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Changes in Soluble and Membrane Proteins of *Chlorella ellipsoidea* during Early Time of Hardening

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Soluble and membrane proteins from *Chlorella ellipsoidea* Gerneck (IAM C-27) during the early time of hardening were separated by two-dimensional gel electrophoresis and visualized by silver staining. The analysis of soluble protein showed that 14 polypeptides newly appeared and 3 polypeptides increased in abundance within 24 h of cold treatment. Three polypeptides having the molecular weight 80 kDa (pI 5.58), 82 kDa (pI 5.46) and 66 kDa (pI 6.11) were detected after a 6-h or a 9-h hardening and markedly increased as the time of hardening proceeded. The result suggests that they may play an important role for the development of cold hardiness. One small polypeptide (29.8 kDa, pI 4.44) was detectable in membrane fraction of 12 h-hardened cells. Membrane protein of 29.2 kDa (pI 5.05) was pronounced after a 12-h hardening. Two major groups of membrane polypeptide (50.8-55.4 kDa, pI 5.29-5.76 and 50.8-55.4 kDa, pI 6.70-7.10) disappeared after a 3-h hardening and were not observed following a longer hardening period, suggesting the rapid degradation of these membrane polypeptides during cold treatment.

INTRODUCTION

Many plants become more resistant to freezing temperature when first exposed to low, nonfreezing temperatures (Graham and Patterson, 1982). A number of reports have shown physiological and biological changes in plants during cold treatment and it has been suggested that these changes are mediated by altered gene expression (Guy *et al.*, 1985 ; Meza-Basso *et al.*, 1986; Mohapatra *et al.*, 1987 ; Harn and Walbot, 1989). Recently mRNAs, which were induced during cold treatment, were cloned from tomato (Schaffer and Fischer, 1988), alfalfa (Mohapatra *et al.*, 1989), and barley (Cattivelli and Bartels, 1990). However, the function and the cellular localization of the proteins encoded by the cloned mRNAs are unknown. The isolation and characterization of genes associated with the development of frost hardiness is important to clarify the regulation of the mechanism. On the other hand, the purification and characterization of proteins associated with the development and maintenance of frost hardiness help to understand their roles and functions during hardening.

We have used *Chlorella ellipsoidea* Gerneck (IAM C-27) for studying on freezing tolerance. *Chlorella* species are suitable for cold-hardiness research : The synchronized cells are uniform and the cell cycle is much shorter than higher plants. In addition, a broad cold tolerance spectrum is available in *Chlorella* species. Our previous papers have demonstrated that RNA and protein syntheses and lipid changes are involved in the hardening process (Hatano, 1978 ; Hatano, *et al.*, 1981). These results suggest that

Chlorella cells can respond to hardening by inducing the synthesis of specific proteins. It is not yet understood what kind of protein is responsible for triggering induction of frost hardiness. We have planned to separate systematically the soluble or membrane proteins from *Chlorella* cells during early phase of hardening and to identify the proteins which involve the initiation and development of frost hardiness. It is requested to know changes in soluble or membrane proteins during hardening by two-dimensional electrophoresis before the systematic separation of their proteins.

We report data that the pattern of soluble or membrane proteins in *Chlorella ellipsoidea* Gerneck (IAM C-27) is changed during early phase of hardening.

MATERIALS AND METHODS

Plant material

Chlorella ellipsoidea Gerneck (IAM C-27) was synchronously grown in the MC medium of Watanabe (1960). Thus, the cells were cultured at 25°C, under 9 to 10 kilolux, with 1.3% CO₂-air, at a concentration of about 5×10^9 cells/l to 15×10^9 cells/l and 16-h light/8-h dark regime. As the cells were hardened most at the L₂ stage (an intermediate stage in the ripening phase of the cell cycle), L₂ cells were used for this experiment. Cold treatment at 3°C was carried out as described for the time periods mentioned in the text or figure legends.

Tests of frost hardiness were carried out as described previously (Hatano et al., 1982).

Protein extraction

The *Chlorella* samples were removed from -80°C storage, and suspended in 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 1 mM PMSF and disrupted at 0°C in a Vibrogen-Zellmühle. The homogenate was filtered through a glass funnel. The filtrate was centrifuged at 13,800 X g for 20 min and the cell debris was removed by the centrifugation. Some of the supernatants were dialyzed against deionized water and lyophilized for SDS-polyacrylamide gel electrophoresis. The remaining supernatant was recentrifuged at 165,000 X g for 90 min. The supernatant obtained was used as the soluble protein fraction and the pellet, which contained mainly microsomes and plasma membranes, was used as the source of membrane proteins. The supernatant was dialyzed against deionized water and lyophilized. Protein was determined by the dye-binding method using bovine serum albumin as a standard (Bradford, 1976).

SDS-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as a Laemmli's method (1970). The samples were dissolved in Laemmli sample solubilizing buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol). The each protein sample (30 µg of protein) from 13,800 X g supernatant was analyzed in 10 % polyacrylamide gel containing 0.1% SDS utilizing a 12 cm separating gel.

For two-dimensional gel electrophoresis, a O'Farrell system (1975) was used. The lyophilized soluble protein and membranes were solubilized in a lysis buffer containing 9 M urea, 3% Nonidet P-40 and 2% Ampholine (pH 3.5-10). Isoelectric focusing was

performed in g-cm-long, 1.5-mm-diameter rod gels using 4.2% polyacrylamide containing 9 M urea, 3% Nonidet P-40, and 2% Ampholine (pH 3.5-10). The isoelectric focusing was run for 4,550 V·h. The samples (60 μ g protein) were loaded on the gels. After migration, the gels were equilibrated in second dimension sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol) and loaded onto SDS-PAGE (10%) gels utilizing a 6 cm separating gel. The gels were fixed and silverstained (Oakley *et al.*, 1980).

RESULTS

SDS-PAGE of crude extract proteins during hardening

Changes in protein patterns of the crude extract during hardening was monitored with SDS-PAGE (Fig. 1). Between 9 and 24 h after beginning of the cold treatment, the polypeptide having molecular weight about 80 kDa markedly increased. However, the resolution of the gels was not good enough to determine how many polypeptides were affected.

Changes in soluble protein patterns during hardening

The samples were compared using a two-dimensional gel electrophoresis system. Changes in the soluble protein population are shown in Fig. 2. About 280 polypeptide spots could be resolved in this system.

Many polypeptides appeared and disappeared during hardening. Since cold tolerance is closely related with induction of specific proteins during cold treatment (Guy

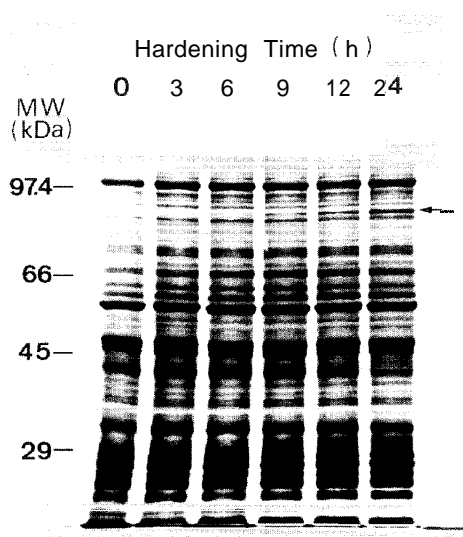


Fig. 1. SDS-PAGE of crude extract proteins from *Chlorella ellipsoidea* during hardening. Changing band is indicated by arrow on the right side of the electrophoregraph. Numbers in the left margin indicate molecular weight of the marker proteins.

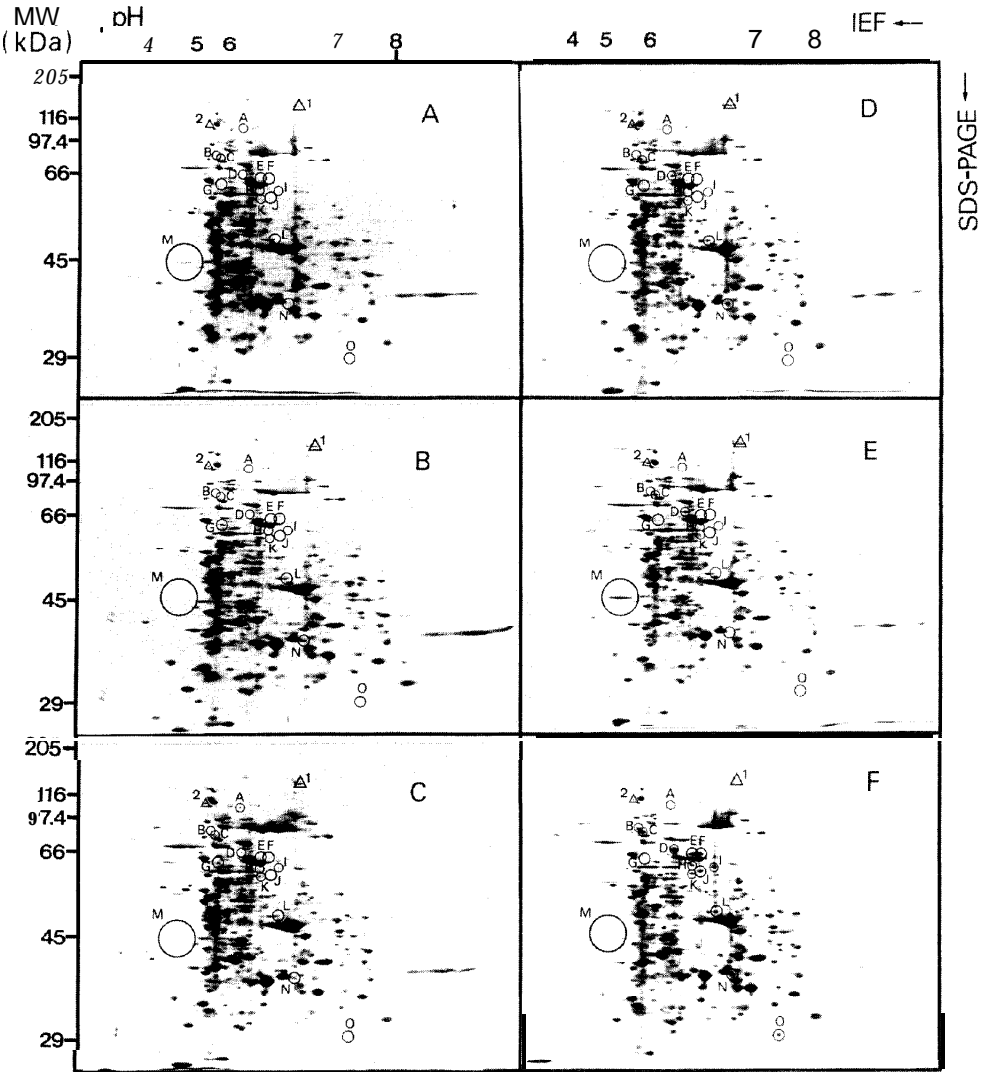


Fig. 2. Two-dimensional electrophoresis of soluble proteins extracted from cold-treated *Chlorella ellipsoidea* cells. Hardening time : A, 0 h ; B, 3 h ; C, 6 h ; D, 9 h ; E, 12 h ; F, 24 h. Circles and triangles indicate polypeptides which newly appeared or increased respectively during hardening.

and Haskell, 1987), we did not summarize the soluble proteins in this report which disappeared during hardening. A summary of the relative molecular weight masses and isoelectric points of the proteins that newly appeared and increased in abundance is given in Table 1. Spots alphabeted A to N (open circles) are undetectable on the gel of unhardened sample and indicate polypeptides newly appeared after a temperature shift. The relative intensity of spots numbered 1 to 3 (open triangles) increased after a cold treatment and these spots were observed on the control gel (hardening 0 h). A majority of the spots from hardened samples were common to proteins isolated from unhardened sample. Seventeen soluble polypeptides newly appeared or increased during a 24-h hardening. Of these, two polypeptides (spots G [63 kDa, pI 5.751; M [38 kDa, pI 6.591) newly appeared after a 3-h hardening. The polypeptide of 63 kDa (pI 5.75) was not observed after a 9-h hardening. Two high molecular weight polypeptides (spots A [106 kDa, pI 6.071 ; C [80 kDa, pI 5.581) were detectable in the sample of a 6-h hardening and consistently induced following a longer hardening period. The 80 kDa polypeptide indicated the marked increase following cold treatment. Two proteins (spots B [82 kDa, pI 5.461; D [66 kDa, pI 6.111) appeared after a 9-h hardening and the abundance increased following a longer hardening period. Especially the intensity of the 82 kDa polypeptide was pronounced after a 24-h hardening. Eight polypeptides (spots E [64 kDa, pI 6.351; F [64 kDa, pI 6.301 ; H [61 kDa, pI 6.301; I [61 kDa, pI 6.441; J [60 kDa, pI 6.371; K [59 kDa, pI 6.301; L [50 kDa, pI 6.481 ; N [27 kDa, pI 7.131) were detectable only in 24-h hardened sample. Of these, seven polypeptides except for the one of 27 kDa (pI 7.13) had the molecular weights ranging from 50 to 64 kDa and isoelectric points ranging from 6.30 to 6.48. Abundance of two high molecular weight polypeptides (spots 1 [141 kDa, pI 6.671; 2 [110 kDa, pI 5.421) increased after temperature shift. However, the abundance of these two polypeptides after a 24-h hardening was depressed at the level of unhardened sample. The polypeptide of 45 kDa

Table 1. Changes in soluble proteins during hardening

spot	MW (kDa)	pI	Hardening time (h)				
			3	6	9	12	24
A	106	6.07					
B	82	5.46				i	
C	80	5.58		i		i	i
D	66	6.11			i		i
E	64	6.35					
F	64	6.30					
G	63	5.75		i			
H	61	6.30					i
I	61	6.44					i
J	60	6.37					i
K	59	6.30					i
L	50	6.48					
M	38	6.59			i		
N	27	7.13					
1	141	6.67	+		+		
2	110	5.42	+				
3	45	4.82					

i, induced ; +, increased.

(spot 3, pI 4.82) temporarily increased after a 12-h hardening.

Changes in membrane protein during hardening

Changes in the membrane protein population are shown in Fig. 3. A summary of the relative molecular weight masses and isoelectric points of the proteins that changed in abundance is given in Table 2. The analysis of membrane polypeptide patterns revealed the remarkable appearance of '29.8 kDa (pI 4.44) after a 12-h hardening. Abundance of 29.2 kDa polypeptide (pI 5.05) increased after 12 h of cold treatment and the accumulation of this polypeptide was more pronounced following a longer hardening period. Both groups of major polypeptides (spots I [50.8-55.4 kDa, pI 5.29-5.76]; II [50.8-55.4 kDa, pI 6.70-7.10]) disappeared after a 3-h hardening and were no longer observed following prolonged treatment.

DISCUSSION

Some reports have shown that Coomassie blue staining of the total proteins extracted from various plants can not detect differences in protein profiles or their relative proportions during the induction of frost hardiness (Johnson-Flanagan and Singh, 1987 ; Cloutier, 1983). Therefore, the silver-staining method was employed in this experiment. In two-dimensional gel electrophoresis, about 280 polypeptides in the soluble fractions were resolved.

We found that, upon exposure to hardening conditions, 14 polypeptides were novel and 3 ones increased in abundance. Of these, five polypeptides having the molecular weight, 106, 82, 80, 63 and 38 kDa were newly induced and 141 kDa and 110 kDa polypeptides increased in abundance between 3 h and 9 h of hardening. The polypeptide (80 kDa, pI 5.58) agreed at the molecular weight and appearance time with the one which markedly increased in SDS-PAGE (Fig. 1). The rapid response of their polypeptides to hardening suggests that they may play an important role for the initiation or the development of frost hardiness.

Induction of high molecular weight polypeptides having 160, 117, and 85 kDa in spinach (Guy and Haskell, 1987), 160 kDa (pI 4.5) in *Arabidopsis thaliana* (Gilmour et al., 1988), 200 kDa (pI 6.85) in wheat (Perras and Sarhan, 1989) have been reported. Five polypeptides (mol wt, 141, 110, 106, 82, and 80 kDa) of 7 polypeptides newly induced or increased during hardening of *Chlorella ellipsoidea*. It is difficult to say whether the mechanism that induces frost hardiness in higher plants and *Chlorella* cell is similar. The cloning of the induced genes is needed to determine the molecular homology of the proteins induced in various kind of plants or *Chlorella ellipsoidea* during cold treatment.

Sadakane et al. (1980) have reported that glucose 6-phosphate dehydrogenase in *Chlorella ellipsoidea* C-27 is highly activated during the early phase of hardening by only low temperature. Recently we have purified dithiothreitol-sensitive glucose 6-phosphate dehydrogenase (55 kDa, pI 5.0 in the two-dimensional electrophoresis) coming from chloroplast (not shown). However, we could not indicate the participation of the enzyme here. This may be due to the sensitivity of the visualization of the spot in the electrophoresed gel.

In membrane fraction, two major membrane polypeptides having molecular

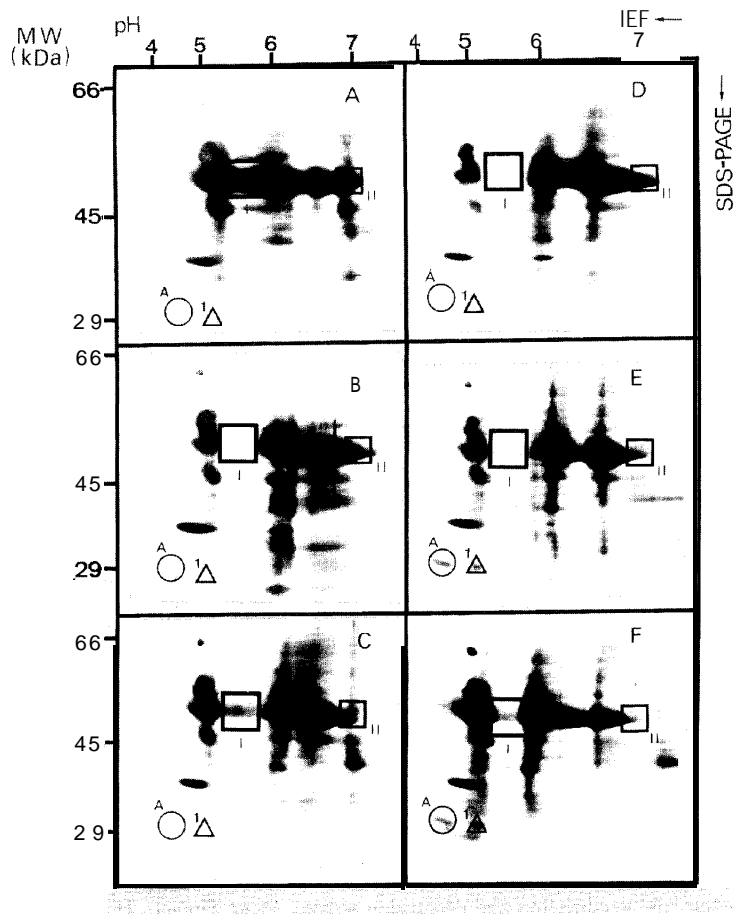


Fig. 3. Two-dimensional electrophoresis of membrane proteins from *Chlorella ellipsoidea* during hardening. Hardening time: A, 0 h; B, 3 h; C, 6 h; D, 9 h; E, 12 h; F, 24 h. Circles and triangles indicate polypeptides which newly appeared or increased respectively during hardening while boxes indicate polypeptides which decreased during hardening.

Table 2. Changes in membrane proteins during hardening

spot	MW (kDa)	PI	Hardening time (h)				
			3	6	9	12	24
A	29.8	5.05				+	+
I	50.8-55.4	5.29-5.76					
II	50.8-55.4	6.70-7.10					

i, induced ; +, increased ; -, decreased.

weight of about 50 to 55 kDa decreased rapidly during hardening, suggesting the degradation of membrane proteins. Steponkus (1984) has reported that plasma membranes are primary site of freezing injury. Shih et al. (1967) have also indicated a positive correlation between membrane protein content and cold hardiness. In spite of the disappearance of both membrane polypeptides, the *Chlorella* cell can maintain and develop frost hardiness. It is possible that newly induced protein may substitute for the disappeared membrane proteins. That is to say, part of frost hardiness in *Chlorella* cells may involve the reorganization membrane proteins by both suppression and induction of specific proteins, resulting in the stabilization of the membrane against low temperature. Mohapatra et al. (1988) have demonstrated that cold acclimation induces two membrane polypeptides in alfalfa seedling membrane. Further experiments are required to clarify the possibility.

In conclusion, the rapid development of frost hardiness in *Chlorella ellipsoidea* was paralleled by changes in soluble and membrane proteins. The appearance of some novel proteins suggests the correlation with frost hardiness. However, the function or the participation of these proteins in the frost hardiness is obscure. Experiments are in progress to separate systematically the proteins induced during early phase of hardening by high performance liquid chromatography. These fractionated proteins will be identified to elucidate the physiological role of the proteins in the induction of cold hardiness in *Chlorella ellipsoidea*. The data concerning the *de novo* synthesis of the novel proteins induced during the early phase of hardening will be reported in the near future.

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