貯蔵されたホウレンソウの黄化に伴う極性脂質及び構成脂肪酸組成の変化

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Polar Lipids Content and their Fatty Acid Composition with Reference to Yellowing of Stored Spinach Leaves[1]

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Summary

Changes in the content of polar lipids and in their fatty acid composition in spinach leaves during storage were studied in order to clarify the mechanism of leaf-yellowing.

The total glycolipids, particularly monogalactosyldiglyceride (MGDG) and digalactosyldiglyceride (DGDG), decreased markedly during storage at 25°C. The total phospholipids, however, decreased moderately during storage at 25°C, the decrease in phosphatidylglycerol (PG) being the most notable.

In the chloroplast lipids (MGDG, DGDG and PG), the relative amounts of hexadecatrienoic acid in MGDG, and palmitoleic acid and linolenic acid in PG decreased during storage at 25°C. Conversely, the relative amount of linoleic acid in MGDG, DGDG and PG increased. The percentage of unsaturated fatty acids in all the polar lipids, however, showed hardly any change.

From these results, it is inferred that the degradation of chlorophylls may be caused by the hydroperoxides of free fatty acids which have been formed by the degradation of polar lipids such as MGDG, DGDG and PG.

Introduction

Foliage yellowing occurs in leaf vegetables soon after harvest. This phenomenon is one of the main factors responsible for deterioration of quality. In a previous paper we reported that the TBA (thiobarbituric acid) value, an index of the degree of lipid peroxidation, increased with progressing senescence of stored spinach leaves, suggesting that the degradation of polar lipids may occur in the leaves and that this change may be involved in chlorophyll degradation(24). Dhindsa *et al.* found that the increase in the TBA value was parallel to that of chlorophyll degradation in senescing tobacco leaves(3). Maruyama *et al.* also found the same change in stored parsley leaves(18).

It has been reported that the content of polar lipids decreases with advancing senescence in leaves (6, 7, 11, 16, 18). Fong and Heath observed that the chloroplast lipids such as monogalactosyldiglyceride, digalactosyldiglyceride and phosphatidylglycerol, showed a marked decrease with senescence in bean leaves(7). Koiwai *et al.* also found a decrease in chloroplast lipids with the progression of senescence in tobacco leaves(16).

In this paper we report the changes in the content of polar lipids and in their fatty acid composition in relation to yellowing of stored spinach leaves.

Materials and methods

Materials

Mature leaves, located from the 8th to 10th from the outside of the true leaves, of each spinach plant (*Spinacia oleracea* L., cv. Atlas) were used. The leaves including leafstalks were stored immediately after har-

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vest in closed containers (0.045 m^3) at 1°C and 25°C in darkness under humidified air flowing at 100 ml per minute. Leaf blades without midribs were used in the following analysis.

Extraction of polar lipids

Spinach leaves (10 g) were frozen with liquid nitrogen, and powdered. The powdered leaves were boiled in 40 ml of methyl alcohol for 3 min. and homogenized after adding 40 ml of chloroform. The homogenate was filtered and the residue was re-extracted with chloroform-methyl alcohol (1 : 1, v/v) solution until no chlorophyll was visible in the residue. The pooled extract was separated into aliphatic and aqueous phases by the addition of distilled water. The aliphatic phase was evaporated to dryness under reduced pressure, dissolved in a small volume of chloroform, and stored in a collodion sealed tube under N_2 at −20°C.

Isolation of polar lipids

Glycolipids

Glycolipids were separated from the polar lipid extract using one-dimensional silica gel TLC (300 μm) according to Maruyama *et al.* (18). The solvent consisted of chloroform-methyl alcohol-distilled water (70 : 22 : 3, v/v). Each glycolipid was identified by spraying with I_2 and anthrone and determining the Rf value(8, 18).

Phospholipids

Phospholipids were separated from the polar lipid extract using two-dimentional silica gel TLC (300 μm) according to Allen and Good(1). The TLC was developed with chloroform-methyl alcohol-distilled water(65 : 25 : 4, v/v) in the first direction and with chloroform-methyl alcohol-isopropylamine-ammonia hydroxide (27%) (65 : 25 : 0.5 : 4, v/v) in the second direction. Each phospholipid was identified by spraying with I_2, Dittmer reagent, ninhydrin reagent and Dragendorff reagent and determining the Rf value(1, 8).

Determination of polar lipids

After each polar lipid was detected by spraying I_2 onto the silica gel plate, the plate was dried in a draft chamber to evaporate the I_2. Each polar lipid area was scraped from the plate, extracted twice with a small volume of chloroform-methyl alcohol (1 : 1, v/v) solution, and analyzed for either Pi for phospholipids(21) or sugar for glycolipids (5).

Total glycolipids and phospholipids were directly estimated from the polar lipid extract by the analytical methods described above.

All the values shown are the average of three measurements.

Fatty acid composition

Fatty acid composition was analyzed by gas-liquid chromatography (GLC) (23). After separating the polar lipid extract by two-dimentional TLC, each glycolipid and phospholipid was detected by spraying with I_2. The plate was then dried in a draft chamber to evaporate I_2. Each polar lipid was scraped, extracted twice with chloroform-methyl alcohol (1 : 1, v/v) solution, and evaporated to dryness under reduced pressure. It was then saponified with 5% KOH-methyl alcohol solution for 24 hrs, and non-saponified substances were removed by the addition diethyl ether and distilled water. After acidifying the aqueous phase, by adding a small volume of conc. HCl, the fatty acids were extracted twice with diethyl ether, and evaporated. The fatty acids were methylated with a solution of diazomethane in diethyl ether and analyzed by GLC using a stainless steel column (3 mm × 1.5 m) packed with 10% DEGS on Chromosorb W. The column temperature was programmed from 160°C to 190°C at 4°C/min., the injection and detector temperatures were 220°C and 260°C, respectively, and the carrier gas was N_2 at a flow rate of 15 ml/min.

All the values shown are the average of three measurements.

Results

Changes of polar lipids in stored spinach leaves

At 25°C, the total glycolipid content decreased continuously from 1 day after the beginning of storage, declining to half the initial value after 6 days. No clear change was found at 1°C (Fig. 1). TLC analysis showed that the major glycolipids of spinach leaves were monogalactosyldiglyceride

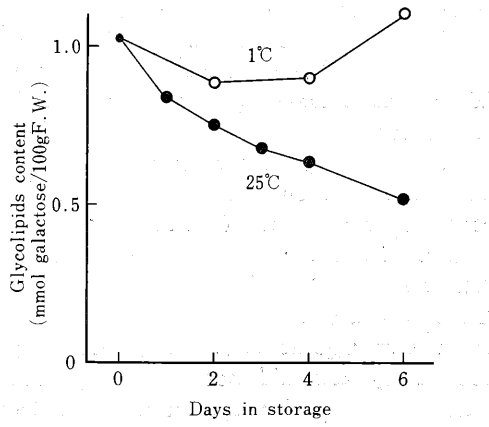

Fig. 1. Changes of total glycolipids content in spinach leaves during storage.

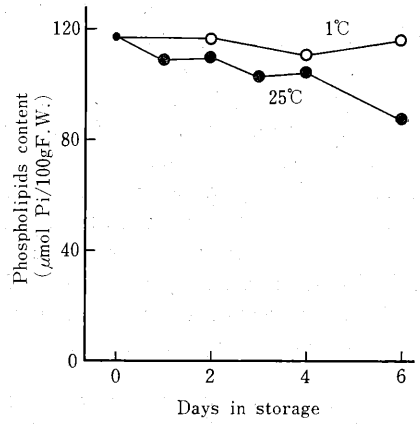

Fig. 3. Changes of total phospholipids content in spinach leaves during storage.

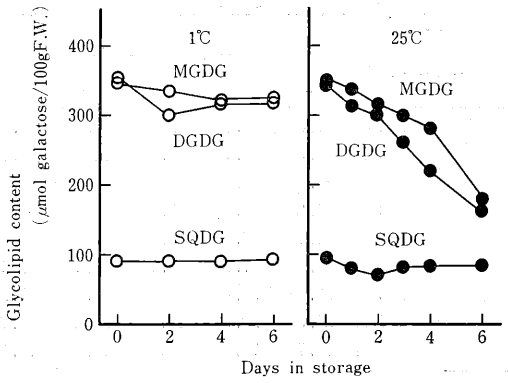

Fig. 2. Changes of each glycolipid content in stored spinach leaves.
MGDG : Monogalactosyldiglyceride, DGDG : Digalactosyldiglyceride, SQDG : Sulfoquinovosyldiglyceride.
DGDG shows duplicate value since it contains two molecules of galactose in a molecule.

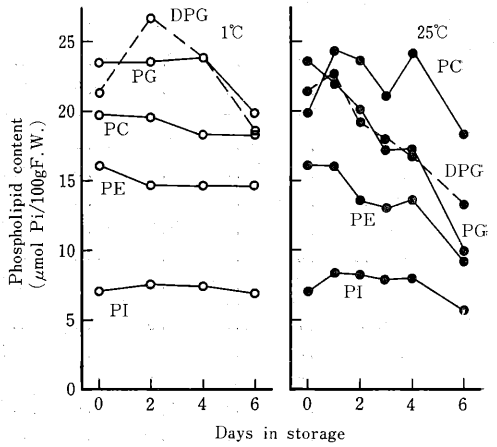

Fig. 4. Changes of each phospholipid content in stored spinach leaves.
PG : Phosphatidylglycerol, DPG : Diphosphatidylglycerol, PC : Phosphatidylcholine, PE : Phosphatidylethanolamine, PI : Phosphatidylinositol.
DPG shows duplicate value since it contains two molecules of Pi in a molecule.

(MGDG), digalactosyldiglyceride (DGDG) and sulfoquinovosyldiglyceride (SQDG). The changes in their contents are shown in Fig. 2. The contents of MGDG and DGDG closely followed the pattern of decline of total glycolipids content during storage, while the content of SQDG showed almost no change even at 25°C. Thus the decrease of total glycolipids at 25°C storage was largely attributable to the decrease of MGDG and DGDG.

As shown in Fig. 3, the content of total phospholipids decreased only moderately during the storage at 25°C and did not change during the storage at 1°C. The major phospholipids were found to be phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The contents of PG, DPG and PE, particularly PG, decreased throughout the storage at 25°C, as shown in Fig. 4. None of the phospholipids showed a clear change during the storage at 1°C.

Changes in fatty acid composition in stored spinach leaves

The polar lipids of spinach leaves contained myristic acid (14 : 0), palmitic acid (16 : 0),

palmitoleic acid (16 : 1), hexadecatrienoic acid (16 : 3), oleic acid (18 : 1), linoleic acid (18 : 2) and linolenic acid (18 : 3) as the major fatty acids (Table 1, 2 and 3). Although the retention time of the peak of 16 : 3 coincided with that of stearic acid (18 : 0) on GLC, it was regarded as being 16 : 3 since it is known that 18 : 0 scarcely occurs in spinach leaves, while 16 : 3 is a fatty acid characteristic to spinach leaves(12).

Table 1 shows the changes in the fatty acid composition of glycolipids in spinach leaves during the storage at 25°C. In MGDG, the proportions of 16 : 3 and 18 : 3, were high and the particularly high level of 16 : 3 was characteristic of this glycolipid. Unsaturated fatty acids (UFA) accounted for most of the fatty acids during storage at 25°C. The relative amount of 18 : 2increased during storage, while that of 16 : 3 decreased gradually. In DGDG, the predominant fatty acid was 18 : 3. The relative amount of UFA was also high, and changed little during the storage at 25°C. The percentage of each fatty acid did not show any marked change, except for the increase in 18 : 2.

Table 1. Changes of fatty acid composition of glycolipids in spinach leaves during the storage at 25°C.

Glycolipid		Days in storage 0	1	2	4	6
MGDG	14 : 0	tr	0.9	0.8	0.9	1.0
	16 : 0	1.4	2.7	3.6	2.5	2.9
	16 : 1	tr	tr	tr	tr	tr
	16 : 3	36.5	35.3	33.4	31.2	28.0
	18 : 1	0.6	0.8	2.4	1.0	1.7
	18 : 2	1.6	1.6	3.5	4.0	10.1
	18 : 3	59.5	58.8	56.3	60.6	56.4
	SFA	1.4	3.6	4.4	3.4	3.9
	UFA	98.6	96.4	95.6	96.6	96.1
DGDG	14 : 0	0.7	1.2	1.0	1.5	1.8
	16 : 0	9.5	8.9	7.0	8.0	6.1
	16 : 1	tr	tr	tr	tr	tr
	16 : 3	5.8	9.5	5.8	4.6	5.5
	18 : 1	3.9	3.2	3.1	2.9	3.2
	18 : 2	6.3	5.0	10.2	7.8	10.8
	18 : 3	73.8	72.2	72.9	75.3	72.6
	SFA	10.2	10.1	8.0	9.5	7.9
	UFA	89.8	89.9	92.0	90.5	92.1
SQDG	14 : 0	5.7	4.5	5.0	3.8	5.2
	16 : 0	45.4	38.2	36.1	37.3	42.9
	16 : 1	tr	tr	tr	tr	tr
	16 : 3	4.7	5.4	5.3	11.7	5.9
	18 : 1	7.1	6.7	7.3	5.9	6.6
	18 : 2	11.2	13.5	13.4	11.4	12.0
	18 : 3	26.1	31.8	33.1	30.0	27.4
	SFA	51.1	42.7	41.1	41.1	48.1
	UFA	48.9	57.3	58.9	58.9	51.9

Fatty acid composition-% of total.
MGDG-Monogalactosyldiglyceride, DGDG-Digalactosyldiglyceride.
SQDG-Sulfoquinovosyldiglyceride, 14 : 0-Myristic acid, 16 : 0-Palmitic acid 16 : 1-Palmitoleic acid, 16 : 3-Hexadecatrienoic acid, 18 : 1-Oleic acid, 18 : 2-Linoleic acid, 18 : 3-Linolenic acid. SFA-Saturated fatty acid, UFA-Unsaturated fatty acid.

Table 2. Changes of fatty acid composition of phosphatidylgycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) in spinach leaves during the storage at 25°C.

Phospholipid		Days in storage 0	1	2	4	6
PG	14 : 0	2.0	1.8	2.1	3.3	3.9
	16 : 0	19.3	18.7	17.6	21.9	24.8
	16 : 1	31.0	31.2	33.3	22.5	22.6
	16 : 3	1.2	1.3	1.3	5.7	3.1
	18 : 1	4.1	4.0	3.8	5.3	6.1
	18 : 2	8.9	7.5	6.7	9.1	12.1
	18 : 3	33.8	35.4	35.5	32.3	27.2
	SFA	21.3	20.5	19.7	25.2	28.7
	UFA	78.7	79.5	80.3	74.8	71.3
PE	14 : 0	3.9	3.0	3.5	3.8	5.3
	16 : 0	28.6	25.7	27.6	24.9	29.7
	16 : 1	0.9	2.3	tr	tr	1.6
	16 : 3	2.0	2.5	4.4	5.7	3.7
	18 : 1	8.7	9.1	14.1	10.9	10.1
	18 : 2	35.1	34.6	32.4	33.6	27.2
	18 : 3	20.9	23.0	18.0	21.2	22.7
	SFA	32.5	28.7	31.1	28.7	35.0
	UFA	67.5	71.3	68.9	71.3	65.0
DPG	14 : 0	3.3	2.2	3.2	3.0	3.1
	16 : 0	23.1	20.5	23.6	22.4	21.7
	16 : 1	1.5	1.3	1.5	1.4	1.2
	16 : 3	3.3	3.0	3.9	7.3	3.4
	18 : 1	13.5	11.3	11.8	10.1	8.7
	18 : 2	31.8	31.0	24.9	24.0	26.0
	18 : 3	23.5	30.9	31.1	31.9	35.9
	SFA	26.4	22.7	26.8	25.4	24.8
	UFA	73.6	77.3	73.2	74.6	75.2

Fatty acid composition-% of total.
14 : 0-Myristic acid, 16 : 0-Palmitic acid, 16 : 1-Palmitoleic acid, 16 : 3-Hexadecatrienoic acid, 18 : 1-Oleic acid, 18 : 2-Linoleic acid, 18 : 3-Linolenic acid.
SFA-Saturated fatty acid, UFA-Unsaturated fatty acid.

In SQDG, the predominant fatty acids were 16 : 0 as well as 18 : 3, and the proportion of UFA was substantially lower than in MGDG and DGDG. As with MGDG and DGDG, the relative amount of UFA in SQDG remained almost unchanged during the storage at 25°C. The relative amount of 16 : 0 decreased gradually for the first 2 days of storage and then increased from the fourth day. Conversely, the relative amount of 18 : 3 increased gradually for the first 2 days of storage and then decreased from the fourth day.

The changes in the fatty acid composition of phospholipids are shown in Table 2 and 3. The proportion of UFA in phospholipids was relatively low compared with that in the glycolipids, MGDG and DGDG. The relative amount of UFA in all the major phospholipids remained almost unchanged during storage at 25°C. Table 2 shows the changes in the fatty acid composition of PG, PE and DPG, the total amounts of which declined during the storage at 25°C. The predominant fatty acids in PG were 16 : 0, 16 : 1 and 18 : 3 ; the particularly high level of 16 : 1 was characteristic of this phospholipid. The relative amounts of 16 : 0 and 18 : 2 decreased slightly for the first 2 days of storage and then increased gradually from the fourth day. Conversely, the relative amounts of 16 : 1 and 18 : 3 increased slightly for the first 2 days of storage and then decreased from the fourth day. PE had relatively high proportions of 16 : 0, 18 : 2 and 18 : 3. While the relative amount of 18 : 2 decreased at the end of storage, others showed almost no change during the storage at 25°C. DPG was found to have relatively high proportions of 16 : 0, 18 : 2 and 18 : 3. The level of 18 : 3 increased and the levels of 18 : 1 and 18 : 2 decreased during storage. Table 3 shows the changes in the fatty acid compositions of PC and PI, the total amounts of which showed almost no decline during the storage at 25°C. PC was found to have relatively high proportions of 16 : 0, 18 : 2 and 18 : 3. Compared with other polar lipids, the fatty acid composition of PC changed markedly during storage, with a continuous increase in 18 : 3, a transient increase in 16 : 0 and a considerable decrease in 18 : 1 and 18 : 2. PI was found to have relatively high proportions of 16 : 0, 18 : 2 and 18 : 3, and the fatty acid composition remained almost unchanged during storage.

Table 3. Changes of fatty acid composition of phosphatidylcholine (PC) and phosphatidylinositol (PI) in spinach leaves during the storage at 25°C.

Phospholipid		Days in storage				
		0	1	2	4	6
PC	14 : 0	3.2	2.5	3.5	3.4	2.7
	16 : 0	20.5	25.2	27.5	24.2	20.6
	16 : 1	tr	tr	tr	tr	1.0
	16 : 3	2.6	2.7	3.0	3.5	3.3
	18 : 1	17.3	12.6	11.4	10.9	6.9
	18 : 2	31.7	28.8	24.7	22.3	24.2
	18 : 3	24.7	28.3	28.7	35.8	42.0
	SFA	23.7	27.7	31.0	27.6	23.3
	UFA	76.3	72.3	69.0	72.4	76.7
PI	14 : 0	5.7	4.3	3.3	5.2	5.4
	16 : 0	34.4	30.7	36.0	37.6	34.7
	16 : 1	tr	tr	tr	1.8	2.0
	16 : 3	3.7	4.9	5.6	8.7	5.6
	18 : 1	11.9	14.3	16.2	11.7	11.1
	18 : 2	27.0	30.3	22.3	20.4	26.2
	18 : 3	17.3	15.6	16.5	14.7	15.2
	SFA	40.1	35.0	39.3	42.8	40.1
	UFA	59.9	65.0	60.7	57.2	59.9

Fatty acid composition-% of total.

14 : 0-Myristic acid, 16 : 0-Palmitic acid, 16 : 1-Palmitoleic acid, 16 : 3-Hexadecatrienoic acid, 18 : 1-Oleic acid, 18 : 2-Linoleic acid, 18 : 3-Linolenic acid.

SFA-Saturated fatty acid, UFA-Unsaturated fatty acid.

Discussion

In the present study, the polar lipids such as MGDG, DGDG and PG decreased noticeably during the storage at 25°C. This decrease occurred before the beginning of chlorophyll degradation, which occurred on the 3rd day of storage, as reported previously (24). MGDG, DGDG and PG are mainly contained in chloroplasts(17, 22), and these chloroplast lipids showed a marked decrease with the progress of senescence of bean leaves(7), cucumber cotyledons(4) and spinach leaves(11). Thus, these findings indicate that the decrease of the lipids is involved in chloroplast disintegration.

It is well known that lipolytic acyl hydrolase (LAH) and lipoxygenase (LOG) are related to the formation and peroxidation of free fatty acids, respectively(9, 10, 19). Anderson *et al.* purified the galactolipid acyl hydrolase from the chloroplasts of pole bean leaves (2). Hosoda *et al* found that in komatsuna (*Brassica campestris* L.) leaf discs incubated at 20°C, the increase in LOG activity was followed by chlorophyll degradation, and they also clarified the localization of LOG in chloroplast of komatsuna and spinach leaves(14). It may be inferred from these results that hydrolysis and peroxidation of polar lipids by LAH and LOG would also occur in spinach chloroplasts.

In the previous paper, we reported that the TBA value increased gradually with the progress of senescence of stored spinach leaves (24). Maruyama *et al.* noted that free fatty acid content and TBA value increased with the advance of senescence in stored parsley leaves(18). Draper also observed that the proportion of 18 : 3 in free fatty acids increased with senescence of cucumber cotyledons, and suggested that the increase might be accounted for by the breakdown of chloroplast lipids, in particular the galactolipids(4). On the other hand, Orthoefer and Dugan observed that chlorophyll a was bleached in a system which consisted of 18 : 2 and LOG (20). Holden demonstrated that chlorophyll was bleached when leaves of various species of plants, which were rich in LOG, were incubated in an aqueous acetone solution (13). Imamura and Shimizu also reported that the leaf tissue extracts of various plants which showed LOG activity bleached chlorophyll in the presence of 18 : 2 or 18 : 3(15).

Our results, together with the evidence mentioned above, indicate that the process of chlorophyll degradation in spinach leaves involves the formation of free fatty acids, by the degradation of polar lipids like MGDG, DGDG and PG, followed by formation of their hydroperoxides, which in turn causes the degradation of chlorophylls. Further study is under way in order to elucidate the relationship between the formation and peroxidation of free fatty acids and the degradation of chlorophyll in spinach chloroplasts.

Koiwai *et al.* reported that in tobacco, the proportions of such fatty acids as 16 : 1 (PG), 16 : 3 (MGDG) and 18 : 3 (MGDG, DGDG and PG) in chloroplast lipids were lower in senescent leaves than in green ones. In the stored spinach leaves used in the present study, the decrease in the relative amounts of 16 : 1, 16 : 3 and 18 : 3 in chloroplast lipids was small compared with that of tobacco leaves, and the decrease of 18 : 3 was observed only in PG, differing from tobacco leaves. Moreover, the relative amount of 18 : 2 in chloroplast lipids increased, and the ratio of UFA to total fatty acids in all the polar lipids remained almost unchanged.

Thus, unlike in tobacco leaves, there was a significant decrease in chloroplast lipids with the progress of senescence in stored spinach leaves, but only a slight change in the fatty acid composition of chloroplast lipids. The reason for the difference between spinach and tobacco leaves is not clear, and is the subject for a future study.

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貯蔵されたホウレンソウの黄化に伴う極性脂質及び構成脂肪酸組成の変化[1]

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摘　要

本研究は，収穫後における葉菜類のクロロフィル分解機構を明らかにするため，ホウレンソウを用い，貯蔵中の葉の黄化に伴う極性脂質及び構成脂肪酸組成の変化について検討した．

ホウレンソウの総糖脂質含量は．25℃貯蔵に伴い急激に減少し，特にモノガラクトシルジグリセリド（MGDG）並びにジガラクトシルジグリセリド（DGDG）の減少が顕著にみられた．一方，総リン脂質含量は，25℃貯蔵に伴い糖脂質とは異なり徐々に減少した．リン脂質中では，特にホスファチジルグリセロール（PG）の減少がみられた．

このように，25℃貯蔵におけるホウレンソウの極性脂質の減少は，クロロフィル分解の開始より以前に生じ，特にクロロプラストに局在している MGDG, DGDG 及び PG の減少が顕著に認められた．

次に，構成脂肪酸組成の変化について検討したところ，MGDG に含まれるヘキサデカトリエン酸及び PG に含まれるパルミトレイン酸並びにリノレン酸の含有割合は，25℃ 貯蔵に伴い減少した．逆に，MGDG, DGDG 及び PG に含まれるリノール酸の含有割合は増大した．しかしながら，すべての極性脂質の不飽和脂肪酸の割合は，25℃ 貯蔵中ほとんど変化が認められなかった．

以上の結果から，クロロフィルの分解は，クロロプラスト脂質である MGDG, DGDG 及び PG の分解によって生じた脂肪酸の過酸化物が関与しているものと推察した．

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