

PURIFICATION AND PROPERTIES OF METAL-CHELATING SUBSTANCE IN CHLOROPHYLL DEGRADATION

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ABSTRACT

The early stage of degradation of chlorophylls includes the loss of phytol, magnesium and the oxidative cleavage (bleaching) of the tetrapyrrole macrocycle by the action of several enzymes or small molecular substance, so called metal-chelating substance (MCS). Pheophorbide formation due to the release of Mg from chlorophyllide in chlorophyll metabolism was studied using extracts of *Chenopodium album* and *Raphanus sativus* L. MCS was highly purified from *C. album* and some properties of MCS were investigated. Molecular weight of MCS was determined to be 452 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. MCS gave a positive reaction with ninhydrin, confirming the presence of an amino group. Since MCS did not react with Ellman's reagent and there was no absorption around 280 nm, it is clear that MCS has no sulfhydryl group including cysteine and aromatic amino acid. MCS chelated not only Mg²⁺, but also other divalent cations such as Zn²⁺ and Ni²⁺. Localization of MCS in radish seedlings showed that the activity of MCS was the highest in leaf and those of root and stem were 84% and 7% of the leaf, respectively. The results of present studies on the Mg-dechelating reaction are discussed in relation to the present understanding of metals in plant body. It is proposed that MCS is a novel chelator in plants and may play not merely Mg-dechelation from chlorophyllide *a*, but also other roles such as micronutrient transport.

Keywords: Chlorophyll degradation, Mg-dechelation, Metal-chelating substance, Micronutrient transport, Pheophorbide formation, Phytometallophore.

INTRODUCTION

Chlorophylls (Chls) are the pigment responsible for photosynthesis, the fundamental life process that converts light energy into chemical energy. Chls as the most important of the three main natural classes of pigments are most widely distributed in green plants, algae and photosynthetic bacteria. Chl metabolism is a universal phenomenon that occurs with seasonal change, and embraces probably the most visually obvious of all biochemical processes. Chl biosynthesis determines the characteristic 'greenness' of plants accompanied by an insertion of Mg which occurs

during the spring season, and its degradation is manifested as the loss of the green pigment from organs such as leaves accompanied by a release of Mg in the autumn. It has been estimated that an excess of 1.1 billion tonnes of Chls are produced and degraded globally each year (Hendry et al. 1987). Chls are considered to be broken down into bilin derivatives called nonfluorescence Chl catabolites (NCCs) (Hörtensteiner 2006) or further into smaller carbon and nitrogen containing fragments such as organic acids via monopyrroles (Llewellyn et al. 1990a, 1990b; Suzuki & Shioi 1999) and these may be finally recycled in the parts of plant as nutrients by sink-source relationship, although the way of Mg is not known. This degradation is thus important for plants in terms of nutrition. Therefore, the knowledge of this degradation mechanism is important to our basic understanding of the physiology and biochemistry of plants.

In Chl degradation pathway, the removal of the central Mg ion from chlorophyllide (Chlide) *a* is expected to follow chlorophyllase (Figure 1), in addition to direct removal from Chl *a* (Hörtensteiner 2006). This Mg-dechelation reaction has been reported in algae and higher plants and is considered to be due to an enzyme that had been designated as “Mg-dechelataase” (Owens & Falkowski 1982; Ziegler et al. 1988; Shimokawa et al. 1990; Shioi et al. 1991; Vicentini et al. 1995). From our previous study, two kinds of “Mg-dechelataase” like activity have been shown with artificial substrate, chlorophyllin (Suzuki & Shioi 2002). One of these was due to a small molecular substance known as metal-chelating substance (MCS), and another was a protein called Mg-releasing protein (MRP). If these were actual Mg-dechelataase, they might catalyze release of Mg from both artificial and native substrates. Our results showed that only MCS was responsible for Mg-dechelation reaction with both substrates, whereas MRPs had activity only for artificial substrate. These findings clearly show that MRP is not a “Mg-dechelataase” in Chl degradation pathway. It is likely that MCS is the only substance that releases Mg from Chlide and is responsible for the reaction in the degradation pathway of Chl.

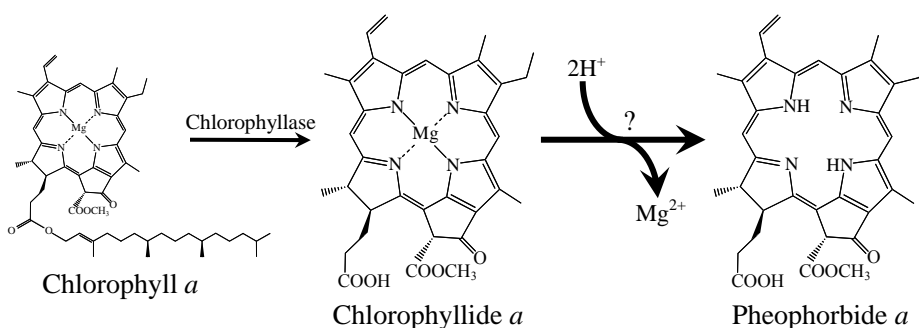


Figure 1. Structures of chlorophyll *a* and its early degradation products in chlorophyllide route. Central Mg ion is considered to be released from chlorophyllide *a* by small molecular compound so called “Metal-chelating substance” and become pheophorbide *a*. This Mg-dechelating reaction is the subject of this study.

MCS was discovered in extracts of goosefoot (*Chenopodium album*) leaves (Shioi et al. 1996). Mg-dechelation activity of MCS was detected in filtrate of 5 kDa cut-off membrane and complete activity was maintained after being boiled. Optimum activity was observed at pH 6.5 to 7.0 using Chlide *a* as a substrate. In addition to Chlide *a*, MCS used Chlide species such as Chlide *b* and bacteriochlorophyllide *a* as substrate, but not Chl esters including Chls *a* and *b*, and porphyrin type species, monovinyl-protochlorophyllide *a* and Mg-protoporphyrin IX dimethyl ester. Interestingly, the activity was stimulated by the addition of chelators such as ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline (Shioi et al. 1996).

Previously, we reported partial purification of MCS from *C. album* using gel filtration chromatography as a main technique (Kunieda et al. 2005). We also demonstrated that MCS was the only substance that has Mg-dechelation activity from Chlide *a* in plant extracts (Suzuki et al. 2005; Kunieda et al. 2005). It was shown that MCS catalyzed the conversion of Chlide to pheophorbide and was involved in Chl degradation pathway, but the mechanism of dechelation reaction is not clear yet. In this article, an improved method for the purification of MCS was developed and a highly purified sample was obtained. The purification and properties of MCS are described here and its tentative structure is also discussed.

MATERIALS AND METHODS

Plant materials

Mature and fully expanded leaves of goosefoot (*C. album*) were collected from uncultivated fields of Shizuoka City, Japan. Collected leaves were instantly frozen with liquid nitrogen and stored at -30°C until further use. For tissue distribution, radish (*Raphanus sativus* L.) seedlings obtained from a local market were also used.

Substrates

Chls were extracted from spinach leaves (about 100 g fresh weight) with acetone and partially purified by precipitation with dioxane according to the method of Iriyama et al. (1974). Chlide *a* was prepared from Chl *a* with enzymatic reaction using a chlorophyllase prepared from leaves of *C. album* (Tsuchiya et al., 1997).

Assay of Mg-dechelation activity

Mg-dechelation activity using Chlide *a* was assayed spectrophotometrically by measuring the difference in absorbance of the reaction product, pheophorbide *a*, before and after incubation. To generate Chlide *a*, subtotal of 140 µL mixture solution containing 30 µM Chl *a*, chlorophyllase and 33 mM Na-phosphate buffer (pH 7.0) was incubated at 30°C in the dark for 30 min. Sixty microliters of MCS preparation was then added to the preincubated solution in a total of 200 µL and incubated again at 30°C in the dark for 0 or 30 min. After incubation, pigments were completely extracted by addition of 500 µL of acetone and measured

spectroscopically for the formation of pheophorbide *a* as the difference (30 min minus 0) in absorption spectra around 540 nm using a spectrophotometer, model UV-2450 (Shimadzu, Kyoto, Japan). To quantify the activity, increase in area between wavelength 525 and 555 nm was calculated using a computer software, UV probe (Shimadzu).

Extraction of MCS

Active fraction of MCS was extracted from acetone powder of leaves. Goosefoot leaves (about 100 g fresh weight) were treated with acetone to prepare an acetone powder as described previously (Shioi et al. 1995). The soluble fractions were extracted from the acetone powder by suspending them in 20 volumes of Milli-Q water. 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 the MCS for further purification.

Purification of MCS

The crude extract was passed through a DEAE-Toyopearl 650M (TOSOH, Tokyo, Japan) column (\varnothing 25 x 50 mm) equilibrated with distilled water to remove most of the proteins. After washing the unbound component with distilled water, cation exchange chromatography was performed according to slightly modified methods of Buděšínský et al. (1980) and Ma and Nomoto (1993). DEAE-unbound fraction was adjusted to pH 3.0 with 10 mM ammonium formate buffer (pH 3.0) and centrifuged at 20,000 x g for 30 min after being stirred for 30 min on ice. The resulting supernatant was charged onto a Macro-Prep High S Support (BIO-RAD, Hercules, CA, USA) column (\varnothing 16 x 50 mm) equilibrated with 10 mM ammonium formate buffer (pH 3.0). After washing the unbound fraction with the same buffer, bound component was eluted with 2 column volumes of 1 M ammonia hydroxide. Eluted fraction was adjusted to pH 7.0 with formic acid and loaded onto a Sephadex G-10 (GE Healthcare, Buckinghamshire, UK) column (\varnothing 25 x 710 mm). The chromatography was performed with distilled water at a flow rate of 0.25 mL min⁻¹ and active fractions were pooled. After concentration, the residue was dissolved with Milli-Q water and the solution was subjected to Superdex peptide HR 10/30 (\varnothing 10 x 300 mm) (GE Healthcare) which was equilibrated with degassed Milli-Q water with an ÄKTA fast protein-liquid chromatography (FPLC) system (GE Healthcare). The chromatography was performed with Milli-Q water at a flow rate of 0.25 mL min⁻¹ and active fractions were pooled and concentrated by lyophilization for next step of high-performance liquid chromatography (HPLC). Purification by HPLC was carried out with a model L-7120 (HITACHI, Tokyo, Japan) using a Polyhydroxyethyl Aspartamide (PHA) (PolyLC Inc., Columbia, MD, USA) column (\varnothing 4.6 x 200 mm) which was equilibrated with 50 mM formic acid. MCS was eluted at a flow rate of 0.5 mL min⁻¹ at room temperature and detected spectrophotometrically at 260 nm with an UV detector, L-7400 (HITACHI). Peak fractions were collected and lyophilized to remove formic acid and dissolved in a small volume of Milli-Q water.

Quantitative determination of sulfhydryl groups (SH)

SH group was determined by the reaction of Ellman's reagent (Ellman 1959) according to the method of Bulaj et al. (1998). Concentration of SH group was determined spectrophotometrically by measuring absorbance at 412 nm with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Wako Pure Chemical, Osaka, Japan) as a substrate. The reaction mixture contained 100 μ M DTNB, 2.5 mM sodium acetate and the purified MCS (10 μ g) in 100 mM Tris-HCl buffer (pH 8.0) in a total volume of 1.0 mL. The reaction mixture was incubated at 37°C for 5 min and absorbance was measured at 412 nm. Cysteine hydrochloride and Milli-Q water were used positive and negative control, respectively. Cysteine hydrochloride was also used for standard SH calibration.

Thin-layer chromatography (TLC)

TLC was performed with Merck Silica gel 60 (Merck, Darmstadt, Germany) using two different solvent systems: *n*-butanol-acetic acid-H₂O (4:1:1, by volume) and *n*-propanol-H₂O (6:7, v/v) according to the method of Shojima et al. (1989b). The ninhydrin reaction was used for detection of samples. MCS sample (20 μ g) was used with alanine and Milli-Q water as positive and negative controls, respectively.

Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

Positive ion mass spectrum of MCS was obtained by MALDI-TOF MS using an Autoflex II (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). The spectrometer was operating in linear mode. Purified MCS (100 μ g) was mixed with α -cyano-4-hydroxycinnamic acid (CCA) (Bruker Daltonics), an electron transfer matrix, dissolved in 0.1% trifluoroacetic acid/acetonitrile mixture 2:1 (v/v), and 1 μ L of the mixture was dried on a stainless steel sample plate for analysis. The nitrogen laser excitation frequency was set at 20 Hz. The laser power was optimized to obtain a good-signal-to-noise ratio after 300 single-shot spectra. The spectra were obtained at 20 kV accelerating voltage and externally calibrated with a peptide mixture.

Metal-chelating activity

It is known that absorption spectrum of MCS is changed after binding of metal ions to form complex as well as other metal-chelators in plants. In this study, therefore, metal-binding activity of MCS was determined spectrophotometrically. Divalent (MgCl₂, ZnCl₂ and NiCl₂), monovalent (NaCl, KCl and LiCl), or trivalent (FeCl₃) cation was added to purified MCS (10 μ g ml⁻¹) at a final concentration of 1 mM and absorption spectra between 200 and 400 nm were measured before and after addition of metal. Metal chelating activity was measured by the change in absorption at prominent band around 254 nm after addition of several concentrations of metal (cf. Fig. 3A, B). MgCl₂ and Milli-Q water were used positive and negative control, respectively.

RESULTS

Purification of MCS

In the previous study, gel filtration was used as a main chromatographic method for the purification of MCS (Shioi et al. 1996; Suzuki & Shioi 2001, 2002; Suzuki et al., 2005). In the present study, we used cation-exchange chromatography (Macro-Prep High S support) and found it was effective for the purification of MCS. At the last step of purification, HPLC with a PHA column was used. Elution was carried out with 50 mM formic acid as an eluent in the size-exclusion chromatography mode with a fractionation range of molecular mass approximately 20 and 1000. In this step, several peaks appeared (Figure 2), but only the first peak at a retention time of 4.4 min had Mg-dechelation activity for Chlide *a*. This peak was thus collected and used for subsequent determination of MCS properties. Summary of the purification of MCS is shown in Table 1. After chromatography with Macro-Prep High S support, specific activity increased four-times. The cation-exchange method was useful for the purification of MCS as in the case of other metal-chelators in plants such as nicotianamine or mugineic acid (Buděšínský et al. 1980; Ma & Nomoto 1993). At the step of DEAE-Toyopearl 650M and Macro-Prep High S Support, most of the proteins and other small substances were removed. A decrease in yield at the first step of Superdex peptide may be due to the removal of a broad side peak that has an activity (data not shown). Finally, MCS was purified 309-fold with a yield of 0.9% after PHA column chromatography.

Absorption spectrum and metal-chelating activity of MCS

Figure 3A shows change of absorption spectrum of MCS before and after binding of Mg^{2+} . Absorption spectrum of non-metal binding form of purified MCS showed a slight shoulder around 254 nm, while addition of Mg^{2+} to MCS resulted in an increase in ultraviolet absorption around 260 nm due to complex formation, similar to the results of phytochelatins and glutathione after binding of metals (Mehra et al. 1995, 1996a, 1996b; Mehra & Mulchandani 1995). Subsequently, binding activity of MCS to metal ions was examined with different cation species. In addition to Mg^{2+} , affinities of MCS to several divalent metal ions, Zn^{2+} and Ni^{2+} , were determined by the increase in absorbance around 260 nm (Fig. 3B) which depended on the species and concentrations of metals. In the case of Mg^{2+} , concentration of half-saturation was 2 mM and saturated at 5 mM. Similarly, half-saturation concentrations of Zn^{2+} and Ni^{2+} were both 0.2 mM, and were saturated at 0.5 and 1 mM, respectively. It is unexpected that the affinities of MCS to other metals were much higher than that of Mg^{2+} , which is a center metal of Chl. This suggests that MCS may play other roles in plant metabolism besides their involvement in Chl degradation pathway. In contrast, addition of monovalent cations such as Na^+ , K^+ and Li^+ and also trivalent ion, Fe^{3+} , had no effect on the absorption spectrum of MCS, suggesting that MCS did not chelate these cations.

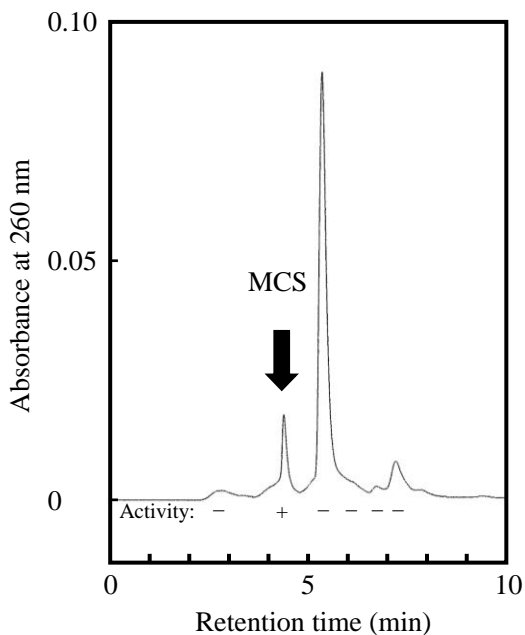


Figure 2. Elution profile of MCS by PHA column. The column was eluted with 50 mM formic acid at a flow rate of 0.5 mL min⁻¹ and MCS was monitored absorbance at 260 nm. MCS was assayed by Mg-dechelation activity using Chlide *a* as a substrate, and also by measuring absorption spectrum at 200 to 400 nm.

Table 1. Summary for purification of MCS. Acetone powder extract was used as the source of the MCS for further purification.

	Total activity (mA ₅₃₅ /min)	Total A ₂₆₀	Specific activity (mA ₅₃₅ /min/A ₂₆₀)	Yield (%)	Purification (-fold)
Crude extract	196	2060	0.10	100	1
DEAE Toyopearl	176	729	0.24	90	3
Macro prep High S	126	104	1.22	65	13
Sephadex G-10	58.2	44.7	1.30	30	14
Superdex peptide (1st)	11.6	7.68	1.51	5.9	16
Superdex peptide (2nd)	4.51	0.90	5.01	2.3	53
PHA	1.76	0.06	29.4	0.9	309

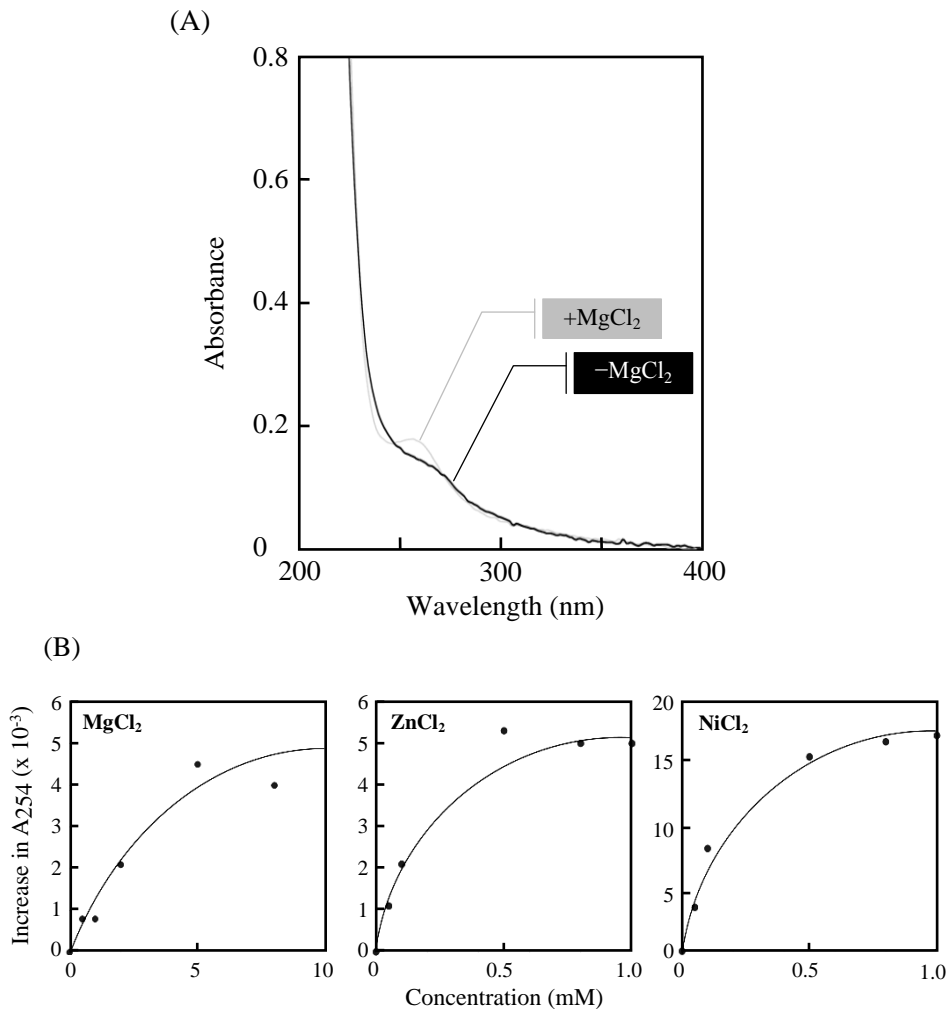


Figure 3. Absorption spectra of purified MCS. The purification of MCS was carried out according to the method described in the text. (A) Change of absorption spectrum of MCS before (black line) and after (grey line) binding of metal. MCS was mixed with MgCl₂ at a final concentration of 10 mM and kept at room temperature for 5 min. (B) Increase in absorption of MCS after binding of metal. MCS was mixed at the indicated concentrations of metals (MgCl₂, ZnCl₂ and NiCl₂) and kept at room temperature for 5 min, then absorbance at 254 nm was measured.

Detection of SH group

From the result of Ellman's reaction, change of absorbance at 412 nm was 0.001 for MCS and it would correspond to the level of 7.5 μM of SH group, if SH group were present. This value of MCS was significantly lower than that expected from the sample. This result supports that MCS did not react with Ellman's reagent, and further that MCS at least lacks SH group. Moreover, this means that MCS does not belong to a class of phytochelatins that highly includes cysteine, known as heavy-metal binding peptides in plants.

Detection of amino group

From the results of above analysis, it is concluded that MCS is not identical to phytochelatins, because of the absence of SH group. However, question arises for MCS on the similarity to nicotianamine or its derivatives, mugineic acids that share the same properties having amino group. In fact, MCS was ninhydrin positive, confirming the presence of amino group. MCS was therefore analyzed using a TLC with two different solvent systems: *n*-butanol-acetic acid- H_2O (4:1:1, by volume) and *n*-propanol- H_2O (6:7, v/v) (Shojima et al. 1989b). The R_f values of MCS were 0.23 and 0.78 in those solvent systems, respectively. These values were different from those of nicotianamine (0.0 and 0.58, respectively) (Shojima et al. 1989b), indicating that MCS is not identical to nicotianamine.

Molecular weight analysis by Superdex Peptide and MALDI-TOF MS

Aromatic hydrocarbons such as CCA is known to form primarily molecular ions upon laser desorption/ionization using near-threshold laser powers. In the present study, therefore, CCA was used as an electron-transfer matrix. In MALDI-TOF MS measurement, MCS was ionized with a single major ion peak at $m/z = 452.96$ and two minor ion peaks at $m/z = 476.48$ and 492.36 (Figure 4). The major peak corresponds to the MH^+ ion of MCS, whereas minor peaks are likely to originate from sodium and potassium adducts of MCS, respectively.

Localization of MCS in radish seedlings

To examine localization of MCS in plants, activity of MCS was analyzed using radish after separating seedlings into three tissues, leaf, stem and root. On a fresh weight basis, the total activities of Mg-dechelation were roughly compared in these three tissues. As shown in Figure 5, the activity of MCS was the highest in leaf and that of stem was about 7% of the leaf. Interestingly, the activity of MCS in root was relatively high and it reached 84% of the leaf. This indicated that MCS activity is present in the root as well as leaf.

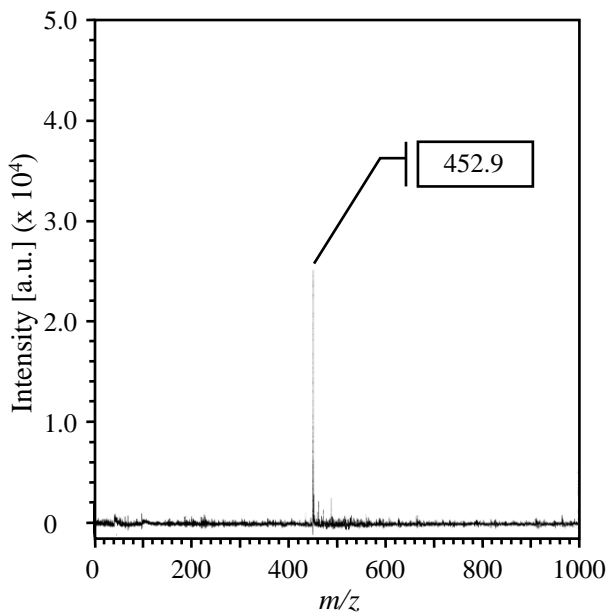


Figure 4. MALDI-TOF MS spectrum of the purified MCS. MCS used was peak fraction at a retention time of 4.4 min in elution of PHA column chromatography. CCA was used for matrix. The nitrogen laser was shot 300 times at single spot and detection was carried out in positive ion mode.

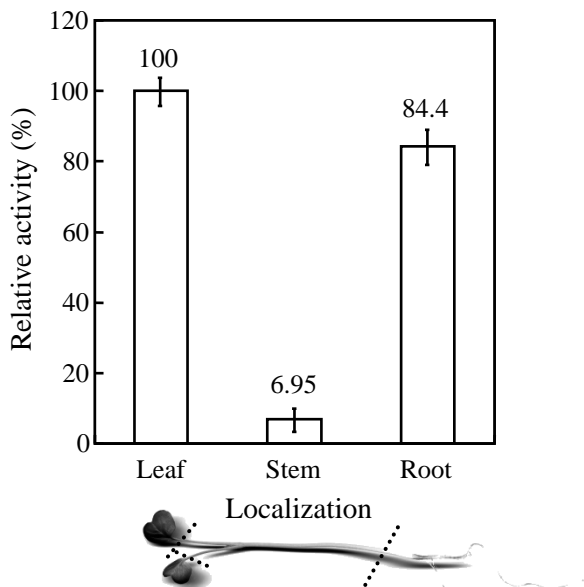


Figure 5. Localization of Mg-dechelating activity. Seedlings of *R. sativus* were detached at dotted lines into leaves, stems and roots and prepared for acetone powder from each part. Extraction was performed with 20 volumes of 20 mM Na phosphate buffer (pH 7.0). Other methods are described in the text. Bars represent standard error (n = 3).

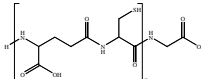
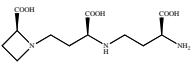
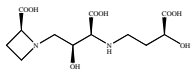
DISCUSSION

In previous study, we clarified that MCS is a possible candidate for a substance that catalyzes the Mg-dechelation reaction in the breakdown pathway of Chl from the point of the use of native Chlide *a* (Suzuki et al. 2005). In this study, MCS was highly purified (Figure 2 and Table 1) and characterized. Molecular weight of MCS determined by MALDI-TOF MS was 452.98 [MH⁺] (Figure 4) which is largely similar to the previous data measured by gel filtration of approximately 400 (Suzuki et al. 2005). At present, attempts to determine structure of MCS by NMR analysis were unsuccessful. Clear assignment signals were not obtained probably due to a low concentration of sample.

Phytochelatin, which is a chelator with a role of heavy metal detoxification in roots and stems, has a structure of (γ -glutamic acid-cysteine)_n-glycin (n = 2–11) (Grill et al. 1989). This compound is synthesized by γ -glutamylcysteine dipeptidyl transpeptidase which is called phytochelatin synthase. The enzyme catalyzes the transfer of the γ -glutamylcysteine dipeptide moiety of glutathione to an acceptor glutathione molecule or a growing chain of (γ -glutamic acid-cysteine)_n-glycin oligomers. Another chelator with a role of metal-transport within the plant body, nicotianamine, and its metabolite, mugineic acid, are synthesized by enzymatic reaction (Mori & Nishizawa 1987; Shojima et al. 1989b). They are formed by the trimerization of three molecules of *S*-adenosyl-L-methionine and that reaction is catalyzed by nicotianamine synthase (EC 2.5.1.43) (Shojima et al. 1989a, 1990; Higuchi et al. 1995). MCS gives a positive reaction with ninhydrin, confirming the presence of an amino group. Judging from their molecular size and amino acid composition, MCS appears to be substance or peptide composed of modified amino acid or amino groups as similar to nicotianamine family, which has also amino groups and synthesized from *S*-adenosyl-L-methionine. However, clear result was not obtained by the analysis of TOF MS for fragment ion or signal of amino acid from MCS after it was hydrolyzed by hydrochloric acid for 24 h (data not shown). This may suggest the presence of modified amino acids in MCS.

A comparison of properties of MCS and known representative chelators from higher plants is shown in Table 2, although structure of MCS is still unclear. As shown above, MCS has no SH group and hence MCS is different from phytochelatins containing cysteine. On the other hand, MCS has amino group as well as nicotianamine because of its reactivity with ninhydrin. The *R_f* values of MCS in TLC were, however, different from those of nicotianamine, which is a metal-transporter and its synthase is especially expressed at reproductive organs such as flowers (Takahashi et al. 2003) and at Fe-deficient graminaceous roots and leaves (Higuchi et al. 1994, 1999). One of its homologs is also expressed at leaves under Fe-sufficient condition (Mizuno et al. 2003). Mugineic acid is a chelator synthesized from nicotianamine. MCS localizes in leaves and roots (Figure 5), but did not chelate monovalent cations such as Na⁺, K⁺ and Li⁺ and also trivalent ions such as Fe³⁺ (data not shown) different from nicotianamine and mugineic acid (Benes et al. 1983; von Wirén et al. 1999; Takagi 1976). Molecular weight of MCS determined

Table 2. Comparison of properties of MCS and other chelators in plants

This study		Known chelators		
	Metal-chelating substance	Phytochelatin	Nicotianamine	Mugineic acid
Structure	Peptide?	(γ -Glu-Cys) $_n$ -Gly (n = 2-11) 	C ₁₂ H ₂₁ O ₆ N ₃ 	C ₁₂ H ₂₀ O ₈ N ₂ 
Molecular weight	452	532 (n = 2)	303	320
Binding metal ion	Mg, Zn, Ni, Cd, Mn, Cu	Cd, Cu, Ag	Fe, Cu, Zn, Mn, Ni	Fe, Cu, Zn, Mn
Ellman reaction	Negative	Positive	Negative	Negative
Distribution	Wide range of plant species and plant tissues	Wide range of plant species and plant tissues	Ubiquitously present in higher plants	Cereal
Localisation	Leaves and roots	Roots and stems	Leaves, flowers and seeds	Roots, xylem, phloem saps
Function	Chlorophyll degradation Heavy metal detoxification? Metal transport?	Heavy metal detoxification	Metal transport to interveinal area Development of pistil and anther Maturation of pollen Maturation of seeds Regulation of metal-requiring protein	Phytosiderophore

by MALDI-TOF MS was different from those of other chelators (Table 2). MCS distributes in a wide range of plant species. Combined together with those results, it is likely that MCS is a novel chelator in higher plants, although structural determination is necessary.

Purified MCS showed high affinity for Zn²⁺ and Ni²⁺ and then Mg²⁺ (Figure 3B). Mg-dechelation activity from Chlide *a* was unexpectedly detected in Chl-less organ such as roots, in addition to leaves of radish (Figure 5). Furthermore, MCS was not induced during senescence (Suzuki et al. 2005). These suggested that MCS may play other roles in plant metabolism besides their involvement in the reaction of Mg-dechelation in Chl degradation pathway. Possibly MCS could perform a role of heavy metal detoxification or act as phytometallophore like other known plant chelators. The typical chelators such as phytochelatins, nicotianamine and mugineic acid have activity of chelating of several divalent and monovalent metal ions and they function in metal transport or heavy metal detoxification in plants.

Further study by NMR analysis is necessary to determine the complete structure of MCS. Elucidation of the structure and function of MCS would provide the understanding of the mechanisms of micro and macronutrient uptake and translocation by plants.

CONCLUSION

Pheophorbide formation by the release of Mg²⁺ from Chlide in Chl metabolism was studied using extracts of *C. alubum* and *R. sativus*. Mg-dechelation reaction is catalyzed by a small molecular weight substance, MCS. MCS was highly purified and its molecular mass was determined to be 452 by MALDI-TOF MS. From the present results, it was indicated that MCS may be a novel chelator in plants and may play not just Mg-dechelation from Chlide *a* in Chl metabolism, but also other roles such as micronutrient transport.

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