S2-022

Degradation of Chlorophylls: Purification and properties of a Mg-releasing protein from *Chenopodium album*

T Suzuki, Y Shioi

Faculty of Science, Shizuoka University, Shizuoka, 422-8529 Japan
Fax: +81-54-238-0986, e-mail: sbysioi@ipc.shizuoka.ac.jp

Keywords: chlorophyll degradation, chlorophyllin, Mg-dechelatase, Mg-releasing protein, pheophorbide formation.

Introduction

The removal of the central Mg ion from chlorophyllide *a* is expected to follow after chlorophyllase as shown in Fig. 1. In fact, an activity catalyzing this reaction has been reported in algae and higher plants and considered to be due to an enzyme that had been designated "Mg-dechelatase" (Owens and Falkowski 1982; Ziegler et al. 1988; Shimokawa et al. 1990; Shioi et al. 1991; Vicentini et al. 1995). Previously, we exploited chlorophyllide *a* as a substrate for Mg-dechelating assay and found that a smaller heat stable substance involved in this metal-releasing process (Shioi et al. 1996). We designated operationally metal-chelating substance (MCS) (former Mg-dechelating substance) and continued to study on the nature of this substance. Our results on the specificity for metal ions of the highly purified substance show that this does not specifically responsible for the reaction of Mg-dechelation in the early stage of chlorophyll (Chl) degradation, but probably for ionophore so called phytometallophores (Morioka et al., in preparation). Recently, Azuma et al. (1999) reported that horseradish peroxidase catalyzed the release of Mg from chlorophyllin in the absence of H_2O_2. There is, however, no consistent view regarding the function of these materials in the Mg-releasing reaction. During the study on MCS using chlorophyllin as a substrate, we found that a heat-labile Mg-releasing activity was present in the extracts of *Chenopodium album* in addition to the heat-stable MCS. In this article, we report that purification and properties of protein that catalyzes the reaction of Mg-releasing from chlorophyllin. Our results show that the purified protein is distinct from heme iron-containing protein, peroxidase from its absorption spectrum.

Materials and Methods

Matured and fully expanded leaves of *Chenopodium album* were collected on the campus of Shizuoka University, Shizuoka, and from the uncultivated fields of Shizuoka City, Shizuoka. Collected leaves were instantly frozen with liquid nitrogen and stored at -30°C until used.

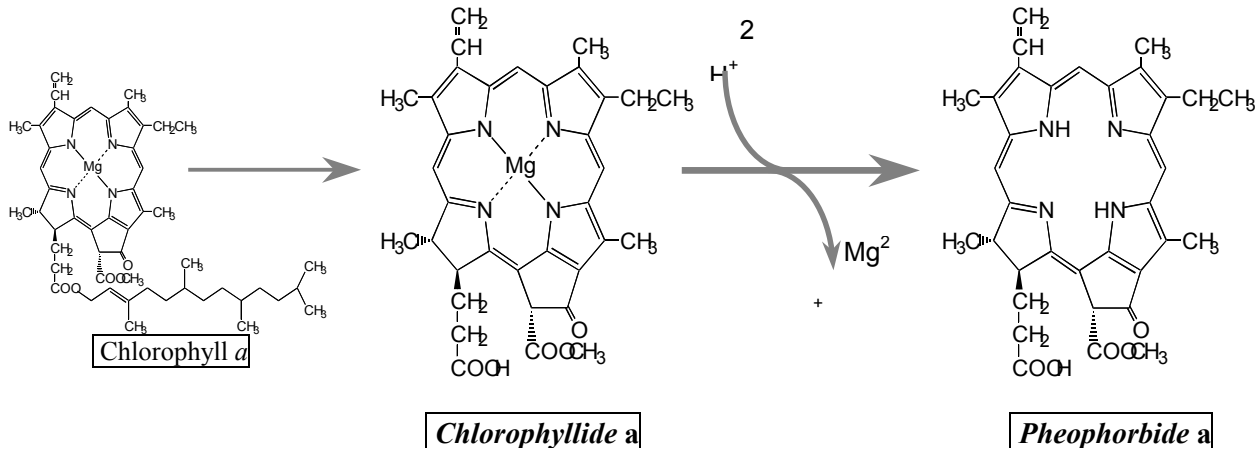

Fig. 1. Structure of chlorophyll *a* and its degradation products. Formation of pheophorbide *a* from chlorophyllide *a* by Mg-dechelation is the subject of this study.

Chl *a*, pheophorbide *a*, and Cu-chlorophyllin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chlorophyllin (Mg-chlorin) was prepared according to the procedure of Vicentini et al. (1995) with a slight modification. We used a purchased Chl *a* instead of the extracted Chl *a* as a starting material. Zn-chlorophyllin was prepared according to the method of Schoch et al. (1995). Chlorophyllide *a* was prepared from Chl *a* by enzymatic reaction using a chlorophyllase isolated from *C. album*.

Mg-dechelating activity was assayed spectrophotometrically by measuring absorbance at 692 nm of reaction product, Mg-released chlorophyllin, pheophorbin. Activity is expressed as increased absorbance at 692 nm. The standard reaction mixture contained the following: 20 mM Tris-HCl buffer (pH 7.5), 20 nM chlorophyllin and enzyme solution in a total volume of 1.0 ml. The enzyme assay was performed at 30°C in the dark.

The soluble proteins were extracted from the acetone powder of leaves by suspending them in 20 volumes of 20 mM Na-K phosphate buffer (pH 7.0). The suspension was stirred for 30 min in an ice-bath and then filtered through six layers of gauze. The filtrate was centrifuged 20,000 x g for 30 min, and the resulting supernatant was used as the source of Mg-releasing protein. The crude extracts were fractionated with ammonium sulfate between 30% and 75% saturation. The resulting yellowish-clear supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and applied to a column (2.5 x 8 cm) of DEAE-Toyoparl (Tosoh, Tokyo) equilibrated with 20 mM Tris-HCl (pH 7.5) (buffer A). Unbound protein was eluted with buffer A, and the bound protein was eluted with a linear gradient of 0 to 0.4 M NaCl in buffer A at a flow rate of 1 ml/min. Fractions (1.5 ml/fraction) were collected and assayed for Mg-releasing activity with chlorophyllin as a substrate. The fractions eluted around 0.15-0.2 M NaCl were pooled (DEAE fraction) and ammonium sulfate was added to 1 M before loading onto a column (1.6 x 5 cm) of Butyl-Toyopearl (Tosoh) previously equilibrated with buffer A containing 1 M ammonium sulfate. The protein was eluted with a reverse-linear gradient from 1 to 0 M ammonium sulfate in buffer A. The active fractions that have been eluted around the concentration of 0.2 M ammonium sulfate were pooled and concentrated by Centriflo CF/24 (Amicon) (Butyl pool). The concentrated protein was chromatographed on a column (2.5 x 58 cm) of Toyopearl HW-55 Fine (Tosoh) equilibrated with Na-K phosphate buffer (pH 7.0) containing 150 mM NaCl. The protein was eluted with the same buffer at a flow rate of 15 ml/h. Fractions (1.5 ml/fraction) were collected and assayed for the activity. Active fractions were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5) and concentrated by Centriflo CF/24 (HW-55 fraction). This fraction was used as a partially purified enzyme for enzymatic analyses.

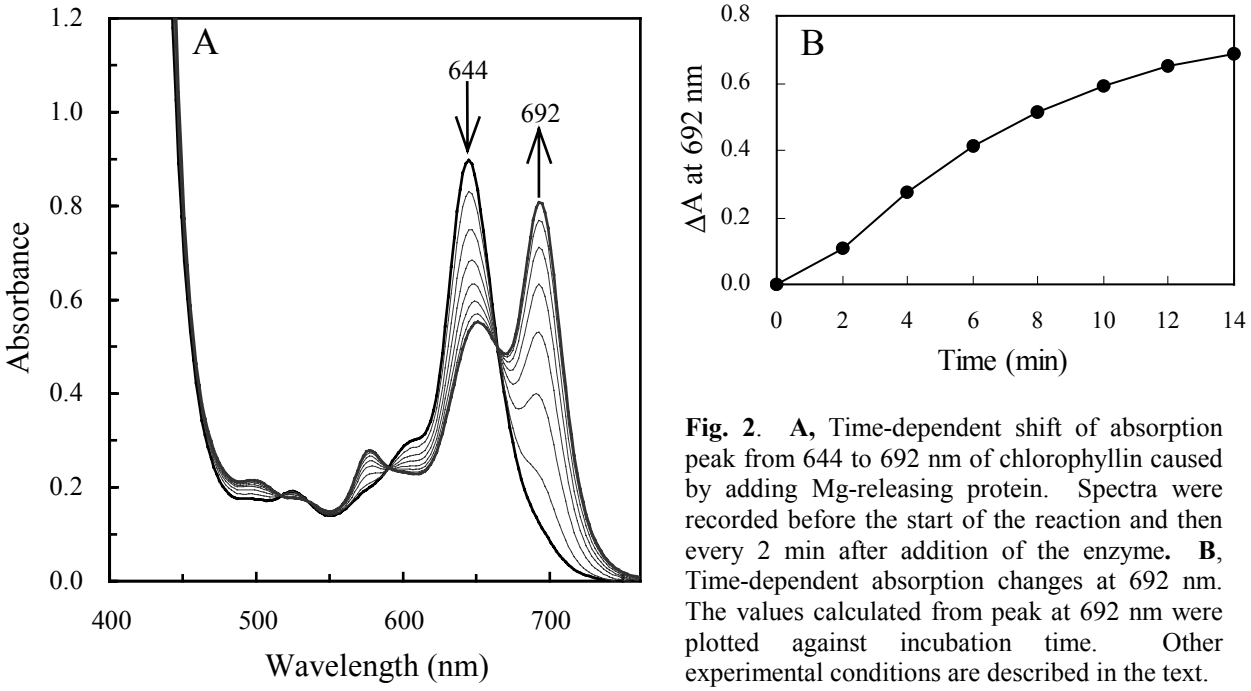

Fig. 2. **A,** Time-dependent shift of absorption peak from 644 to 692 nm of chlorophyllin caused by adding Mg-releasing protein. Spectra were recorded before the start of the reaction and then every 2 min after addition of the enzyme**.** **B**, Time-dependent absorption changes at 692 nm. The values calculated from peak at 692 nm were plotted against incubation time. Other experimental conditions are described in the text.

Molecular weight of the enzyme was estimated using Toyopearl HW-55 Fine (Tosoh) column (2.5 x 58 cm) described above by comparison with standard proteins. The standards used are shown in the Figure. Protein was determined using Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

Results and Discussion

In the present study, we purified and characterized the protein catalyzing the activity of Mg-release from the extracts of *C. album* using chlorophyllin as a substrate. As shown in Fig. 2A, upon addition of Mg-releasing protein, the absorption peak at 644 nm of chlorophyllin began to decrease concomitant with the appearance of a new peak at 692 nm. This peak progressively increased with incubation time (Fig. 2B). Mg-releasing protein was purified 28-fold from the initial crude extracts by ammonium sulfate fractionation (30-75% saturation), DEAE, Butyl and HW-55 chromatography. pH profile of the purified enzyme showed a single, but broad pH optimum at a pH value of 7.0-7.5 in Mes-Hepes-Tricine buffer (Fig. 3). Molecular weight was estimated to be 20 k by HW-55 gel filtration as shown in Fig. 4. The K_m value for substrate, chlorophyllin, was 95.1 nM as measured by the formation of pheophorbin calculated from the absorption peak at 692 nm. However, Mg-releasing protein had no activity for chlorophyllide *a*, a native substrate. It is known that horseradish peroxidase shows a similar Mg-releasing activity towards Mg-chlorophyllin (Azuma et al. 1999). This enzyme showed no activity against chlorophyllide *a* as similar to Mg-releasing protein from *C. album*, although these enzymes had an activity for Zn-chlorophyllin. The enzyme reaction was strongly inhibited by sodium ascorbate, propyl gallate and hydroquinone as in the case of horseradish peroxidase (Azuma et al. 1999). However, an absorption spectrum of the purified Mg-releasing protein from *C. album* was clearly distinct from that of the horseradish peroxidase, suggesting that Mg-releasing protein is not a heme iron-containing protein. Interestingly, a smaller heat-resistant substance, MCS, isolated from *C. album* had activity for both substrates. It is therefore concluded that the Mg-releasing protein

and horseradish peroxidase does not involve in Mg-dechelating reaction. Thus, at present, MCS may be one of candidates as a substance that catalyzes Mg-dechelating reaction in the Chl breakdown pathway. Further study is needed to clear the role of MCS.

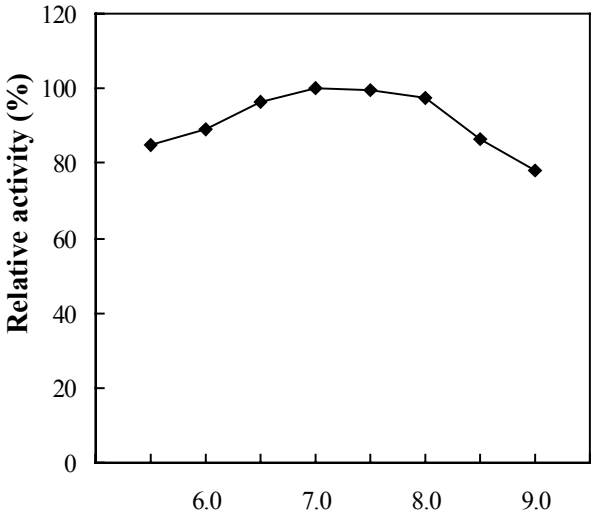

Fig. 3

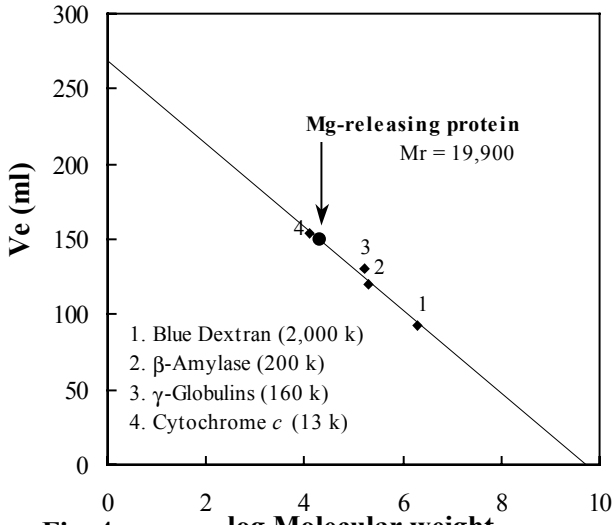

Fig. 4

Fig. 3. pH dependency of the activity of Mg-releasing protein. The enzyme assay was done in 50 mM Mes-Hepes-Tricine buffer at the indicated pH values. Assay and other experimental conditions are described in the text.

Fig. 4. Determination of the molecular weight of the purified Mg-releasing protein by gel filtration using Toyopearl HW-55 Fine. For details, see text.

Acknowledgments

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

References

Azuma, R., Takahashi, Y., Kurata, H., Kawano, T., Shimokawa, K. and Adachi, M. (1999) Does peroxidase act as a 'Mg-dechelatase'? *Plant Peroxidase Newslett*. **13**, 145-151.

Owens, T. G. and Falkowski, P. G. (1982) Enzymatic degradation of chlorophyll *a* by marine phytoplankton in vitro. *Phytochemistry* **21**, 979-984.

Schoch, S., Helfrich, M., Wiktorsson, B., Sundqvist, C., Rüdiger, W. and Ryberg, M. (1995) Photoreduction of zinc protochlorophyllide *b* with NADPH-protochlorophyllide oxidoreductase from etiolated wheat (*Triticum aestivum* L.). *Eur. J. Biochem*. **229**, 291-298.

Shimokawa, K., Hashizume, A. and Shioi, Y. (1990) Pyropheophorbide *a*, a catabolite of ethylene-induced chlorophyll *a* degradation. *Phytochemistry* **29**, 2105-2106.

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

Shioi, Y., Tomita, N., Tsuchiya, T. and Takamiya, K. (1996) Conversion of chlorophyllide to pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. *Plant Physiol. Biochem.* **34**, 41-47.

Vicentini, F., Iten, F. and Matile, P. (1995) Development of an assay for Mg-dechelatase of oilseed rape cotyledons, using chlorophyllin as the substrate. *Physiol. Plant*. **94**, 57-63.

Ziegler, R., Blaheta, A., Guha, N. and Schönegge, B. (1988) Enzymatic formation of pheophorbide and pyropheophorbide during chlorophyll degradation in a mutant of *Chlorella fusca* SHIHIRA et KRAUS. *J. Plant Physiol*. **132**, 327-332.