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Lipolytic Enzyme Treatment of chloroplasts and Characterization of Lipid-Depleted Chloroplasts

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葉緑体の脂質分解酵素処理ならびに脱脂質葉緑体の特性

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Several researches[1-5] in photosynthesis have been performed on the localization and functions of lipids in the thylakoid membranes using lipolytic enzymes. In the previous paper[6], we reported a simple procedure for preparation of phospholipid-depleted chloroplasts by phospholipase A_2 treatment.

In the present study, we have attempted to remove both of galacto- and phospholipids by treatment of chloroplasts with potato lipolytic acyl-hydrolase. This paper describes characteristics of the potato enzyme reaction on spinach chloroplast membranes, and preparation and characterization of the lipid-depleted chloroplasts.

MATERIALS AND METHODS

Preparations of chloroplasts and lipoyltic acyl-hydrolase. Class II chloroplasts were prepared from spinach leaves, as described previously.[2] Potato lipolytic acyl-hydrolase was extracted and purified from potato tubers.[7] Enzyme assay was made by determining free fatty acids released.[8]

Enzyme treatments of chloroplasts. The basal mixture (1 ml) for enzyme treatment contained 0.1 M phosphate buffer (pH 5.5), 10 mM NaCl, chloroplasts (0.5 mg Chl), and enzyme (1 mg protein). Sucrose, $MgCl_2$ or BSA was added to the basal mixture when required. After incubation under the conditions indicated, 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 were separated by centrifugation, and the photoactivity and the remaining lipids were assayed.

Measurements of photochemical activities. Photosynthetic electron flow activities of the $H_2O \rightarrow$ DCIP[2] and DPCO disproportionation[9] were measured as described previously. The light-induced H^+ uptake was evaluated by measurement of pH[10], and the photophosphorylation activity was assayed by determining pH change.[11]

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Abbrevations : Chl, chlorophyll ; BSA, bovine serum albumin ; DCIP, 2, 6-dichlorophenyl-indophenol ; DPC, diphenylcarbazide ; DPCO, diphenylcarbazone ; TLC-FID, thin-layer chromatography with flame ionization detector ; MV, methyl viologen.

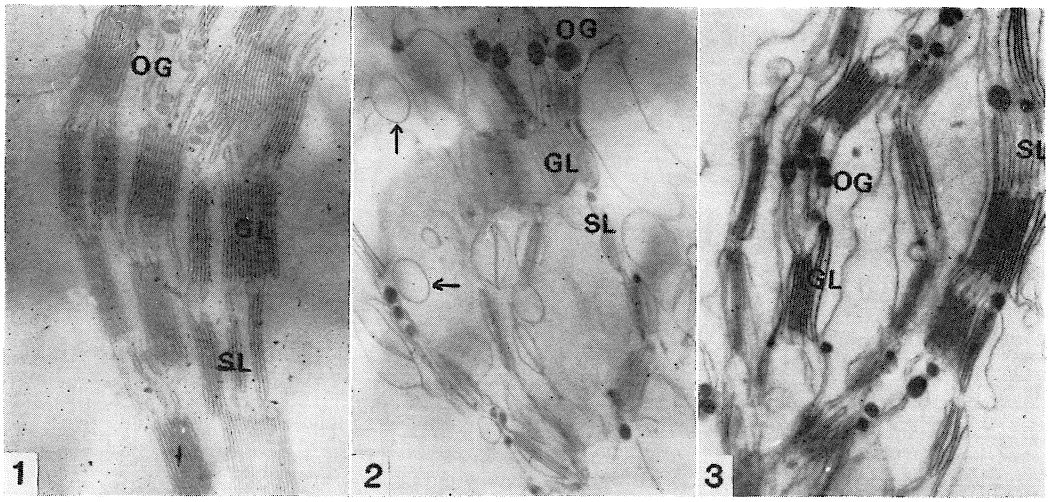

Fig. 1. Thin sectoins of potato enzyme-treated chloroplasts. The chloroplasts were treated with potato enzyme at 20°C for 30 min in the basal mixture (see Text) containing 0.4 M sucrose in the absence (2) and in the presence (3) of 3 % BSA. The control chloroplasts (1) were prepared under the same conditions as in (2) without enzyme. OG, osmiophilic globule ; GL, grana lamellae ; SL, stroma lamellae. ×20,000

Lipid analysis. The chloroplasts were extracted by the isopropanol-chloroform method[12] with a complete recovery of lipids. The lipid extract was analyzed by TLC[13] and TLC-FID.[12] The rate of lipid hydrolysis was determined by measuring free fatty acids and remaining lipids using TLC-FID.[12]

Other analytical methods. Protein was determined by the method of Lowry et al. as modified by Hartree.[14] Chlorophyll concentrations were measured by the method of Arnon.[15]

Electron microscopy. The chloroplast samples were centrifuged, and the resulting pellets were fixed with 3 % glutaraldehyde in 50 mM phosphate buffer (pH 7.5) containing 0.5 M sucrose and 10 mM KCl, and post-fixed with 1 % OsO_4. Samples were then dehydrated and embeded in Epon 812. Thin sections were examined with a Hitach HU-12A.

RESULTS AND DISCUSSION

Effects of some factors on potato lipolytic acyl-hydrolase action on the chloroplast membrane lipids.

Table I shows the effects of $MgCl_2$, BSA and sucrose on the extent of lipid hydrolysis in chloroplasts. The presence of 5 mM $MgCl_2$ in the reaction mixture induced a high inhibition of the enzyme in the early stage of incubation. This effect would be related to a stimulation of the grana stacking with $MgCl_2$. The addition of 0.4 M sucrose depressed the enzyme at a higher level than that of 0.1 M sucrose, indicating that the osmotic pressure which controls the membrane swelling is closely related to the reaction. The presence of 3 % BSA also inhibited the enzyme through all processes of incubation. Fig. 1 shows an electron-microscopic observation in this respect. In the absence of BSA the enzyme caused a marked swelling and destruction of all thylakoid membranes, whereas in the presence of BSA it brought about a little swelling limited in the outersurface membranes. This is interpreted as due to a specific function of BSA to bind and remove

Table I. Effects of Some Factors on the Extents of Lipid Hydrolysis in Chloroplasts with Potato Lipolytic Acyl-Hydrolase

Conditions of enzyme reaction				Incubation time, min				
Temp. °C	Sucrose M	$MgCl_2$ mM	BSA %	6	10	20	30	60
				lipid hydrolysis, %				
18	0.4	5	—	10	23	47	85	96
18	0.4	—	—	92	96	96	96	96
6	0.4	—	—	19	25	33	33	33
6	0.1	—	—	62	62	62	62	75
18	0.4	5	—	10	23	47	85	96
18	0.4	5	3	5	7	15	20	40

The chloroplasts were treated with potato enzyme as described in Methods under the conditions indicated. The rates of lipid hydrolysis were determined by measuring both the contents of free fatty acids released and the remaining lipids.

Table II. Characterization of Lipid-Depleted Chloroplasts

Lipid contents and functions of chloroplasts	Chloroplast preparations		
	Control	lipid-depleted	% of control
Lipid contents (mg/mgChl)	4.12	1.28	31
Electron flow (μmol/mg Chl·hr)			
DPCO	177	156	88
DPCO+plastocyanin	463	229	49
$H_2O \longrightarrow$DCIP	126	52	41
DPC$\longrightarrow$DCIP	151	95	63
H^+ uptake (meq/mg Chl)	1610	0	0
Photophosphorylation (μmol/mg Chl·hr)	180	0	0

The lipids of chloroplasts were removed by potato enzyme treatment and subsequent separation were made by sucrose-density gradient centrifugation as in Methods. The resulting lipid-depleted chloroplasts were assayed and compared with the control chloroplasts which were prepared by the same procedure without enzyme. H^+ uptake and photophosphorylation were both measured by using MV as the electron acceptor.

free fatty acids, which were released by the enzyme and would easily cause a large swelling of the membrane if BSA was missing.

Fig. 2 shows the effect of temperature on the enzyme reaction. In the treatments of chloroplasts at varying temperatures, the lipid hydrolysis increased stepwise with increase of temperature. In contrast, the lipid extract from chloroplasts was hydrolyzed almost linearly with increasing temperature except around 35°C. It seems likely that the stepped increase of lipid hydrolysis comes from differences in the localization and the existing forms of the lipid components in the membranes. At present, a possible explanation is as follows : at a low temperature the gross membrane structure of chloroplasts is stable and therefore the outer-layer lipids of the membrane, accessible to the enzyme, are preferentially hydrolyzed in the first step (0°−10°C, in Fig. 1). In the second step (10°−32°C), all the fluid lipids in both the outer and inner layers are decomposed, and finally all the lipids containing the bound lipids are degraded in the third step (32°−35°C).

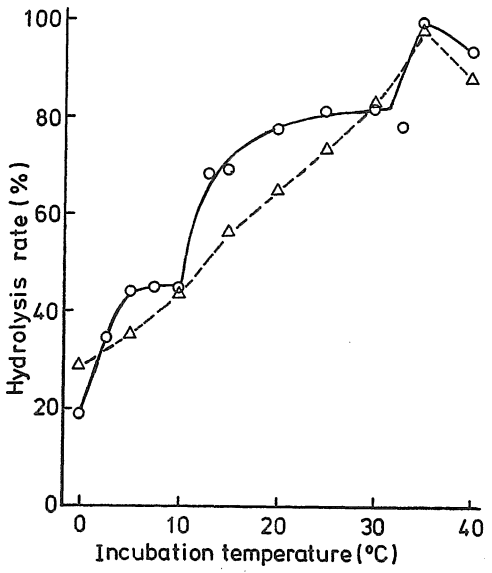

Fig. 2. Effect of incubation temperature on the hydrolysis rate of chloroplast lipids with potato enzyme. The chloroplasts were treated with the enzyme in the presence of 5 mM $MgCl_2$ and 0.4 M sucrose at varying temperatures for 30 min. Other conditions and analysis were the same as in Methods. Solid line shows the treatment of chloroplasts, and broken line the treatment of the lipid extract from chloroplasts emusified by sonication.

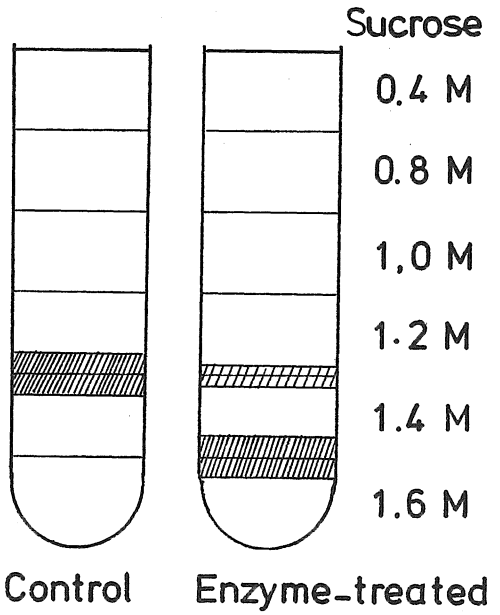

Fig. 3. Patterns of sucrose-gradient centrifugation. The chloroplasts were treated extensively with double amounts of potato enzyme (2 mg protein/ml) in the presence of 3 % BSA and 0.4 M sucrose at 20°C for 30 min. The reaction mixture was loaded on a sucrose-density column containing six layers of sucrose solutions in the concentrations indicated, and cenrifuged at 80,000 ×g for 60 min. The control chloroplasts were treated in the same way without enzyme only.

Preparation and characterization of lipid-depleted chloroplasts.

The chloroplasts were treated extensively with more of potato enzyme in the presence of 3 % BSA and 0.4 M sucrose at 20°C for 30 min. The treated chloroplasts were separated into two bands on the sucrose-density gradient column as shown in Fig. 3. The heavy band was collected and analyzed as the lipid-depleted chloroplasts. The results were summarized in Table II, compared with those of the control chloroplasts. In spite of lipid removal to about 70 %, the preparation still retained nearly half of the initial electron flows. This agrees with the report of Shaw et al.[3] that the major galactolipids could be removed from the chloroplast membranes without a strong damage to the electron flow activity. On the other hand, both activities of H^+ uptake and photophosphorylation were completely lost. This suggests that the lipid depletion from the chloroplast membranes lead to a loss of the permearbility barrier of the membranes, and the H^+ leakage due to it and a possible damage to CF_1 resulted in a complete loss of the photophosphorylation.

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SUMMARY

Potato galactolipase treatment of spinach chloroplasts were investigated. The enzyme reaction was inhibited with $MgCl_2$, bovine serum albumin and sucrose, to varying levels with their specific action on the chloroplast grana stacking. The reaction increased in three steps with increase of temperature, suggesting that there are different localization and existing forms of lipids. By the enzyme treatment and subsequent centrifugation on density-gradient column, lipid-depleted chloroplasts were prepared. Analysis revealed that the preparation retained nearly half portion of the initial electron flows, but H^+ uptake and phosphorylation activities were completely lost.

摘　　要

ホウレンソウ葉緑体の馬鈴薯ガラクトリパーゼ処理について調べた．この葉緑体脂質の酵素分解反応は，$MgCl_2$，牛清血アルブミンあるいはショ糖の存在で抑制され，その程度は葉緑体グラナ構造に与える効果に依存していた．また，この反応は温度の上昇とともに3段階に分れて増加した．これは膜脂質の分布，存在状態に差異があることを示唆している．酵素処理後密度勾配遠心分離によって脱脂質葉緑体を調製した．これはもとの半分近くの電子伝達活性を保持していたが，H^+ とり込みおよび光リン酸化活性は完全に消失していた．