Mechanism of Inhibition of the Photochemical Activities Caused by Lipolytic Enzyme Treatments of Spinach Chloroplasts

Osamu HIRAYAMA※, Takashi MATSUI※ and Haruo SUMI※

ホウレンソウ葉緑体の脂質分解酵素処理における
光化学活性の阻害機構

平 山　修※・松 井　隆※・角　治 夫※

Selective degradation and depletion of the chloroplast lipids by enzymes are a valuable approach to study the function and distribution of the membrane lipids in the chloroplasts.[1-4] Various lipolytic enzymes have been used in studies of the photochemical electron flows,[5] phosphorylation,[1,6] fluorescence,[2] and the morphorogy of the grana.[1] Okayama and Mautai[3] showed that pancreatic lipase inhibits photosystem II reaction to varying degree and is less effective for inhibiting photosystem I reaction. Recently, Anderson et al.[4,7] have demonstrated that galactolipase from *Phaseolus vulgaris* leaves causes a rapid inhibition of photosynthetic electron flow, and this inhibition is largely prevented in the presence of a high concentration of BSA, suggesting that the lipase inhibition is due to release of fatty acids and their interaction with the membranes.

In a previous work,[8] we have shown that potato galactolipase hydrolyzes monoglactolipid at a fast rate and causes a rapid decrease of photosystem I activity. In contrast, venom phospholipase A_2, which preferentially attacks phosphatidylglycerol, resulted in a rapid decrease of photosystem II activity. In other previous paper,[9] we have also revealed that, in the enzyme treatments of the extracted lipids from chloroplasts, potato galactolipase generates fatty acids with trace of lyso-monogalactolipid, and venom phospholipase A_2 forms fatty acids and lyso-phosphatides as the lipid-soluble hydrolysis products. These products are known to cause a marked inhibition of the photochemical electron flow in chloroplasts.[10-14]

If it is true that the enzymic inhibitions are principally caused by the lipid degradation products, there are two possible mechanisms to be expceted for the above contrasted inhibition by the galactolipase and the phospholipase. One is different components of the degradation products, and the other is different locality of the degradation products in the membrane (or the membrane damages), which can be in turn attributed to the lipid distribution in the chloroplast membranes.

The present study was undertaken to get insight into the mechanism of the inhibitory

※ Laboratory of Food Chemistry, Faculty of Agriculture, Shimane Univesity, Matsue 690, Japan
　　Abbrevations : BSA, bovine serum albumin ; DCIP, 2, 6-dichlorophenyl-indophenol ; DCMU, 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea.

action of lipolytic enzymes on the photosynthetic electron flow. First, effects of BSA in the enzyme treatments were investigated on both sides of the inhibition of the electron flow and of the removal of the lipid degradation products. Second, the inhibitory patterns by the enzyme-digested lipids were determined and compared with those by the enzyme own action. Finally, spectral changes of the chloroplasts treated with the lipolytic enzymes or the digested lipids were determined for following fine alterations of the membrane structure of chloroplasts.

MATERIALS AND METHODS

Chloroplast preparation. Spinach leaves were homogenized in a medium containing 0.35 M NaCl, 10 mM Tris-HCl (pH-8.0) and 5 mM $MgCl_2$. The pellets which sedimented between $200 \times g$ and $2,000 \times g$ were washed, and resuspended in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 50 % glycerol. The suspension was stored at $-20°C$ until used.

Lipolytic enzyme preparations and enzyme assays. Potato lipolytic acyl-hydrolase (potato galactolipase) having both galactolipase and phospholipase (B-like) activities was extracted and purified from potato tubers.[9] Phospholipase A_2 was purified from snake venom from *Habu*. Neither of the lipolytic enzyme preparations had proteolytic activity. Enzyme assays were carried out by determining the free fatty acid released by the enzymes according to the method reported previously.[8,9]

Enzymic treatments of chloroplasts. The reaction mixture (1 ml) for treatment with potato galactolipase contained 0.1 M phosphate buffer (pH 5.5), 0.4 M sucrose, chloroplasts (1 mg chlorophyll), and the enzyme (0.37 mg protein). With venom phospholipase A_2, the mixture (1 ml) contained 0.1 M Tris-HCl (pH 8.0), 0.4 M sucrose, 0.1 mM $CaCl_2$, chloroplasts (1 mg chlorophyll), and the enzyme (0.43 mg protein). The mixtures were incubated at 30°C with stirring. After incubation, the mixtures were rapidly cooled to 0°C, and assayed for the photosynthetic electron flows.

Treatments of chloroplasts with lipids and enzyme-digested lipids. The lipid materials used were prepared by the following procedure. Lipid extracts from spinach chloroplasts were separated on silica gel column. After elution of neutral lipids (containing free fatty acids) with chloroform, glycolipids were eluted with acetone, and then phospholipids with chloroform-methanol (1 : 1, v/v) and methanol containing 1 % HCl. The polar lipids (total mixture of glycolipid and phospholipid fractions) were treated with potato galactolipase as described in Methods. The mixture was extracted with chloroform, giving potato galactolipase-digested lipids. Phospholipase-digested lipids were also prepared by treatment of the phospholipid fraction with venom phospholipase A_2. Non-digested polar lipid and phospholipid fractions were used as the control lipids, respectively. A solution (50 μl) of non-digested or digested lipids in ethanol was added to 1.0 ml of the chloroplast suspension containing 1 mg chlorophyll, then incubated for 20 min at 20°C, followed by spectrophotometry for measurements of the electron flow activities.

Measurements of the photochemical electron flow and difference spectra. DCIP photoreduction was measured spectrophotometrically at 610 nm. The reaction mixture (3 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.1 mM DCIP, and chloroplasts (30 μg chlorophyll). NADP photoreduction was determined from the absorbance change at 340 nm. The reaction mixture (3 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 8.5 μM spinach ferredoxin, and chloroplasts (40 μg chlorophyll). When the reduced DCIP was used as the electron donor, 0.1 mM DCIP, 5 mM ascorbate, and 10 μM DCMU were added to the

above basal mixture.　All the reactions were carried out under light illumination (40,000 lux) from a projector lamp at 20°C.　Spectral change of chloroplasts, caused by treatments with enzymes and lipids, were measured by difference spectrophotometry using a mixture of chloroplasts with enzymes or lipids on the sample side of a spectrophotometry and a chloroplast suspension without enzyme or lipids on the reference side.　Spectral measurements were made by Hitachi 124 recording spectrophotometer.

TLC of lipids and densitometry.　The chloroplasts were extracted with isopropanol (1 vol.) and then chloroform (2 vol.).[15]　The extracted lipids were separated on Silica Gel G plate with chloroform-methanol-acetic acid-water (70 : 20 : 2 : 2, v/v) as the developing solvent.　The chromatograms were visualized by charring with 50 % H_2SO_4, and scanned by Asuka Ozmor 82 densitometer.

RESULTS

Effect of BSA on the enzymic inhibition of the photoactivities and on the removal of lipid degradation products

Spinach chloroplasts were treated with lipolytic enzymes, potato galactolipase and venom phospholipase A_2, as shown in Methods, and the inhibitory patterns of the electron flow and the effects of BSA were investigated.　Fig. 1 shows the results with potato galactolipase and Fig. 2 with venom phospholipase A_2.　Potato galactolipase inhibited photosystem I activity (DCIPH$_2$→NADP) more rapidly than photosystem II activity (H$_2$O→DCIP).　In contrast, venom phospholipase inhibited photosystem II activity much faster than photosystem I activity.　These results are consistent with those of the previous work.[8]　The marked inhibitions by these enzymes, however, were largely prevented, when a high concentration of BSA (30 mg/ml) was added to the reaction mixture.　The protective effect of BSA was greater in venom phospholipase than in potato galactolipase.　The temporary stimulation of the electron flow (DCIPH$_2$→NADP, in Fig. 2) is probably due to an uncoupling between the photosynthetic electron flow and the photophosphorylation.

Table I shows the effects of BSA on the rate of the enzymic fatty acid release and on the removal of the fatty acids from the chloroplast membranes.　In the Table, the effect of BSA on the extent of fatty acid release was examined by determining the total fatty acids enzymatically released in the absence of BSA (the control) or in the presence of BSA (added before the enzyme treatments).　Next, the removal of free fatty acids from the chloroplast membranes were estimated by comparing the amounts of free fatty acids between the chloroplasts (the precipitate) and the supernatant obtained by centrifugation of the reaction mixture after enzyme treatments.　In addition, the effect of BSA on the fatty acid removal was tested in both cases of BSA addition before and after enzyme treatments. The data indicate that potato galactolipase action (fatty acid release) was somewhat inhibited by addition of 3 % BSA, while the phospholipase action was rather stimulated.　In the absence of BSA (the control), almost all fatty acids released by the enzymes remained in the chloroplasts.　However, in the presence of BSA, which was added before enzyme treatments, all or most of fatty acids were transferred to the supernatant.　It was further observed that the fatty acid removal by BSA was greater in the phospholipase than in the galactolipase treatment. This just coincides with the above finding that a higher prevention of inhibition by BSA took place in the phospholipase than in the galactolipase treatment (Figs. 1 and 2).
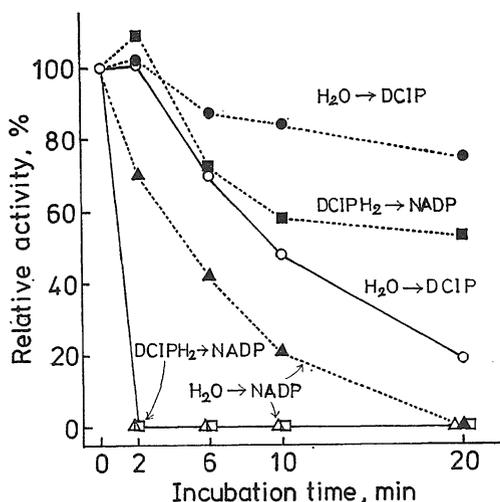
Fig. 1. Inhibition of the photochemical activities by potato galactolipase and effect of BSA on the enzymic inhibition. The enzyme reaction mixtures (1 ml) contained 0.1 M phosphate buffer(pH 5.5), 0.4 M sucrose, chloroplasts (1 mg chlorophyll), and the enzyme (0.37 mg protein), with or wothout 30 mg BSA. The mixtures were incubated at 30°C with shaking, and the photochemical activities were assayed as described in Methods. Solid lines are the reaction without BSA, and dotted lines with BSA. The control rates (μmoles/mg chlorophyll·hr) of photochemical electron flows of the chloroplasts used : $H_2O \rightarrow DCIP$, 101 ; $DCIPH_2 \rightarrow NADP$, 59 ; $H_2O \rightarrow NADP$, 65.
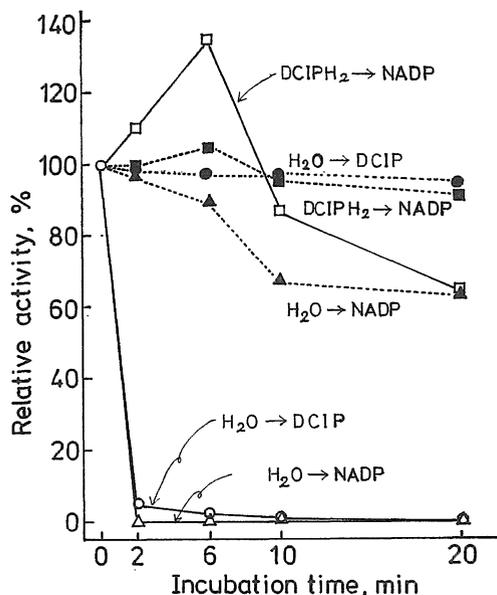

Fig. 2. Inhibition of the photochemical activities by venom phospholipase A_2 and effect of BSA on the enzymic inhibition. The reaction mixtures (1 ml) contained 0.1 M Tris-HCl (pH 8.0), 0.4 M sucrose, 0.1 mM $CaCl_2$, chloroplasts (1 mg chlorophyll), and the enzyme (0.43 mg protein), with or without 30 mg BSA. The mixtures were incubated at 30°C with shaking, and the photochemical activities were assayed as descriebed in Methods. Solid lines are the reaction without BSA, and dotted lines with BSA. The control rates (μmoles/mg chlorophyll·hr) of photochemical electron flows of the chloroplasts used : $H_2O \rightarrow DCIP$, 98 ; $DCIPH_2 \rightarrow NADP$, 57 ; $H_2O \rightarrow NADP$, 66.

The densitometric analyses of the lipid extracts from the treated chloroplasts showed that lysolecithin and lyso-phosphatidylglycerol as well as free fatty acids could be removed by BSA from the chloroplast membranes (Fig. 3). Evidence also indicates that BSA does not inhibit potato galactolipase but rather stimulates it when it act on the extracted chloroplast lipids (Table II). This suggests that the BSA inhibition of the galactolipase as shown in Table I is not due to its direct action on the enzyme itself but probably due to some conformational change of the substrates or the membranes caused by adsortion of BSA.

Effect of the enzyme-digested lipids on the photosynthetic electron flow

The enzyme-digested lipids prepared as described in Methods were analyzed by TLC. The results showed that potato galactolipase-digested lipids contained a large portion of fatty acids and trace of lyso-monogalactolipid with the original polar lipids, and venom phospholipase-digested lipids had large portions of fatty acids and lyso-phosphatides with the original phospholipids (the data not presented). Figs. 4 and 5 show effect of the digested lipids on the electron flow when they were added to the chloroplast suspension.

Table I. Effects of BSA on the Fatty Acid Release by Enzymes and on the Removal of Fatty Acids from Chloroplasts

Spinach chloroplasts (1 mg chlorophyll) were treated with potato galactolipase or venom phospholipase as described in Methods, by adding BSA (30 mg/ml) before or after enzyme treatment. The mixture was centrifuged at $27,000 \times g$ for 20 min, and the resulting precipitate (chloroplasts) and supernatants were subjected to determination of free fatty acids. The control was carried out by treating chloroplasts with enzymes in the absence of BSA.

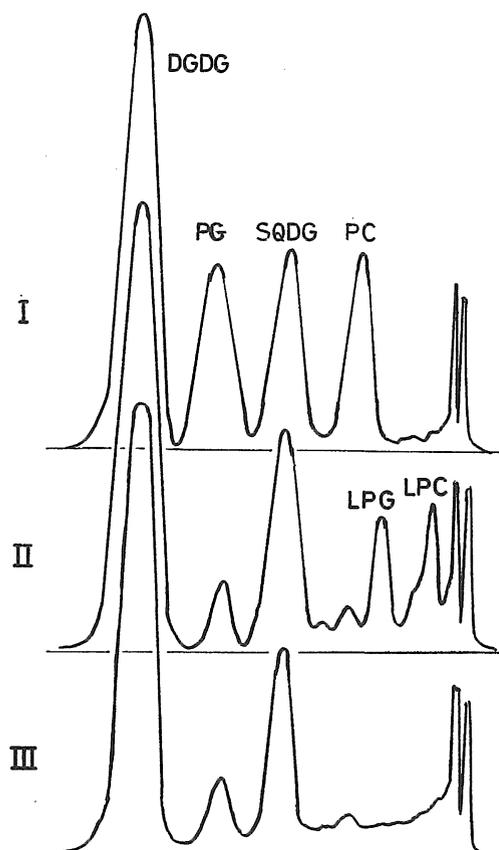| Fractions	Control	BSA addition	
		Before treatment	After treatment
		μmoles of free fatty acids	
Potato galactolipase			
chloroplasts	2.83	0.84	1.33
supernatants	0.05	1.16	1.35
total	2.88	2.00	2.68
Venom phospholipase			
chloroplasts	0.51	0.00	—
supernatants	0.00	1.06	—
total	0.51	1.06	—

Fig. 3. Densitometry of TLC of the lipids from the treated chloroplasts. The lipid extracts from the treated chloroplasts were separated on silca gel plates, visualized, and scanned by desitometer as described in Methods. I, non-treated chloroplasts ; II, chloroplasts treated with potato galactolipase in the presence of 3 % BSA ; III, chloroplasts treated with potato galactolipase in the presence of 3 % BSA, followed by separation by sucrose-density centrifugation. DGDG, digalactosyldiglyceride ; PG, phosphatidylglycerol ; SQDG, sulfoquinovosyldiglyceride ; PC, lecithin ; LPG, lysophosphatidylglycerol ; LPC, lysolecithin.

Table II.　Effect of BSA on Enzymic Hydrolysis of the Extracted Lipids from Chloroplasts
The extracted lipids from the chloroplasts were treated with potato galactolipase for 30 min in the absence or in the presence of BSA.　The hydrolysis rates were determined by estimating free fatty acids released by the enzyme.

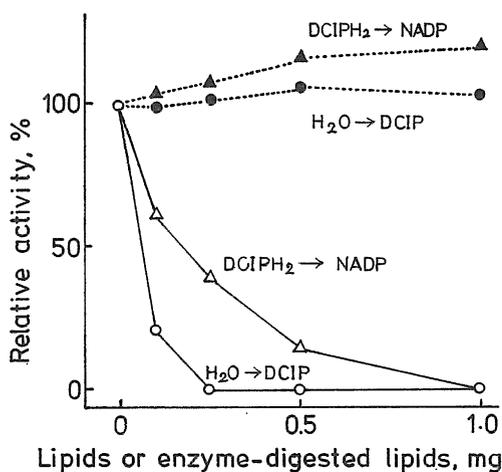| Treatments of chloroplasts	Hydrolysis rate, %
Enzyme	76
Enzyme + 3 % BSA	91
Enzyme + 5 % BSA	96

Fig. 4.　Inhibitory effect of potato galactolipase-digested lipids on the photochemical electron flows.　Spinach chloroplast polar lipids were separated and treated with potato galactolipase. The chloroform extract from the digestion mixture was added to the chloroplasts (1 mg chlorophyll) in concentrations as shown in the Figure. After incubation at 20°C for 20 min, the photochemical electron flow rates of the treated chloroplasts were assayed (solid lines). Dotted lines were the control, which was treated with non-digested polar lipids under the same conditions. The control rates (μmoles/mg chlorophyll·hr) of photochemical electron flows of the chloroplasts used : $H_2O \rightarrow DCIP$, 95 ; $DCIPH_2 \rightarrow NADP$, 55 ; $H_2O \rightarrow NADP$, 62.
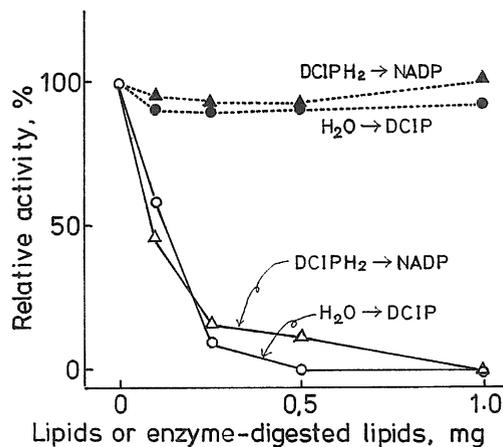

Fig. 5.　Inhibitory effect of venom phospholipase-digested lipids on the photochemical electron flows. Spinach chloroplast phospholipids were separated and treated with venom phospholipase. The chloform extract from the digestion mixture was added to the chloroplasts (1 mg chlorophyll) in concentrations as shown in the Figure. After incubation at 20°C for 20 min, the photochemical electron flow rates of the treated chloroplasts were assayed (solid lines). Dotted lines are the control, which was treated with non-digested phospholipids.　The control rates (μmoles/mg chlorophyll·hr) of the photochemical electron flows of the chloroplasts used : $H_2O \rightarrow DCIP$, 97 ; $DCIPH_2 \rightarrow NADP$, 53 ; $H_2O \rightarrow NADP$, 60.

Although the original lipids, i. e.　non-digested lipids, had little effect on the electron flow, the digested lipids exhibited a strong inhibition. The figures also indicate that the digested lipids were much different from the enzyme own in the inhibitory pattern, in both cases of potato galactolipase and venom phospholipase. In treatment with the galactolipase-digested lipids, photosystem II reaction was inhibited somewhat faster than photosystem I reaction (Fig. 4), while, in the galactolipase treatment, photosystem I reaction decreased more rapidly than photosystem II reaction (Fig. 1). In the case of the phospholipase, its digested lipids caused a nearly similar inhibifion of the two photoreactions (Fig. 5),
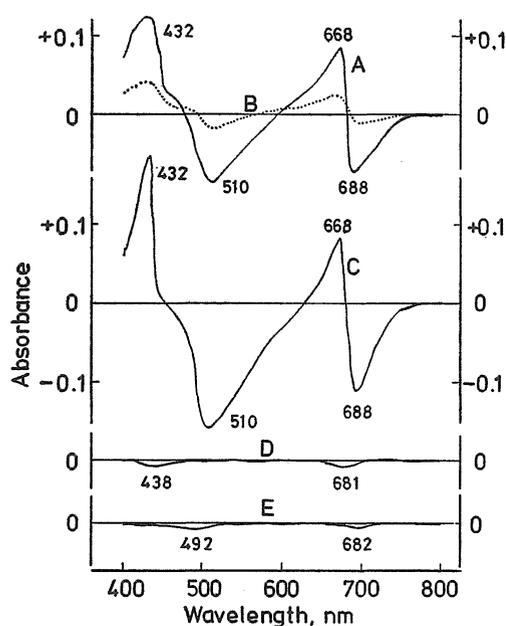
Fig. 6. Spectral changes of the chloroplasts treated with enzymes or enzyme-digested lipids. Spinach chloroplasts were treated with lipolytic enzymes or enzyme-digested lipids at various degrees as shown in Methods. Difference spectra (treated minus non-treated) and the rates of photochemical electron flows were determined. The spectra at 50 % inhibition of the original electron flow rates were obtained by interpolation from several spectra at various extents of inhibition. Curve A, linolenic acid treatment (50 % inhibition of photosystem I activity) ; Curve B, linolenic acid treatment (50 % inhibition of photosystem II activity) ; Curve C, lysolecithin treatment (50 % inhibition of photosystem I activity) ; Curve D, potato galactolipase treatment (50 % inhibition of photosystem I activity) ; Curve E, venom phospholipase treatment (50 % inhibition of photosystem II activity). The spectrum of lysolecithin treatment at 50 % inhibition of photosystem II activity was almost all similar to Curve C.

whereas the enzyme suppressed photosystem II reaction preferentially (Fig. 2). These show that a specific inhibition for either photosystem I or II reaction occurs with the lipolytic enzymes, and a less specific inhibition takes place with their digested lipids.

On the other hand, the two inhibitory patterns by the galactolipase- and phospholipase-digested lipids were rather similar to each other (Figs. 4 and 5). Although some difference can be observed, it likely comes from the different components in the digested lipids. As shown above, the galactolipase-digested lipids contain fatty acids and the phospholipase-digested lipids have fatty acids and lyso-phosphatides as the main components.[9] Moreover, fatty acids tend to inhibit photosystem II activity faster than photosystem I activity, while lysolecithin inhibits the two activities at a similar rate.[13] Thus, Fig. 4 reflects mainly the effect of fatty acids, and Fig. 5 shows the mixed effect of lyso-phosphatides and fatty acids.

Spectral change of the treated chloroplasts

In order to examine alteration of the membrane structure of the treated chloroplasts, spectral changes were investigated by determining difference spectra as well as the photochemical activities. The difference spectra at 50 % inhibition of the photoactivity were obtained by interpolation from several spectra at various extents of inhibition. Major components in the enzyme-digested lipids i. e. free linolenic acid and lysolecithin, caused strong changes of absorbances at 688, 668, 510, and 432 nm. This indicates that chlorophyll and also probably carotenoid molecules drastically changed their existing states in the chloroplast membranes. On the contrary, both the enzymes of potato galactolipase and venom phospholipase induced only a little effectwith some what different spectral patterns. The results make us possible to presume that the two types of inhibitions by the lipolytic enzymes and the lipid degradation products occur in different sites of the chloroplast membranes at least around the photosynthetic pigments.

DISCUSSION

The present early work has shown that potato galactolipase and venom phospholipase A₂ cause a marked inhibition of the photosynthetic electron flow in the chloroplasts. The inhibitions were, however, largely prevented in the presence of BSA (Figs. 1 and 2). It may be possible that the prevention is caused by direct inhibition of the enzymes with BSA, but the possibility is ruled out because BSA rather stimulated the phospholipase action (Table I). Although the lipid-hydrolysis rate with potato galactolipase was reduced by BSA at some extent (Table I), the reduction is not due to its inhibitory aciton on the enzyme but probably due to the formation of grana stacking with BSA as shown by Okamoto et al.[16] The data also proved that BSA can remove fatty acids (Table I) and also lyso-phosphatides (Fig. 3) from the chloroplast membranes. These findings with both potato galactolipase and venom phospholipase are fundamentally consistent with those reported by Anderson et al.[4,7] working with *Phaseolus vulgaris* galactolipase. Thus, we could summarize that the inhibitions of the electron flow of the chloroplasts by lipolytic acylhydrolase are principally caused by the lipid-soluble degradation products such as fatty acids and lyso-compounds, and that the protective effect of BSA on the photoactivities is mostly the results of binding the lipid degradation products and removing them from the chloroplast membranes. Although some other contribution of BSA by a direct protection of the functional proteins can be presumed, it would be a little.

The enzyme-digested lipids, when separated and added to the chloroplast suspension, brought about a much different pattern of inhibition from that induced by the enzyme treatment, in both cases of potato galactolipase and venom phospholipase, respectively (Figs. 1 and 4, and Figs. 2 and 5). The data show that a specific inhibition for either photosystem I or II reaction is caused by the enzymes, while a less specific inhibition occurs with the enzyme-digested lipids. The examination on the spectral change also exhibited a great difference between the enzyme and the digested lipid treatments (Fig. 6), indicating that the exogenous digested lipids caused a strong change of the existing state of the pigment molecules in the membranes, while the endogenous digested lipids induced only a little effect. On the basis of these findings it appears likely that the two types of inhibitions by lipolytic enzymes and their digested lipid products are partially different in the mechanism of the inhibitory reaction.

When the digested lipids are added to the chloroplast suspension, they may be adsorbed freely all over the exposed surface of the chloroplast membranes, probably depending on the charge and/or lipophile characteristic of the membrane surface. In contrast, the lipolytic enzymes will attack the membrane lipids and produce lipid degradation products on the specific sites of the membranes, depending on their substrate specificity and lipid distribution in the membranes. Thus, the locality of exogenous and endogenous lipid hydrolysates in the membranes will be different, and this difference would cause the different patterns of inhibition. In this case, some contribution by different components of lipid degradation products may be involved, but it would be less in the light of the results that the inhibitory patterns between enzymes and their digested lipids were much different (Figs. 1 and 4, and Figs. 2 and 5), and that the patterns between the digested lipids derived by the two different enzymes were rather similar (Figs. 4 and 5). Still, it seems that the water-soluble degradation products derived from lipids by potato galactolipase, i. e. galactosyl-

glycerol and glycerylphosphorylcholine, are released into the aqueous phase during the enzymic reaction. Accordingly, they may be hardly concerned with the inhibitory reaction.

From the above findings and deduction, the contrasting inhibitory patterens between potato galactolipase and venom phospholipase treatments could be attributed to the different locality of the lipid degradation products, in its turn, to the lipid distribution in the chloroplast membranes. Consequently, one experimental result[8] that potato galactolipase caused a decrease of photosystem I reaction with rapid degradation of monogalactolipid, can be interpreted to indicate that physiologically more important monogalactolipid occurs near photosystem I. Similarly, the other result[8] that venom phospholipase produced a rapid decay of photosytem II reaction with selective degradation of phospholipids, can be assumed that more functional phospholipid is located around photosystem II. In fact, we have recently found special monogalactolipid bound with photosystem I complex and also peculiar phospholipids bound with photosystem II complex, which were closely related to the electron flow including photosystem I or II, respectively. The detail will be published in the near future.

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SUMMARY

The mechanism of inhibition of the photoactivity of spinach chloroplasts by lipolytic enzymes was investigated. Potato galactolipase and venom phospholipase A_2 caused a rapid inhibition of the photoactivity. These inhibitions were, however, largely prevented by addition of bovine serum albumin to the mixtures. Evidence also indicated that the fatty acids and lyso-lipids produced by the enzymes can be removed from the chloroplast membranes by bovine serum albumin. These exhibit that the enzymic inhibitions occur principally due to the lipid digestion products. Thus, a comparison between the digested lipids and enzymes in the inhibitory reactions was then performed. The inhibition by the digested lipids was less specific but the enzymic inhibition was specific for either photosystem I or II reaction. Also, the digested lipids induced a large spectral change of the chloroplasts but the enzymes gave a little. From the findings it seems that lipolytic enzymes attack specific site of the membranes and inhibit the electron flow near the site, while the added digested lipids are freely adsorbed all over the exposed surface of the chloroplast membranes and bring about a less specific inhibition.

摘　　　　要

　　ホウレンソウ葉緑体における 光化学活性の脂質分解酵素による阻害機構を調べた．馬鈴薯ガラクトリパーゼおよび蛇毒ホスホリパーゼA_2 は葉緑体の光化学活性を強く阻害した．しかし，反応液に牛血清アルブミンを加えると，酵素反応が進んでいるにも拘らず活性阻害が大きく低下した．酵素で生成される脂質分解物（脂肪酸，リゾ脂質など）は牛血清アルブミンによって葉緑体膜から除去されることが見出され，従って葉緑体光化学活性の酵素的阻害は，本質的には酵素で生成される脂質分解物の作用に基づいていることが明らかになった．そこで，次に葉緑体に対する酵素処理効果と脂質酵素分解物の添加効果とを比較した．前者は後者に比較して系Ｉ・系Ⅱ活性に対する阻害の特異性が高く，また両者は葉緑体の吸収スペクトル変化に大きな差異を示した．以上の結果から，脂質分解酵素は葉緑体膜の脂質分布に従って膜の特定部位を攻撃し脂質分解物を生成することによって攻撃部位近くの電子伝達系を阻害するのに対し，添加された分解脂質は，葉緑体膜の露出面に自由に吸着され特異性の低い阻害をおこすことを考察した．