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Distribution and Function of Lipids in Spinach Chloroplast Membranes as Revealed by Lipolytic Enzyme Treatment

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脂質分解酵素処理法によって調べたホウレンソウ葉緑体膜における脂質の分布と機能

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Enzymic degradation of the chloroplast lipids is a valuable approach to study the function and distribution of the component lipids in the membranes. Anderson et al.[1,2] have demonstrated that a galactolipase from *Phaseolus vulgaris* leaves causes a strong inhibition of the photosynthetic electron flow with hydrolysis of the major part of the chloroplast lipids, and that the inhibition is largely prevented in the presence of a high concentration of BSA. They further reported that the lipase inhibition is due to release of fatty acids, and the major galactolipids can be removed without a strong damage to the photoactivity. We have prepared phospholipid-depleted chloroplasts[3] by a treatment with venom phospholipase A_2 in the presence of BSA, and found that the treated chloroplast preparation losing 82.6% of the original phospholipids has still a large portion of the chloroplast functions.

In the present study, a prolonged treatment of spinach chloroplasts and subchloroplasts with potato lipolytic acyl-hydrolase was carried out to hydrolyze the chloroplast lipids as fully as possible. The enzymic hydrolysis of lipids occurred through two steps, and the lipids hydrolyzed at each step were characterized. From the results the distribution and function of the chloroplast lipids were discussed.

MATERIALS AND METHODS

Preparations of chloroplast and subchloroplasts. Spinach chloroplasts were prepared as described before.[3] The resulting class II chloroplasts were then treated with digitonin according to the method reported by Ohki and Takamiya.[4] After differencial centrifugation followed by washing twice to remove digitonin, 15,000x*g*- and 200,000x*g*-pellets were used as PS-II and PS-I particles, respectively.

Potato lipolytic acyl-hydrolase. Potato lipolytic acyl-hydrolase (EC 3·7·7·26) having galactolipase, phospholipase, and sulfolipase activities, was extracted and purified to homogenity.[5]

Enzymic treatments of chloroplasts and subchloroplasts. The reaction mixture (1 ml) for

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Abbreviations : PS, photosystem ; MGDG, monogalactosyldiglyceride ; DGDG, digalactosyldiglyceride ; SQDG, sulfoquinovosyldiglyceride ; PG, phosphatidylglycerol ; PC, phosphatidylcholine.

treatment with the potato enzyme, contained 0.1 M phosphate buffer (pH 5.5), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, substrate (0.5 mg Chl), and enzyme (1 mg protein). The mixtures were incubated at 20°C with stirring. After incubation, the mixtures were rapidly cooled to 0°C, and aliquots of a 10% BSA solution were added to give a final concentration of 2%. The treated chloroplasts and subchloroplasts were precipitated by centrifugation, and the photoactivity and lipids were assayed.

Measurements of phothchemical activities. DPC-mediated DCIP photoreduction (PS-II photoactivity) was measured specrtophotometrically at 610 nm.[3] Diphenylcarbazone disproportionation (PS-I photoactivity) was measured according to the method reported by Vernon et al.[7]

Analytical methods. The chloroplast preparations were extracted by the isopropanol-chloroform method.[8] The lipid extracts were separated quantitatively by TLC[9] and TLC-FID (TLC with flame ionization detector).[8] Fatty acid compositions were analyzed by GLC, and the free fatty acid contents were determined by the rhodamine method[6] and TLC-FID.[8] Microdetermination of the lipid contents in the samples was performed by use of TLC-FID.[8] The rate of lipid hydrolysis with enzyme were determined by measurements of the free fatty acids released and the remaining lipids were estimated according to TLC-FID.[8] Molecular species of the lipid classes were analyzed by $AgNO_3$-TLC,[10] using $CHCl_3/CH_3OH/H_2O$ (120 : 47 : 8, v/v) as a developing solvent. The chromatograms were visulalyzed with 2′, 7′-dichlorofluorescein, and the band lipids separated were extracted and assayed for component fatty acids by GLC.[6] Densitometry of the chromatograms was performed by charring with 50% H_2SO_4 and scanning by Asuka Ozmor 82 desitometer. Protein was determined by the method of Lowry et al. as modified by Hartree.[11] Chlorophyll concentrations were measured by the method of Arnon.[12]

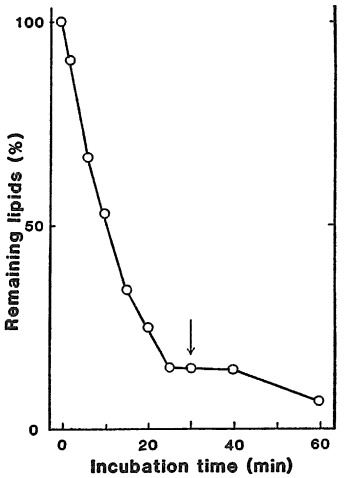

Fig. 1. Enzymic degradation of the chloroplast lipids. The chloroplasts were treated with potato lipolytic acyl-hydrolase as described in Methods. The arrow shows a point at which the lipids were separated into two groups.

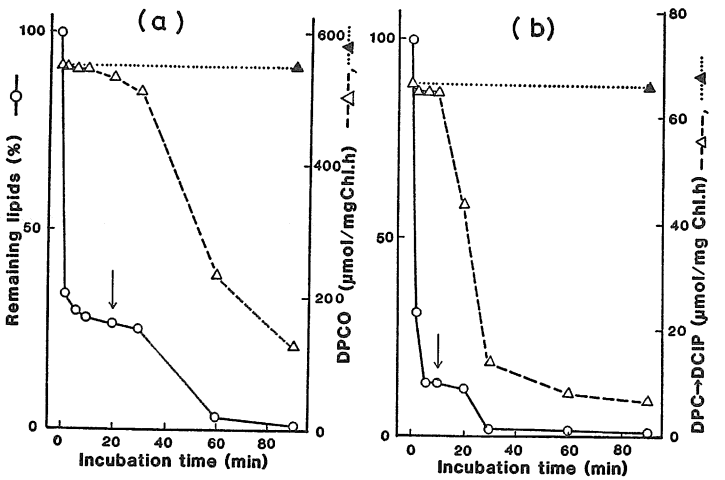

Fig. 2. Enzymic degradation of the lipids in PS-I (a) and PS-II (b) particles and their effect on the photoactivity. Subchloroplast particles were treated with the potato enzyme under the same conditions as in Fig. 1. Solid line shows the remaining lipids, broken line the photoactivity (DPCO, PS-I activity ; DPC→DCIP, PS-II activity), and dotted line activity of the control without the enzyme. The arrow shows a point at which the lipids were separated into two groups.

RESULTS

Treatments of chloroplasts and subchloroplasts with potato lipolytic acyl-hydrolase.

The subchloroplasts, PS-I and PS-II particles were prepared from chloroplasts in yields of 14% and 49% on the chlorophyll basis, respectively. Fig. 1 shows a typical pattern of lipid hydrolysis in the chloroplasts with the potato enzyme. In the early stage of the reaction (the first 25 min), major lipids were rapidly hydrolyzed, and then the remaining lipids were decomposed slowly, indicating that there are two steps for the lipid degradation. Fig. 2 shows the enzyme treatments of the subchloroplasts. Both the lipids of PS-I and PS-II particles were also hydrolzed through two phases in a similar way as shown in the chloroplasts. Most lipids were rapidly hydrolyzed in the early stage of the reaction (Type I lipids), and after that the remaining lipids were attacked slowly to an almost complete hydrolysis (Type II lipids). From Figs. 1 and 2 the contents of the Type II lipids were estimated to be 15% of the total polar lipids for chloroplasts, 28% for PS-I particles, and 13% for PS-II particles. In both of the subchloroplast particles it was found that the first step of the enzyme reaction did not affect the electron flow activities in spite of much lipid degradation, but the second step reaction caused a rapid decrease of the electron flows. The control particles, which were incubated without enzyme, did not show any change of the electron flow activities through all the incubation processes.

Lipid components of Type I and Type II lipids.

The chloroplasts and subchloroplasts were treated with potato lipolytic acyl-hydrolase at 20°C. After the incubation period for the degradation of Type I lipids (for 30 min for chloroplasts, 20min for PS-I particles, and 10 min for PS-II particles according to the results in Figs. 1 and 2), the remaining Type II lipids were extracted for analysis of lipid contents and compositions as described in Method. The total lipids, containing Type I lipids as the major components, were also obtained from the original preparations of chloroplasts and subchloroplasts, and assayed in the same way. The results were summarized in Table I. Compared with chloroplasts, the subchloroplasts had a low content of lipids, especially in PS-II particles. As for Type II lipids, the contents were 2.4% for PS-I and 0.6% for PS-II particles, and the components were much different between the two subchloroplast particles. The main components of PS-I particles were MGDG, and those of PS-II particles were PG. The chloroplast Type II lipids were observed to have both the features of the two subchloroplast Type II lipids.

Table I. Lipid Analysis of the Total and Type II Lipids from Chloroplasts and Subchloroplasts

Polar lipids	Chloroplasts		PS-I particles		PS-II particles	
	Total	Type II *	Total	Type II *	Total	Type II *
Contents (%)	33.0	6.6	9.0	2.4	4.3	0.6
Compositions (%)						
MGDG	56	23	51	51	44	19
DGDG	26	29	24	26	33	21
SQDG (+PC)	8	18	15	12	6	4
PG	10	30	10	11	17	56

* Type II lipids, see text.

Component fatty acids and molecular species of the total and Type II lipids from chloroplasts.

Table II shows the component fatty acids of the chloroplast lipid classes, which were

Table II. Component Fatty Acids of the Lipid Classes Separated from the Total and Type II Lipid Fractions from Chloroplasts

Fatty acids	MGDG		DGDG		SQDG		PG		PC	
	Total	Type II	Total	Type II	Total	Type II	Total	Type II	Total	Type II
14 : 0 14 : 1	1	6	1	2	1	3	2	2	1	2
16 : 0	6	15	6	6	34	34	20	19	14	24
16 : 1		9		2	4	8	35	33	3	4
16 : 3	11	28	4	8						
18 : 0		3		1	2	1	1	2	1	5
18 : 1			1		4	15	3	6	11	20
18 : 2	1	5	1	3	4	5	3	6	27	21
18 : 3	81	34	87	78	51	35	36	32	43	42

Type IIIipids, see text.

separated from both the total and Type II lipids from chloroplasts. In general, the lipid classes from Type II lipids had less 18 : 3 acid than those from the total lipids. However, the former contained more of saturated, monoenoic or shorter chain-length acids than the latter. These characteristics of fatty acid distribution between Type I and II lipids decreased in the following order : MGDG, PC > SQDG > DGDG, PG. Fig. 3 shows a separation of the molecular species by $AgNO_3$-TLC of the galactolipid classes from chloroplasts. It was found that MGDG class has a clear difference in molecular species between the total and Type II lipids. Fatty acid analysis indicated that the MGDG from the total lipids has 18 : 3/18 : 3 species (Band No. 2) and the one from Type II lipids contains 18 : 3/16 : 3 species (Band No. 1), in addition to the two common bands of No. 3 and No. 4. The molecular species of DGDG class were also separated into five bands on the $AgNO_3$-plate (Fig. 3), but there was no difference in quantities of each band between the two types of lipids on the densitograms.

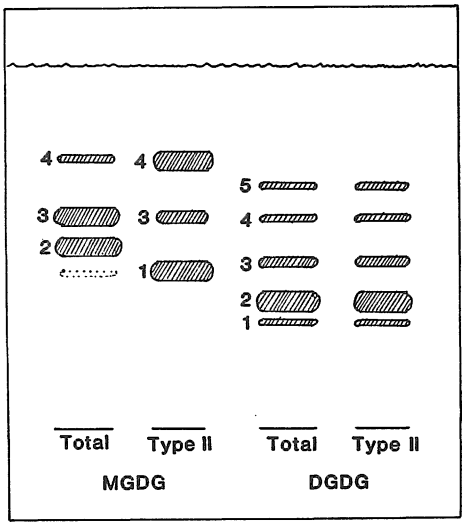

Fig. 3. Separation of molecular species of galactolipids from chloroplasts. The chloroplasts were treated for 30 min with theenzyme under the same conditions as in Fig. 1, and the remaining lipids (Type II) were extracted. From the Type II and the total lipid fractions, MGDG and DGDG were isolated by TLC. Their molecular species were separated on $AgNO_3$-TLC.

DISCUSSION

The prolonged treatments of spinach chloroplasts and subchloroplasts with potato liplytic acyl-hydrolase showed two steps in the hydrolysis pattern of the membrane lipids. In the first step of the reaction most lipids were rapidly hydrolyzed and in the second step the remaining lipids were slowly decomposed, suggesting that there are two types

of lipids, the enzyme-sensitive (Type I) and enzyme-resistant (Type II) lipids in the membrane. For the occurrence of these two types of lipids, three possible factors may be involved. One is the lipid components which are closely related to the substrate specificity of the lipolytic enzyme used. Another is the molecular species of the lipid classes which induce the liquid or solid phase of the membranes, affecting the rate of the enzyme reaction. The third is the distribution and state of lipids in the membranes, in which the lipids would exist as the buound lipids associating with proteins or the free lipids forming lipid bilayer.

The Type II lipids of PS-I particles were much different from those of PS-II particles in lipid composition, although the total lipids from the two subchloroplast particles were not so different from each other. On the other hand, the lipid compositions of Type II lipids from PS-I and PS-II particles were different from those to be expected from the substrate specificity of the potato enzyme.[6] The enzyme also tends to display a lower substrate specificity for the mixture of several lipids than for a single lipid substrate. Therefore, it seems that the Type II lipids observed do not occur due to only the substrate specificity of the enzyme. The molecular species with shorter and unsaturated fatty acids are more rapidly attacked by enzyme than those with longer and saturated acids,[13] because the former do not make a solid phase more easily than the latter. However, the enzyme-resistant Type II of MGDG has more 16 : 3 acid than the enzyme-sensitive Type I, and each lipid class of DGDG, SQDG, and PG showed a similar content of 16 : 0 acid between the two types (Table II). These suggest that the moleculatr species of lipids are not a dominant factor for the occurring of the two types of lipids.

Based on the above consideration with the effect of lipid hydrolysis on the electron flow activities (Fig. 2), we favor the idea that the two types of lipids occur mostly due to the difference in lipid distribution and lipid existing state in the chloroplast membranes. Presumably, Type II lipids would be the bound lipids, which contain the boundary lipids and the tightly bound lipids reported by Jost et al.[14] and Robinson and Capaldi,[15] respectively. On the other hand, Type I lipids can be considered to be the fluid bilayer lipids in the chloroplast membranes, and some parts of them still remain in the subchloroplast particles even after disintegration of the thylakoid membranes.

As shown in Fig. 2, the Type II lipids (or the bound lipids) were closely associated with the photosynthetic electron flow activities which were measured by the diphenylcarbazone disproportionation and DPC-mediated DCIP photoreduction, but Type I lipids (or the fluid bilayer lipids) did not concerned with the electron flow activities. This is consistent with the experimental results that the phospholipid-depleted chloroplasts, losing the fluid phospholipids, still retain a large portion of the photoactivities.[3] The finding also coincides with the report of Shaw et al.[2] that the major galactolipids can be removed from the chloroplast membranes without a strong damage to the activities.

Rawyer and Siegenthaler[16] performed a similar study on chloroplasts and subchloroplasts with potato enzyme, and reported that there is no quantitative correlation between lipid hydrolysis and photochemical activities. This discrepancy may be concerned with the fact that in their study the subchloroplast particles prepared with Triton X-100 were treated with a small amount of the enzyme. As known well, the subchloropalasts prepared with Triton have a large amount of Triton as contaminant, which would possibily obscure the effect of lipid degradation. Furthermore, their employed method for lipid extraction might be somewhat incomplete in lipid recovery compared with the present method. This would

suggest a possibility that most of the bound lipids described here remain unestimated.

It is interestingly observed that the bound lipid of MGDG (Type II) has a characteristic moleular species of 18 : 3/16 : 3, while the fluid lipid of it (Type I) contains 18 : 3/18 : 3 species (Fig. 4). However, it seems that such a difference in molecular species between the two type lipids is rather rare in other lipid classes of the chloroplasts. Further detailed studies will be required in the region.

SUMMARY

Spinach chloroplasts and subchloroplasts were treated with potato lipolytic acyl-hydrolase. The lipids were hydrolyzed through two steps. In the first step a major part of lipids (Type I) was rapidly hydrolyzed without affecting the photoactivity. In the second step the remaining lipids (Type II) were decomposed with a decrease of the photoactivity. The main components of Type II lipids in the photosystem I particles were monogalactosyldiglyceride, and those of the photosystem II particles were phosphatidylglycerol. Molecular species were clearly different between Type I and Type II of monogalactosyldiglyceride, but not in those of other lipid classes. It seems likely that Type II lipids are the bound lipids relating to the photoactivity and Type I lipids are the fluid bilayer lipids which have no concern with the activity.

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摘　　要

ホウレンソウ葉緑体および亜葉緑体粒子を馬鈴薯脂質アシル水解酵素で処理し，膜脂質の分布および機能を調べた．葉緑体，系Ⅰ粒子および系Ⅱ粒子のいずれの場合も，反応の初期に大部分の脂質（Type I）が分解し，ついで残りの脂質（Type II）が徐々に分解された．電子伝達活性は，Type I 脂質の分解ではほとんど影響を受けないが，Type II 脂質の分解に伴って急速に低下した．系Ⅰおよび系Ⅱ粒子の Type II 脂質の組成は大きく異なり，前者の主成分はモノガラクト脂質であり後者はホスファチジルグリセロールであった．また，モノガラクト脂質の Type I および II の間には分子種の差異が認められた．以上の結果から，Type I 脂質は流動性脂質，Type II 脂質は結合性脂質であり，前者は光化学活性に直接関与していないが後者はこの活性発現に寄与していることが推測された．