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Changes in Fatty Acid Composition of Membrane Fractions during Hardening of *Chlorella ellipsoidea**

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Chlorella ellipsoidea cells at an intermediate stage in the ripening phase of the cell cycle were hardened at 3℃ for 48 hr. Chloroplast and E. R. fractions were prepared from the homogenate of *Chlorella* cells on sucrose density gradients. Changes in fatty acid composition of chloroplast and E. R. fractions and whole cells during the development of frost hardiness were determined by gas-liquid chromatography. In whole cells and chloroplast fraction, myristic, palmitic and linoleic acids decreased while oleic and linolenic acids increased. In E. R. fraction, myristic, palmitic, oleic and linoleic acids decreased while linolenic acids increased. The percentage of unsaturated fatty acid increased during hardening from 70.1 % to 80.5 % in chloroplast fraction, from 70.4 % to 78.0 % in E. R. fraction and from 73.6% to 81.5% in whole cells. The increase in unsaturation ***was*** mainly due to the increase in linolenic acid.

INTRODUCTION

Recent papers suggest that some profound membrane changes are involved in the development of frost hardiness (Garber and Steponkus, 1976; Steponkus ***et al.,*** 1977; Yoshida, 1976). Since lipids are essential component of membranes, lipid changes during hardening of higher plants have been studied intensively (Gerloff ***et al.,*** 1966; Willemot ***et al.,*** 1977). Particularly, a preferential synthesis of unsaturated fatty acids during hardening has been reported (de la Roche ***et al.,*** 1972; Grenier and Willemot, 1974; de la Roche ***et al.,*** 1975). In young shoots of wheat and rye an increase in linolenic acid was observed, while in alfalfa roots the proportion of linoleic acid increased (de la Roche ***et al.,*** 1972; Grenier and Willemot, 1974; de la Roche ***et al.,*** 1975). Willemot(1977) indicated that low temperature stimulation of linolenic acid synthesis was a prerequisite for the development of freezing resistance in wheat. However, de la

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ABBREVIATION

E. R. : endoplasmic reticulum; DPCO : diphenylcarbazone; DCIP: 2, 6-dichlorophenolindophenol; DCMU: 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea.

Roche (1979) demonstrated that an enrichment in linolenic acid is not a prerequisite for low temperature acclimation in wheat. The question arises whether an increase in fatty acid unsaturation is a prerequisite for the development of frost hardiness in plant or not.

Previous studies have shown that hardened cells of *Chlorella ellipsoidea* are able to survive slow freezing to -196°C (Hatano *et al.*, 1976 a, b) and that amounts of glycolipids, phospholipids and the nonpolar lipid in the cells increase with an increase in the algal hardiness (Kabata *et al.*, 1979).

As a first step in studying the necessity of an increase in fatty acid unsaturation for the development of frost hardiness, changes in fatty acid composition were determined in chloroplast and E. R. fractions and whole cells during hardening of *Chlorella ellipsoidea*.

MATERIALS AND METHODS

Plant materials

Chlorella ellipsoidea Gerneck (IAM C-27) was grown in synchronous culture at 25°C, under 9-10 Klx, with 1 % CO_2-air, and a 28-hr light-14-hr dark regime as described previously (Hatano *et al.*, 1976 a). Since the cells were most hardened at the L_2 stage (an intermediate stage in the ripening phase of the cell cycle), L_2 cells were used in this study.

Hardening

Algal cells synchronized at 25°C were directly hardened at 3°C for 48 hr. During treatment, the culture was aerated with air enriched to about 1 % CO_2 and kept in the light (9–10 Klx) as described previously (Hatano *et al.*, 1976a).

Determination of viability

The viability of algal cells was determined with the growth curve on the basis of A_{420}. Previous study has demonstrated that the viability determined with the growth curve coincided with that determined by both colony count and packed cell volume.

Sucrose gradient centrifugation

About 5 x 10^9 cells of the algae were suspended in 7.5 ml of 0.05 M phosphate buffer (pH 7.8) containing 0.4 M sucrose and 0.01 M NaCl. The suspension was homogenized with glass beads of 0.5 mm diameter in a reciprocal shaker, Vibrogen-Zellmtihle (Edmund Bühler Co., Tübingen, Germany), at 4,500 rpm at 3°C for 10 min in unhardened cells and for 6min in hardened cells. The rate of disrupted cells was about 80-90 % in unhardened and hardened cells. The homogenate was centrifuged at 200×g for 10 min to remove the glass beads, whole cells and cell debris. The supernatant was centrifuged at 9, 000×g for 30min at 4°C.

The supernatant obtained by centrifugation at 9, 000×g was diluted twice with 0.05 M phosphate buffer (pH 7.8) containing 0.01 M NaCl and centrifuged at 105, 000×g for 1 hr at 4°C in an RP 50-2 rotor with a Hitachi 55 P–2 ultracentrifuge. The precipitate was used as E. R. fraction.

The pellet obtained by centrifugation at 9, OOOxg was resuspended in 0.9 ml of 0.05 M phosphate buffer (pH 7.8) containing 0.4 M sucrose and 0.01 M NaCl. The suspension (0.3 ml) was loaded onto a discontinuous sucrose gradient. Discontinuous sucrose gradients were prepared by layering in succession 1 ml each of 1. OM, 1.5 M, 2. OM and 2.5 M sucrose solution in 0.05 M phosphate buffer (pH 7.8) containing 0.01 M NaCl. The gradients were centrifuged at 64, 000 × g for 1.5 hr at 4°C in a Hitachi RPS-50 rotor. After centrifugation, each fraction was collected for subsequent photochemical activity assays.

Measurements of photochemical activities

Photosystem I activity was measured by DPCO disproportionation at 487 nm according to the method described by Vernon (1972). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 2.5 mM DPCO, 10 μM DCMU and chloroplasts (30 μg chlorophyll). Photosystem II activity was measured by DCIP photoreduction at 610 nm according to the method described by Hirayama and Matui (1976). The reaction mixture (3 ml) contained 50 mM Tris-HCl (pH 8.0), 0.1 mM DCIP, 10 mM NaCl and chloroplasts (30 μg chlorophyll). Chlorophyll was measured by the method of Mackinney (1941).

Lipid extraction and fatty acid assay

Total lipids were extracted from chloroplast and E. R. fractions and whole cells and the nonlipid contaminants were removed as previously reported (Kabata *et al.*, 1979). Fatty acid composition of each extract was determined by gas chromatography of the methyl esters. Methanolysis of fatty acids was performed according to the method of Stoffel et al. (1959) with a slight modification. After cooling, the methyl ester samples were extracted with n-hexane and chromatographed on a Hitachi model K53 equipped with a hydrogen flame ionization detector at 0.7 kg/cm^2 N_2 flow. The column (1 m x 3 mm) was packed with 15 % ethylene glycol succinate polyester (80/100 mesh). The column temperature was programmed from 180 to 230°C at a rate of 5°C/min.

RESULTS AND DISCUSSION

The membrane fragments of chloroplasts in unhardened and hardened cells obtained by centrifugation at 9, OOOxg were subdivided into fractions 1, 2 and 3 by discontinuous sucrose gradient centrifugation at 64, OOOxg, as shown in Fig. 1. The volume of fraction 1 in hardened cells was more than that in unhardened cells, while the volumes of fractions 2 and 3 in hardened cells were less than those in unhardened cells. These results suggest that a specific gravity of membrane fragments decrease during the development of the algal hardiness.

Tables 1 and 2 show photochemical activities of three fractions in the 9, OOOxg pellet of unhardened and hardened cells, respectively. In both photosystem I and II activities, fraction 1 showed higher values than fractions 2 and 3. Therefore, fraction 1 was used as chloroplast fraction to assay the fatty acid composition of chloroplast membranes. However, the O_2-uptake ac-

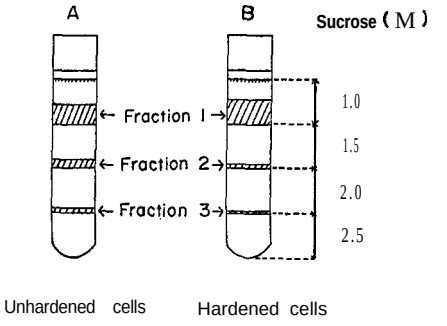

Fig. 1. Profile of fractions, on discontinuous sucrose gradient, obtained after disruption of *Chlorella* cells.

Table 1. Photochemical activities of three fractions in the 9,000×g pellet of unhardened cells.

	Photosystem I activity (DPCO reacting)	Photosystem II activity (DCIP reduced)
	μmoles/mg chl./hr	
Fraction 1	292	41
Fraction 2	217	38
Fraction 3	206	28

Table 2. Photochemical activities of three fractions in the 9,000×g pellet of hardened cells.

	Photosystem I activity (DPCO reacting)	Photosystem II activity (DCIP reduced)
	μmoles/mg chl./hr	
Fraction 1	307	46
Fraction 2	221	39
Fraction 3	—	—

tivity and ***cytochrome c*** oxidase activity were widespread in sucrose gradient solutions in both unhardened and hardened cells (data not shown). Fraction 1 contains some of mitochondria.

Table 3 shows the fatty acid composition of total lipids from unhardened and hardened cells. In *Chlorella ellipsoidea*, palmitic, oleic and linolenic acids were the major constituents of fatty acids. During hardening, myristic, palmitic and linoleic acids decreased while oleic and linolenic acids increased. The percentage of unsaturated fatty acid increased from 73.6 % to 81.5 % during hardening. The increase in unsaturation was due to oleic and linolenic acids.

Table 4 shows the fatty acid composition of chloroplast fraction from unhardened and hardened cells. In chloroplast fraction, myristic, palmitic and linoleic acids decreased while oleic and linolenic acids increased during hardening. The percentage of unsaturated fatty acid increased from 70.1% to 80.5 % during hardening. The increase in unsaturation was mainly due to the

Table 3. Relative fatty acid composition of unhardened and hardened cells. Relative percent: mean value with standard error (n=3).

Fatty acid	Relative percent of fatty acid content	
	Unhardened cells	Hardened cells
14 : 0	2.5±0.15	0.7±0.03"
16 : 0	23.7±0.18	17.7±0.26*
18 : 0	0.2±0.03	0.1±0.03
18 : 1	26.6±0.20	33.6±0.49*
18 : 2	12.7±0.33	8.4±0.12*
18 : 3	34.3±0.20	39.4±0.23*
Saturated %	26.4±0.28	18.5±0.21*
Polyunsaturated %	73.6±0.34	*81.51-o.* 27*

(*P<0.001)

Table 4. Relative fatty acid composition of chloroplast fraction from unhardened and hardened cells. Relative percent: mean value with standard error (n=3).

Fatty acid	Relative percent of fatty acid content	
	Unhardened cells	Hardened cells
14 : 0	7.8±0.15	2.5±0.10"
16 : 0	21.6±0.16	16.9±0.26*
18 : 0	0.6±0.05	0.2±0.03*
18 : 1	34.3±0.21	35.7±0.32***
18 : 2	7.6±0.10	6.2±0.35***
18 : 3	28.1 ±0.18	38.6±0.46*
Saturated %	29.9±0.21	19.5±0.31*
Polyunsaturated %	70.1±0.25	80.5±0.42*

(*P<0.001. *** P<0.05)

linolenic acid. Peoples ***et al.*** (1978) reported the hypothesis that the photosynthetic response is influenced by the unsaturated fatty acid composition of the chloroplast membrane which affect temperature-induced phase changes in chloroplast membrane lipids in alfalfa. The results in Table 4 support the hypothesis.

Table 5 shows the fatty acid composition of E. R. fraction from unhardened and hardened cells. During hardening, linolenic acid increased but myristic, palmitic, oleic and linoleic acids decreased. The difference in fatty acid composition between chloroplast fraction and E. R. fraction suggests that the fatty acid composition of cellular membrane for freezing tolerance differs in organelles. The percentage of unsaturated fatty acid increased from 70.4% to 78.0% during hardening. The large increase in linolenic acid contributed toward changing the composition of membrane lipids in *Chlorella ellipsoidea*. These results suggest that the increase in fatty acid unsaturation due to linolenic acid plays an important role in the hardening process.

Willemot (1977) indicated that low temperature stimulation of linolenic acid synthesis was a prerequisite for the development of freezing resistance in wheat.

Table 5. Relative fatty acid composition of E. R. fraction from unhardened and hardened cells. Relative percent: mean value with standard error (n=3).

Fatty acid	Relative percent of fatty acid content	
	Unhardened cells	Hardened cells
14 : 0	4.0±0.10	1.6±0.29**
16 : 0	25.5±0.25	20.1±0.28"
18 : 0	0.2±0.05	0.3±0.06
18 : 1	41.7±0.35	32.2±0.72*
18 : 2	10.0±0.20	9.4±0.12***
18 : 3	18.6±0.26	36.4±0.90*
Saturated %	29.6±0.25	22.0±0.32*
Polyunsaturated %	70.4±0.35	***78.0***±0.36*

(* P<0.001. ** P<0.01, *** P<0.05)

However, de la Roche (1979) demonstrated that an enrichment in linolenic acid is not a prerequisite for low temperature acclimation in wheat. Their experimental conditions differ from each other. Willemot (1977) hardened winter wheat ***(Triticum aestivum)*** in an 8-hr light-16-hr dark regime while de la Roche (1979) in the dark. At present, we can not determine whether an increase in fatty acid unsaturation is the necessity for the development of frost hardiness in ***Chlorella.*** A previous study demonstrated that ***Chlorella*** cells are also hardened in the dark in the presence of 0.1% glucose (Hatano *et al., 1978).* Comparative studies between the hardening process in the dark and light are required to elucidate the necessity of an increase in fatty acid unsaturation for the development of frost hardiness.

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