島根大農研報 (Bull. Fac. Agr. Shimane Univ.) 24 : 227−232, 1990

Release of Ferredoxin-NADP Reductase and Plastocyanin from Spinach Chloroplast Membranes by Potato Galactolipase

Osamu HIRAYAMA, Takashi NOMOTOBORI and Haruo SUMI

馬鈴薯ガラクトリパーゼ処理による葉緑体膜からのフェレドキシン
―NADP還元酵素ならびにプラストシアニンの脱離

平山　修・野元堀　隆・角　治夫

Spinach class II chloroplasts were trearted with purified potato galactolipase, and the effects on the photoactivities and the functional proteins were investigated A treatment of chloroplasts with the enzyme in the absence of bovine serum albumin caused a marked decrease in NADP photoreduction and a great increase in NADPH-diaphorase activity. This indicates that the ferredoxin-NADP reductase was released from the membranes by the enzyme action. The rate of the release by the lipolytic enzyme was much higher than those of release by sonication with or without Triton X-100. Other treatment with the enzyme in the presence of bovine serum albumin, followed by a separation by density gradient centrifugation, also caused a decrease in photosystem I activity, but the decrease was recovered with addition of plastocyanin. This indicates a release of plastocyanin by the enzyme.

INTRODUCTION

Selective lipid degradation of the chloroplast membranes by enzyme is a valuable approach to understanding of lipid functions in the chloroplasts. Several workers[1-3] reported the effects of lipolytic enzyme treatments on the photochemical electron flow in the chloroplasts. Anderson et al.[2] demonstrated that a treatment of subchloroplast particles with a galactolipase from *Phaseolus vulgaris* leaves resulted in a rapid inhibition of the photosynthetic electron transport, but this inhibition was largely prevented in the presence of a high concentration of BSA. Shaw et al.[3] further described that the electron flow through photosystems I and II (DPC→methyl viologen) was not affected by the galactolipase treatment in the presence of BSA, while the oxygen evolution and photophosphrylation were partially inhibited.

In the previous paper, we reported[4] that a treatment of spinach chloroplasts with

* Labolatory of Food Chemistry, Faculty of Agriculture, Shimane University, Matsue 690, Japan.

Abbrevation: BSA, bovine serum albumin; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-p-benzoquinone; DPC, diphenylcarbazide; TLC, thin-layer chromatography

potato galactolipase in the absence of BSA caused a decrease in photosystem I electron flow ($DCIPH_2 \rightarrow NADP$) with a rapid degradation of monogalactosyldiacylglycerol in the chloroplasts. The present work revealed that the enzymic inhibition of photosystem I electron flow was partly due to liberations of the ferredoxin-NADP reductase and plastocyanin. The functions of lipids in the chloroplasts were also discussed.

MATERIALS AND METHODS

Preparation of chloroplasts

Spinach leaves were homogenized in a mediun containing 0.35 M NaCl, 5 mM $MgCl_2$ and 50 mM Tris•HCl (pH 8.0). The pellets which sedimented between 200 xg and 2000 xg were washed, and then resuspended in 10 mM Tris•HCl (pH 8.0), 5 mM NaCl and 50% glycerol. The suspension was stored at −20°C until used.[5)]

Preparation of potato galactolipase and enzyme assay

Potato galalctolipase having both galactolipase (E. C. 3.1.1.26) and phospholipase B-like activities was extracted and purified from potato tubers.[6)] The enzyme assay was carried out by determining free fatty acid released from galactolipids or phospholipids according to the method as reported previously.[7)]

Enzyme treatments of chloroplasts

The reaction mixtrue (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 (1 mg protein), with or without 3% BSA. The mixture was incubated at 30°C for 30 min (unless indicated) with stirring. After incubation, the mixture was rapidly cooled to 0°C, and then used for photoactivity measurement or density gradient centrifugation.

Sucrose-density gradient centrifugation

A discontinuous sucrose-density gradient of five layers was prepared by pipetting 5 ml of each of 0.8 M, 1.0 M, 1.2 M, 1.4 M, and 1.6 M sucrose in 10 mM Tris•HCl buffer (pH 7.5) containing 20 mM NaCl and 5 mM $MgCl_2$. A sample solution was layered on the top of the gradient, and centrifuged at 80,000 xg for 60 min.

Measurements of photoactivities and NADPH-diaphorase activity

The rate of O_2 evolution ($H_2O \rightarrow DMBQ$) was measured polarographically with a YSI 4004 Clark oxygen electrode. The reaction mixture (3 ml) contained 50 mM Tricine/KOH (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, 1 mM NH_4Cl, 200 μM NaN_3, 1 mM DMBQ, and chloroplasts (30 μg chlorophyll). Methyl viologen-mediated O_2 uptake ($DCIPH_2 \rightarrow$ methyl viologen) was measured polarographically by the Clark oxygen electrode described above. The reaction mixture (3 ml) contained 50 mM Tricine/KOH (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, 5 mM NaN_3, 0.1 mM methyl viologen, 5 mM NH_4Cl, 0.1 mM DCIP, 1 mM ascorbate, 10 μM DCMU, and chloroplasts (20 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, 1 mM NH_4Cl, 8.5 μM spinach ferredoxin, and chloroplasts (40 μg chlorophyll). When using reduced DCIP 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 white light illumination (40,000 lux) from a projector lamp at 20°C.

NADPH-diaphorase activity was followed by determining the absorbance change at 610 nm for 1 min spectrophotometrically.[8] The assay system contained 50 mM Tricine/KOH (pH 7.5), 0.5 mM NADPH, 55 μM DCIP, and the enzyme in a final volume of 3 ml.

Lipid analysis

The chloroplast suspension (1 ml) was extracted three times with 1 ml of water-saturated n-butanol.[9] The extracts were collected, evaporated under reduced pressure, and then redissolved in 1 ml of chloroform. The resulting lipid solution was used for silicagel TLC analysis[10] or silicagel micro-column analysis.[11]

RESULTS AND DISCUSSION

Effect of potato galactolipase on ferredoxin-NADP reductase in the chloroplast membranes

Chloroplasts were treated with potato galactolipase in the absence of BSA as described in Methods. About 80% of total polar lipids in the chloroplasts was hydrolyzed

TABLE I EFFECT OF THE ENZYME TREATMENT ON NADP PHOTOREDUCTION AND NADP-DIAPHORASE ACTIVITIES OF THE CHLOROPLASTS

Chloroplasts were treated with potato galactolipase in the absence of BSA as described in Methods, using 0.1 M phosphate buffer containing 0.6 M sucrose (A) or no sucrose (B). After incubation, aliquots of the mixture were centrifuged at 200,000 xg for 30 min. The whole mixture and the precipitated fraction were both subjected to activity measurements. NADP photoreduction and NADP- diaphorase activity were measured as described in Methods, except that the former was carried out with saturated plastocyanin.

Treatments of chloroplasts	NADP photoreduction ($DCIPH_2$→ NADP)		NADPH-diaphorase (NADPH→ DCIP)	
	A	B	A	B
	μmoles/mg chlorophyll·h			
Incubation without enzyme, mixture	58	44	31	46
precipitate	53	26	46	8
Incubation with enzyme, mixture	8	3	192	180
precipitate	2	2	46	20
Original chloroplasts	63		28	

TABLE II
A COMPARISON OF EFFECT OF POTATO GALACTOLIPASE WITH THOSE OF OTHER TREATMENTS ON FERREDOXIN-NADP REDUCTASE IN THE CHLOROPLASTS

Chloroplasts were suspended in distilled water with or without Triton X-100 in varying concentrations to give final suspensions of a chlorophyll concentration of 1 mg per ml. The suspensions were sonicated at 0°C for 5 min. The treatment with the enzyme was carried out in the medium containing no sucrose as described in TABLE I. NADPH-diaphorase activity was directly measured for the treatment mixtures.

Treatments of chloroplasts	NADPH-diaphorase activity
	μmoles DCIP reduced/mg chlorophyll·h
Original chloroplasts	27
Sonication	40
Sonication with	
0.1% Triton X-100	43
0.5% 〃	50
1.0% 〃	55
2.0% 〃	66
Potato galactolipase	185

by the treatment. As shown in Table I, the enzyme-treated chloroplasts lost most of NADP photoreduction mediated with reduced DCIP. The control incubation also induced a slight decrease in the activity probably with the endogenous enzyme. In the both cases, the decrease in NADP photoreduction was higher in chloroplasts treated in a hypotonic medium than in those treated in a hypertonic, and the higher inhibition was also observed in the precipitated chloroplasts than in the mixture. These suggest that the decay of the activity was caused by a release of some components required for the electron flow, in addition to the inhibition by the lipid hydrolysis products induced with the enzyme.

In contrast with NADP photoreduction, NADPH-diaphorase activity of the chloroplasts was remarkably enhanced by the enzyme treatment. The marked increase, however, was not observed in the precipitated chloroplasts, although the precipitated ones

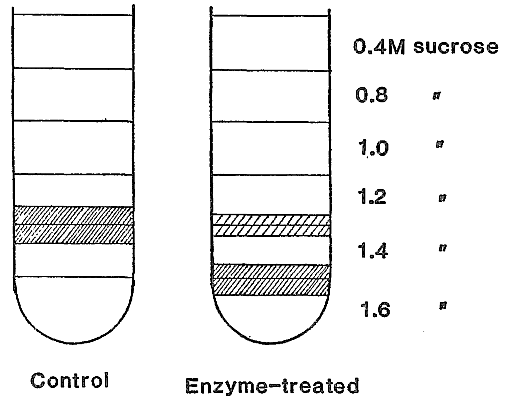

Fig. 1. Sucrose-density gradient centrifugation patterns of spinach chloroplasts and enzyme-treated ones. The chloroplasts (1 mg chlorophyll) were treated with potato galactolipase (2 mg protein) at 30°C for 15 min in the presence of 3% BSA. The reaction mixture was rapidly cooled to 0°C, and diluted three times with cold medium used for enzyme reaction. The resulting mixture was loaded on the top of sucrose-density gradient column, and then centrifuged at 80,000 xg for 60 min.

TABLE III
LIPID ANALYSIS OF THE ENZYME-TREATED CHLOROPLASTS

Chloroplasts were treated with potato galactolipase and then centrifuged on sucrose-density gradient column as described in Fig. 2. The resulting heavy band was separated and subjected to lipid analysis. The lipid contents were shown as percentages of the total polar lipid content in the control chloroplasts. The control chloroplasts were prepared by the same procedure without the enzyme.

Lipid components	Lipid contents in chloroplasts		Removal rate %
	Control	Enzyme-treated	
Total polar lipids	100	63	37
Monogalactosyldiacylglycerol	44	20	54
Digalactosyldiacylglycerol	32	26	18
Sulfoquinovosyldiacylglycerol	10	5	50
Phosphtidylglycerol	9	8	11
Phosphtidylcholine	5	4	20

TABLE IV
PHOTOCHEMICAL ACTIVITIES OF THE ENZYME-TREATED CHLOROPLASTS

The enzyme-treated chloroplasts prepared as described in TABLE III, were assayed for the photoactivities as in Methods. The control chloroplasts were prepared by the same procedure without the enzyme.

Reactions	Photochemical activities		
	Control	Enzyme-treated	% of control
	μmoles/mg chlorophyll·h		
$H_2O \rightarrow$ DMBQ (O_2 evolution)	72	70	97
$DCIPH_2 \rightarrow$ methyl viologen	262	186	71
+plastocyanin	335	350	104
$DCIPH_2 \rightarrow$ NADP	50	22	44
+plastocyanin	65	58	89
$H_2O \rightarrow$ NADP+plstocyanin	74	62	84

treated in the hypertonic medium showed a slight increase in the activity. These indicate that the membrane bound ferredoxin-NADP reductase, having NADPH-diaphorase activity at a strongly limited level, was released by the lipolytic enzyme. The liberation from the membranes resulted in a marked increase in the total activity. Most of the released enzyme proteins were transferred in the medium, but some of them remained in the chloroplasts when chloroplasts were treated in the hypertonic medium containing 0.6 M sucrose.

Table II shows a comparison of the effect of potato galactolipase with those of sonication and detergent treatments on a release of ferredoxin-NADP reductase in the chloroplasts. The sonication with or without Triton X-100 was found to produce a little release unexpectedly, while the enzymic treatment caused a remarkable release. This indicates that most of the reductase are tightly bound through the lipids in the chloroplast membranes. By the previous work,[12] it has been confirmed that the reductase can be extracted only by homogenizing spinach leaves in ice water in a Waring blender. Thus, it seems that there are two types of the reductase, easily released and tightly bound.

Effect of potato alactolgipase on plastocyanin in the chloroplast membranes

It was well known that, when the chloroplasts are treated with lipolytic enzymes, the electron flow is markedly inhibited by lipid hydrolysis products such as fatty acids and lyso-compounds. Thus, for the investigation on an enzymic release of plastocyanin is required a preparation of enzyme-treated chloroplasts which are freed of lipid digestion products, because the release of plastocyanin must be estimated by measuring the rates of photosynthetic electron flow. So, the method for preparing the lipid-depleted chloroplasts as reported previously[11] was adopted for the above purpose. The chloroplasts were treated with potato galactolipase at 30°C for 15 min in the presence of 3% BSA. The mixture was rapidly cooled to 0°C, diluted three times with cold medium used for enzyme reaction, and then centrifuged on sucrose-density gradient as described in Methods.

Fig. 1 shows the patterns of the sucrose-density gradient centrifugation, indicating that the treated chloroplasts were separated into two bands. A large portion of the treated chloroplasts was located on the interface between 1.4 M and 1.6 M sucrose layers (heavy band), and a small portion was located between 1.2 M and 1.4 M (light band) which was the same position as that of the control chloroplasts. The heavy band was separated and then subjected to lipid analysis. The results are shown in Table III. The chloroplast preparation lost 37% of the total polar lipids, in which monogalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol were prominently hydrolyzed. Most of BSA added and the lipid hydrolysis products occurred were removed from the treated chloroplast preparation (heavy band).

Table IV shows the photochemical activities of the enzyme-treated chloroplasts. The rate of photosystem II electron flow (O_2 evolution) was not affected by the enzyme treatment, but the rates of photosystem I electron flow measured by methyl

viologen-mediated O_2 uptake and NADP photoreduction were both reduced, especially in the latter to a more extent. However, the loss of photosystem I electron flow was largely recovered by addition of plastocyanin, particularly in the electron flow of $DCIPH_2$→methyl viologen. This suggests that the enzymic hydrolysis of the membrane lipids causes disruption of the thylakoid membranes, which stimulates a release of plastocyanin from the membranes, because it is trapped in water-soluble state into the lumina of the thylakoids.

Shaw et al.[3] reported that photosystem I electron flow (diaminodurene→methyl viologen) was not affected by treatment with a galactolipase from *Phaseolus vulgaris* leaves in the presence of BSA. In their experiments, the enzyme treatment was carried out at 20°C, and the treatment mixture was directly used for the measurements of the photoactivity without a separation of the treated chloroplasts from the released components. The contrasted results with ours might be attributed to the presence of plastocyanin released in the assay mixtures and the extent of lipid hydrolysis. Kochubeĭ et al.[13] measured the absorbance difference spectra, ESR, and delayed light emission of chloroplast fragments, which were treated with a galactolipase. They suggest that the functional link between photosystem I reaction center and the donor site of the electron transport was deteriorated by the enzyme treatment. This is another site damaged by lipolytic enzyme in photosystem I.

REFERENCES

1) Mantai, K. E., *Plant Physiol.*, **45**, 563–566, 1970.
2) Anderson, M. M., McCarty, R. E. and Zimmer, E. A., *Plant Physiol.*, **53**, 699–704, 1974.
3) Shaw, A. B., Anderson, M. M. and McCarty, R. E., *Plant Physiol.*, **57**, 724–729, 1976.
4) Hirayama, O. and Matsui, T., *Biochim. Biophys. Acta*, **423**, 540–547, 1976.
5) Asada, K. and Takahashi, M., *Plant & Cell Physiol.*, **12**, 709–715, 1971.
6) Hirayama, O. Matsuda, H., Takeda, H., Maenaka, K. and Takatsuka, H., *Biochim. Biophys. Acta*, **384**, 127–137, 1975.
7) Hirayama, O. and Matsuda, H., *Agric. Biol. Chem.*, **36**, 1831–1833, 1972.
8) Jagendorf, A. J., "Methods in Enzymology", ed. by Colowick, S. P. and Kaplan, N. O., Academic Press, New York and London, 1963, Vol. 6, p. 430–434.
9) Bjerve, K. S., Daae, L. N. W. and Bremer, J., *Anal. Biochem.*, **58**, 238–245, 1974.
10) Hirayama, O. and Matsuda, H., *Agric. Biol. Chem.*, **36**, 2593–2596, 1972.
11) Hirayama, O. and Nomotobori, T., *Biochim. Biophys. Acta*, **502**, 11–16, 1978.
12) Shin, M., "Methods in Enzymology" ed. by Pietro, A. S., Academic Press, New York and London, 1971, Vol. 23 part A, p. 440–447.
13) Kochubeĭ, S. M., Shadchina, T. M., Kononenko, A. A., Lukashev, E. P., Timofeev, K. N. and Matorin, D. N., *Photosynthetica*, **9**, 255–260, 1975.

摘 要

ホレンソウのクラスII葉緑体を精製した馬鈴薯ガラクトリパーゼで処理し，光化学活性ならびに機能蛋白質に与える影響を調べた．牛血清アルブミンの無存在下で酵素処理すると，NADP 光還元活性が著しく減少し，NADP ジホラーゼ活性が大きく増加した．これはフェレドキシン—NADP 還元酵素が葉緑体膜から脂質分解酵素処理により遊離したことを示す．この酵素による離脱速度は，トリトン X-100 の存在下超音波処理する場合よりも極めて速い．また，牛血清アルブミンの存在下で酵素処理し，ショ糖密度勾配遠心で処理葉緑体膜を分離すると，系I活性が減少し，プラストシアニンが遊離することが分かった．