increase in freezing tolerance of higher plants.

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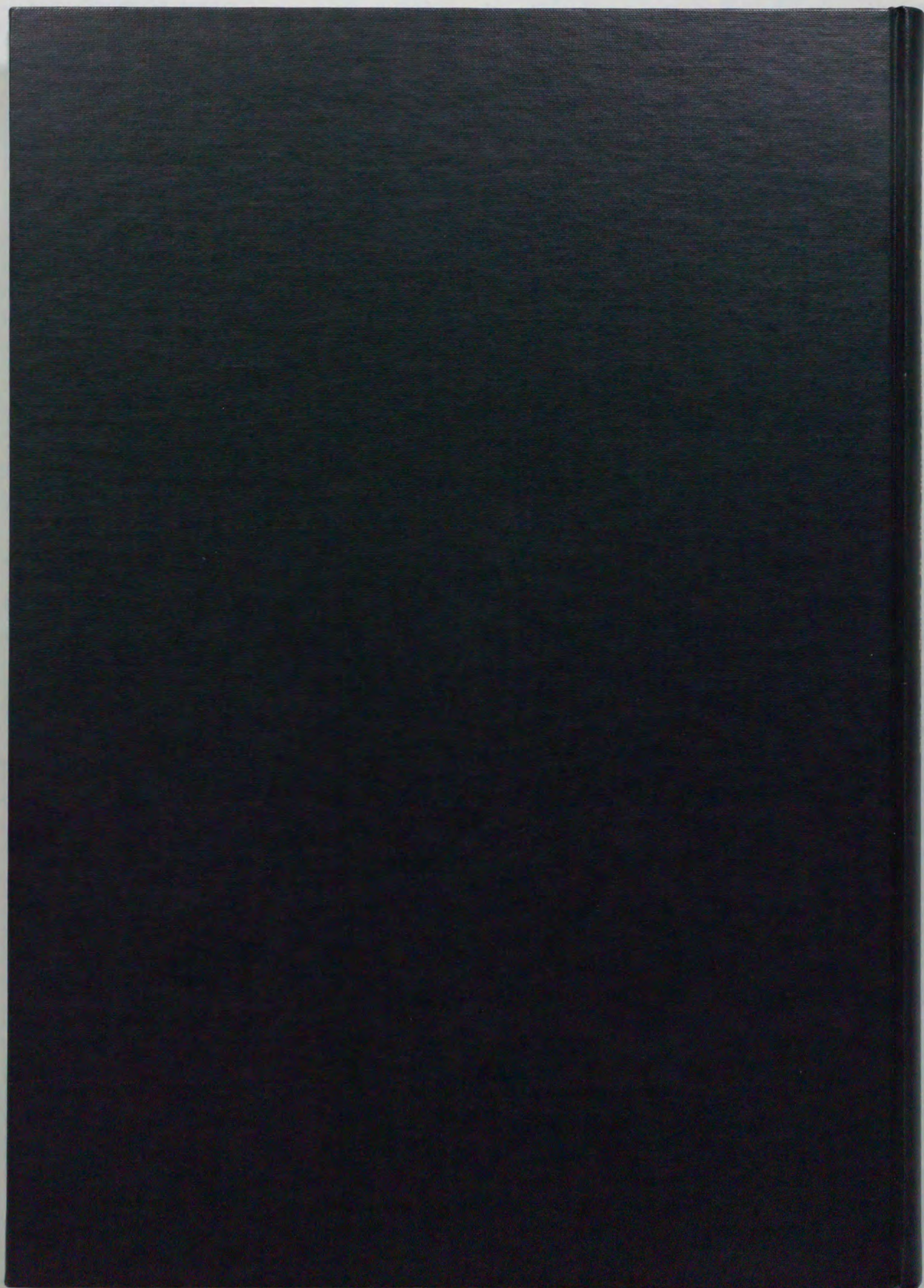
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