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Genes and Biochemical Characterization of Three Novel Chlorophyllase Isozymes from *Brassica oleracea*†

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Three full length cDNAs (*BoCLH1*, 1140 bp; *BoCLH2*, 1104 bp; *BoCLH3*, 884 bp) encoding putative chlorophyllases were cloned from the cDNA pools of broccoli (*Brassica oleracea*) florets and characterized. The amino acid sequence analysis indicated that these three BoCLHs contained a highly conserved lipase motif (GXSXG). However, only BoCLH3 lacked the His residue which is the component of the catalytic triad (Ser-His-Asp). N-terminal sequences of BoCLH1 and BoCLH2 were predicted to have typical signal sequences for the chloroplast, whereas the plasma membrane-targeting sequence was identified in BoCLH3. The predicted molecular masses of BoCLH1, 2, and 3 were 34.7, 35.3, and 23.5 kDa, respectively. The recombinant BoCLHs were successfully expressed in *Escherichia coli* for the biochemical characterization. The recombinant BoCLH3 showed very low chlorophyllase activity possibly due to its incomplete catalytic triad. BoCLH1 and BoCLH2 showed significant differences in biochemical properties such as pH stability and temperature optimum. Kinetic analysis revealed that BoCLH1 preferably hydrolyzed Mg-free chlorophyll, while BoCLH2 hydrolyzed both chlorophyll and Mg-free chlorophyll at a similar level. Different characteristics between BoCLH1 and BoCLH2 implied that they may have different physiological functions in broccoli. The catalytic triad of recombinant BoCLH2 was identified as Ser141, His247, and Asp170 by site-directed mutagenesis. It suggested that the three broccoli chlorophyllase isozymes were serine hydrolases.

KEYWORDS: Chlorophyllase; isozyme; Broccoli (*Brassica oleracea*)

INTRODUCTION

Chlorophyllase (chlorophyll chlorophyllidohydrolase, Chlase, EC 3.1.1.14), which catalyzes the hydrolysis of chlorophyll (Chl) to chlorophyllide (Chlide) and phytol (dephytylation), was proposed to be the first enzyme in Chl breakdown during the degreening processes of plants such as leaf senescence, pathogen infection, and fruit ripening (*1*−*4*). Although the enzyme has long been known to exist in diverse species, including diatoms (*5*), chlorella (*6*, *7*), and some angiosperms (*6*), only a few Chlase genes were isolated. Tsuchiya et al. cloned the first Chlase gene (*CaCLH*) from the mature leaves of *Chenopodium album* and then isolated two homologues of Chlase genes (*AtCLH1* and *AtCLH2*) in *Arabidopsis thaliana* leaves (*8*). One *Citrus sinensis* Chlase cDNA (*CsCLH*) was obtained from ethylene-treated citrus fruit peel (*9*). The *Triticum aestivum* (wheat) Chlase cDNA (*TaCLH*) was identified among expressed sequence tags generated from wheat leaves of etiolated seedlings (*10*). The recombinant Chlase from *Ginkgo biloba* (GbCLH) was recently characterized, and its subcellular localization was analyzed by transient expression (*11*).

The deduced amino acid sequences of all cloned Chlase genes share a GXSXG lipase motif containing an active site residue of serine. *AtCLH2* and *CsCLH* encode proteins of 318 and 329 amino acids, respectively, and both contain a predicted N-terminal signal peptide for the chloroplast. *AtCLH1* encodes a protein of 324 amino acids, which has no typical signal sequence. In contrast, *CaCLH* encodes a 347 amino acid protein that contains a typical signal peptide for the endoplasmic reticulum (ER) and a (NPIR) vacuolar-sorting determinant, suggesting that CaCLH is a vacuolar enzyme produced via ER transport. It is still not clear whether various plants or tissues evolve different isoenzymes which are targeted to various organelles for regulating different types of Chl degradation.

Broccoli (*Brassica oleracea*) is an important vegetable crop. The florets senesce and turn yellow rapidly after harvest. Chlase is presumably involved in the breakdown of the Chl. The cloning and characterization of the Chlase gene is essential to understanding the physiological role of Chlase and preventing Chl

†GenBank accession numbers: *BoCLH1*, AF337544; *BoCLH2*, AF337545; *BoCLH3*, AF337546.

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degradation by transgenic antisense Chlase genes. We have reported three broccoli Chlase genes *BoCLH1*, *BoCLH2*, and *BoCLH3* (GenBank accession numbers AF337544, AF337545, and AF337546). Expression of the antisense constructs of gene *BoCLH1* in broccoli plants using *Agrobacterium tumefaciens*-mediated transformation resulted in a phenotype with slower postharvest yellowing during storage (*12*). In the present article, we describe the cloning, expression, and characterization of these three Chlase cDNAs from mature broccoli florets. The primary structures of three deduced proteins were analyzed and compared with other plant Chlases.

MATERIALS AND METHODS

Plant Material. Plants of broccoli (*Brassica oleraceae*, cv. Green king) were grown from seeds in a growth chamber. Tissue samples were harvested 2 weeks after the first cluster of florets appeared. The tissue samples of florets were collected separately. The collected tissue samples were frozen in liquid nitrogen and stored at −70 °C for further use.

Cloning of Broccoli Chlase cDNAs. The total RNA isolated from a freshly harvested broccoli floret was used for cDNA synthesis by a CapFinder cDNA synthesis system (Clonetech, Palo Alto, CA, USA). The cDNA pool and a pair of degenerate primers derived from conserved sequences of reported Chlases were used for PCR amplification. The sequence of the degenerate primers are as follows: CLHdN (5′-GGY-CAYAGYMGMGGWGGVAAAWC-3′) and CLHdC (5′-ARCATR-TCCAWRTGHCCRTAMT-3′). A cDNA fragment of about 336 bp in length was amplified and subcloned into a pGEMT-EASY vector (Promega, Madison, WI) for DNA sequence analysis. The sequence of the cloned cDNA fragment that shared high homology to the known Chlase genes was used to design nested primers for cloning 5′ and 3′ ends of the full length cDNA by the 5′/3′ RACE technique. Specific primers corresponding to each terminal sequence was designed for further cloning of various full length cDNA through PCR. The primers derived from the 5′ and 3′ untranslated regions of each Chlase cDNA were used to amplify the full length cDNAs from the cDNA pool. These primers are Bo-CLHaN (5′-ATACAAATGGCGGGGAAGGA-3′) and Bo-CLHaC (5′-GAGT-TTGGAGTCTTTCTGGGGATAC-3′) for generating *BoCLH1*; Bo-CLHbN (5′-AGAGAAAAAAAGTGAAAAAAATAAC-3′) and Bo-CLHbC (5′- GTCAAGATGTCACTTGAGTAAAGAC-3′) for generating *BoCLH2*; and Bo-CLHcN (5′-ACACAAAAAAATATATAACACAAAG-3′) and Bo-CLHcC (5′-CACAAGAGGTTTCTTACCTCATA-3′) for generating *BoCLH3*. In order to bypass the misincorporated bases associated with PCR, both strands of the cDNA sequence were confirmed by a direct sequencing strategy using automated DNA sequencers (DSQ-1, Shimadzu; 373A, Applied Biosystems) with commercially available kits (Perkin-Elmer; Amersham Pharmacia Biotech; Wakunaga; Takara Shuzo). The DNA and deduced amino acid sequences were analyzed by the program of SeqWeb version 2 of the Genetics Computer Group (Accelrys Inc.) software package.

Expression Plasmid Construction. The specific forward primers for amplifying the open reading frames (ORFs) of *BoCLH1*, *BoCLH2*, and *BoCLH3* were designed to create an *Eco*RI restriction site upstream from the translation start site. The specific reverse primers were designed to contain a *Xba*I site downstream from the end of the open reading frame of *BoCLH1* and the *Hin*dIII site for both *BoCLH2* and *BoCLH3*. The PCR conditions were as follows: 95 °C for 3 min; 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; and 72 °C for 5 min. PCR products of the amplified three Chlase ORFs were purified by agarose gel electrophoresis and then subcloned in the *E. coli* expression vectors pMAL-c2x (New England BioLabs Inc., Beverly, MA) following the manufacturer's instructions. The recombinant vectors were transformed into *E. coli* Rosseta-Gami B (DE3). Transformants were used to produce recombinant Chlases.

Protein Expression and Purification. *E. coli* transformant with the pMAL-c2x-BoCLH plasmid expressed recombinant Chlases with an N-terminal fusion of maltose-binding protein (MBP). The protein expression and purification were performed according to the procedure recommended by the manufacturer (New England BioLabs, Inc.). The expression of each MBP-fusion Chlase was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside into the refreshed culture at 37 °C for 6 h. The cells were then harvested and lysed by sonication in TE buffer containing 20 mM Tris-HCl (pH 7.4) and 2 mM EDTA. After high-speed centrifugation (10,000*g* for 10 min at 4 °C), the resultant supernatant was used as the enzyme source.

Table 1. Oligonucleotide Primers Used for Site-Directed Mutagenesis of *BoCLH2*

mutagenic primer	nucleotide sequence (5′ → 3′)[a]
	reversed primers
CLH2-H66A	G GTA ACC **AGC** GAG GAG CAT C
CLH2-S141A	TTT GCC GCC GCG **GGC** ATG GCC GG
CLH2-D170A	ATC CAC CGG **AGC** TAC ACC GA
	forward primers
CLH2-H226A	GGA GTG AAC **GCC** CGA GAG TT
CLH2-H239A	T CCA GCG TGG **GCT** TTT GTC GCA
CLH2-H247A	AG GAT TAC GGA **GCT** TTG GAC ATG

[a] Underlining denotes the mismatched nucleotide, and bold facing denotes the mutant codon.

MBP-fusion Chlases were purified using an amylose resin affinity column. Native forms of Chlases were prepared from MBP-BoCLH recombinant proteins by cleavage with protease under the following conditions. Purified fusion protein from amylose resin was directly cleaved with Factor Xa protease (0.5 U/10 μg of fusion protein) (Novagen, Madison, WI) in the column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA) with 10 mM maltose for 2.5−3.5 h at room temperature. After protease cleavage, Chlases were further purified by ion exchange column chromatography using a DEAE Sepharose Fast Flow column (Amersham, Pharmacia Biotech, Buckinghamshire, UK) equilibrated with TE buffer (20 mM Tris-HCl, pH 7.4, and 1 mM EDTA). After loading the cleavage reaction mixture onto the column, pure Chlases were eluted with TE buffer containing 200 mM NaCl. Protein concentration was determined with the Bradford dye-binding assay (BioRad, Hercules, CA, USA) using bovine serum albumin (Pierce Biotechnology, Rockford, IL) as a standard.

Chlase Assay. The standard reaction mixture for the activity assay of Chlase contains 10 μL of enzyme sample, 65 μL of reaction buffer (100 mM sodium phosphate, pH 7.4, and 0.24% Triton X-100), and 7.5 μL of acetone-dissolved Chl *a* (at a final concentration of 500 μM) (Sigma, St. Louis, MO, USA). The reaction mixture was incubated in a shaking water bath at 40 °C. The amount of product formed had a linear relationship with reaction time within 30 min. Therefore, in the following assay we carried out the reaction for 30 min to measure the initial velocity. Enzyme reaction was stopped by transferring the reaction mixture to a centrifuge tube containing 1 mL of the mixture of acetone/hexane/10 mM KOH = 4:6:1 (v/v). The mixture was vigorously vortexed and centrifuged at 12000*g* for 2 min for phase separation. The Chlide *a* formed remained in the aqueous acetone layer, while the unreacted Chl *a* was extracted into the *n*-hexane layer (*13*). The absorbance of the aqueous acetone phase was measured at 667 nm for Chlide *a* with a spectrophotometer. The amount of each product in the acetone layer was estimated from the millimolar extinction coefficient of 81.0 mM^{-1} cm^{-1} for Chlide *a* (*14*). One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of Chl *a* per min at 40 °C.

Enzyme Characterization. The pH effect on the activity of the purified recombinant Chlase was measured between pH 3.0 to 10.0, using Good's buffer (50 mM Bicine, 50 mM CHPS, sodium acetate, and 50 mM bis-Tris propane). For this purpose, 10 μL of enzyme mixed with 65 μL of Good's buffer with different pH values and 7.5 μL of Chl *a* (500 μM) was incubated at 40 °C for 5 min. The pH stability assay was performed with the enzyme which was incubated with Good's Buffer containing 0.2% Triton X-100 at different pH values from 3.0 to 10.0 for 10 min at 40 °C. Chlase activity was subsequently measured as described above.

The temperature dependence of chlase activity was measured between 20 and 80 °C using the standard Chlase assay. Thermal stability profiles were performed with enzymes, which were incubated at different temperatures ranging from 20 to 80 °C for 10 min and then chilled on ice. Chlase activity was subsequently measured as described above.

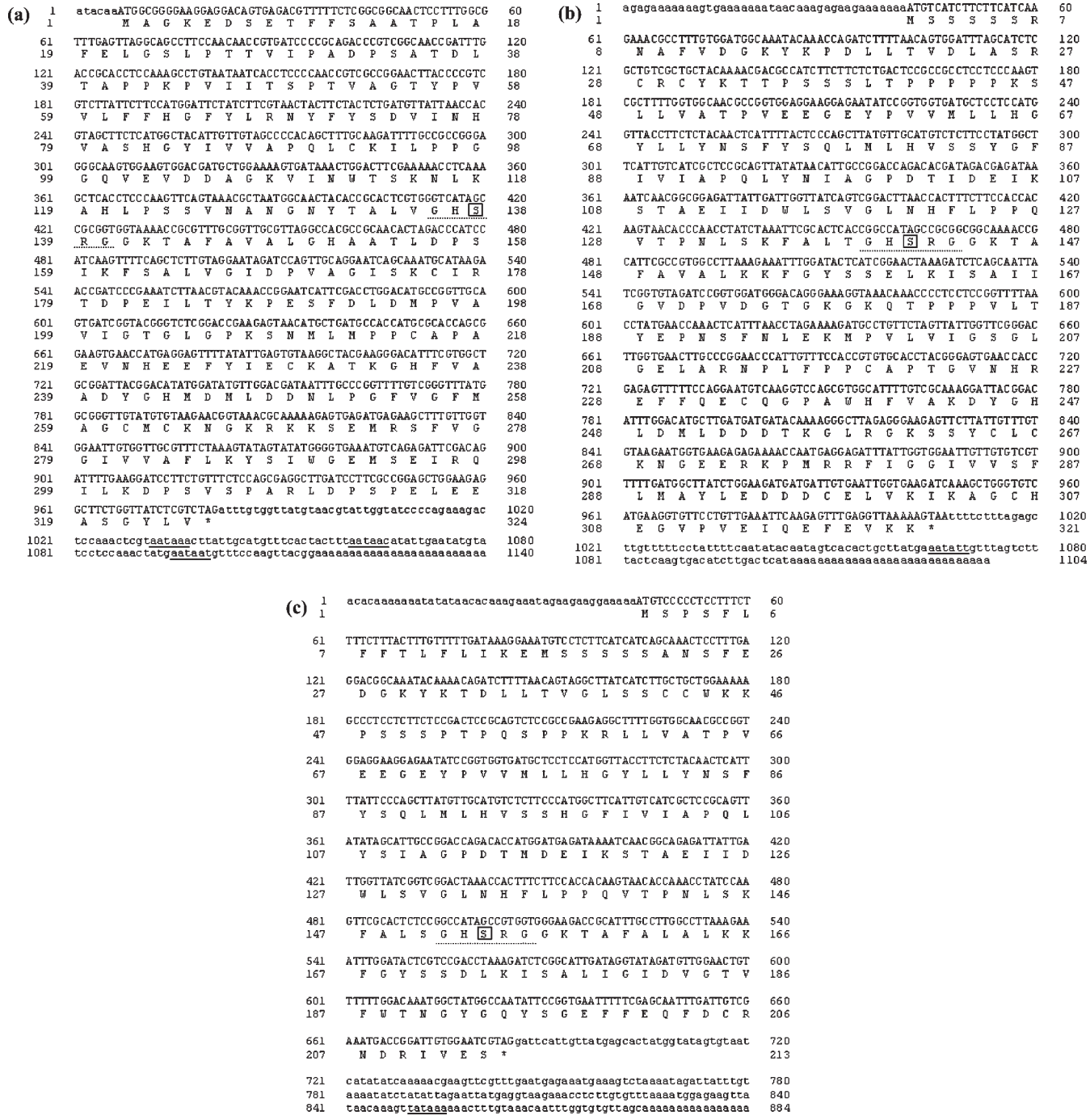

Figure 1. Nucleotide and deduced amino acid sequences of (**a**) *BoCLH1*, (**b**) *BoCLH2*, and (**c**) *BoCLH3*. The sequences of the 5′ and 3′ untranslated regions are shown in lower case. The predicted polyadenylation signal sequences are underlined. The conserved lipase motif is shown in dashed underlining, and the putative active site of the Ser residue is boxed.

Substrate specificity of the purified recombinant Chlase was determined with different substrates such as Chl *a*, Chl *b* (Sigma, St. Louis, MO, USA), and pheophorbide (Pheide) *a* methyl ester (an Mg-free chlorophyll analogue) (Wako Pure Chemical Industries, Japan). The assay reaction contained 10 μL of enzyme, 65 μL phosphate buffer, and 7.5 μL acetone dissolved substrate with concentrations ranging from 50 to 500 μM. All reactions were carried out at 40 °C for 5 min in triplicate and average values reported. The absorbance of the aqueous acetone phase was measured at 651 nm for Chlide *b* and 405 nm for Pheide *a* with a spectrophotometer. The amount of each product in the acetone layer was estimated from the millimolar extinction coefficient of 47.6 mM^{-1} cm^{-1} for Chlide *b* and 77.3 mM^{-1} cm^{-1} for Pheide *a* (*14*).

Enzyme kinetic parameters (V_{max}, K_m, and k_{cat}) were determined for three specific substrates. To determine the kinetic parameters, initial velocities were measured for at least five concentrations of each specific substrate. Product formation had a linear correlation with time under the assay conditions. The data were fitted to the Michaelis−Menten equation, and kinetic parameters were calculated through nonlinear regression using Origin software (version 6.1, UK).

Site-Directed Mutagenesis. The putative catalytic Ser141, Asp170, and four conserved His residues (His66, His226, His239, and His247) of BoCLH2 were altered to alanine by the PCR-based megaprimer method (*15*). Megaprimer was synthesized by PCR using a forward BoCLH2-NdeI primer (5′-GGAATTCCATATGTCATCTTCTTCATC-3′) and a reversed mutagenic primer (**Table 1**) or using a forward mutagenic primer (**Table 1**) and a reversed BoCLH2-Xho-HT primer (5′-CCGCTCGAGCT-TTTTAACCTCAAACTC-3′). These mutagenic primers were synthesized by Mission Biotech (Taipei, Taiwan). The resulting PCR products contained

BoCLH2 ORF with single amino acid mutation and were purified with an agarose gel-extraction kit (Quiagen II, Madison, WI). The purified PCR products were digested with *Nde*I and *Xho*I and ligated to an *Nde*I/*Xho*I-predigested pET-20b vector. The sequences of resulting DNA were confirmed by full-length DNA sequencing. A hexapeptide His_6-Tag was located in frame behind the BoCLH2 and facilitated the purification of recombinant wild-type and mutant BoCLH2s by single-step affinity chromatography.

Table 2. Pairwise Identity of the Amino Acid Sequence between BoCLHs and Other Plant Chlases

	BoCLH1	BoCLH2	BoCLH3	CaCLH	CsCLH	AtCLH1	AtCLH2	TaCLH	GbCLH
BoCLH1	100[a]	38	22	37	42	83	39	43	38
BoCLH2		100	47	31	33	38	85	41	48
BoCLH3			100	19	22	22	47	23	25
CaCLH				100	35	38	30	36	29
CsCLH					100	41	34	39	33
AtCLH1						100	39	44	38
AtCLH2							100	40	49
TaCLH								100	38
GbCLH									100

[a] Percentage of identity was calculated using a ClustalW multiple alignment program with a BLOSUM62 score matrix.

RESULTS

cDNA Cloning of the Broccoli Chlases. Degenerate primers derived from the consensus regions of reported Chlase proteins were used to amplify a partial fragment of *BoCLH* ORF from the broccoli cDNA pools. Three full-length cDNAs (named *BoCLH1*, *BoCLH2*, and *BoCLH3*) were obtained after 5′/3′ RACE. As shown in **Figure 1a**, the BoCLH1 is an 1140 bp cDNA which contains a 6-bp 5′-untranslated region, followed by a 972 nucleotide open reading frame and a 162-bp 3′-untranslated sequence. Three putative polyadenylation site sequences were observed in the 3′-untranslated region. The open reading frame encodes a protein of 324 amino acids with a predicted molecular mass of 34.7 kDa. The *BoCLH2* cDNA is an 1104 bp cDNA which contains a 41-bp 5′-untranslated region, followed by a 963 nucleotide open reading frame and a 100-bp 3′-untranslated sequence (**Figure 1b**). One putative polyadenylation signal sequence was observed in the 3′-untranslated region. The open reading frame encodes a protein of 321 amino acids with a predicted molecular mass of 35.3 kDa. The *BoCLH3* is an 884 bp cDNA which contains a 43-bp 5′-untranslated region, followed by a 639 nucleotide open reading frame and a 202-bp 3′-untranslated sequence (**Figure 1c**).

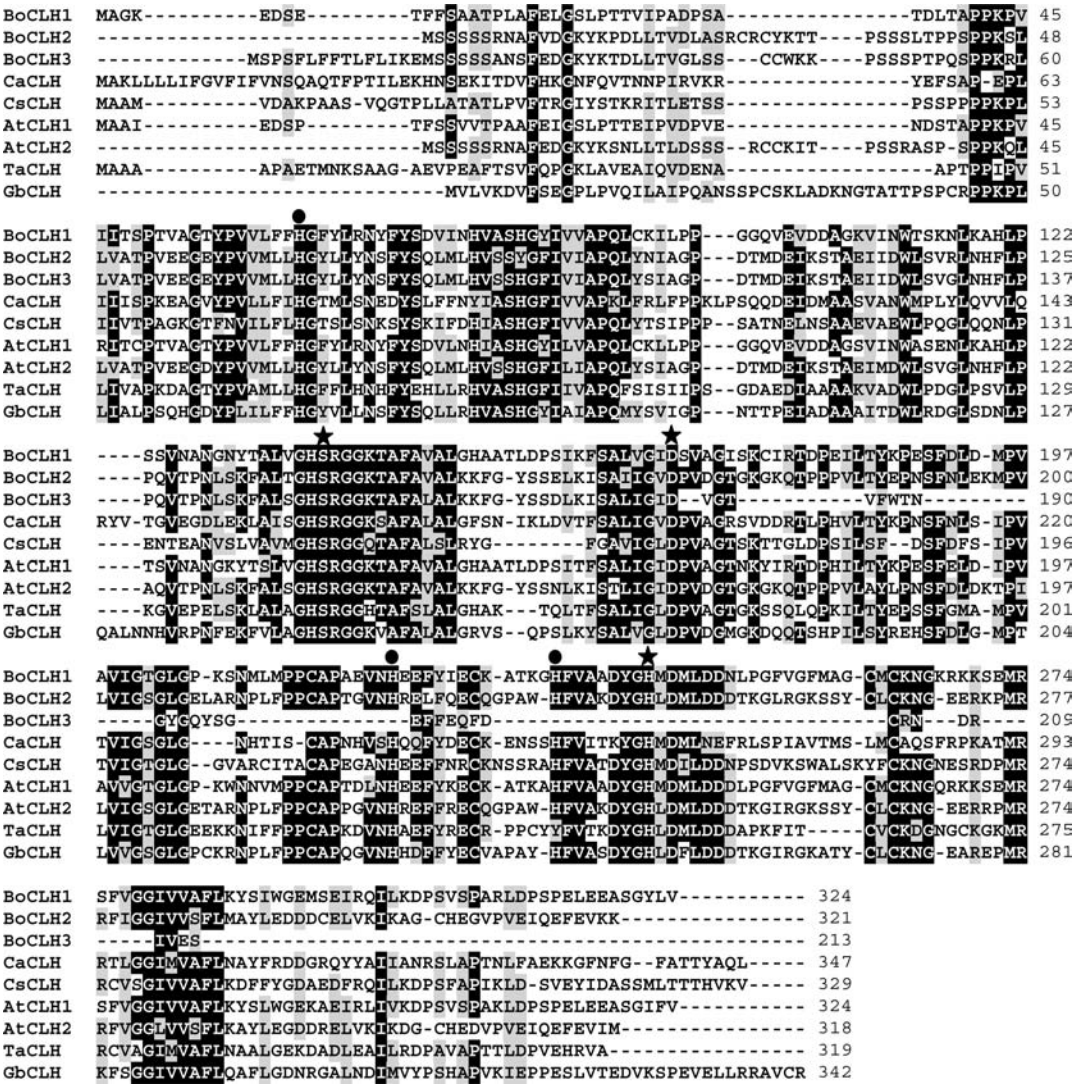

Figure 2. Alignment of amino acid sequences of various Chlases from *B. oleracea* (BoCLH), *C. album* (CaCLH), *C. sinensis* (CsCLH), *A. thaliana* (AtCLH), *T. aestivum* (TaCLH), and *G. biloba* (GbCLH). Protein sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The identical amino acids among at least six out of nine sequences are shaded in black and similar amino acids in gray. The conserved catalytic residues (Ser-His-Asp) are indicated by an asterisk, and the other three conserved His residues are indicated by circle.

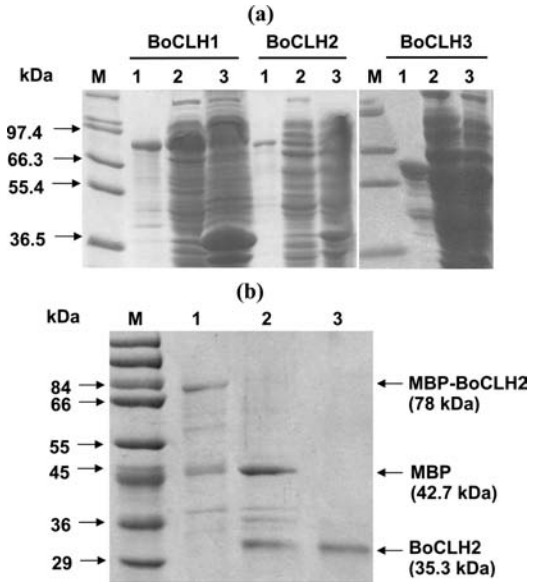

Figure 3. SDS−PAGE of the recombinant BoCLH1, BoCLH2, and BoCLH3. (**a**) Expression and purification of BoCLH-MBPs. Lane 1, purified MBP-BoCLHs with amylose resin; lane 2, soluble fraction of total cell protein; and lane 3, total cell protein. (**b**) Purification of the recombinant BoCLH2. Lane 1, recombinant BoCLH2-MBP fusion protein purified with amylose resin; lane 2, proteolytic cleavage of purified BoCLH2-MBP with Factor Xa; and lane 3, purified native BoCLH2 enzyme after proteolytic cleavage; lane M, molecular weight standards. SDS−PAGE was performed in a 12% polyacrylamide slab gel. Protein bands were detected by staining with Coomassie Brilliant Blue.

One predicted polyadenylation signal sequence was observed in the 3′-untranslated region. The open reading frame encodes a protein of 213 amino acids with a predicted molecular mass of 23.5 kDa.

Analysis of the Deduced Amino Acid Sequence. As shown in **Table 2**, the deduced amino acid sequences of three BoCLHs show 22% to 47% of pairwise identity. The broccoli BoCLH1 and BoCLH2 have high homology with *Arabidopsis* AtCLH1 and AtCLH2, respectively. BoCLH1 shows the highest identity (83%) to the *Arabidopsis* Chlase AtCLH1, and BoCLH2 shows the highest identity (85%) to AtCHL2. This means that BoCLH1 and AtCHL1 may be orthologues with similar function and that the same relationship might occur between BoCHL2 and AtCLH2. However, BoCLH3 appears to be a unique novel type of Chlase since it is much smaller. In the N-terminal regions, BoCLH3 (residues 1−185) has a higher identity percentage (79%) with BoCLH2 (residues 1−175) than that (35%) with BoCLH1 (residues 1−173); however, it has a shorter C-terminal sequence compared with that of BoCLH2. It is possible that BoCLH2 and BoCLH3 evolved from the same ancestor gene. The N-terminal sequences of BoCLH1 and BoCLH2 were predicted to have typical signal sequences for the chloroplast by CELLO v.2.5, a subcellular localization predictive system (*16*), whereas BoCLH3 was predicted to be a plasma membrane protein.

The three deduced protein sequence of broccoli Chlases were aligned with those of other reported Chlases using the ClustalW program (*17*) (**Figure 2**). The core domain, containing the lipase Ser active site motif GHSRG, is highly conserved among the members of Chlase family. However, the N- and C-terminal sequences are quite variable in length and in composition. Several crystal structures of lipases show conserved spatial arrangement of the catalytic serine in a sharp turn between a β-strand and α-helix, which is called the β-Ser-α motif (*18*). From the secondary structure prediction using the PREDICTPROTEIN server

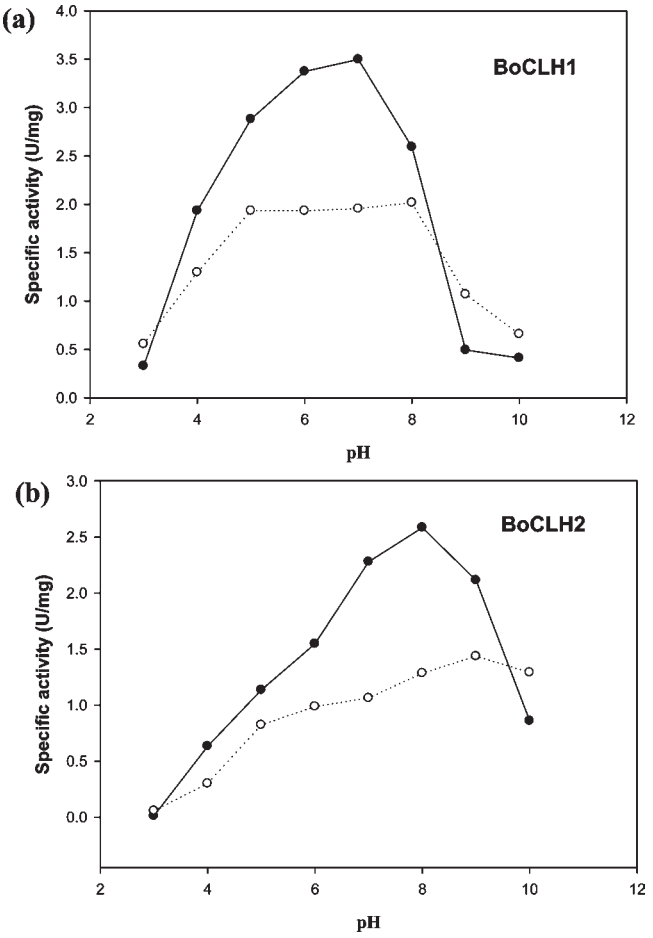

Figure 4. Effect of pH on the activity and stability of (**a**) BoCLH1 and (**b**) BoCLH2. The activity and the stability are indicated by solid circles and open circles, respectively. Specific activity was measured according to the standard Chlase assay.

(http://www.predictprotein.org/) (*19*), the three broccoli Chlases showed the conserved β-Ser-α folding pattern. The phylogenetic analysis of esterases and lipases using the peptide fragment sequence revealed that the Chlases are a distinct group of α/β hydrolases with active site serine.

Chlase Protein Expression. Three recombinant broccoli Chlases were expressed in *E. coli* for enzyme activity study. We subcloned the full-length ORFs of Chlase genes under the control of the *tac* promoter in the pMAL-c2x expression vector. The transformants of *E. coli* Rosseta-Gami B (DE3) harboring a recombinant vector expressed recombinant Chlases in a high yield after IPTG induction. We obtained soluble and functional BoCLH1, 2, and 3 as MBP-fusion proteins of molecular sizes 77.4, 78.0, and 66.2 kDa, respectively. After protease cleavage to remove the MBP moiety, we obtained the native BoCLHs (**Figure 3**). We noticed that the BoCLH3 enzyme showed very low Chlase activity; therefore, all further experiments were performed with BoCLH1 and 2.

Biochemical Characterization. Experiments were performed to determine the biochemical properties of the purified recombinant Chlases. The optimal pH was investigated at a pH range between pH 3.0 and 10.0. **Figure 4** shows that the pH optima for BoCLH1 and 2 were pH 7.0 and pH 8.0, respectively. BoCLH1 was more acid-active because about 50% of activity was found at pH 4.0, and broad pH stability was found between pH 5.0 and 8.0. On the contrary, BoCLH2 was more basic-active because about 80% of activity was found at pH 9.0, while BoCLH1 showed only 12% of

activity at the same pH value. The pH stability also showed that BoCLH2 was stable between pH 8.0 and 10.0.

The temperature optimum was measured between 20 and 80 °C (**Figure 5**). BoCLH1 and BoCLH2 showed optimal enzyme activities at 30 and 60 °C, respectively. BoCLH1 showed a 50% decrease in activity at 45 °C, while BoCLH2 showed it at 65 °C. However, these two enzymes showed similar thermostability because after incubation at 60 °C for 10 min, they all lost about 50% of activity.

Kinetic Studies. The kinetic parameters were investigated with various substrates including Chl *a*, Chl *b*, and Pheide *a* methyl ester. The steady-state kinetic parameters such as V_{max}, K_m, and k_{cat} for various substrates were calculated by fitting the data to the Michaelis−Menten equation and summarized in **Table 3**.

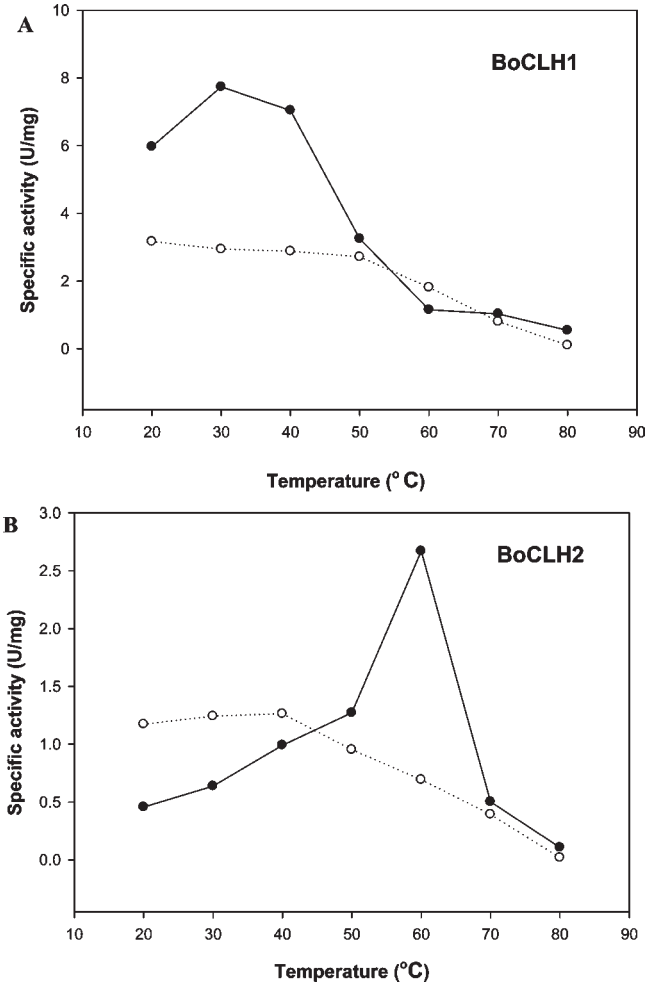

Figure 5. Effect of temperature on the activity and stability of (**a**) BoCLH1 and (**b**) BoCLH2. The activity and the stability are indicated by solid circles and open circles, respectively. Specific activity was measured according to the standard Chlase assay.

Kinetic analysis revealed that BoCLH1 hydrolyzes three substrates more rapidly than BoCLH2. Furthermore, BoCLH1 showed distinct substrate specificity for the Pheide *a* methyl ester. The catalytic efficiency (k_{cat}/K_m) for the Pheide *a* methyl ester is about 20 to 30 times higher than that for the other two substrates due to BoCLH1's high binding affinity for the Pheide *a* methyl ester (i.e., the lowest K_m).

Site-Directed Mutagenesis. Because BoCLHs have a lipase motif (G-X-S-X-G), which is conserved in the alignment of various Chlases (**Figure 4**), we believed that BoCLHs might have a catalytic triad (Ser-His-Asp/Glu) similar to lipases, and therefore we introduced mutations into the putative catalytic residues of BoCLH2. The recombinant proteins of mutant and wild-type BoCLH2s were purified for the standard Chlase assay. **Table 4** shows the specific activities of various recombinant BoCLH2s using Chl *a* as a substrate. The mutations at the putative catalytic residues (Ser141 and Asp170) caused the loss of Chlase activity as expected. Four mutant BoCLH2s with a single mutation at different conserved His also showed significantly decreased activities. In particular, H247A almost lost activity just as S141A and D170A did. These results showed that three residues (S141, D170, and H247) are important for the Chlase activity in BoCLH2 and that they might be the members of catalytic triad.

DISCUSSION

Chlase plays an essential role in Chl degradation. The isolation and characterization of three Chlase genes from broccoli florets represent an important step toward understanding the molecular mechanism of the Chl breakdown process and the biological function of Chlase isozymes in floret development.

The subcellular localization prediction revealed that BoCLH1 and BoCLH2 might be chloroplast enzymes. However, BoCLH3 did not contain any typical chloroplast signal sequence. In vitro, BoCLH1 and BoCLH2 had significantly higher Chlase activity than BoCLH3. Our present study suggested that in broccoli chlorophyll may be degraded in the chloroplast as previously proposed (*20*). Chl was found to secrete from senescing chloroplast to cytoplasm (*21*), which implies that Chl could be also degraded in the cytosol. Tsuchiya et al. speculated that Chl degradation in *C. album* might exist in the vacuole since CaCLH

Table 4. Specific Activity of Wild-Type and Mutant BoCLH2s

mutation	specific activity[a] (U × mg^{-1})
H66A	0.372
S141A	ND
D170A	ND
H226A	0.52
H239A	0.149
H247A	0.074
Wild type	7.43

[a] The enzyme activity was determined by measurement of the absorbance of the aqueous acetone phase with Chlide *a* at 667 nm. One unit of Chlase activity was defined as described in Materials and Methods.

Table 3. Steady-State Kinetic Parameters of BoCLH1 and BoCLH2

	substrate	V_{max} (mM/min × mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^{-5} × s^{-1} μM^{-1})
BoCLH1	Chl *a*	12.40 ± 0.75[a]	465.92 ± 64.46	0.072 ± 0.005	15.51
	Chl *b*	11.23 ± 0.51	651.63 ± 113.25	0.072 ± 0.003	11.05
	Pheide *a* methyl ester	44.80 ± 0.52	76.37 ± 2.72	0.259 ± 0.003	339.67
BoCLH2	Chl *a*	4.33 ± 0.06	307.93 ± 6.88	0.0024 ± 0.00	0.78
	Chl *b*	6.57 ± 0.11	310.73 ± 8.75	0.0037 ± 0.00	1.19
	Pheide *a* methyl ester	6.77 ± 0.21	143.14 ± 8.42	0.0039 ± 0.00	2.72

[a] Values are the means ± SD from three independent experiments.

contains a typical signal peptide for ER in the N-terminal region (*8*). Therefore, various plants might evolve different Chl degradation mechanisms and different Chlase regulations. Immunolocalization and Chlase transport experiments are currently underway to confirm our hypothesis.

Previously, Tsuchiya et al. have identified the catalytic triad of CaCLH as Ser162, Asp191, and His262, which is essential for Chlase activity (*22*). In our present study, introduction of mutations into the putative catalytic triad of BoCLH2 (Ser141, Asp170, and H247) also showed the loss of Chlase activity (**Table 4**). Sequence alignment reveals that these amino acid residues are conserved with the catalytic triad of CaCLH and also conserved in all other Chlases except BoCLH3, which does not have the catalytic His (**Figure 2**). This strongly suggested that BoCLHs may be similar to those of serine hydrolases. The absence of the catalytic His residue in BoCLH3 may explain why it had very low Chlase activity. BoCLH3 may have some other substrate specificity or enzyme function other than Chlase, and it might play a different role in Chl degradation from the other two BoCLH isozymes. Further molecular and biochemical investigations are needed to elucidate the actual function of BoCLH3.

Kinetic analysis revealed that BoCLH1 preferably hydrolyzed the Pheide *a* methyl ester which resembles the Mg-free chlorophyll pigment, pheophytin (Phein), and that BoCLH2 hydrolyzed both the Pheide *a* methyl ester and Chl at a similar level. Recently, Schelbert et al. found that pheophytinase (PPH), a chloroplast-located and senescence-induced hydrolase which specifically dephytylates Phein yielding Pheide, is an important component of the chlorophyll breakdown pathway in *Arabidopsis thaliana* (*23*). We propose that as a whole, BoCLH1 resembling PPH degrades Chl into Pheide via Phein, not Chlide, in broccoli, even though the amino acid sequence similarity is very low between these two enzymes. However, BoCLH2 may degrade Chl into Pheide via both Phein and Chlide. It suggests that different Chlase isozymes may be involved in different Chl breakdown pathways.

Isoforms of enzymes presumably have different biochemical properties and/or regulations. Previously, we have shown that isoforms of *Candida rugosa* lipase have different catalytic efficiencies, substrate specificities, and thermostabilities and show differential expression in the presence of different culture conditions (*24*, *25*). C-terminal deletion of rubisco activase has been shown to have a regulatory effect on both ATP hydrolysis and rubisco activation (*26*). Therefore, it is logical to assume that the three broccoli Chlase isoforms should have different kinetic properties and regulatory mechanisms. Experiments are currently underway to confirm the actual subcellular localization of BoCLHs and the roles played during the postharvest senescence of broccoli.

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