Characterization and Subcellular Localization of Chlorophyllase from *Ginkgo biloba*

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Chlorophyllase (Chlase) catalyzes the initial step of chlorophyll (Chl)-degradation, but the physiological significance of this reaction is still ambiguous. Common understanding of its role is that Chlase is involved in de-greening processes such as fruit ripening, leaf senescence, and flowering. But there is a possibility that Chlase is also involved in turnover and homeostasis of Chls. Among the de-greening processes, autumnal coloration is one of the most striking natural phenomena, but the involvement of Chlase during autumnal coloration is not clear. Previously, it was shown that Chlase activity and expression level of the Chlase gene were not increased during autumnal coloration in *Ginkgo biloba*, indicating that Chlase does not work specially in the de-greening processes in *G. biloba.* In this study, we characterized the recombinant Chlase and analyzed its subcellular localization to understand the role of the cloned Chlase of *G. biloba* (*Gb*CLH). *Gb*CLH exhibited its highest activity at pH 7.5, 40 °C. Kinetic analysis revealed that *Gb*CLH hydrolyzes pheophytin (Pheo) *a* and Chl *a* more rapidly than Pheo *b* and Chl *b*. Transient expression analysis of 40 N-terminus amino acids of *Gb*CLH fused with GFP (green fluorescent protein) and subcellular fractionation showed that *Gb*CLH localizes within chloroplasts. Together with our previous results, property of *Gb*CLH and its location within the chloroplasts suggest that *Gb*CLH plays a role in the turnover and homeostasis of Chls in green leaves of *G. biloba.*

Key words: Chlorophyllase, *Ginkgo biloba*, Subcellular Localization

Introduction

Chlorophyll (Chl)-degradation is obviously occurring in leaves of deciduous trees when they show their dramatic autumnal coloration. Some biochemical studies have been conducted on Chl-degradation during this phenomenon (Matile, 2000), however, molecular biological studies on Chl-degradation in the leaves of deciduous trees are much less numerous than those about senescing leaves of annual grasses and ripening fruits (Hörtensteiner, 1999; Matile *et al.*, 1999; Takamiya *et al.*, 2000).

Chlorophyllase (chlorophyll-chlorophyllide hydrolase, Chlase; EC 3.1.1.14) catalyzes the hydrolysis of Chls into chlorophyllides (Chlides) and phytol, which is thought to be the first step of Chl-degradation (Holden, 1961), as the increase of Chlase activity is correlated with the loss of Chls in many plants (Gong and Mattheis, 2003; Johnson-Flanagan and McLachlan, 1990; Ketsa *et al.*, 1998; Looney and Patterson, 1967; Rodríguez *et al.*, 1987; Shimokawa, 1981; Trebitsh *et al.*, 1993). However, there are contrary reports that indicate only a tenuous role for Chlase in the loss of Chls. Majumdar *et al.* (1991) have reported that the activity of Chlase decrease as the amounts of Chls decline, and Todorov *et al.* (2003) have reported that Chlase activity shows no correlation with the amount of Chls. Chlase activity is found in both normal green leaves and developing fruits, indicating that Chlase may play a role in Chl-turnover and homeostasis (Todorov *et al.*, 2003; Mínguez-Mosquera and Gallardo-Guerrero, 1996). It was shown that when Chlase was overexpressed or knocked-down, the ratio of Chlides to Chls and Chl *a* to Chl *b* were altered without a substantial change in the total amount of Chls (Benedetti and Arruda, 2002). These contradictory results leave Chlase a vague role in Chl-catabolism.

Numerous experiments have been conducted to purify and characterize Chlases from various ma-

terials (Bacon and Holden, 1970; Böger, 1965; Fernandez-Lopez *et al.*, 1992; Garcia and Galindo, 1991; Ichinose and Sasa, 1973; Khalyfa *et al.*, 1995; Klein and Vishniac, 1961; Shimokawa, 1982; Shioi and Sasa, 1986; Tanaka *et al.*, 1982; Tsuchiya *et al.*, 1997). From the results of these early attempts and lately revealed information about amino acid sequences, there are some candidates for Chlase location, *i.e.* chloroplast, endoplasmic reticulum (ER) and vacuole. However, no direct indication of its localization in the intact plant cells has been done. Indirectly, Chlase has been shown to localize within the chloroplast envelope membrane by means of a membrane fractionation technique (Matile *et al.*, 1997). On the other hand, Chlase appears to be a glycoprotein, suggesting that Chlase is transported via the ER system (Terpstra, 1981; Terpstra *et al.*, 1986). Chlase was the first enzyme, whose gene was successfully cloned, among the enzymes involved in Chl-degradation (Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999). Sequence analysis revealed that *Ca*CLH from *Chenopodium album* has a putative signal sequence for ER at the N-terminus (Tsuchiya *et al.*, 1999). From studies on the Chl-transport, Takamiya *et al.* (2000) have proposed that vacuoles contain Chlase and are responsible for Chl-degradation.

In the previous study, we cloned a Chlase gene (*Gb*CLH; accession no. AY29252) from leaves of *Ginkgo biloba* to elucidate its participation during autumnal yellowing. Because *G. biloba* is an early diverging gymnosperm, it should be also helpful in understanding the evolutionary origin of angiosperm Chlase function. The expression of *Gb*CLH was greatest in green leaves and significantly declined during the process of leaf yellowing, suggesting that *Gb*CLH is involved in Chl-turnover and homeostasis in the green leaves of *G. biloba* rather than the de-greening process (Tang *et al.*, 2004). In the present study, characterization and subcellular localization, respectively, were analyzed using recombinant *Gb*CLH and sGFP fused with 40 amino acids at the N-terminus of *Gb*CLH. The results showed its highest activity at pH 7.5, 40 °C and higher maximum catalytic rate with Chl *a* and pheophytin (Pheo) *a* as a substrate than with Chl *b* and Pheo *b*. Subcellular localization analysis revealed that *Gb*CLH localizes within the chloroplast. Accordingly, *Gb*CLH should play an important role in Chl-turnover and homeostasis in *G. biloba.* To our knowledge, this is the first study that directly visualizes the location of Chlase in the intact cells.

Materials and Methods

Enzyme expression in Escherichia coli

*Gb*CLH (accession no. AY292526) was expressed in *E. coli* using a pET expression system (Novagen, Inc., Madison, WI, USA) as described previously (Tang *et al.*, 2004). The recombinant protein was extracted using BugBuster™ HT Protein Extraction Reagent (Novagen, Inc.) and the crude extracts were used for further characterization of *Gb*CLH.

Enzyme assay

The enzymatic assay was conducted as described previously (Tang *et al.*, 2000). The enzyme solution (0.2 ml) was mixed with a reaction buffer containing 50 μM Chl *a* and 50 mM lauryldimethylamine *N*-oxide (0.8 ml) and incubated at 30 °C for 30 min as a standard condition and at 20–70 °C to determine the optimum temperature at pH 7.0. To determine the effect of pH value on the activity, buffer systems were used as follows: 25 mM citrate-NaOH (pH 3.5 to 5.5), 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH (pH 5.5 to 7.0), 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.0 to 7.5), 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS)-NaOH (pH 7.5 to 8.5), 25 mM [(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]-1-propanesulfonic acid (TAPS)-NaOH (pH 8.5 to 9.0), 25 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES)-NaOH (pH 9.0 to 10.0), and 25 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-NaOH (pH 10.0 to 12.5). The reaction was stopped by the addition of 3 ml of acetone/*n*-hexane [1:2 (v/v)] and 0.1 ml of 2 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)-HCl, pH 9.0. After shaking the mixture vigorously, the concentration of Chlide *a* in the aqueous layer was spectrophotometrically determined using an absorption coefficient of 76.79 mM^{-1} cm^{-1} at 667 nm (Porra *et al.*, 1989).

Measurement of substrate specificity of GbCLH

25 mM Chl *a*, 25 mM Chl *b*, 25 mM Pheo *a*, 25 mM Pheo *b*, and 25 mM bacteriochlorophyll *a* (BChl *a*) were used to examine the substrate specificity of *Gb*CLH. The enzymatic reaction was stopped by

chilling the solution in liquid nitrogen. Amounts of substrate decrease were determined by HPLC [LC-10AT pump , CTO-10A column oven, and SPD-M10AVP photodiode array detector (SHIMADZU Co., Kyoto, Japan)]. Detection of each substrate was performed by absorption at an appropriate wavelength (Chl *a*: 660 nm; Chl *b*: 645 nm; Pheo *a*: 667 nm; Pheo *b*: 655 nm; BChl *a*: 775 nm). The samples were loaded on a 5 μm YMC-PACK ODS-A column (4.6 i.d. × 250 mm, YMC Co. Ltd., Kyoto, Japan) and eluted by a two-solvent system (A: 50% methanol; B: 100% acetone) with a linear gradient of 50–90% solvent B at a flow rate of 1.0 ml/min over 40 min while maintaining the column temperature at 40 °C.

sGFP constructs

For transient expression of the 40 N-terminus amino acids of *Gb*CLH (*Gb*CLH$_{1-40}$) C-terminally fused to the N-terminal of sGFP, we used the CaMV35S-sGFP(S65T)-NOS3′/pUC18 vector, which was kindly provided by Dr. Yasuo Niwa of the University of Shizuoka. The cDNA encoding *Gb*CLH$_{1-40}$ was amplified using a forward primer (5′-CGC CAT GGT TTT AGT GAA GGA TGT G-3′) and a reverse primer (5′-CGC CAT GGG AAG GCG TGG TTG CAG TTC C-3′) including *Nco* I sites. The PCR products were digested by *Nco* I restriction enzymes. The digested fragment was ligated into the *Nco* I site of the CaMV35S-sGFP(S65T)-NOS3′/pUC18 vector giving CaMV-35S-*Gb*CLH$_{1-40}$-sGFP(S65T)-NOS3′/pUC18. The CaMV35S-sGFP(S65T)-NOS3′/pUC18 vector was used as negative control.

Transient transformation of Arabidopsis thaliana protoplasts

A. thaliana ecotype Columbia (Col-0) was provided by Phytoculture Control Co., Ltd. (Osaka, Japan). Transient transformation of *A. thaliana* protoplasts by a polyethylene glycol method was conducted as described by Sheen (2001). 0.2 ml of protoplast suspension (2×10^5 per ml) was transfected with 20 μg of plasmid DNAs. The transfected protoplasts were incubated at 22 °C for 12 h in the dark. sGFP and chlorophyll-fluorescence was observed by a microscope (BX-50, Olympus Optical Co. Ltd., Tokyo, Japan) with a fluorescence illuminator (BX-FLA, Olympus Optical Co.).

Subcellular fractionation

Green leaves of *G. biloba* (5 g fresh weight) harvested on the campus of Osaka University were chopped by a razor with 3 ml of 150 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)-KOH (pH 7.5) containing 1 mM EDTA and 0.5 M sucrose. The sample was centrifuged at about 70 × *g* for a few seconds. The supernatant was filtered through three layers of gauze and the filtrate was layered on a 16 ml gradient (30% to 60% sucrose, 1 mM EDTA, pH 8.0). The gradient was centrifuged at 85,000 × *g* for 2.5 h at 4 °C. After centrifugation, fractions (1 ml) were collected and the Chlase activity of each fraction was measured.

The activities of subcellular marker enzymes were assayed as described previously: catalase activity as a marker enzyme for microbodies (Aebi, 1983) and cytochrome c oxidase for mitochondria (Briskin *et al.*, 1987). Chl-contents were determined by measuring the absorption at 645 and 663 nm [Chl $(a + b) = (A_{645} \times 20.2 + A_{663} \times 8.02)$ μg/ml] (Arnon, 1949) and used as a marker for chloroplast thylakoid membranes.

Results and Discussion

Optimum temperature and pH for GbCLH activity

We used the recombinant protein expressed in *E. coli* for characterization of *Gb*CLH. Although the recombinant protein could not be glycosylated, appreciable enzymatic activity was observed from 30 to 50 °C and the maximal enzymatic activity was at 40 °C (Fig. 1A). The optimum pH was measured at pH 7.5 (Fig. 1B). The optimum temperature and pH values are similar to those of Chlases from other species of plants such as sugar beat (40 °C, pH 7.1) (Bacon and Holden, 1970), *Citrus limon* (pH 7.8) (Fernandez-Lopez *et al.*, 1992), *Capsicum annuum* L. fruits (50 °C, pH 8.5) (Hornero-Méndez and Mínguez-Mosquera, 2001), greened rye seedlings (30 °C, pH 7.0–8.5) (Tanaka *et al.*, 1982), *Citrus unshiu* (pH 7.0) (Shimokawa, 1981), *Citrus sinensis* L. (45 °C, pH 7.5) (Trebitsh *et al.*, 1993), *Chenopodium album* (pH 7.0) (Tsuchiya *et al.*, 1997), olive fruits (50 °C, pH 8.5) (Mínguez-Mosquera *et al.*, 1994) and parsley (pH 6–8) (Klein and Vishniac, 1961); and algae such as *Phaeodactylum tricornutum* [31 °C and 35 °C (Gaffer *et al.*, 1999), pH 8.0 (Khalyfa *et al.*, 1995)], *Chlorrella vulgaris* (pH 7.2–7.3) (Böger, 1965) and *Chlorella protothecoides* (45 °C,

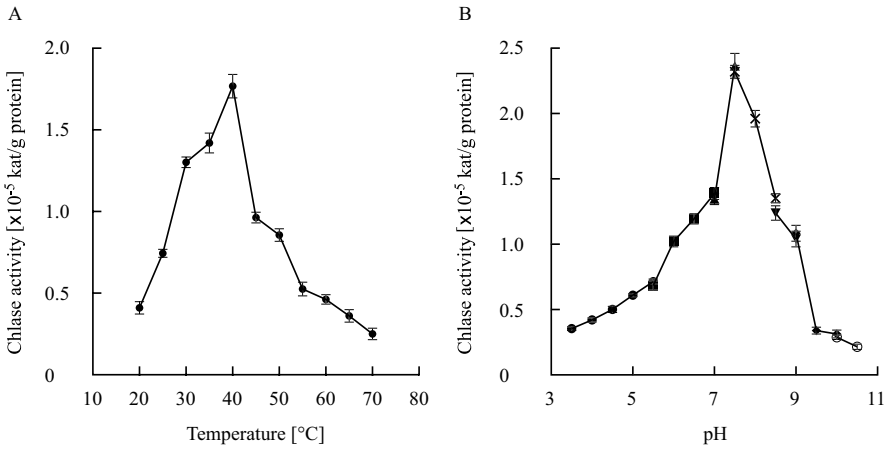

Fig. 1. Effect of temperature (A) and pH value (B) on the activity of crude recombinant *Gb*CLH. Data are mean values ± SD, n = 5. (B) The buffer systems were citrate-NaOH (●), MES-NaOH (■), MOPS-NaOH (▲), EPPS-NaOH (**X**), TAPS-NaOH (▼), CHES-NaOH (◆), and CAPS-NaOH (**O**).

pH 6.0–8.5) (Tamai *et al.*, 1979), indicating similar characteristic of Chlases in algae, gymnosperm and angiosperms. The optimum condition for *Gb*CLH suggests that the environment where the enzyme works is neutral or weakly basic, such as in cytoplasm and chloroplast. In contrast, acidic optimum pH values have been reported for Chlase from tea leaves [30–35 °C, pH 5.8 (Ogura, 1972) and pH 5.5 (Kuroki *et al.*, 1981)].

Substrate specificity of GbCLH

Substrate specificity was studied for Chl *a* and *b*, Pheo *a* and *b*, and BChl *a*. The parameters (Table I) were similar to those reported previously, for example, K_m values for Chl *a* are 7 μM (Ogura, 1972) and 10 μM (Kuroki *et al.*, 1981) in tea leaves, 12 μM in greened rye seedlings (Tanaka *et al.*, 1982), 10.7 μM in *C. annuum* L. fruits (Hornero-Méndez and Mínguez-Mosquera, 2001), 16.1 μM in olive fruits (Mínguez-Mosquera *et al.*, 1994), 13 μM in *C. limon* leaves grown under normal nutritional status (Fernandez-Lopez *et al.*, 1992), and 7.13 μM in de-greening canola seeds (Johnson-Flanagan and McLachlan, 1990). Lower K_m values for Chl *a* have been observed for Chlases in other sources, for example, 2.65 μM in *C. unshiu* fruits (Shimokawa, 1982), 2 μM in *C. protothecoides* (Ichinose and Sasa, 1973), 4.0 and 4.6 μM in *C. album* (Tsuchiya *et al.*, 1997), 1.76 μM in *C. sinensis* (Trebitsh *et al.*, 1993), while higher K_m values for Chl *a* have also been reported in algae (*P. tricornutum*) and in chlorotic *Citrus* leaves (278 μM; Fernandez-Lopez *et al.*, 1992).

Table I. Substrate specificity of *Gb*CLH.

Substrate	K_m [μM]	V_{max}	r^2 [a]
Chl *a*	11.3	1.97	0.993
Chl *b*	11.0	0.746	0.992
Pheo *a*	12.3	3.44	0.990
Pheo *b*	5.50	0.949	0.991
BChl *a*	33.4	1.69	0.998

[a] Correlation coefficient was derived from Lineweaver-Burk plot analysis.

The K_m values indicated that *Gb*CLH has the highest affinity for Pheo *b*, although its V_{max} is much lower than those of Chl *a* and Pheo *a*. Substrate inhibition was observed when the Pheo *b* concentration was more than 20 μM due to the high affinity and low transformation rate. Substrate inhibition by Pheo *b* has also been reported for Chlase from *C. annuum* L. (Hornero-Méndez and Mínguez-Mosquera, 2001). Affinity for Chl *a*, Chl *b* and Pheo *a* is on the same level, V_{max} for Pheo *a* is the highest while V_{max} for Chl *b* is less than half of Chl *a* and Pheo *a*. These results indicate a faster transformation of Pheo *a* and Chl *a* to pheophorbide *a* and Chlide *a*, respectively, by *Gb*CLH. The substrate specificity is similar to that of Chlase from olive fruits except for its higher affinity to Chl *b* than *Gb*CLH (Mínguez-Mosquera *et al.*, 1994). Partially purified Chlases from *C. album* have a similar affinity for Chl *a* and Chl *b* (Tsuchiya *et al.*, 1997). However this tendency conflicts with other reports. For example, Chlase from *C. annuum* L. shows the greatest affinity for Pheo *a*, and the fastest transformation for Chl *a* (Hornero-Méndez and Mínguez-Mosquera, 2001). Chlase in de-greening canola seeds shows fast transformation for Pheo *a* and *b* (Johnson-Flanagan and McLachlan, 1990).

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GbCLH      1:--------------MVLVKDVFSEGPLPVQILAIPQANSSPCSKLADKNGTATTPSPCRP: 46
AtCLH1     1:----------MAAIEDSPTFSSVVTP----------AAFEIGSLPTTEIPVDPVENDSTAP: 41
AtCLH2     1:------------MSSSSSRNAFEDGKYKSNLLTLDSS--SRCCKITPSSRASPS-----P: 41
Chlase1    1:--------MAAMVDAKPAASVQGTPLLATAT---LPVFTRGIYSTKRITLETSSPSSPPP: 49
CaCLH      1:MAKLLLLIFGVFIFVNSQAQTFPTILEKHNSEKITDVFHKGNFQVTNNPIRVKR-YEFSA: 59

GbCLH     47:PKPLLIALPSQHGDYPLILFFHGYVLLNSFYSQLLRFVASHGYIAIAPQMYSVIGP---N:103
AtCLH1    42:PKPVRITCPTVAGTYPVVLFFHGFYLRNYFYSDVLNHIASHGYILVAPQLCKLLPP---G: 98
AtCLH2    42:PKQLLVATPVEEGDYPVVMLLHGYLLYNSFYSQLMLHVSSHGFILIAPQLYSIAGP---D: 98
Chlase1   50:PKPLIIVTPAGKGTFNVILFLHGTSLSNKSYSKIFDFLASHGFIVVAPQLYTSIPP--PS:107
CaCLH     60:PEPLIIISPKEAGVYPVLLFIHGTMLSNEDYSLFFNYIASHGFIVVAPKLFRLFPPKLPS:119
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Fig. 2. Alignment of partial N-terminal amino acid sequences of Chlases. *At*CLH1 (AAC13947) and *At*CLH2 (AAF27046) are from *A. thaliana* (Tsuchiya *et al., 1999) Chlase1 (AAF59834) is from Citrus sinensis* cv. Valencia (Jacob-Wilk *et al.*, 1999). *Ca*CLH (AFF27045) is from *C. album* (Tsuchiya *et al.*, 1999). The amino acid sequence fused with sGFP is underlined.

Subcellular localization of GbCLH

There are a number of reports in the literature that indicate the existence of different isozymes of Chlase with different subcellular localizations and potentially different functions. It is reported that several isozymes of Chlase are located within plastids (Garcia and Galindo, 1991), especially within the plastid envelope (Matile *et al.*, 1997), but it is not clear that all isozymes are located within the plastids and it has been proposed that Chlase is transported via the ER system (Terpsta, 1981; Terpsta *et al.*, 1986) or exists in vacuoles (Takamiya *et al.*, 2000). To reveal the subcellular localization of *Gb*CLH, transient expression analysis with *A. thaliana* protoplasts was conducted. It is reported that all cloned Chlases contain their pu-

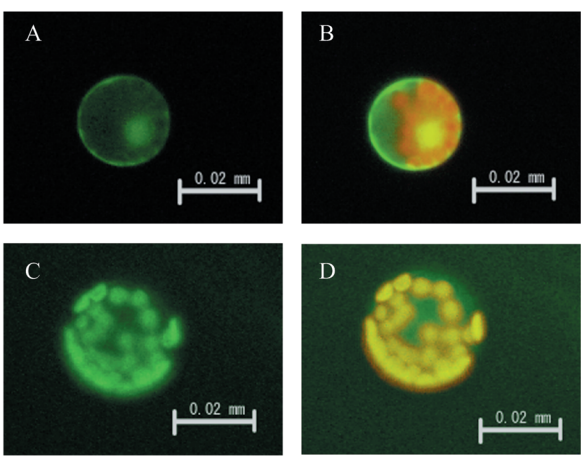

Fig. 3. Fluorescence imaging of the transient expression assay. (A, B) pUC18-sGFP as negative control. (C, D) pUC-GbCLH1–40sGFP. (A, C) Exitation and detection of the green fluorescence from sGFP were performed with a U-MWIBA/GFP filter cube (Olympus Optical Co. Ltd.). (B, D) Excitation and detection of the red fluorescence from Chls and the green fluorescence from sGFP were performed with a U-MWIB/GFP filter cube (Olympus Optical Co. Ltd.).

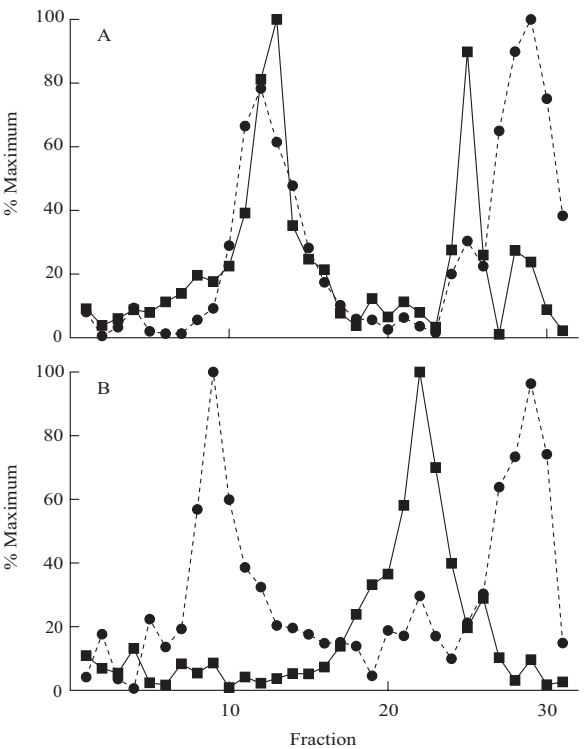

Fig. 4. Subcellular fractions from *G. biloba* leaves. Subcellular fractions were separated on a 30% to 60% sucrose density gradient. (A) Relative Chlase activity (■ with solid line) and Chl (● with dashed line) content in each fraction. (B) Relative activity of the marker enzymes, cytochrome c oxidase (■ with solid line) and catalase (● with dashed line).

tative transit peptides at their N-termini. For example, *At*CLH2 has a putative transit peptide to chloroplast and *Ca*CLH has a transit peptide to ER (Takamiya *et al.*, 2000). Since proline 46 is well conserved among Chlases, and a transit peptide may be involved in front of this position, the 40 N-terminus amino acids in *Gb*CLH were fused with sGFP (Fig. 2). The results of the fluorescence imaging are shown in Fig. 3. Only green fluorescence from GFP was detected in Figs. 3A and C, while green fluorescence from GFP and red fluorescence from Chls were merged in Figs. 3B and D, the yellow color in Fig. 3D indicates that the green fluorescence of GFP and red fluorescence from Chls are emitted from the same places. It was clearly revealed that $GbCLH_{1-40}$:sGFP is localized within the chloroplasts (Figs. 3C, D) while the control sGFP is localized within the cytoplasm (Figs. 3A, B).

To confirm this result, subcellular fractionation of Chlase activity from *G. biloba* was conducted. Fig. 4 shows the result of subcellular fractionation of the Chlase activity. The highest Chlase activity was detected in the fractions where Chls were present (Fig. 4A, fraction nos. 11–14), indicating that Chlase is located within the chloroplast thylakoid membranes. In our previous study, Southern analysis revealed that *Gb*CLH is encoded by a single gene (Tang *et al.*, 2004). But, additionally, there was another peak in those fractions of less density than that of the mitochondrion (Fig. 4B, fraction nos. 20–25). This may be the Chlase in the intact chloroplast or other isozymes that are localized in a different compartment such as ER or vacuole. Further studies are required to prove the existence of isozymes.

These results lead us to conclude that *Gb*CLH is localized within chloroplast. Our previous report indicated that the expression of *Gb*CLH and Chlase activity were high in green leaves and low in yellowing leaves (Tang *et al.*, 2004). That together with these results suggests that *Gb*CLH is involved in the turnover of Chls in green leaves in *G. biloba*.

Aebi H. E. (1983), Catalase. In: Methods of Enzymatic Analysis, Vol. III (Bergmeyer H. U., ed.). Verlag Chemie, Weinheim, pp. 273–286.

Arnon D. I. (1949), Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**, 1–15.

Bacon M. F. and Holden M. (1970), Chlorophyllase of sugar-beet leaves. Phytochemistry **9**, 115–125.

Benedetti C. E. and Arruda P. (2002), Altering the expression of the chlorophyllase gene *ATHCOR1* in transgenic *Arabidopsis* caused changes in the chlorophyll-to-chlorophyllide ratio. Plant Physiol. **128**, 1255–1263.

Böger P. (1965), Chlorophyllase of *Chlorella vulgaris* Beijerinck. Phytochermistry **4**, 435–443.

Briskin D. P., Leonard R. T., and Hodges T. K. (1987), Isolation of the plasma membrane: Membrane markers and general principles. Methods Enzymol. **148**, 542–558.

Fernandez-Lopez J. A., Almela L., Almansa M. S., and Lopez-Roca J. M. (1992), Partial purification and properties of chlorophyllase from chlorotic *Citrus limon* leaves. Phytochemistry **31**, 447–449.

Gaffer R., Kermasha S., and Bisakowski B. (1999), Biocatalysis of immobilized chlorophyllase in a ternary micellar system. J. Biotechnol. **75**, 45–55.

Garcia A. L. and Galindo L. (1991), Chlorophyllase in *Citrus* leaves. Localization and partial purification of the enzyme. Photosynthetica **25**, 105–111.

Gong Y. and Mattheis J. P. (2003), Effect of ethylene and 1-methylcyclopropene on chlorophyll catabolism of broccoli florets. Plant Growth Regul. **40**, 33–38.

Holden M. (1961), The breakdown of chlorophyll by chlorophyllase. Biochem. J. **78**, 359–364.

Hornero-Méndez D. and Mínguez-Mosquera M. I. (2001), Properties of chlorophyllase from *Capsicum annuum* L. fruits. Z. Naturforsch. **56c**, 1015–1021.

Hörtensteiner S. (1999), Chlorophyll breakdown in higher plants and algae. Cell. Mol. Life Sci. **56**, 330–347.

Ichinose N. and Sasa T. (1973), Studies on chlorophyllase of *Chlorella protothecoides* III. Purification and catalytic properties. Plant Cell Physiol. **14**, 1157–1166.

Jacob-Wilk D., Holland D., Goldschmidt E. E., Riov J., and Eyal Y. (1999), Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the *Chlase1* gene from ethylene-treated *Citrus* fruit and its regulation during development. Plant J. **20**, 653–661.

Johnson-Flanagan A. M. and McLachlan G. (1990), The role of chlorophyllase in degreening canola (*Brassica napus*) seeds and its activation by sublethal freezing. Physiol. Plant. **80**, 460–466.

Ketsa S., Phakawatmongkol W., and Subhadrabhandhu S. (1998), Peel enzymatic activity and colour changes in ripening mango fruit. J. Plant Physiol. **154**, 363–366.

Khalyfa A., Kermasha S., Marsot P., and Goetghebeur M. (1995), Purification and characterization of chlorophyllase from alga *Phaeodactylum tricornutum* by preparative native electrophoresis. Appl. Biochem. Biotechnol. **53**, 11–27.

Klein A. O. and Vishniac W. (1961), Activity and partial purification of chlorophyllase in aqueous systems. J. Biol. Chem. **236**, 2544–2547.

Kuroki M., Shioi Y., and Sasa T. (1981), Purification and properties of soluble chlorophyllase from tea leaf sprouts. Plant Cell Physiol. **22**, 717–725.

Looney N. E. and Patterson M. E. (1967), Chlorophyllase activity in apples and bananas during the climacteric phase. Nature **214**, 1245–1246.

Majumdar S., Ghosh S., Glick B. R., and Dumbroff E. B. (1991), Activities of chlorophyllase, phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase in the primary leaves of soybean during senescence and drought. Physiol. Plant. **81**, 473–480.

Matile P. (2000), Biochemistry of Indian summer: physiology of autumnal leaf coloration. Exp. Gerontol. **35**, 145–158.

Matile P., Schllenberg M., and Vicentini F. (1997), Localization of chlorophyllase in the chloroplast envelope. Planta **201**, 96–99.

Matile P., Hörtensteiner S., and Thomas H. (1999), Chlorophyll degradation. Ann. Rev. Plant Physiol. Plant Mol. Biol. **50**, 67–95.

Mínguez-Mosquera M. I. and Gallardo-Guerrero L. (1996), Role of chlorophyllase in chlorophyll metabolism in olives cv. Gordal. Phytochemistry **41**, 691–697.

Mínguez-Mosquera M. I., Gandul-Rojas B., and Gallardo-Guerrero L. (1994), Measurement of chlorophyllase activity in olive fruit (*Olea europaea*). J. Biochem. **116**, 263–268.

Ogura N. (1972), Studies on chlorophyllase in tea leaves III. Properties of soluble and insoluble chlorophyllases. Plant Cell Physiol. **13**, 971–979.

Porra R. J., Thompson W. A., and Kriedemann P. E. (1989), Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta **975**, 384–394.

Rodríguez M. T., González M. P., and Linares J. M. (1987), Degradation of chlorophyll and chlorophyllase activity in senescing barley leaves. J. Plant Physiol. **129**, 369–374.

Sheen J. (2001), Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol. **127**, 1466–1475.

Shimokawa K. (1981), Purification of ethylene-enhanced chlorophyllase from *Citrus unshiu* fruits. Agric. Biol. Chem. **45**, 2357–2359.

Shimokawa K. (1982), Hydrophobic chromatographic purification of ethylene-enhanced chlorophyllase from *Citrus unshiu* fruits. Phytochemistry **21**, 543–545.

Shioi Y. and Sasa T. (1986), Purification of solubilized chlorophyllase from *Chlorella protothecoides*. Methods Enzymol. **123**, 421–427.

Takamiya K.-I, Tsuchiya T., and Ohta H. (2000), Degradation pathway(s) of chlorophyll: what has gene cloning revealed? Trends Plant Sci. **5**, 426–431.

Tamai H., Shioi Y., and Sasa T. (1979), Studies on chlorophyllase of *Chlorella protothecoides* IV. Some properties of the purified enzyme. Plant Cell Physiol. **20**, 1141–1145.

Tanaka K., Kakuno T., Yamashita J., and Horio T. (1982), Purification and properties of chlorophyllase from greened rye seedlings. J. Biochem. **92**, 1763–1773.

Tang L., Okazawa A., Fukusaki E., and Kobayashi A. (2000), Removal of magnesium by Mg-dechelatase is a major step in the chlorophyll-degrading pathway in *Ginkgo biloba* in the process of autumnal tints. Z. Naturforsch. **55c**, 923–926.

Tang L., Okazawa A., Itoh Y., Fukusaki E., and Kobayashi A. (2004), Expression of chlorophyllase is not induced during autumnal yellowing in *Ginkgo biloba*. Z. Naturforsch. **59c**, 415–420.

Terpstra W. (1981), Identification of chlorophyllase as a glylcoprotein. FEBS Lett. **126**, 231–235.

Terpstra W., Lambers J. W. J., and Levine Y. K. (1986), Studies on chlorophyllase. II. Interaction of the enzyme with concanavalin A and with *N,N′*-dicyclohexylcarbodiimide. Photobiochem. Photobiophys. **11**, 249–255.

Todorov D. T., Karanov E. N., Smith A. R., and Hall M. A. (2003), Chlorophyllase activity and chlorophyll content in wild type and *eti 5* mutant of *Arabidopsis thaliana* subjected to low and high temperatures. Biol. Plant. **46**, 633–636.

Trebitsh T., Goldschmidt E. E., and Riov J. (1993), Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. Proc. Natl. Acad. Sci. USA **90**, 9441–9445.

Tsuchiya T., Ohta H., Masuda T., Mikami B., Kita N., Shioi Y., and Takamiya K. (1997), Purification and characterization of two isozymes of chlorophyllase from mature leaves of *Chenopodium album*. Plant Cell Physiol. **38**, 1026–1031.

Tsuchiya T., Ohta H., Okawa K., Iwamatsu A., Shimada H., Masuda T., and Takamiya K. (1999), Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. Proc. Natl. Acad. Sci. USA **96**, 15362–15367.