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# Cryoprotective Activities of Group 3 Late Embryogenesis Abundant Proteins from *Chlorella vulgaris* C-27

Ken-ichi HONJOH,*,† Hiroko MATSUMOTO,* Hideyuki SHIMIZU,* Kanae OOYAMA,* Kageyuki TANAKA,* Yuichi ODA,* Ryoji TAKATA,* Toshio JOH,** Koushirou SUGA,*,*** Takahisa MIYAMOTO,* Masayoshi IIO,* and Shoji HATANO****

**Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University, Fukuoka 812-8581, Japan*
***Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, 2-Ikarashi, Niigata 950-2181, Japan*
****Chlorella Industries Co. Ltd., 1343 Hisatomi, Chikugo, Fukuoka 833-0056, Japan*
*****Graduate School of Health and Social Welfare Science, Nishikyushu University, 4490-9 Ooazaozaki, Kanzaki-machi, Kanzaki-gun, Saga 842-8585, Japan*

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**The nucleotide sequence of *hiC12*, isolated as a cDNA clone of *h*ardening-*i*nduced *Chlorella* (*hiC*) genes, was identified. The clone encodes a late embryogenesis abundant (LEA) protein having six repeats of a 11-mer amino acid motif, although in a slightly imperfect form. To overexpress the *hiC6*[1] and *hiC12* genes, their coding regions were PCR amplified and subcloned into a pGEX-1λT vector. The HIC6 and HIC12 proteins were expressed as GST fusion proteins in *E. coli*, then purified. The two HIC proteins were found to be effective in protecting a freeze-labile enzyme, LDH, against freeze-inactivation. On a molar concentration basis, they were about $3.1 \times 10^6$ times more effective in protecting LDH than sucrose and as effective as BSA. Cryoprotection tests with five kinds of chain-shortened polypeptides, synthesized based on the 11-mer amino acid motif of the HIC6 protein showed that the cryoprotective activity decreased with a decrease in the repeating units of the 11-mer motif. In fact, cryoprotective activities of three kinds of single 11-mer amino acids were very low even at high concentrations. All the results suggested that the sufficiently repeated 11-mer motif is required for the cryoprotective activities of *Chlorella* LEA proteins.**

**Key words:** *Chlorella vulgaris* C-27; cryoprotection; freezing tolerance; LEA protein

Some plants can acclimate to a variety of environmental stresses for optimal function and survival.[2,3] It has been reported that changes in gene expression occur during cold acclimation, and cold-regulated genes have been isolated from a number of plant species.[2,3] Many genes are thought to be involved in the development of freezing tolerance.[2–5] However, as little is known of the functions of cold-induced proteins, it is difficult to effectively improve the freezing tolerance of plants through genetic manipulation procedures.

We studied the development of freezing tolerance in plants, using *Chlorella vulgaris* C-27. Hardened cells of *Chlorella vulgaris* C-27 survive slow freezing to −196°C.[6] Seventeen cDNA clones corresponding to *h*ardening-*i*nduced *Chlorella* (*hiC*) genes were isolated from 6-h hardened *Chlorella* cells.[1] Out of these, the protein encoded by the most abundant cDNA clone, *hiC6*, was similar to a late embryogenesis abundant (LEA) protein.[1]

LEA proteins were first characterized in cotton as a set of proteins that accumulate highly in embryos at the late stage of seed development.[7] Based on their common amino acid sequence domains, LEA proteins are classed into three major groups.[8,9] Of these, Group 3 LEA proteins have a characteristic of repetition of an 11-mer amino acid motif. The motif is characterized by apolar residues in positions 1, 2, 5, and 9 and charged or amide residues in positions 3, 6, 7, 8, and 11.[10] The regions containing repeats of the 11-mer amino acid motif seem to form an amphiphilic α-helix structure.[8–10] Based on the correlation of

† To whom correspondence should be addressed. Tel: +81-92-642-3025; Fax: +81-92-642-3025; E-mail: honjoh@agr.kyushu-u.ac.jp
*Abbreviations*: GST, glutathione S-transferase; *hiC*, *h*ardening-*i*nduced *Chlorella*; LDH, L-lactate dehydrogenase; LEA, late embryogenesis abundant; PCR, polymerase chain reaction

*lea* gene expression with physiological and environmental stresses and on the predicted structures of the LEA proteins, it has been hypothesized that the proteins may play a protective role in plant cells exposed to various stress conditions. Moreover, this protective role may be essential for the survival of the plant under extremely stressful conditions.[8,9,11,12]

In this study, we analyzed the nucleotide sequence of the *hiC12* cDNA clone and the cryoprotective activities of HIC6 and HIC12 from *Chlorella vulgaris* C-27 were given attention. Furthermore, we constructed shortened HIC6 polypeptides and, by investigating their cryoprotective activities, the involvement of the 11-mer amino acid motif repeats in *Chlorella* Group 3 LEA (HIC6) proteins in cryoprotection was also examined.

## Materials and Methods

*Plant materials.* Cells of *Chlorella vulgaris* Beijerinck IAM C-27 were grown synchronously at 25°C in MC medium,[13] under a photosynthetic photon flux density of about 250 $\mu$mol $m^{-2}$ $s^{-1}$, with aeration with 1.3% $CO_2$ in air, to a concentration of about $1.0–1.5 \times 10^{10}$ cells $liter^{-1}$ under a 16-h photoperiod, as described.[6] Since the cells were hardened to the greatest extent at the $L_2$ stage (the intermediate stage of the ripening phase of the cell cycle),[6] $L_2$ cells were studied.

*Hardening. Chlorella* cells the growth of which had been synchronized at 25°C were directly hardened by incubation at 3°C for 24 h. During hardening, the culture was aerated with air enriched to about 1.3% $CO_2$ and kept in the light. At intervals, about $1.5 \times 10^{10}$ cells were collected by centrifugation at $1,000 \times g$ for 5 min, washed with distilled water, and resuspended in an extraction buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, and 1% SDS). The suspension of cells was frozen in liquid nitrogen and stored at −80°C.

*Isolation and nucleotide sequence analysis of hiC12 cDNA clone.* Poly(A)$^+$ RNA was prepared from the suspension of 6-h hardened *Chlorella* as described.[1] A cDNA library corresponding to poly(A)$^+$ RNA obtained from 6-h hardened cells was constructed in a $\lambda$gt10 vector using a commercial cDNA synthesis and cloning kit (Amersham, Buckinghamshire, England). The *hiC12* was cloned as described.[1] For further characterization, the *hiC12* clone was subcloned into the *Eco*RI site of pUC119. The nucleotide sequence of the *hiC12* clone was identified as described by Sanger *et al.*[14] 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 analyzed in both directions. Analysis of nucleotide and amino acid sequences was done with MacVector computer software (Oxford Molecular Ltd., Oxford, UK). Nucleotide and deduced amino acid sequences were compared with those of proteins in the PRF, PIR, and GenBank databases, using the BLAST program.[15]

*Construction of pGEX-1λT/hiC6 and pGEX-1λT/hiC12.* The *hiC6*[1] 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 protein,[16] and an *Eco*RI restriction site just after the stop codon. For this purpose, four kinds of oligonucleotide fragments were synthesized as primers for *hiC6* and *hiC12*. The first oligonucleotide, 5′-TGGTTGGATCCGCCCTCGGGGAGGAGT-3′, was homologous to the coding strand of *hiC6*, with the exception of 4 nucleotides (underlined) of the introduced *Bam*HI site. The second oligonucleotide, 5′-GCTGCGAATTCTCAGAGCTTGTTAGCCTC-3′, was complementary to the coding strand, with the exception of 4 nucleotides (underlined) of the introduced *Eco*RI site. The third oligonucleotide, 5′-CACAGGATCCATGGCCGGCAAC-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′-CTAGTGAATTCGGGCAAGGAGAGG-3′, was complementary to the coding strand, with the exception of the 3 nucleotides (underlined) of the introduced *Eco*RI site. The open reading frames of *hiC6* and *hiC12* were amplified by PCR, and the amplified fragments were subcloned into a pUC119 vector (Takara, Kyoto, Japan), respectively. After confirming these sequences, the inserts were digested with *Bam*HI and *Eco*RI and then subcloned into the *Bam*HI-*Eco*RI-digested pGEX-1λT plasmid vector (Pharmacia, Uppsala, Sweden). The fragments of *hiC6* and *hiC12* were downstream from the *tac* promoter to allow for 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 using the method of Hanahan and Meselson.[17]

*Construction of pGEX-1λT/hiC6A and pGEX-1λT/hiC6B.* To study the involvement of the unit numbers of the amino acid in cryoprotection, two kinds of shortened HIC6 protein were expressed by gene engineering. Two shortened polypeptide fragments were designated as HIC6A and HIC6B protein, respectively. Two genes coding for the HIC6A and HIC6B proteins (Fig. 1) were mutated by PCR, using the *hiC6* gene as a template. For this purpose, three oligonucleotides were synthesized as primers for *hiC6A* and *hiC6B*. The first oligonucleotide, 5′-TGGTTGGATCCGCCCTCGGGGAGGAGT-3′, was the same primer as that used for amplification of

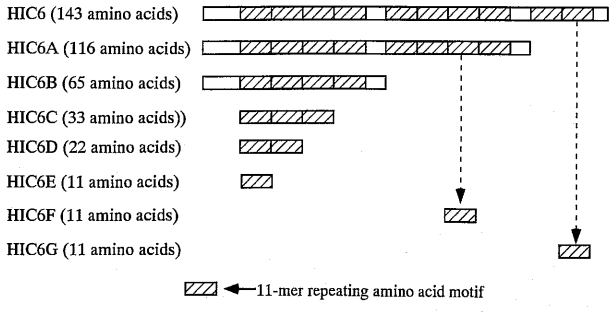


**Fig. 1.** Design of Artificially Shortened HIC6 Polypeptide Fragments.

The polypeptides were designated as HIC6, HIC6A, HIC6B, HIC6C, HIC6D, HIC6E, HIC6F, and HIC6G, respectively. HIC6, HIC6A, and HIC6B were overexpressed in *E. coli* using pGEX1λT vector. HIC6C, HIC6D, HIC6E, HIC6F, and HIC6G were chemically synthesized.

*hiC6*. The second oligonucleotide, 5′-CTCCCTTGAATTCGGCAAATCAGTTGCTA-3′, was complementary to the coding strand of *hiC6A*, with the exception of 6 nucleotides (underlined) of the introduced *Eco*RI site. The third oligonucleotide, 5′-CTTGGGAATTCCCAGTCAGAAGCAG-3′, was homologous to the coding strand of *hiC6B*, with the exception of 6 nucleotides (underlined) of the introduced *Bam*HI site. The amplified fragments were digested with *Bam*HI and *Eco*RI, then ligated into the *Bam*HI-*Eco*RI-digested pGEX-1λT plasmid vector. These fragments were downstream from the *tac* promoter to allow for 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.

*Expression and purification of recombinant 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). After 1 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and shaken for 3–4 h.

Bacterial cells collected by centrifugation at 9,000 × *g* for 10 min were suspended in 5–10% of the culture volume of PBS (phosphate buffered saline; 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 addition of Triton X-100 to a final concentration of 1%, the lysate was centrifuged at 9,000 × *g* for 20 min. Fusion proteins in the supernatant were purified using a glutathione Sepharose 4B column (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The fusion proteins were then 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.[18] The protein concentrations were measured by the method of Bradford,[19] using a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.).

*Construction of shortened polypeptide fragments of hiC6.* Based on the sequence of the deduced HIC6 protein (Fig. 1), five kinds of polypeptides (HIC6C, HIC6D, HIC6E, HIC6F, and HIC6G) were synthesized (Biologica Co. Nagoya, Japan) to measure cryoprotective activity. These were suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM PMSF and used for the cryoprotective assay.

*Cryoprotection assay for a freeze-labile enzyme, lactate dehydrogenase (LDH).* 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. A freeze-labile enzyme solution (0.5 ml) was put into a test tube and 0.5 ml of the test compound suspended in 25 mM potassium phosphate buffer (pH 7.5) was added. The sample was frozen at −20°C for 24 hours, thawed at room temperature for 5–10 min and immediately used to assay for the enzymatic activity.

Cryoprotective activities of LEA proteins and of various substances were assayed as described by Tamiya *et al.*[20] The reaction mixture consisted of 2.5 ml of 96 mM Tris-HCl buffer (pH 7.5) and 120 mM KCl, 0.25 ml of 24 mM sodium pyruvate, and 0.25 ml of 3.6 mM NADH. The enzyme reaction was started by adding 60 μl of the enzyme solution. Activity of the enzyme was measured by the absorbance change at 340 nm, depending on the oxidation of NADH with a Shimadzu UV-160 spectrophotometer at 25°C. $CP_{50}$ (50% cryoprotection) was defined as the concentration of the additive required to give 50% residual enzyme activity after the freeze-thaw cycle, then average values were compared. All samples were assayed in triplicate.

## Results

*Nucleotide and deduced amino acid sequences of hiC12*

The nucleotide sequence of *hiC12* was analyzed. The full-length of *hiC12* was 585 bp long and contained an open reading frame coding for 104 amino acid residues with a molecular mass of about 10.8 kDa (Fig. 2). Sequence analysis found that the deduced HIC12 protein had a high level of alanine (21%) and was devoid of cystein. The relative level of hydrophilic amino acid residues (A, R, N, D, Q, E, G, H, K, P, S, and T) in the HIC12 protein was 87.4%. The nucleotide sequence has been submitted to DDBJ/EMBL/GenBank under the accession number AB035642.

A computer search of the databases revealed amino acid sequence homology between the HIC12 protein and a Group 3 late embryogenesis abundant

(LEA) protein from *Hordeum vulgare*, HVA1 (Fig. 3).[21] The highest degree of conservation was found between amino acid residues 9 to 84 of the HIC12 protein and residues 21 to 109 of the HVA1 protein. Alignment of the sequences revealed 29% identical and 43% biochemically similar amino acids. A repeating motif of the 11-mer amino acid, a feature of Group 3 LEA proteins, was found in the amino acid sequence of the HIC12 protein (Fig. 2), although the motif was not necessarily perfect.

*SDS-PAGE of HIC6, HIC12, HIC6A, and HIC6B proteins*

To investigate cryoprotective activities of the HIC6 and HIC12 proteins, these proteins were produced in *E. coli*. A transcriptional fusion was made between the objective gene and a combination of the *tac* promoter and the GST gene translational leader in pGEX-1λT. Thus, use of a pGEX-1λT vector resulted in the incorporation of two amino acids (GlySer-) into the N-terminus of the expressed proteins. These constructed plasmids 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, consistent with the size of the GST-HIC6 and GST-HIC12 proteins, respectively (data not shown). The GST fusion proteins were purified from *E. coli*, on a glutathione Sepharose 4B column, and cleaved with thrombin. Then, the objective proteins were purified by affinity chromatography on a glutathione Sepharose 4B column. SDS-PAGE analysis showed that the molecular masses of the purified HIC6 and HIC12 proteins were 14 kDa and 10 kDa, respectively (Fig. 4).

HIC6A and HIC6B proteins were also expressed and purified, as described above. SDS-PAGE analysis confirmed that the molecular masses of HIC6A and HIC6B proteins were 12 kDa and 6 kDa, respectively (Fig. 4).

*Cryoprotection of LDH by HIC6 and HIC12*

Without the addition of a cryoprotectant, a freeze-thaw cycle resulted in the loss of over 80% of LDH activity (data not shown). However, the addition of the HIC6 or HIC12 protein at a concentration of 0.5 μM resulted in an almost complete protection for LDH against freeze-inactivation.

A comparison of the cryoprotective activities of the HIC6 or HIC12 proteins with those of other agents indicated that both the proteins had high cryoprotective activities, while sucrose, which is commonly regarded as an effective cryoprotectant and protein-stabilizer, had a $CP_{50}$ of about $3.4 \times 10^7$ nM, $CP_{50}$ of the HIC6 and HIC12 proteins were about 7.4 and 11 nM, respectively (Fig. 5, Table). Thus, on a molar concentration basis, LEA proteins of *Chlorella* were approximately $3.1 \times 10^6$ times more effective than sucrose in protecting LDH from freeze-inactivation. Further, the cryoprotection of both the HIC6 and HIC12 proteins was as effective as BSA, known to be effective in protecting LDH against freeze-inactivation.

*Effects of the unit number of the 11-mer amino acid motif on cryoprotection*

To examine the relationship between cryoprotection and the length of the HIC6 protein, cryoprotective activities of five kinds of polypeptides designated as HIC6A, HIC6B, HIC6C, HIC6D, and HIC6E, each different in the unit number of the motif, were assayed. At 50 nM concentration, the HIC6 protein retained about 70% of LDH activity and the HIC6A protein approximately 50% of the activity (Fig. 6). The $CP_{50}$ of the HIC6, HIC6A, and HIC6B proteins were 7.4, 11, and 51 nM, respectively (Table). The residual LDH activity increased with an increase in the repeat number of the motif. On a weight concentration basis, HIC6C ($CP_{50}$; 0.52 μg/ml) and HIC6D ($CP_{50}$; 0.46 μg/ml) were also as effective as BSA ($CP_{50}$; 0.44 μg/ml) or lactalbumin (0.40 μg/ml). The $CP_{50}$ of HIC6E (one unit of the motif) was at least 20

```
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  GCACAGGCCGCAAGCCTGACCGGCACCGCCGAGCAGAAGGCCACTGAGGCCAAGCACGAT  180
      A  Q  A  A  S  L  T  G  T  A  E  Q  K  A  T  E  A  K  H  D
181  GCCAACCGCCAGGGCGGTGGTGTTGTCGACGACATCAAGGGTGCTGCTGCTGAGGCCCAG  240
      A  N  R  Q  G  G  G  V  V  D  D  I  K  G  A  A  A  E  A  Q
241  CACCGCGCAGGCGAGACTGCGGAGAAGGCCAAGCACAACGTGCAGGAGGGCTGGACCGAG  300
      H  R  A  G  E  T  A  E  K  A  K  H  N  V  Q  E  G  W  T  E
301  ACCAAGCACAAGGTCGATGAGGCGCGACCGAACGCGACCCGCTAAGTGTACAGCTTTAGC  360
      T  K  H  K  V  D  E  A  R  P  N  A  T  R  *
361  ACCACGGCCATATGTACTGTCTATCCTTTTAGCACATTCCAGAAGCCAGGCACAGCAGCA  420
421  TGTTATTGGGCATACGGCATGCCGCTAATTTTAGCCGTTTATCGATGCATGTCCTTGACC  480
481  TCCCCTCCTCCCTCTCCTTTGCCCCAAGGCACTAGTGCCATTGCACTATGTACTTGCCTC  540
541  CATCCATGTAAGCAGATGTAGAAAAAAAAAAAAAAAAAAAAAAAA                585
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**Fig. 2.** Nucleic Acid and Deduced Amino Acid Sequences of *hiC12*.

Underlining represents an imperfect 11-residue repeat in the amino acid sequence.

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HIC12:  9 EQISDAVGAAGQKVGETFEAAKAQAASLTGTAEQKATEAKHDANRQGGGVVDDIKGAAAE 68
          •+    +••  •• ••+• •• • +•       +••  •    • ++     •     •
HVA1:  21 EKTGQMMGATKQKAGQTTEATKQKAGETAEATKQKTGETAEAAKQKAAEAKDKTAQTAQA 80

HIC12:69 AQHRAGETAEKAKHNVQEGWTETKHKVDE 97
          •+ +  •••+ ••    +•  +•   + •
HVA1:  81 AKDKTYETAQAAKERAAQGKDQTGSALGE 109
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**Fig. 3.** Alignment of the Deduced Amino Acid Sequence of the HIC12 Protein with that of HVA1 from *Hordeum vulgare*.[21]

Identical amino acids are shown by dots and similar amino acids by crosses.

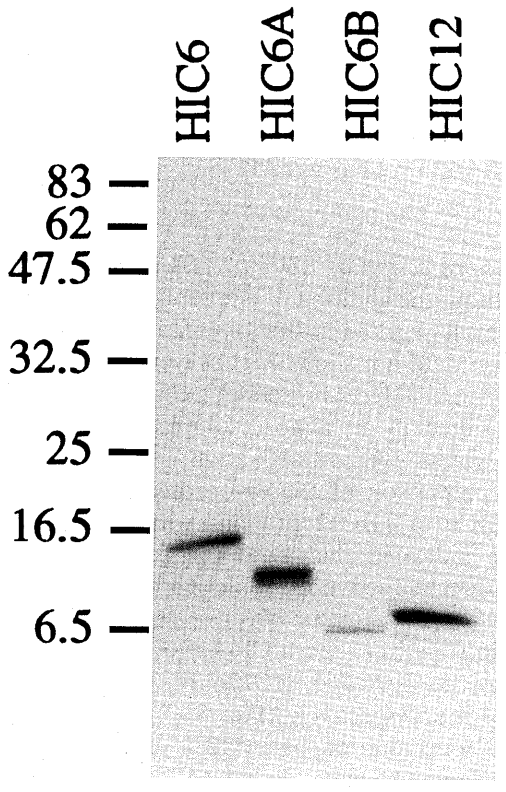


**Fig. 4.** SDS-PAGE Analysis of Purified HIC Proteins from *E. coli*.

Lane 1, HIC6; lane 2, HIC6A; lane 3, HIC6B; lane 4, HIC12. The proteins were separated on a 15% polyacrylamide gel and stained with Coomassie blue R-250.

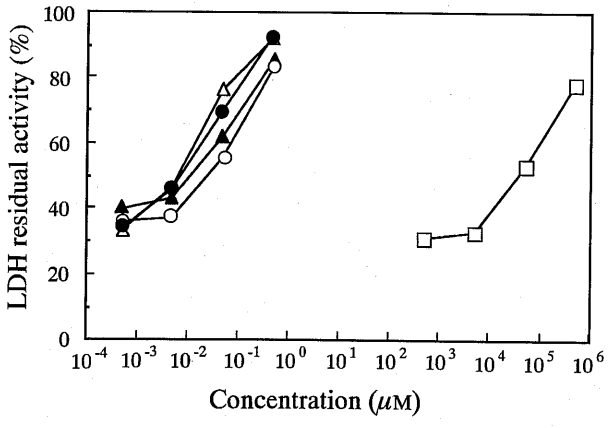


**Fig. 5.** Cryoprotective Activity of HIC6 and HIC12 Proteins on L-Lactate Dehydrogenase.

The curve shows the percentage LDH activity remaining after a freeze/thaw cycle in the presence of different concentrations of HIC6 and HIC12. The concentration of each protein solution was the final concentration during freezing. Each experiment (n) included at least three replicate samples per point.

Symbols: ●, HIC6; ▲, HIC12; △, BSA; ○, Lactalbumin; □, sucrose.

times higher than the other five polypeptides (HIC6, HIC6A, HIC6B, HIC6C, and HIC6D), even on a molar concentration basis (Table). These results suggest that the unit number of the 11-mer amino acid motif is important in the cryoprotection shown by LEA proteins and that the cryoprotective activities of Group 3 LEA proteins were very high because they have larger unit numbers of the 11-mer amino acid motif.

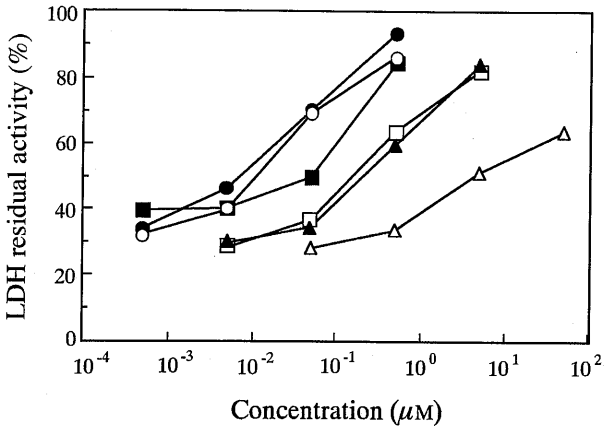


**Fig. 6.** Cryoprotective Activity of Six Kinds of Various Lengths of Polypeptides, Which Were Synthesized Based on HIC6 Protein, for L-Lactate Dehydrogenase.

For other details, see legends to Figs. 1 and 5.

Symbols: ●, HIC6; ○, HIC6A; ■, HIC6B; □, HIC6C; ▲, HIC6D; △, HIC6E.

*Effects of the kinds of the 11-mer motif units on cryoprotection*

Three kinds of single units of the 11-mer motif, each different in amino acid components, were selected (Fig. 1), and their cryoprotective activities were assayed similarly by using LDH (Fig. 7). At 5 μM, the HIC6E has approximately 55% cryoprotection. The $CP_{50}$ of HIC6E, HIC6F, and HIC6G were about 4.3, 6.3, and 7.2 μM, respectively. There were no significant differences in cryoprotection among the three kinds of the 11-mer motif units.

## Discussion

The amino acid sequence of the *hiC12* clone was deduced from its nucleotide sequence (Fig. 2). As we reported,[16] the amino-terminal amino acid sequence of 10-kDa protein, a hardening-inducible protein separated by 2D-HPLC, is AGNKPITEQISDAV-GAAGQKVG- 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 amino acid position 2 of the deduced amino acid sequence. Thus, the HIC12 protein does not seem to have an amino acid sequence like a signal peptide and would be located in the cytoplasm of *Chlorella*.

A computer search showed that the putative HIC12 protein is similar to HVA1 protein, a Group 3 LEA protein (Fig. 3).[21] The HVA1 protein has ten

**Table.** Comparison of $CP_{50}$ of HIC Proteins and Various Agents

| Agents | $CP_{50}$ (nM) | $CP_{50}$ ($\mu$g/ml) | Molecular mass |
|---|---|---|---|
| HIC6 | 7.4 | 0.11 | 14,720 |
| HIC6A | $1.1\times10$ | 0.13 | 11,750 |
| HIC6B | $5.1\times10$ | 0.33 | 6,459 |
| HIC6C | $1.6\times10^2$ | 0.52 | 3,219 |
| HIC6D | $2.1\times10^2$ | 0.46 | 2,204 |
| HIC6E | $4.3\times10^3$ | 5.13 | 1,208 |
| HIC6F | $6.3\times10^3$ | 8.08 | 1,289 |
| HIC6G | $7.2\times10^3$ | 8.72 | 1,215 |
| HIC12 | $1.1\times10$ | 0.11 | 10,800 |
| Bovine Serum Albumin | 6.6 | 0.44 | 66,000 |
| Lactalbumin | $2.5\times10$ | 0.40 | 16,000 |
| Sucrose | $3.4\times10^7$ | $1.2\times10^4$ | 340 |

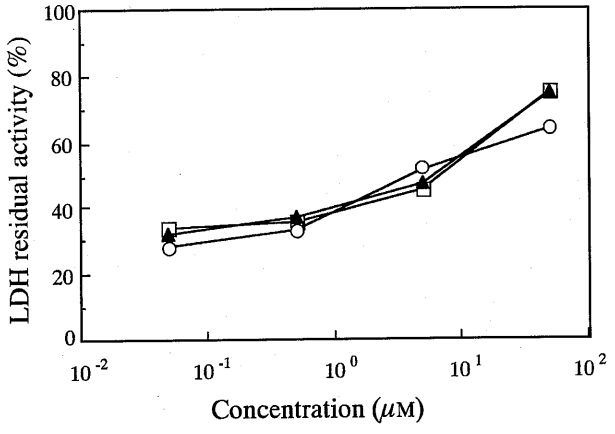


**Fig. 7.** Cryoprotective Activity of Three Kinds of 11-mer Amino Acid Motif for L-Lactate Dehydrogenase.
For other details, see legends to Figs. 1 and 5.
Symbols: ○, HIC6E; ▲, HIC6F; □, HIC6G.

repeating units of the 11-mer amino acid, a characteristic of Group 3 LEA proteins. The HIC12 protein also seems to have six repeats of the 11-mer amino acid motif. However, the motifs of the HIC12 protein are not as clear as those of other Group 3 LEA proteins,[8] in that the amino acid sequences of the motif units did not strictly obey the principle of the 11-mer amino acid motif mentioned in the introduction.

LEA proteins are very hydrophilic[8] and remain soluble even after boiling.[4,22] As we reported,[16] the amino-terminal amino acid sequence of the 14-kDa protein separated by 2D-HPLC is identical with that of the deduced HIC6 protein. Furthermore, both the 10-kDa and 14-kDa proteins remain soluble even after boiling.[16] The solubility of the proteins in a boiling aqueous solution might be due to high hydrophilicity.[23] 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.[5] 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.[5] Since *Chlorella* LEA (HIC6 and HIC12) proteins have the three characteristics described above, the cryoprotective activities of these two proteins was investigated.

As Fig. 5 shows, the two HIC proteins showed effective cryoprotective activities in protecting LDH from freezing inactivation. On a molar concentration basis, they were as effective as BSA, which is recognized as an effective cryoprotectant and stabilizer.[20] Lin and Thomashow[24] also reported that COR15, a cold-regulated polypeptide encoded by the *cor15* gene, has cryoprotective activity. Since the $CP_{50}$ of HIC6 and HIC12 are 7.4 nM and 11 nM, respectively, they are almost equal to that of COR15 (5.6 nM). The enzyme denaturation caused by freezing is considered to be due to aggregation and unfolding or transconformation.[20] Tamiya *et al.*[20] suggested that cryoprotectants, such as BSA, surrounding the enzyme molecules give rise to increased hydration and protect them from aggregation and transconformation. The HIC proteins seem to play a similar role *in vivo*, as one of their possible functions.

To make clear the mechanism of cryoprotection of LDH by the HIC proteins, we tried to investigate the interaction between LDH and the HIC proteins by using gel filtration chromatography. However, we could not obtain a result indicating any direct interaction between them (data not shown). The HIC proteins do not appear to affect LDH directly for their cryoprotective activities. The relative contents of hydrophilic amino acids in HIC6, HIC12, BSA, and lactalbumin were 85.2, 87.4, 65.9, and 58.4%, respectively.[1,25,26] On a weight concentration basis, cryoprotective activities of the two HIC proteins were approximately four times higher than those of BSA and lactalbumin (Table). Thus, the higher the content of hydrophilic amino acids in a protein is, the more cryoprotective the protein seems to be. The cryoprotection by the HIC proteins seems to be relative to the levels of their hydrophilicity, so they might protect LDH by preventing the removal of water molecules on freezing.

The HIC6 and HIC12 proteins were also found to be effective in protecting malate dehydrogenase against freeze-inactivation. $CP_{50}$ of HIC6 and HIC12 were about 7.5 nM and 9.2 nM on malate dehydrogenase, respectively (data not shown). In contrast, HIC6 or HIC12 at a concentration of approximately 1 $\mu$M led to no cryoprotection for alcohol dehydrogenase (ADH; data not shown). Out of the three enzymes, only ADH is a metalloenzyme; it contains a zinc-thiolate center at the active site.[27] It was reported that the zinc-thiolate center is essential for maintaining the enzyme activity since ADH is inactivated by zinc release and thiol oxidation,[27] which might not be protected by HIC6 and HIC12 during frozen storage.

The relationship between cryoprotection and the unit number of the 11-mer amino acid motif in HIC6 was investigated by using various lengths of polypeptides, synthesized based on the sequence of the HIC6 protein (Fig. 1). Cryoprotective activity decreased with a decrease in the unit number of 11-mer motifs (Fig. 6). Polypeptides with a length of more than one unit of the motif had higher cryoprotective activities. On a weight concentration basis, cryoprotective activity of a dimer (HIC6D) was similar to that of BSA (Table). As Tamiya *et al.*[20] suggested, cryoprotective proteins would be necessary to have a sufficient length of polypeptide in order to surround the freeze-labile enzymes and protect them.

Three kinds of polypeptides (HIC6E, HIC6F, and HIC6G), each having only one unit of the motif with different amino acid components, were selected and assayed for cryoprotection (Fig. 1). There were little differences in cryoprotective activities among these peptides (Fig. 7), indicating that the cryoprotective activities of the peptides were independent of the components making up the 11-mer amino acid motif. Furthermore, their cryoprotective activities were much lower than those of the HIC proteins. Actually, the $CP_{50}$ of the three peptides were at least 20 times higher than those of HIC proteins. The lack of cryoprotection for LDH by either single unit of the 11-mer motif even at high levels indicated that more than one unit of the motifs is required for the high activity of cryoprotection. In other words, simple presence of single motif units in large amounts is ineffective for cryoprotection.

Group 3 LEA proteins are also correlated with water-deficit stress.[8] Dure[10] proposed that D-7, a group 3 LEA protein from cotton, functions as an ion sequestrator. Imai *et al.*[28] suggested that LE25, a group 4 LEA, protein functions as an ion scavenger. Sequestration or scavenging of ions seems to be one of the important functions of LEA proteins, although the functions of LEA proteins have not been clarified. This study showed that the Group 3 LEA (HIC6 and HIC12) proteins have cryoprotective activities, as does BSA.

This paper also showed that the repeat number of the 11-mer amino acid motif in group 3 LEA proteins is responsible for the cryoprotective activity of the LEA proteins. The construction of transgenic plants expressing the LEA (HIC6) protein, and further characterization of the protein will be presented soon.

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