S2-021

Degradation of chlorophylls: Two reaction pathways in the formation of pyropheophorbide *a*

Y Suzuki[1], M Doi[2], Y Shioi[1]

[1]*Faculty of Science, Shizuoka University, Shizuoka, 422-8529 Japan*
Fax: +81-54-238-0986, e-mail: sbysioi@ipc.shizuoka.ac.jp
[2]*Reserch Center of Higher Education, Kyushu University, Fukuoka, 810-8560 Japan*

Keywords: Chlamydomonas*, chlorophyll degradation, pheophorbidase, pheophorbide demethoxycarbonylase, radish.*

Introduction

Previously, we reported the enzymatic conversion of pheophorbide to pyropheophorbide in higher plant, *Chenopodium album* (Shioi et al. 1991, 1996) and characterized the enzyme (Watanabe et al. 1999). The enzyme so called pheophorbidase (Phedase) of this plant catalyzes the conversion of pheophorbide to C-13^2-carboxylpyropheophorbide, followed by the chemical decarboxylation of C-13^2-carboxylpyropheophorbide to pyropheophorbide (see Fig. 1). During the study of Chl degradation, we found the same enzymatic activity in *C. reinhardtii* and partially purified the enzyme (Doi et al. 2001). In the reaction, however, the intermediate that appeared in Phedase was not detected. This seems that the enzyme catalyzes a direct release of methoxycarbonyl moiety from pheophorbide. We therefore distinguish this enzyme from the former Phedase and called pheophorbide demethoxycarbonylase (PDC). These two alternative pathways in the formation of pyropheophorbide from pheophorbide are illustrated in Figure 1. In this report, we compare the properties of these enzymes, Phedase purified from radish and PDC from *C. reinhardtii*. Our results show that the action of reaction product, methanol, on the activity is different between Phedase and PDC. It is, therefore, likely that two alternative pathways exist in the demethoxycarbonyl reaction of pheophorbide. Purification and characterization of these enzymes will be published elsewhere.

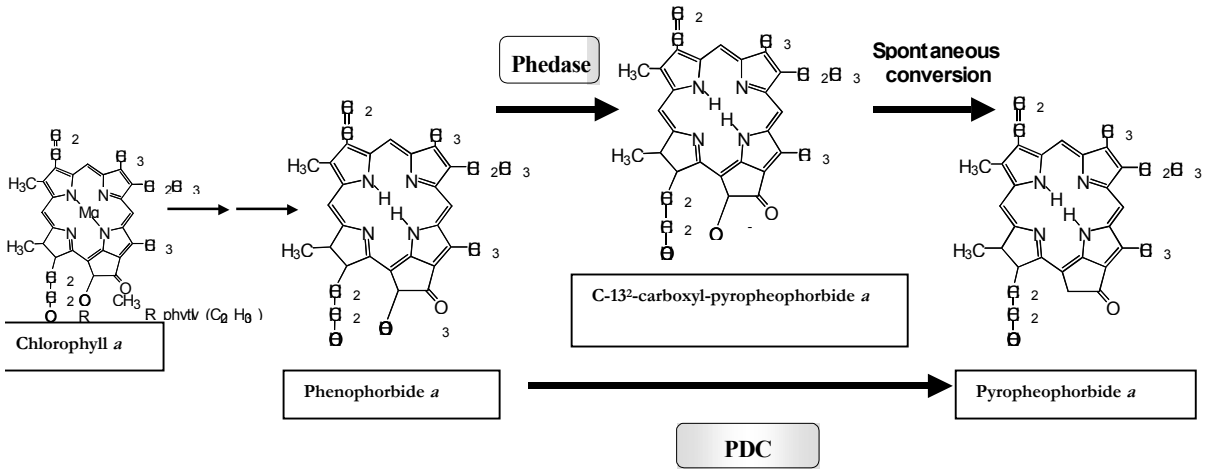

Fig. 1. Demethoxycarbonyl reaction of pheophorbide *a*. Two types of the reaction are considered. One catalyzed by pheophorbidase is composed of two steps: conversion of pheophorbide *a* to precursor, followed by spontaneous conversion of the precursor to pyropheophorbide. The other catalyzed by PDC is considered to be direct conversion of pheophorbide to pyropheophorbide. Phedase, pheophorbidase; PDC, pheophorbide demethoxycarbonylase.

Materials and Methods

Cotyledons of radish (*Raphanus sativus* L.) were purchased from a local market. A Chl *b*-less mutant NL-105 of *Chlamydomonas reinhardtii* was used. The algal cells were grown for 4 days in the light and followed by 3 days in the dark as described previously (Doi et al. 1997). Chl *a*, pheophorbide *a* and pyropheophorbide *a* were purchased from Wako Pure Chemical Industries (Osaka, Japan). The concentration of pheophorbide *a* and pyropheophorbide *a* were determined spectrophotometrically as described previously (Doi et al. 1997).
Analysis of pheophorbide *a* and pyropheophorbide *a* was carried out according to our previous method with a slight modification (Shioi et al. 1996). Briefly, HPLC was performed using a Zorbax ODS column (250 x 4.6 mm) (Du Pont). Pigments were eluted isocratically with methanol-2 M ammonium acetate (95/5, v/v) at a flow rate of 1.0 ml per min at room temperature (ca. 25°C). The pigments were monitored spectrophotometri- cally at 410 nm and quantified by an integrator. Peak areas were used for the calculation of enzyme activity.
For routine analysis and monitoring of activity in the purification steps, activity of both enzymes was assayed basically according to the methods described by Shioi et al. (1996) for Phedase and by Doi et al. (2001) for PDC. In assay of Phedase, substrate, pheophorbide *a* dissolved in acetone (20% final concentration) was used, whereas in 1% Triton X-100 (final 0.1%) was used PDC assay. Protein concentrations were determined using a Protein Assay Kit (Bio-Rad) with bovine serum albumin as a standard.
Phedase from cotyledons of radish was purified 3,397 and 3,017-fold for constitutive (type 1) and senescence-induced types (type 2), respectively, with successive chromatography by DEAE, Butyl, hydroxyapatite and gel filtration using a Toyopearl HW-55 Fine (Suzuki, Y. et al., in preparation). PDC was purified 76.4-fold from the crude extracts of mutant cells of *C. reinhardtii* according to the method described previously (Doi et al. 2001).

Table 1. Comparison of some properties of Phedase and PDC.

Property	Phedase[a]		PDC[b]	
	type 1	type 2		
Enzyme type	Senescence-induced	Constitutive	Senescence-induced	Constitutive
Molecular weight	113,000	113,000	170,000	Trace
Subunit	Dimer	Dimer	Dimer	
K_m (Phed *a*)	14 μM	15 μM	283 μM	
pH optimum	pH 7.0-7.5	pH 6.5	pH 7.2	
Activation energy	15.9 kcal	12.3 kcal	8.5 kcal	
Thermostability (50% lost)	67.5°C	67.5°C	56.0 °C	

[a]Purified from radish; [b]Purified from *C. reinhardtii.*

Results and Discussion

The enzyme catalyzing demethoxycarbonylation of pheophorbide *a* exists two types, Phedase and PDC, both consisted of senescence-induced and constitutive enzymes. We highly purified Phedase from radish and PDC from *C. reinhardtii*, except for the constitutive PDC because it was too trace to purify. Some properties of these enzymes were compared (Table 1). Molecular mass of PDC was much higher than that of Phedase, although they were dimer. Phedase had about 20-fold higher affinity for pheophorbide *a* than PDC. However, other properties were almost comparable.

We reported that in the Phedase reaction, methanol is produced as a reaction product and inhibits Phedase activity (Shioi et al. 1996). If PDC has the same reaction mechanism as Phedase, the enzyme activity might be inhibited by methanol. Therefore, effects of methanol and reaction-product analogues were tested. As shown in Fig. 2, both Phedases, types 1 and 2 were inhibited by methanol almost similarly to 20% at 1 M (4.0%, v/v), but not by ethanol. PDC was inhibited equally both by methanol and ethanol. This may be due to a simple inactivation of the enzyme by these alcohols. Addition of methyl formate had no difference among three enzymes. These results suggest that the action of methanol is different between Phedase and PDC, indicating the difference of the reaction mechanism.

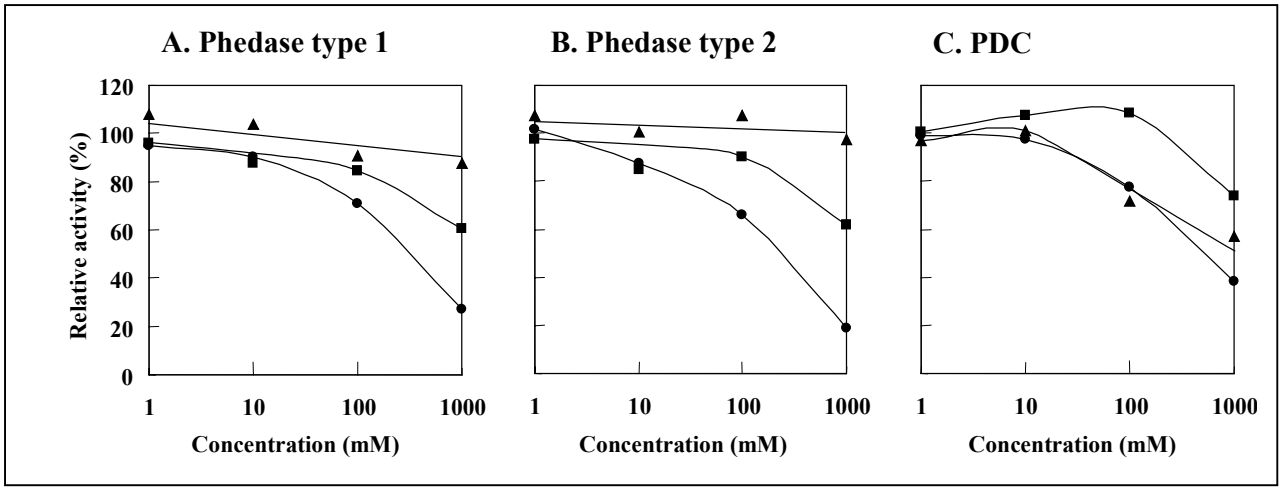

Fig. 2. Effect of possible reaction products on the enzyme activity. ●, methanol; ▲, ethanol; ■, methyl formate.

Table 2. Effect of various inhibitors on the enzyme activity.

Inhibitor	Concentration (mM)	Relative Activity %		
		Phedase[a]		PDC[b,c]
		type 1[c]	type 2[d]	
Control	- □	100	100	100
Ebelactone A	5 (mg/l)	85.6	102.0	93.7
PMSF	1	38.9	73.6	74.1
Antipain	0.1	98.1	108.3	100.3
E-64	0.1	132.2	93.7	99.5
NEM	1	89.0	96.4	24.4
EDTA	1	122.2	109.2	108.7

[a]From *R. sativus*; [b]From *C. reinhardtii*; [c]Senescence-induced; [d]Constitutive

In order to understand the reaction mechanism further, effects of various inhibitors on the enzyme activities were examined. Most conspicuously, the activity of Phedase type 1 decreased to 39% by PMSF and PDC was reduced to 24% by NEM whereas Phedase type 2 was not inhibited by these reagents. These results indicate that serine for Phedase type 1 and cysteine for PDC may play an important role in the reaction. Distribution of these enzymes was studied in a variety of plants. Among 21 species from 14 different families, Phedase activity was found in 9 species. PDC activity was not detected from the plants lacking Phedase activity, but was only detected in *C. reinhardtii* among species tested. PDC seems to be specific for algae. From these, it is conclude that in the demethoxycarbonyl reaction, there are two alternative pathways that are catalyzed by different enzymes, Phedase and PDC.

Acknowledgments

This study was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan (no. 12640631 and no. 07856).

References

Doi, M., Shima., S., Egashira, T., Nakamura, K. and Okayama, S. (1997) New bile pigment excreted by a *Chlamydomonas reinhardtii* mutant: a possible breakdown catabolite of chlorophyll *a*. *J. Plant Physiol.* **150**, 504-508.

Doi, M., Inage, T. and Shioi, Y. (2001) Chlorophyll degradation in a *Chlamydomonas reinhardtii* mutant: An accumulation of pyropheophorbide *a* by anaerobiosis. *Plant Cell Physiol*. **42**, 469-474

Shioi, Y., Tatsumi, Y. and Shimokawa, K. (1991) Enzymatic degradation of chlorophyll in *Chenopodium album*. *Plant Cell Physiol*. **32**, 87-93.

Shioi, Y., Watanabe, K. and Takamiya, K. (1996) Enzymatic conversion of pheophorbide *a* to the precursor of pyropheophorbide *a* in leaves of *Chenopodium album*. *Plant Cell Physiol*. **37**, 1143-1149.

Watanabe, K., Ohta, H., Tsuchiya, T., Mikami, B., Masuda, T., Shioi, Y. and Takamiya, K. (1999) Purification and some properties of pheophorbidase in *Chenopodium album*. *Plant Cell Physiol*. **40**, 104-108.