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EFFECTS OF LIPOLYTIC ENZYMES ON THE PHOTOCHEMICAL ACTIVITIES OF SPINACH CHLOROPLASTS

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SUMMARY

1. Spinach class II chloroplasts were treated with purified potato lipolytic acyl-hydrolase and venom phospholipase A_2, and their lipid degradations and the effects on the photochemical activities were followed.

2. Potato lipolytic enzyme hydrolyzed monogalactosyldiacylglycerol at a faster rate than phospholipids such as phosphatidylglycerol and phosphatidylcholine. The treatment caused a rapid decrease of Photosystem I activity, and a less change of Photosystem II activity.

3. Venom phospholipase A_2 which preferentially hydrolyzed phosphatidylglycerol, caused a rapid decrease of Photosystem II activity and only a slight decrease of photosystem I activity.

4. Potato enzyme and phospholipase A_2 degraded the membrane lipids of glutaraldehyde-fixed chloroplasts at a rather slightly higher rate than those of non-treated chloroplasts.

5. The results suggested a possible correlation between monogalactosyldiacylglycerol degradation and decay of Photosystem I activity and between phosphatidylglycerol degradation and decay of Photosystem II activity. A possible mechanism is discussed.

INTRODUCTION

Among the numerous methods which have been developed to elucidate structure and function of biological membranes are the use of lipolytic [1, 2] and proteolytic [3, 4] enzymes. Since the lamellar membranes of chloroplasts are about 50 % protein and 50 % lipid, treatments which alter or destroy proteins and lipids might be useful in determining the role and importance of these substances in maintaining over-all membrane integrity and photosynthetic activity.

Various lipolytic enzymes have been used in studies of the Hill reaction [2, 5, 6, 7], photophosphorylation [8], fluorescence [6, 9], and the morphology [5]

Abbreviation: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

of the grana. Lipolytic enzymes inhibited the Photosystem II reaction to varying degrees and were less effective inhibiting the Photosystem I reaction [2, 6]. In such experiments, however, the effects closely depend on the enzyme preparations and the reaction conditions used. Thus it is desirable that lipolytic enzymes as pure as possible are employed under mild conditions.

In the present report, spinach chloroplasts were treated with purified lipolytic enzymes, and the lipid degradations and the effects on photochemical activities were investigated. The enzymes used were lipolytic acyl-hydrolase from potato tubers and venom phospholipase A_2.

MATERIALS AND METHODS

Chloroplast preparation

Spinach leaves were homogenized in a medium containing 0.4 M sucrose, 10 mM Tris · HCl (pH 8.0), 20 mM NaCl and 5 mM $MgCl_2$ for class I chloroplast preparation, and a medium containing 0.35 M NaCl, 10 mM Tris · HCl (PH 8.0) and 5 mM $MgCl_2$ for class II chloroplast preparation. The pellets which sedimented between $200 \times g$ and $2000 \times g$ were washed, and then 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 having both galactolipase and phospholipase activities was extracted from potato tubers and purified to homogenity using DEAE-Sephadex and Sephadex G-100 columns and isoelectric focusing [10]. The preparation had specific activities of 8.5 and 8.1 μmol fatty acid released per mg protein per min for monogalactosyldiacylglycerol and phosphatidylcholine, respectively. Phospholipase A_2 was purified from snake venom of Habu, *Trimeresurus flavoviridis*, by DEAE-Sephadex column (specific activity, 6.0 μmol fatty acid released per mg protein per min). Neither of the lipolytic enzyme preparations had proteolytic activity. Enzyme assays were carried out by determining free fatty acid released according to the method reported previously [10, 11], using spinach monogalactosyldiacylglycerol and egg phosphatidylcholine as substrates.

Enzymic treatments of chloroplasts

The reaction mixture (1 ml) for treatment with potato lipolytic enzyme, 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). Mixtures were incubated at 35 °C with stirring. After incubation, the mixtures were cooled to 0 °C rapidly, and assayed for photochemical activities and assayed for lipid degradation after extraction with chloroform/methanol (2 : 1, v/v). Lipid degradation was followed by determining free fatty acid released, lipid sugar, lipid phosphorus, and individual lipid components.

Measurements of photochemical activities

DCIP photoreduction was measured spectrophotometrically at 610 nm. The

reaction mixture (3 ml) contained 50 mM Tricine-KOH (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 Tricine-KOH (pH 8.0), 10 mM NaCl, 8.5 μM spinach ferredoxin, and chloroplasts (40 μg chlorophyll). When using reduced DCIP as 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 white light illumination (40 000 lux) from a projector lamp at 20 °C.

Fixation of chloroplasts

Chloroplasts were fixed essentially by the method of Hallier and Park [12] with partial modification. Glutaraldehyde solution (1 %, 5 % or 10 %) containing 0.4 M sucrose and 10 mM Tris · HCl (pH 7.0) was added to an equal volume of chloroplast suspension (1 mg chlorophyll/ml) containing the sucrose buffer. The suspension was stirred thoroughly at 0 °C for 30 min, and then diluted with the sucrose-buffer and centrifuged for 30 min at 15 000 $\times g$. The precipitate was washed twice with the sucrose-buffer.

Analytical methods

Lipids analyses were carried out by the method previously reported [13] using thin-layer chromatography and gas-liquid chromatography. Free fatty acids were determined by the rhodamine method as described previously [11]. Protein was determined by the method of Lowry et al. modified by Hartree [14]. Lipid sugar was measured by the method of Dubois et al. [15], while lipid phosphorus and inorganic phosphorus were followed by the procedure of Allen [16] and by a modification [17] of Allen's procedure, respectively. Chlorophyll was measured by the method of Arnon [18]. Electron microscopy was carried out by fixing chloroplasts with buffered OsO_4, embedding in Epon, and examining with a Hitachi HS-6 electron microscope.

RESULTS

It has been reported that spinach chloroplasts have weak endogenous activities

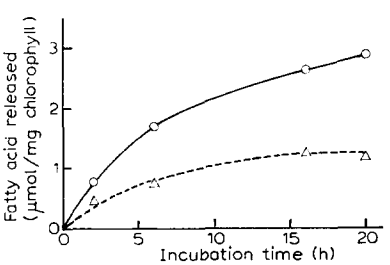

Fig. 1. Time course of release of free fatty acids in aging of isolated chloroplasts. Class I or II chloroplasts from spinach leaves were incubated at 35 °C. The incubation mixture (1 ml) contained 50 mM phosphate buffer (pH 5.5), chloroplasts (1 mg chlorophyll), and 0.4 M sucrose. After incubation the mixture was extracted with chloroformmethanol (2 : 1, v/v), and free fatty acids released were determined by the rhodamine method described in Methods. ○–○, class I chloroplasts; △–△, class II chloroplasts.

of lipolytic enzymes [19, 20]. Thus, the rates of release of fatty acid during aging of class I and II chloroplasts were compared. The results (Fig. 1) show that class I chloroplasts liberated free fatty acids at a faster rate than class II chloroplasts which had lost stroma proteins. It seems that lipolytic enzymes are localized in the stroma and easily lost. Therefore, class II chloroplasts were used as a favorable material for the following experiments.

Potato lipolytic enzyme digestion and its effects

When spinach class II chloroplasts were treated with potato lipolytic enzyme under the condition described in Methods, there was a marked increase of free fatty

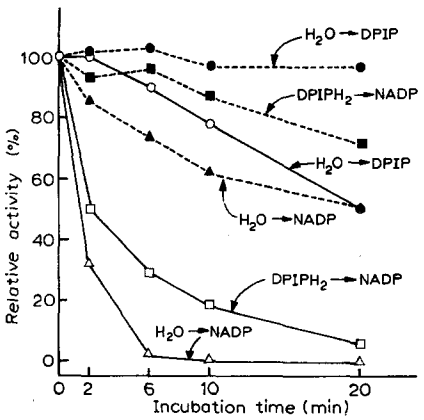

Fig. 2. Effects of potato lipolytic enzyme on photochemical activities. The reaction mixture (1 ml) contained 0.1 M phosphate buffer (pH 5.5), 0.4 M sucrose, chloroplasts (1 mg chlorophyll), and potato enzyme (0.37 mg protein). The mixture was incubated at 35 °C with shaking, and the photochemical activities were assayed as described in Methods. Dotted line is the control without the enzyme.

TABLE I

HYDROLYSIS RATES OF CHLOROPLAST MEMBRANE LIPIDS ON TREATMENT WITH LIPOLYTIC ENZYMES

Spinach class II chloroplasts (1 mg chlorophyll) were treated for 10 min at 35 °C with potato lipolytic enzyme (0.37 mg protein) and with venom phospholipase A_2 (0.43 mg protein) under conditions described in Methods, and lipid degradation was followed. Hydrolysis rates are expressed as percentages of the initial lipid contents.

Lipids	Hydrolysis rates, %	
	Potato lipolytic enzyme	Phospholipase A_2
Total glycolipids	27	–
Total phospholipids	21	84
Monogalactosyldiacylglycerol	40	–
Digalactosyldiacylglycerol	20	–
Phosphatidylglycerol	10	100
Phosphatidylcholine	29	71
Phosphatidylethanolamine	5	44

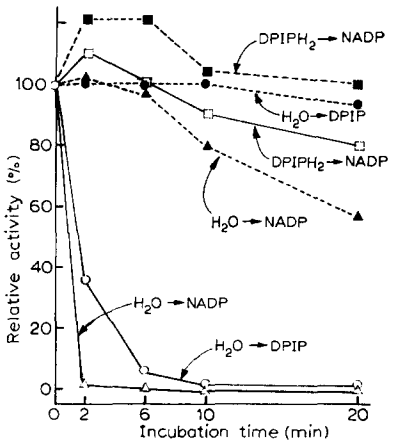

Fig. 3. Effects of phospholipase A_2 on photochemical activities. The reaction mixture (1 ml) contained 0.1 M Tris · HCl (pH 8.0), 0.1 mM $CaCl_2$, 0.4 M sucrose, chloroplasts (1 mg chlorophyll), and the enzyme (0.43 mg protein). The mixture was incubated at 35 °C with shaking, and the photochemical activities were assayed. Dotted line is the control without the enzyme.

acids and a corresponding decrease of lipid sugar and phosphorus. Lipid analysis revealed that monogalactosyldiacylglycerol was hydrolyzed at a faster rate than phosphatidylcholine and phosphatidylglycerol (Table I). Fig. 2 shows the rates of decay of photochemical activities during incubation with enzyme. The treatment caused a marked decrease of electron flow from both water and $DCIPH_2$ to NADP at an early stage of incubation, whereas DCIP reduction was more resistant throughout the incubation.

Venom phospholipase A_2 digestion and its effects

Phospholipase A_2 hydrolyzed selectively the phospholipids in the chloroplast thylakoid membranes, resulting in the release of free fatty acids and the accumulation of lyso-phospholipids. Determination of the hydrolysis rates of individual lipids indicated that phosphatidylglycerol was preferentially attacked (Table I). During the

TABLE II

ACTION OF LIPOLYTIC ENZYMES ON GLUTARALDEHYDE-FIXED CHLOROPLASTS

Chloroplasts were fixed with glutaraldehyde (GA) as described in Methods. Fixed and unfixed chloroplasts were treated for 20 min with potato lipolytic enzyme and phospholipase A_2 as described in Fig. 2, and release of free fatty acids was followed.

Treatments	Fatty acid released (μmol/mg chlorophyll 20 min)	
	Potato enzyme	Phospholipase A_2
Unfixed	1.10	0.60
Fixed		
1 % glutaraldehyde	1.17	–
5 % glutaraldehyde	1.43	0.86
10 % glutaraldehyde	1.41	0.64

TABLE III

EFFECTS OF FIXATION ON ENZYMIC HYDROLYSIS OF CHLOROPLAST MEMBRANE LIPIDS

Chloroplasts were fixed with 10 % glutaraldehyde as described in Methods. The fixed and unfixed chloroplasts were treated for 20 min with potato lipolytic enzyme or phospholipase A_2 as described in Figs. 2 and 3, and lipid degradation was followed. Hydrolysis rates were expressed as percentages of the initial lipid contents.

Lipids	Hydrolysis rates (%)			
	Potato lipolytic enzyme		Phospholipase A_2	
	Unfixed	Fixed	Unfixed	Fixed
Monogalactosyldiacylglycerol	76	62	–	–
Digalactosyldiacylglycerol	56	82	–	–
Phosphatidylglycerol	54	100	92	91
Phosphatidylcholine	49	100	57	65

digestion, there was a rapid inactivation of electron flow from water to DCIP and from water to NADP, although electron flow from $DCIPH_2$ to NADP was found to decrease only slightly (Fig. 3). Digestions with potato enzyme and phospholipase A_2 respectively had contrasting effects on photochemical activities.

Effects of the lipolytic enzymes on the morphology of chloroplast membranes

In order to elucidate whether the membrane structure of chloroplasts was affected by enzyme digestions, the enzyme-treated chloroplasts were observed by electron microscopy. It was noted that most chloroplasts treated with potato enzyme for 20 min still maintained their gross membrane structure, although a remarkable swelling of end granal thylakoids occurred. The chloroplasts treated with phospholipase A_2 for 20 min maintained membrane structure more similar to that of non-treated chloroplasts. Chloroplasts were also fixed with glutaraldehyde and the effects of fixation on enzymic lipid hydrolysis were examined. The results are presented in Tables II and III. It was surprisingly observed that the lipolytic enzymes attacked the fixed chloroplasts at a rather higher rate than the unfixed ones, significantly changing the relative hydrolysis rates of galacto- and phospholipids. These results suggest that enzymic degradation of chloroplast lipids proceed without disrupting the gross membrane structure.

DISCUSSION

In general, lipolytic enzymes employed here easily attacked chloroplasts, and degraded chloroplast membrane lipids at faster rates than extracted lipids under the conditions described. As mentioned in Results, electron microscopic observations and enzymic treatments of the fixed chloroplasts demonstrated that enzymic hydrolysis of the membrane lipids occurred without destroying the membrane structure at least at earlier stage of the digestion. This is a favourable situation for studying lipid hydrolysis since it avoids complications due to changes in gross membrane structure.

The treatment with potato lipolytic enzyme brought about a rapid decrease

of $DCIPH_2$-supported NADP reduction (Photosystem I activity), while it caused only a slight inhibition of DCIP reduction (Photosystem II activity). On the contrary, phospholipase A_2 digestion caused a strong inhibition of photosystem II activity and less change of photosystem I activity. Potato lipolytic enzyme rapidly hydrolyzed monogalactosyldiacylglycerol and degraded phosphatidylcholine and phosphatidylglycerol more slowly, while phospholipase A_2 decomposed selectively degraded phospholipids, predominantly phosphatidylglycerol as shown in Table I. Thus, it seems that the decrease of Photosystem I activity correlated with degradation of galactolipids, particularly monogalactosyldiacylglycerol, and decay of Photosystem II activity to degradation of phospholipids, particularly phosphatidylglycerol.

Moreover, in enzymic hydrolysis of chloroplast membrane lipids, two different mechanisms are expected for the enzymic inhibition of photochemical activities. One is an inhibitory action of lipid-hydrolyzing products such as free fatty acids [21–23], lyso-phospholipids etc., and the other is a local damage of the membrane structure, which may cause changes in the conformation of membrane proteins and in their orientation and arrangement. It has been established that potato enzyme hydrolyzed galactolipids to form free fatty acids, galactosyl-glycerol and a small amount of free galactose, and simultaneously degraded phospholipids, e.g. phosphatidylcholine, resulting in formations of free fatty acids, glycerophosphate and free choline [10]. On the other hand, phospholipase A_2 formed free fatty acids and lyso-phospholipids from the membrane lipids as hydrolysis products. Such a difference of lipid degradation products from the enzyme digestions may partially explain the contrasting pattern of decay of photochemical activities.

Recently Anderson et al. [2] have reported that, in chloroplast membranes, inhibition of electron flow by a lipolytic enzyme was prevented in the presence of high concentration of bovine serum albumin. They suggested that the enzymic inhibition of electron flow was mainly due to free fatty acids released enzymatically since bovine serum albumin was able to bind free fatty acids. However, there are other suggestions for the protective function of bovine serum albumin toward chloroplasts apart from the binding of free fatty acids [24, 25]. Thus it might be possible that the enzymic inhibition of chloroplast electron flow was partially due to a local damage of the membrane, and the damage was simultaneously repaired or protected with the bovine serum albumin. If this is true, the results in the present paper would lead to a speculation that there are specific lipid components required for Photosystem I and II activities. The investigation of a functional role of chloroplast membrane lipids is incomplete but a worthwhile area of reserach. Further work on this problem is in progress in this laboratory.

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