Involvement of chloroplast peroxidase on chlorophyll degradation in postharvest broccoli florets and its control by UV-B treatment

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ABSTRACT

Chlorophyll (Chl)-degrading peroxidase (POX) was purified from green and green-yellow broccoli florets to elucidate the physiological role and cell localization of Chl-degrading POX. Effect of UV-B treatment on Chl-degrading POX activity was also determined. The Chl-degrading POX activity was separated by an ion exchange chromatography into 3 fractions, Chl-POX1, Chl-POX2 and Chl-POX3. The activities of Chl-POX2 and Chl-POX3, but especially that of Chl-POX3, were found in green-yellow florets and were clearly suppressed by UV-B irradiation. The protein molecular sizes of cPOX1 and cPOX3 purified from Chl-POX1 and POX3 were 43 and 34 kDa, respectively. The cPOX1 was not located within chloroplast fraction, whereas the cPOX3 was found in intact chloroplasts extracted from senescing broccoli florets. We propose that cPOX3 is a chloroplast POX and the activity was suppressed by UV-B treatment, suggesting that cPOX3 might be involved in Chl degradation in stored broccoli florets.

1. Introduction

Postharvest senescence in broccoli florets is one of main quality deteriorations during transportation and storage. The symptom of senescing broccoli is floret yellowing, resulting from chlorophyll (Chl) loss in sepals. The degradation of Chl occurs markedly with the progress of senescence. Recently, Chl *a* degradation has been shown to follow different putative pathways depending on the commodity. First, the removal of phytol from Chl *a* and the formation of chlorophyllide (Chlide) *a* by chlorophyllase (Chlase) (Harpaz-Saad, Azoulay, Arazi, Ben-Yaakov, Mett, Shiboleth, Hörtensteiner, Gidoni, Gal-On, Goldschmidt, & Eyal, 2007) are followed by the removal of the Mg atom by the Mg-dechelating substance (MDS) (Shioi, Tomita, Tsuchiya, & Takamiya, 1996) . Subsequently, pheophorbide (Pheide) *a* is degraded to fluorescent Chl catabolites, which are primarily colorless catabolites, including a red Chl catabolite generated by Pheide *a* oxygenase (PAO) and a red Chl catabolite reductase (RCCR) (Matie, Hörtensteiner , & Thomas, 1999). An alternative process that differs in the first step of the above pathway proceeds by Chl *a* removing the Mg atom before the phytol group, with pheophytin (Phy) *a* as an intermediate (Tang, Okazawa, Fukusaki, & Kobayashi, 2000). Afterward, the dephytilation of Phy *a* is catalyzed by Chlase (Heaton & Marangoni, 1996) or pheophytinase (PPH) (Schelbert, Aubry, Burla, Agne, Kessler, Krupinska, & Hörtensteiner, 2009). Chl degradation seems to occur mainly within the chloroplast (Hörtensteiner , 2013), but it may also occur in a vacuole since plastoglubuli, which are lipoprotein particles contain Chl and carotenoids, were formed from the thylakoid membrane and present in the chloroplast (Steinmüller & Tevini, 1985). Recently, PPH and PAO were reported to be a co-expression with *At5g17450*, which was identified as a gene involved in the removal of the Mg atom from Chl *a*, with a protein located in the plastoglobuli (Lundquist, Poliakov, Bhuiyan, Zybailov, Sun, & van Wijk, 2012). Guiamét, Pichersky, and Noodén

(1999) found that plastoglubuli moved through a chloroplast envelope and emerged into the cytoplasm during senescence. Furthermore, Martínez, Costa, Gomez, Otegui, and Guiamet (2008) reported that the senescence-associated vacuoles (SAVs) of tobacco leaves contained Chl *a*.

Plant peroxidases (POXs) are a high number of isozymes, with remarkable catalytic versatility that allows them to be involved in a broad range of physiological and developmental processes all along the plant life cycle (Parrardi, Cosio, Penel, & Dunand, 2005), including lignin polymerization, the catabolism of auxin, and the formation of reactive oxygen species. Class III POX of cationic type was also found to degrade Chl *a* in the presence of hydrogen peroxide and phenolic compounds such as *p*-coumaric acid and flavonoids, which have a hydroxyl group at *p*-position, *in vitro* (Yamauchi, Funamoto, & Shigyo, 2004). The intermediate product of this degrading process is 13^2-hydroxychlorohyll (OHChl) *a*, which is the primary Chl derivative in plants (Funamoto, Yamauchi, & Shigyo, 2003; Hynninen, Kaartnien & Kolehminen, 2010; Johnson-Flanagan & McLachlan, 1990; Martínez, Civello, Chaves, & Añón, 2001; Yamauchi et al., 2004). An accumulation of OHChl *a* were reported to be present in different plant species such as barley leave, parsley, spinach and broccoli (Maunders, Brown, & Woolhouse, 1983; Yamauchi et al., 2004; Yamauchi & Watada, 1991). In plants, hydrogen peroxide is continually produced from various sources such as chloroplast, mitochondria and peroxisomes, and diffuses freely into the vacuoles (Neill, Desikan, & Hancock, 2002). Flavonoids are also known to be present in chloroplast and vacuole (Hernández, Alegre, Breusegem, & Munné-Bosch, 2009; Saunders & McClure, 1976), inferring that POX-mediated Chl degradation could occur in the chloroplast and vacuole.

Martinoia, Dalling, and Matile (1982) demonstrated that Chl-degrading POX was identified in the thylakoid membrane of barley seedlings. Abeles, Dunn, Morgens, Callahan, Dinterman,

and Schmidt (1988) reported that, in cucumber cotyledons, ethylene treatment resulted in an increase of cationic peroxidase, which might be involved in Chl degradation. Thereafter, Abeles and Dunn (1989) found that the cationic peroxidase is hardly related to Chl degradation because the inhibitor of heme synthesis and protein glycosylation had no effect on Chl degradation and reduced the synthesis of cationic peroxidase. In contrast, Kuroda, Ozawa, and Imagawa (1990) proved that the cationic peroxidase involved in Chl degradation was located in the chloroplasts of barley leaves. Further analysis showed that POX was present in isolated thylakoid from green canola (*Brassica napus* L.) seeds and degraded the thylakoid-bound pigments in the presence of H_2O_2 and 2, 4-dichlorophenol. A high activity of POX was found with rapid degreening of seeds (Johnson-Flanagan & McLachlan, 1990). Gandul-Rojas, Roca, and Mínguez-Mosquera (2004) showed that POX activity associated with the thylakoid membranes was involved in the transformation of Chl *a* to OHChl *a*. On the other hand, a small activity of Chl-degrading POX was found in intact chloroplasts of radish cotyledons, which could indicate that the degradation of Chl by Chl-degrading POX is mainly present outside the chloroplast (Akiyama & Yamauchi, 2001). Previously, a high activity of Chl-degrading POX was found in cytosolic and microsomal fractions in fresh broccoli florets, whereas a low activity of the enzyme was seen in the chloroplast fraction (Funamoto et al., 2003). These findings suggest that Chl-degrading POX may occur in the chloroplast, the vacuole, or both. Currently, not much data on Chl-degrading POX has been published, and the issue remains unclear.

In the previous papers, Chl-degrading POX increased with floret yellowing in stored broccoli and the increase of the activity was clearly suppressed by UV-B treatments in accordance with the inhibition of Chl degradation by UV-B treatment, indicating that Chl-degrading POX might be involved in Chl degradation (Aiamla-or, Kaewsuksaeng, Shigyo, & Yamauchi, 2010). Therefore, here, we discuss the purification, characterization, and

126 localization of Chl-degrading POX in broccoli florets to clarify the physiological role of the
127 enzyme in Chl degradation. Moreover, the effect of UV-B irradiation on Chl-degrading POX
128 activity was determined, since UV-B treatment delayed Chl degradation in broccoli florets.
129
130 **2. Materials and Methods**
131
132 *2.1. Plant material*
133 Broccoli (*Brassica oleracea* L. Italica Group, cv. endeavor) heads were harvested in Fukuoka
134 Prefecture and transported to the Horticultural Science Laboratory at Yamaguchi University.
135 Broccoli florets were kept in polyethylene film bags (0.03 mm in thickness) with the top
136 folded over. The bags were then placed on a plastic tray and stored at 15 °C in the dark.
137 Yellow florets were collected on day 3 for the determination of localization of Chl-degrading
138 POX in chloroplast or day 4 for the purification of Chl-degrading POX in Section 2.5.
139
140 *2.2 Chemical reagents*
141 Specific polyclonal antibodies of cPOX1 and cPOX3 were generated in rabbit, using
142 purified protein from broccoli florets as described in Section 2.5. *N*,*N*-dimethylformamide,
143 sodium chloride (NaCl), calcium chloride ($CaCl_2$), dithiothreitol (DTT), ammonium
144 sulfate($(NH_4)_2SO_4$), manganese chloride ($MnCl_2$), D-glucose, hydrogen peroxide (H_2O_2), *p*-
145 coumaric acid, copper chloride ($CuCl_2$), trichloroacetic acid (TCA), dioxane, petroleum ether,
146 coomassie brilliant blue (CBB) R-250, bovine serum albumin (BSA), tris(hydroxymethyl)
147 aminomethane hydrochloride (Tris-HCl), glycine, ethylenediaminetetraacetic acid (EDTA), 2-
148 mercaptoethanol, sodium dodecyl sulfate (SDS), bromophenol blue, 4-(2-
149 polyvinylpolypyrrolidone (PVPP), sodium acetate and acetic acid, which were of analytical

reagent grade, were obtained from WAKO Pure Chemical Ind., Ltd (Japan). Methanol and ethyl acetate of high performance liquid chromatography (HPLC) grade were purchased from KANTO Chemical Co., INC (Japan). Ponceau S was purchased from Sigma Aldrich Co. (USA).The molecular weight markers were obtained from Thermo Fisher Scientific Inc. (USA). All of the other chemical reagents were of analytical reagent grade.

2.3. UV-B treatment

After broccoli florets were transported to the laboratory, the heads were immediately irradiated with UV-B (spectral peak value: 312 nm, T-15M, VL). Each broccoli head was placed vertically under UV-B lamps at a distance of 15 cm, resulting in UV-B energy doses of 19 kJ m^{-2}. After UV-B treatment, broccoli florets were stored at 15 °C in the dark, as previously described in Section 2.1. UV-B-treated broccoli florets were kept separately from untreated broccoli florets.

2.4. Preparation of acetone powder

Ten grams of florets were homogenized twice in 200 mL of cold acetone (–20 °C). The homogenate was filtered through filter paper No.2 (ADVANTEC, Japan). The residue was washed with cold acetone. Subsequently, 50 mL of diethyl ether was added to remove the acetone. The residues were completely dried at room temperature using a desiccator jar and air suction and stored at –20 °C.

2.5. Purification of chlorophyll-degrading peroxidase

All purification procedures were performed at 0–4 °C. Chl-degrading POX protein was extracted from three grams of acetone powder of green-yellow broccoli florets in 100 mL of a 20 mM phosphate buffer (pH 7.0) containing 2% (w/v) PVPP and 2 mM DTT. The protein

extraction solution was partially purified using a saturated $(NH_4)_2SO_4$ (60–90%) precipitation. The solution was then centrifuged at 15,000 g for 15 min at 4 °C, and the precipitate was dissolved in a 10 mM acetated buffer (pH 5.5). Afterward, the partially purified protein was desalted using an ultrafiltration system (ADVANTEC, Japan) with a polysulfone membrane (MW, 10,000 cutoff; diameter, 43 mm, ADVANTEC, Japan). The desalted protein was subsequently fractionated by ion exchange chromatography (CM-Sepharose Fast Flow, GE Healthcare, UK). The column size was approximately 7.0 cm^2×15 cm (BIO-RAD, USA). CM-Sepharose resins were equilibrated using a 10 mM acetate buffer (pH 5.5). The Chl-degrading POX protein combined with CM-Sepharose resin was eluted by a linear gradient of 0–1 M NaCl in a 10 mM acetate buffer (pH 5.5). The active fractions from CM-Sepharose were collected and continually fractionated using hydrophobic chromatography (HIC) (butyl-650, Toyopearl, Japan). The resins of HIC were equilibrated with 2 M $(NH_4)_2SO_4$ in a 10 mM acetate buffer (pH 5.5). The proteins combined with resins were eluted by a linear gradient of 2–0 M $(NH_4)_2SO_4$ in a 10 mM acetate buffer (pH 5.5). Afterward, the active fractions from HIC were applied into an affinity chromatography column (Concanavalin A (ConA)-Sepharose 4B, GE Healthcare, UK). The resins were equilibrated with a 10 mM acetate buffer (pH 5.5) containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. A linear gradient of 0–0.8 M glucose in the same buffer was applied. The protein from active fractions was collected and continually applied to molecular exclusion chromatography (Sephacryl S-200, GE Healthcare, UK). The active fractions obtained from Sephacryl S-200 column were concentrated using ultrafiltration with the membrane cutoff at 10 kDa (ADVANTEC, Japan) and finally purified by ion exchange chromatography (SP-650, Toyopearl, Japan). SP-650M resins were equilibrated with a 10 mM acetate buffer (pH 5.5). A linear gradient of 0–0.3 M NaCl in the same buffer was used as a buffer to elute binding proteins from a column. The purity of Chl-

degrading POX isozyme proteins was assessed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with highly sensitive silver staining.

2.6. Enzyme assay

Chl-degrading POX was determined as previously described (Aiamla-or et al., 2010). The reaction mixture contained 0.2 mL of an enzyme solution, 0.08% Triton X-100, 2 mM *p*-coumaric acid, 40 μg mL^{-1} Chl *a* acetone solution, 64 mM phosphate buffer (pH 6.0), and 0.012% H_2O_2 in total volume 1.25 mL. Activity was determined spectrophotometrically by measuring the decrease of Chl *a* at 668 nm per unit per mg protein at 25 °C. One unit of Chl-degrading POX was defined as a change of 1.0 μg Chl *a* degradation per min.

The cytokinin oxidase/dehydrogenase (CKK) activity was determined using the Libreros-Minotta and Tipton's method (Libreros-Minotta & Tipton, 1995). Assays of CKK activity were carried out in 0.5 mL total volume solutions containing 100 mM imidazole (pH 6.5), 1 mM $CuCl_2$, and 40 μM isopentenyladenine or zeatin. The reaction mixtures were incubated at 37 °C for 2 h. Subsequently, the reactions were stopped by addition of 0.3 mL of 40% (w/v) TCA and 0.2 mL of a 3% (w/v) *p*-aminophenol reagent and then measured an absorbance at 352 nm.

2.7. Preparation of chlorophyll a

Spinach leaves were homogenized for 3 min in cold acetone (–20 °C). The homogenate was filtrated through two layers of Miracloth (Calbiochem, USA). The filtrates were treated with dioxane and distilled water and then kept for 1 h on ice. The filtrates were centrifuged at 10,000 × g for 15 min at 4 °C. After centrifugation, the pellets were treated again with acetone, dioxane, and distilled water and then kept for 1 h on ice. Afterwards, the soluble pellets were centrifuged at 10,000 × g for 15 min at 4 °C and then subsequently dissolved in petroleum

ether. Soluble chlorophyll in petroleum ether was stored at –20 °C until the individual pigments were separated using sugar powder column chromatography (Perkins & Roberts, 1962). Finally, 500 μg mL^{-1} of Chl *a* was prepared in acetone.

2.8. Protein determination

The concentration of protein was determined with a bicinchoninic acid (BCA) assay kit (Sigma-Aldrich, USA) using BSA as the protein standard. CBB R-250 or silver staining was used for visualizing proteins on acrylamide gel. Protein molecular weight markers were obtained from Thermo Fisher Scientific Inc. (USA) (beta-galactosidase of 116 kDa, bovine serum albumin of 66.2 kDa, ovalbumin of 45 kDa, lactate dehydrogenase of 35 kDa, REase Bsp98l of 25 kDa, beta-lactiglobulin of 18.4 kDa, and lysozyme of 14.4 kDa).

2.9. SDS-PAGE preparation

SDS-PAGE was carried out according to Laemmli's procedure (Laemmli, 1970). The gel consisted of a stacking gel of 3% acrylamide in a Tris-HCl buffer (pH 6.8) and a separating gel of 10% acrylamide in Tris-HCl (pH 8.8). The running buffer (pH 8.3) contained 3 g Tris base, 14.4 g glycine, and 1 g SDS, which were dissolved in 1,000 mL distilled water. Protein samples were mixed with a stacking buffer, 10% SDS, 2-mercaptoethanol, 1% bromophenol blue, and about five grains of sucrose. Subsequently, the mixture was heated at 95 °C for 4 min and then loaded into a PAGE well. The gel PAGE loaded with samples was performed at 20 mA per gel. Proteins were visualized by CBB R-250 or silver staining.

2.10. Determination of glycoprotein

After acrylamide gel was electrophoresed, each protein on the acrylamide gel was visualized using a periodic acid-Schiff (PAS) staining kit (Sigma-Aldrich, USA) with a

249 slightly modified version of the method of Segrest and Jackson (Segrest & Jackson, 1972).
250 The gel was soaked in PAS fixative solution overnight at 4 °C. Afterward, the gel was rinsed
251 with distilled water twice and immediately soaked in a periodic solution for 3 h at room
252 temperature. The gel was rinsed with distilled water twice more and subsequently immersed
253 in Schiff`s reagent. The purplish-pink color of the glycoprotein in gel developed in 12–18 h.
254 After Schiff`s reagent was removed, distilled water was replaced. Finally, the gel was
255 counterstained in hematoxylin solution for 90 sec and rinsed with distilled water.
256
257 *2.11. N-terminal amino acid sequence analysis*
258 The purified cPOX3 protein was subjected to SDS-PAGE and electrophoretically
259 transferred to a polyvinylidene difluoride (PVDF) membrane. After the membrane was briefly
260 stained by 0.1% Ponceau S, the individual band of cPOX3 on the PVDF membrane was
261 excised, and the protein N-terminal amino acid sequence was determined by Edman
262 degradation on an automated sequencing system PPSQ-21 (SHIMADZU, Japan). The
263 sequence homology of protein was searched on available databases (NCBI BLASTP search
264 tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi).
265
266 *2.12. Chloroplast separation*
267 A broccoli head was divided into small branches, and about 25 g of only florets were
268 ground with a stainless steel grater in a cooled grinding medium (A medium). The grinding
269 medium (pH 7.5) consisted of 0.5 M sorbitol, 2.5% Ficoll 400 (GE Healthcare, UK), 1 mM
270 $MnCl_2$, 2 mM EDTA, 10 mM KCl, 1 mM cysteine, and 50 mM tricine. Subsequently, the
271 extraction was filtered through two layers of Miracloth, and the filtrate was then centrifuged
272 according to Funamoto et al. (2003). The chloroplast fraction was suspended in a B medium
273 (pH 7.6) consisting of 0.33 M sorbitol, 1 mM $MnCl_2$, 2 mM EDTA, 1 mM $CaCl_2$, 5 mM

pyrophosphoric acid, 0.5 mM KH_2PO_4, and 50 mM HEPES. Afterward, the suspension was layered on the Percoll gradient, which consisted of four layers: 10% (1.5 mL), 15% (2.5 mL), 35% (2.5 mL), and 60% (1.5 mL). The Percoll gradient medium (C medium, pH 7.6) consisted of 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, and 50 mM HEPES (Nakano & Asada, 1980). The chloroplast layered on gradient was centrifuged at 3,700 × g for 15 min at 4 °C. The intact chloroplast between 15 and 35% Percoll (Fig. 4) was removed and diluted with the B medium to remove Percoll and then re-centrifuged at 300 × g for 7 min at 4 °C. The pellet was re-suspended with 1 mL of B medium and about 86% of chloroplasts were intact according to a ferric cyanide assay (Heber & Santarius, 1970).

2.13. Antibody preparation and Western blot analysis

cPOX1 and cPOX3 were separated and purified from broccoli floret protein as described in Sections 2.5 and 2.9, and specific polyclonal antibodies of cPOX1 and cPOX3 were generated in rabbit using purified those enzymes. After electrophoresis, Western transfer from the gel onto PVDF membranes (pore size 0.45 μm, Millipore) was performed using a transfer buffer system in conjunction with the trans blot system at a constant current (Marysol Corp., Japan) of 63 V for 2 h. The transfer buffer consisted of 150 mL of 100 mM N-cyclohexyl-3-aminopropanesulfonic acid (cAPS) (pH 11), 150 mL of 100% methanol, and 1,200 mL deionized water. After Western transfer, the protein on the PVDF membrane was soaked for 1 h in a 0.1 M Tris-HCl buffer (pH 7.4) containing 4% (w/v) BSA and 0.1% Tween-20 and then incubated with diluted rabbit Chl-degrading POX (cPOX1 or cPOX3) (1:2,000) as the first polyclonal antibody for 1 h with gentle shaking at room temperature. After the PVDF membrane was washed with a 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1% Tween 20, it was soaked in a Tris-HCl buffer (pH 7.4) containing 4% BSA and incubated with horseradish

peroxidase (1:10,000) as the second antibody for 1 h. The immunoblot color was then developed using ECL plus the Western blotting detection system (GE Healthcare, UK). Subsequently, the blot was exposed to film.

2.14. Chlorophyll and derivative analysis

Chl content was determined usung *N*,*N*-dimethylformamide (Moran, 1982). Chl and the resulting derivatives were analyzed by HPLC using a Hitachi L-700 pump with an automated gradient controller and a Hitachi L-2450 diode array detector or a Hitachi L-7240 UV-visible spectrophotometer. The absorption spectrum of the pigment was recorded at 665 nm. Pigments were separated on a LiChropher C18 column (MERCK), 4×250 mm, using two solvents: solvent A, 80% methanol (methanol : milipore water, 80:20, v/v); and solvent B, 100% ethyl acetate in a gradient. Solvent B was added to solvent A at a linear rate until a 50:50 mixture was attained at the end of 20 min. The 50:50 mixture was then used isocratically for an additional 20 min as described by Eskin and Harris (1981). The flow rate was 1.0 mL min^{-1}, and the injection volume was 100 μL. The identification of Chl and the resulting derivatives was based on the retention time and the visible absorption spectra. Chl derivative standards, such as OHChl *a* and Phy *a*, were prepared by the method reported previously (Aiamla-or et al., 2010).

3. Results

3.1. Changes in chlorophyll content and chlorophyll-degrading peroxidase in stored broccoli florets

As shown in Fig. 1, the total Chl content decreased with floret yellowing during storage at 15°C and the Chl-degrading POX activity in the control increased significantly with floret

yellowing. UV-B treatment delayed the reduction of Chl level and effectively suppressed the enhancement of Chl-degrading POX activity.

Chl-degrading POX extracted from the fresh green florets and yellowing florets was partially purified by ammonium sulfate precipitation and ion exchange chromatography (CM-Sepharose). On the CM-Sepharose chromatography column, three activity peaks of Chl-degrading POXs (Chl-POX1, Chl-POX2, and Chl-POX3) were separated and found in stored broccoli florets. Only Chl-POX1 was clearly revealed in fresh green broccoli florets, and its activity increased on day 4 of storage. Chl-POX2 and Chl-POX3, but especially the latter, were found on day 4 in broccoli florets without UV-B treatment, whereas both were effectively suppressed in broccoli florets with UV-B treatment (Fig. 2).

3.2. Purification and characterization of chlorophyll-degrading peroxidase

Chl-degrading POX isozymes, especially cPOX1 and cPOX3, were purified using acetone powder prepared from green-yellow florets of broccoli after 4 days of storage. To remove Chl-degrading POX from the acetone powder extract, 60–90%-saturated ammonium sulfate was used as the optimum concentration for enzyme precipitation. Chl-degrading POX purification was pursued by successive chromatographic steps using ammonium sulfate precipitation, as shown in Table 1. In the first step, ion exchange chromatography (CM-Sepharose) was used, and three activity peaks of Chl-degrading POXs, namely Chl-POX1, Chl-POX2 and Chl-POX3, but especially those of Chl-POX1 and Chl-POX3, were found on CM-Sepharose in the order of the linear gradient (Fig. 2). A 464-fold increase in Chl-POX1 and a 42-fold increase in Chl-POX3 in specific activity were obtained. Chl-POX1 was higher in specific activity than Chl-POX3. For further purification, partial purified Chl-POX3 was pursued by means of hydrophobic chromatography (butyl-650), and the specific activity obtained by chromatography increased about 10 times more than that by CM-Sepharose. In

addition to purification, ConA-Sepharose chromatography, which is an affinity chromatography, was used. However, Chl-POX3 was hardly adsorbed by ConA resins. The enzyme activity of Chl-POX3 was detected in the early fraction before a linear gradient of 0–0.8 M glucose (data not shown). Subsequently, molecular exclusion chromatography (Sephacryl S-200) was continually applied, and, finally, active fractions of Chl-POX3 separated by molecular exclusion chromatography were purified by an ion exchange chromatography column (SP-650).

The molecular weight size of cPOX3, which was purified from Chl-POX3, was 34 kDa using SDS-PAGE with molecular weight markers, while that of cPOX1, which was purified from Chl-POX1, was 43 kDa, as shown in Fig. 3A. In addition, partial purified Chl-POX1, unlike Chl-POX3, was adsorbed by the ConA-Sepharose column, and the development of pink color by the glycoside chain, which was contained in cPOX1, was observed using a periodic acid-Schiff (PAS) staining system after SDS-PAGE (Fig. 3B). Using cPOX1 and cPOX3, a kinetic constant was determined. The cPOX3 (K*m*-0.64 mM) had a low K*m* value corresponding to H_2O_2 as compared to that of cPOX1 (K*m*-2.32 mM). cPOX1 (K*m*-0.6 mM) and cPOX3 (K*m*-0.63 mM) had similar values to K*m* corresponding to *p*-coumaric acid.

3.3. Cell localization of chlorophyll-degrading peroxidase

As shown in Fig. 4, green (G) and green-yellow (GY) broccoli florets were used for chloroplast isolation, and the intact chloroplast was separated by Percoll gradient centrifugation. Intact chloroplast proteins were run on SDS-PAGE and visualized by Coomassie brilliant blue R-250 (Fig. 4A). The chloroplast proteins on SDS-PAGE were also blotted to the membrane and hybridized with the rabbit cPOX1 and cPOX3 antibodies, which were prepared from purified Chl-degrading POX (cPOX1 and cPOX3) of broccoli florets. As shown in Fig.4B, the band of cPOX3 was observed only in the intact chloroplast protein from

GY broccoli florets (lane No. 5), whereas that did not appear in the intact chloroplast protein of G broccoli (lane No. 4). The band of cPOX1 was not observed with intact chloroplast protein fraction from G and GY broccoli florets, and the cPOX3 band was not detected in the broken chloroplast fraction from G or GY broccoli florets (data not shown).

Moreover, we determined N-terminal amino acid sequence of cPOX3 protein, which was represented as Ala-Arg-Ala-Asp-Ala-Asp-Ala-Met-Ala-Trp, following an increase in cPOX3 in the chloroplast of senescing broccoli. This sequence was compared with that of other protein by NCBI BLAST search. The homologous sequence with 90% homology was found in that of cytokinin oxidase/dehydrogenase from wheat (accession no. ADK56162.1 position 19^{th} –Ala-Arg-Ala-Asp-Ala-Asp-Ala-Trp-Ala-Trp–position 28^{th}, *Triticum aestivum*). However, cPOX3 did not have any cytokinin oxidase activity (data not shown).

3.4. Chlorophyll and 13^2-hydroxychlorophyll a in an intact chloroplast fraction

Chl and their derivative levels in an intact chloroplast fraction of G and GY broccoli florets were measured. Intact chloroplasts of G broccoli florets had higher Chl *a* and OHChl *a* levels than those of GY broccoli florets. Surprisingly, we found that the OHChl *a*: Chl *a* ratio increased with senescing broccoli florets (Fig. 5).

4. Discussion

Chl-degrading POX, which is a cationic type, has been reported to be involved in the Chl degradation of broccoli florets (Funamoto et al., 2003). The Chl-degrading POX activity was markedly increased with floret senescence, but an increase of the activity was suppressed by heat treatment. The suppression of Chl-degrading POX activity was also found in broccoli with UV-B treatment (Aiamla-or et al., 2010). The three activity peaks of Chl-degrading

POXs, Chl-POX1, Chl-POX2 and Chl-POX3, were separated by CM-Sepharose ion exchange chromatography. Chl-POX1 was detected in fresh broccoli florets, and the activity gradually increased with floret yellowing. On the other hand, the activity of Chl-POX1 was not clearly inhibited by UV-B treatment. Moreover, the activities of Chl-POX2 and Chl-POX3, but especially the latter, were detected in broccoli florets on day 4, but these two types were not found in fresh broccoli florets, indicating that Chl-POX2 and Chl-POX3, but especially the latter, might be involved in Chl degradation. The activities of Chl-POX2 and Chl-POX3 were also suppressed by UV-B treatment.

By CM-Sepharose ion exchange chromatography, Chl-POX1 was purified, whereas Chl-POX3 was one-tenth of the specific activity as compared with activity of Chl-POX1, as shown in Table 1. Therefore, Chl-POX3 was pursued by means of butyl-Toyopearl hydrophobic and ConA-Sepharose affinity chromatographies. However, cPOX3, which was purified from Chl-POX3, was hardly absorbed by ConA resins. In contrast, cPOX1, which was purified from Chl-POX1, was absorbed by ConA resins, which bind to a glycoside chain containing D-mannose and D-glucose (Bonner, 2007). The glycosides in purified cPOX1 and cPOX3 were also examined using PAS-staining, and only cPOX1 was stained with the PAS reagent, suggesting that cPOX1 could be a kind of glycoprotein. The molecular sizes of cPOX1 and cPOX3 were examined on SDS-PAGE, and those of purified proteins were 43 and 34 kDa, respectively. The molecular size of cPOX3 was close to that of the cationic peroxidase (33 kDa) in the chloroplast of cucumber cotyledon that Abeles et al. (1988) reported to be involved by *in vitro* Chl degradation. Furthermore, these researchers demonstrated that the cationic peroxidase is hardly related to Chl degradation because the inhibitor of heme synthesis and protein glycosylation had no effect on Chl degradation and reduced the synthesis of cationic peroxidase (Abeles & Dunn, 1989). The cPOX1 and cPOX3 had the same substrate affinity corresponding to *p*-coumaric acid. However, cPOX3 had a

lower K*m* value, corresponding to H_2O_2, than cPOX1, which suggests that cPOX3 has a high substrate affinity to H_2O_2 and plays an important role on Chl degradation. To elucidate the physiological role of cPOX3 on Chl degradation in broccoli florets, the localization in the chloroplast of cPOX3 was determined. As previously reported, POX activity was found in isolated thylakoids from green canola (*Brassica napus* L.) seeds, and this activity degraded the thylakoid-bound pigments in the presence of H_2O_2 and 2,4-dichlorophenol (Johnson-Flanagan & McLachlan, 1990). On the other hand, a small degree of activity of Chl-degrading POX was found in the intact chloroplast of radish cotyledons, which could imply that Chl degradation by Chl-degrading POX is mainly present outside the chloroplast (Akiyama & Yamauchi, 2001). In this study, the intact chloroplast was isolated from G and GY broccoli florets by Percoll gradient centrifugation. Protein immunoblot using antibodies obtained from purified Chl-degrading POXs (cPOX1 and cPOX3) showed that only cPOX3 was found in the intact chloroplast fraction isolated from GY broccoli florets. The cPOX1 was not detected in any intact chloroplast isolated from G or GY broccoli florets. In addition, neither cPOX1 nor cPOX3 was localized in the broken chloroplast fraction containing mainly thylakoid membranes. These results demonstrated that cPOX3 of Chl-degrading POX could be in the stroma and/or chloroplast envelope and be related to Chl degradation in the chloroplast.

We reported previously that OHChl *a* was formed in broccoli florets and it was not accumulated during senescing process of broccoli florets (Aiamla-or et al., 2010). In present study, the levels of Chl *a* and OHChl *a* in intact chloroplast were examined. The levels of Chl *a* and OHChl *a* in intact chloroplast showed a decrease, but the OHChl *a* : Chl *a* ratio in intact chloroplast increased approximately 1.36 times in senescing intact chloroplast, and correlated with the enhancement of cPOX3 activity with yellowing broccoli florets. Kuroda et al. (1990) noted previously that two cationic isoperoxidase activities (C3 and C4) were found in the chloroplast of barley leaf segments. Only C4 peroxidase functioned as a Chl-degrading

449 enzyme during barley leaf senescence, since its activity was repressed by kinetin and
450 glucosamine, and these treatments delayed Chl degradation. C4 peroxidase in barley leaves
451 and cPOX3 in broccoli florets might play the same role, which relates to Chl degradation in
452 the chloroplast. Furthermore, Funamoto et al. (2003) demonstrated that, in fresh broccoli
453 florets, a high activity of Chl-degrading POX was not found in the chloroplast fraction but in
454 the cytosolic and microsomal fractions. With senescence, plastoglubuli, which are formed
455 from the thylakoid membrane and contain Chl and carotenoid pigments (Steinmüller & Tevini,
456 1985), seem to be present not only in the chloroplast but also in the vacuole, suggesting that
457 cPOX1 might be involved in Chl degradation outside the chloroplast or in the vacuole.
458 Martínez et al. (2008) found Chl *a* in SAVs, which were prepared from a senescent leaf
459 containing chloroplasts, and suggested that the presence of Chl *a* in SAVs may be an
460 alternative non-plastic pathway for Chl degradation. Therefore, the degradation of Chl by
461 cPOX1 in vacuole cells could be demonstrated in the near future.
462 We found that the activity of cPOX3 was particularly exposed with GY broccoli florets
463 and located within the chloroplast. Hence, we attempted to determine the N-terminal amino
464 acid sequences of the purified cPOX3 protein and compared them with other known N-
465 terminal amino acid sequences by homology search of available databases (NCBI BLASTP
466 search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequence of cPOX3 had
467 90% similarity to cytokinin oxidase/dehydrogenase from wheat (*Triticum aestivum*). An
468 identical difference of insertion was found in the eighth amino acid of cPOX3, Trp.
469 Unfortunately, cPOX3 did not have any cytokinin oxidase activity. Although POXs represent
470 a large protein group, the sequences of cationic POXs may not be as widely available in
471 current databases.
472 Postharvest environmental stress is known to induce reactive oxygen species (ROS)
473 (Toivonen & Hodges, 2011). We found that the level of total peroxide including H_2O_2, which

contained a certain amount during storage, showed almost no changes in stored broccoli florets. Total peroxide content increased by UV-B treatment, whereas an ascorbate-glutathione cycle, which relates to H_2O_2 elimination, activated simultaneously during storage (Takino, Yamauchi, Aiamla-or, & Shigyo, 2009), inferring that the control of floret yellowing by UV-B treatment might be involved in the activation of ROS-scavenging enzymes and ascorbate-glutathione cycle as well as the suppression of enhancement of Chl-degrading POX activity.

5. Conclusions

Three peaks of Chl-degrading POX activity were separated from senescent broccoli florets by an ion exchange chromatography column, particularly Chl-POX2 and Chl-POX3. The activity of these two types was clearly suppressed by UV-B irradiation. Chl-POX1 activity was not clearly inhibited by UV-B irradiation. Only cPOX3, which was purified from Chl-POX3 by column chromatography, localized in the chloroplast. Finally, we would imply that cPOX3 might be involved in Chl degradation in the chloroplast of senescent broccoli. Further study is necessary to elucidate the level of H_2O_2 in relation to cPOX3 activity in broccoli florets.

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621 **Figure legends**

622 Fig. 1. Changes in total Chl content (A) and Chl-degrading POX activity (B) in control and
623 UV-B treated broccoli florets during storage at 15 °C. Broccoli florets were irradiated with 19
624 kJ m^{-2} at 25 °C. Vertical bars represent the average values with SE (n=3).

625 Fig. 2. Elution profile of Chl-degrading POX activities on ion exchange chromatography
626 column (CM-Sepharose). Three peaks of Chl-degrading POX activities were noted as Chl-
627 POX1, Chl-POX2, and Chl-POX3 by the order of a linear gradient of 0–1 M NaCl in a 10
628 mM acetate buffer (pH 5.5). The activity of Chl-POX1 was found in green or fresh broccoli
629 florets (A) and green-yellow broccoli florets (B). The activities of Chl-POX2 and Chl-POX3
630 were exposed in green-yellow broccoli florets.

631 Fig. 3. SDS-PAGE of purified cPOX1 and cPOX3 proteins. Proteins on acrylamide gel were
632 visualized by silver staining; the molecular weight of cPOX1 and cPOX3 was 43 and 34 kDa,
633 respectively (A). The proteins on acrylamide gel were visualized with periodic staining,
634 which detected glycoprotein (B). Lanes 1 and 4, molecular weight markers; lanes 2 and 5,
635 purified cPOX1 protein; lanes 3 and 6, purified cPOX3 protein.

636 Fig. 4. Chloroplast suspensions were prepared from green broccoli (G) and green- yellow
637 (GY) florets, which were days 0 and 3, respectively. Green and green-yellow broccoli florets
638 were used for chloroplast isolation and separation of intact chloroplast on the Percoll gradient
639 as described in Section 2.12. Intact chloroplast proteins were separated on SDS-PAGE and
640 visualized by CBB R-250 (A). Each well contained 15 µg intact chloroplast proteins. After
641 electrophoresis, intact chloroplast proteins were blotted to the PVDF membrane and
642 hybridized with Chl-degrading POX (cPOX1 and cPOX3) antibody (B). Lane 1, molecular

643 weight markers; lane 2, intact chloroplast protein of green broccoli florets; lane 3, intact
644 chloroplast protein of green-yellow broccoli florets; lanes 4 and 5, intact chloroplast protein
645 of green and green-yellow broccoli florets probed with cPOX3 antibody, respectively.
646
647 Fig. 5. The levels of Chl *a* (A), OHChl *a* (B), and OHChl *a*: Chl *a* ratio (C) in intact
648 chloroplast of green and green-yellow broccoli florets during storage at 15 ºC. Each level of
649 Chl *a* or OHChl *a* was calculated using peak area amount of HPLC chromatogram. Vertical
650 bars represent the average values with SE (n=3).
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Table 1. Summary of purification of chlorophyll-degrading peroxidase from acetone powder of broccoli florets on day 4, when the florets began to turn yellow. For protein extraction, acetone powder of green-yellow broccoli florets was suspended in a 20 mM phosphate buffer (pH 7). The extracted protein was continually used for purification.

Purification step	Total protein (μg)	Total activity (unit)	Specific activity (unit mg^{-1} protein)	Recovery (%)	Purification fold
Crude enzyme	635,960	13,241	21	100	1
60-90% ammonium sulfate	115,190	12,466	108	94	5
IEC-CM-Sepharose					
Chl-POX1	967	9,420	9,741	71	464
Chl-POX3	2,564	2,262	882	17	42
HIC-butyl					
Chl-POX3	< 9	84	> 9,333	0.63	444
AC-ConA-Sepharose					
Chl-POX3	< 3.5	80	> 22,857	0.60	1,088
MEC-S200					
Chl-POX3	< 2	76	> 38,000	0.57	1,810
IEC-SP650					
Chl-POX3	< 1.3	63	> 48,461	0.48	2,308

One unit of activity was defined as the amount of enzyme degrading 1 μg of chlorophyll *a* per min at 25 °C. IEC; ion exchange chromatography column, HIC; hydrophobic chromatography column, AC; affinity chromatography column, MEC; molecular exclusion chromatography column. The single protein for Chl-POX1 was achieved and replaced the name with cPOX after CM-Sepharose chromatography, whereas single protein for Chl-POX3 was performed after ion exchange chromatography column (SP-650). The single protein for Chl-POX3 was then named cPOX3.

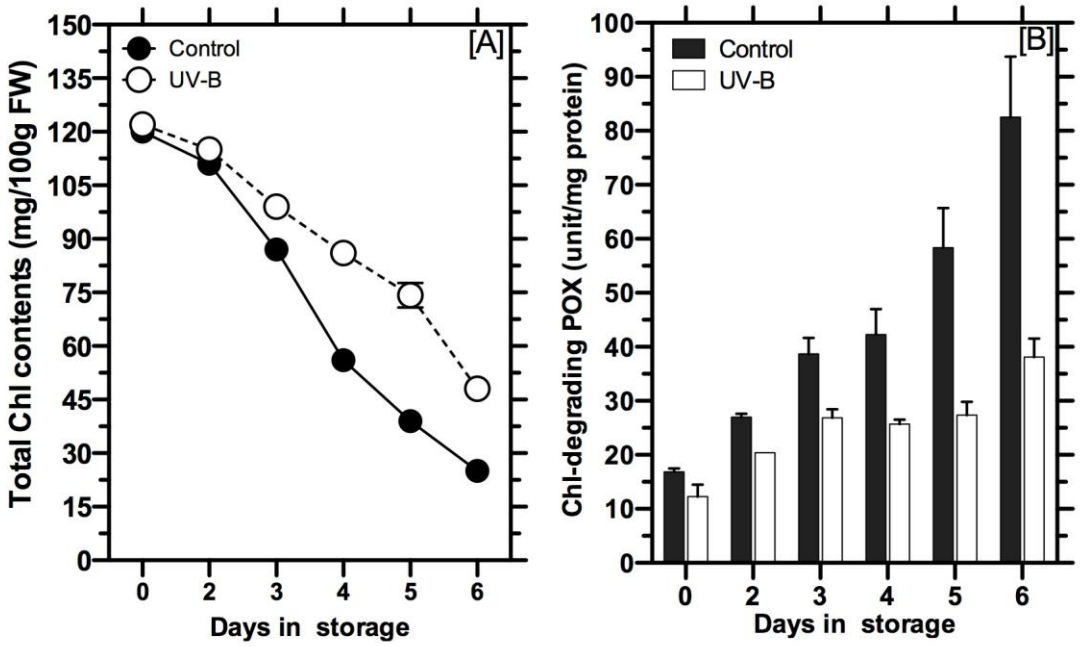
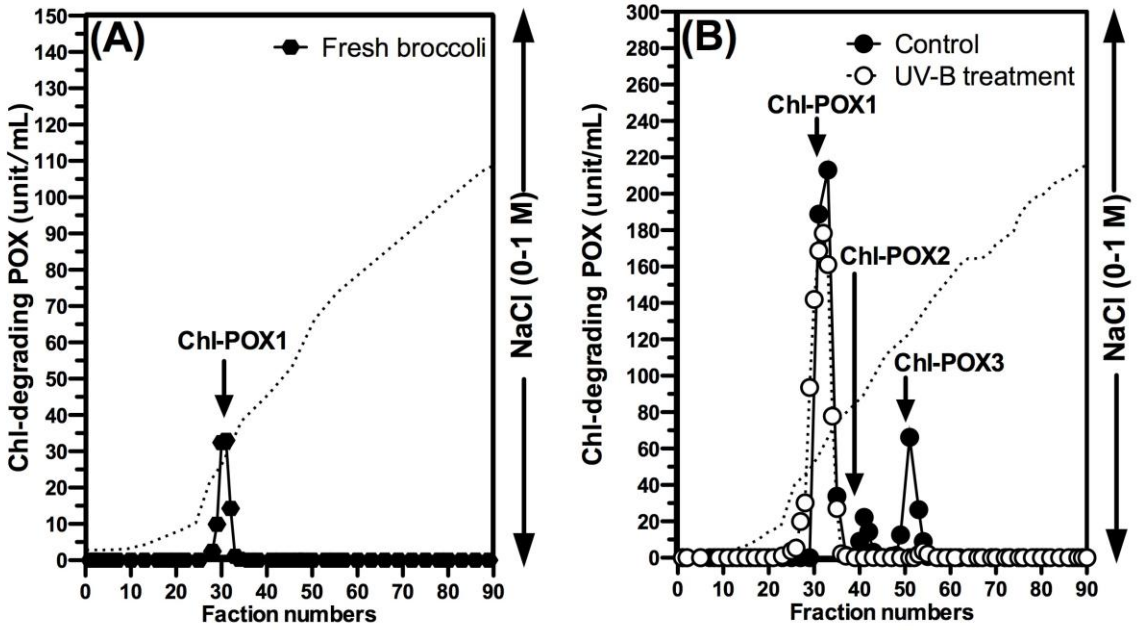

[A]
Control
UV-B
Total Chl contents (mg/100g FW)
150
135
120
105
90
75
60
45
30
15
0
0
2
3
4
5
6
Days in storage
[B]
Control
UV-B
Chl-degrading POX (unit/mg protein)
100
90
80
70
60
50
40
30
20
10
0
0
2
3
4
5
6
Days in storage

Fig. 1



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680 Fig. 2

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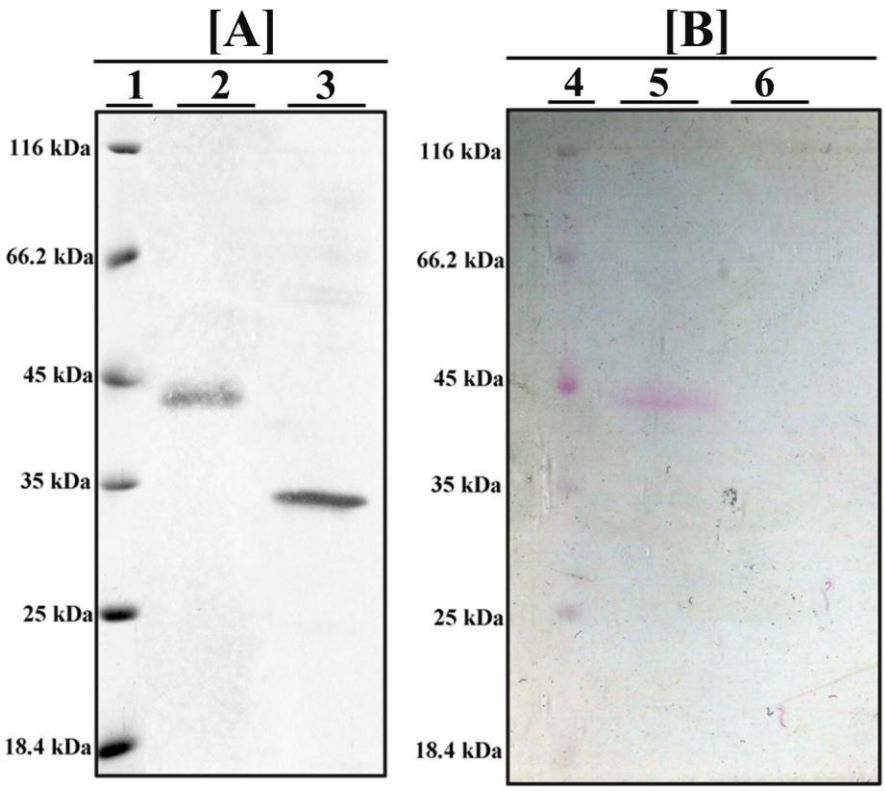

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691 Fig. 3

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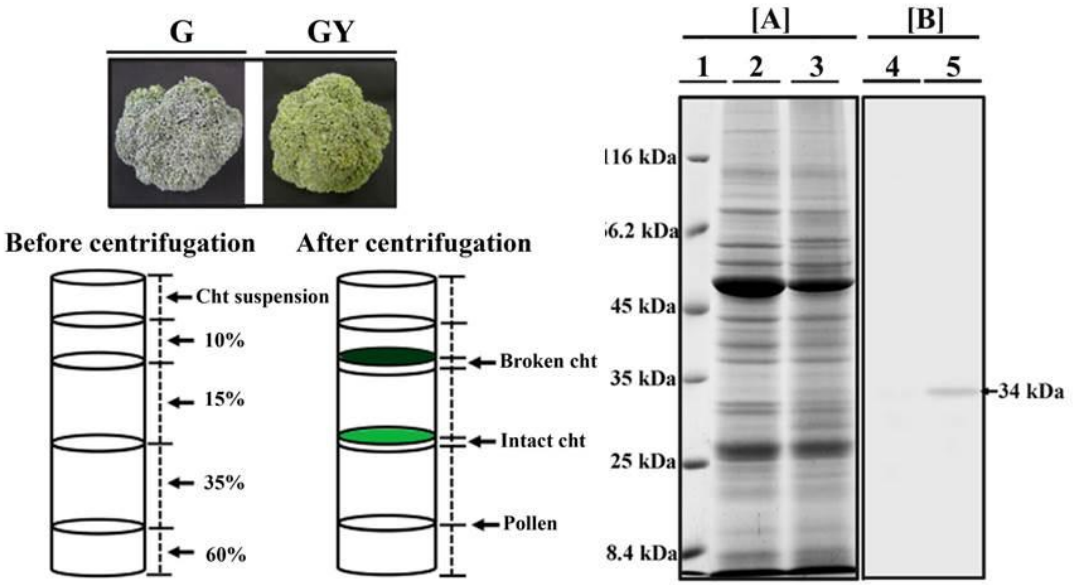

699

700 Fig. 4

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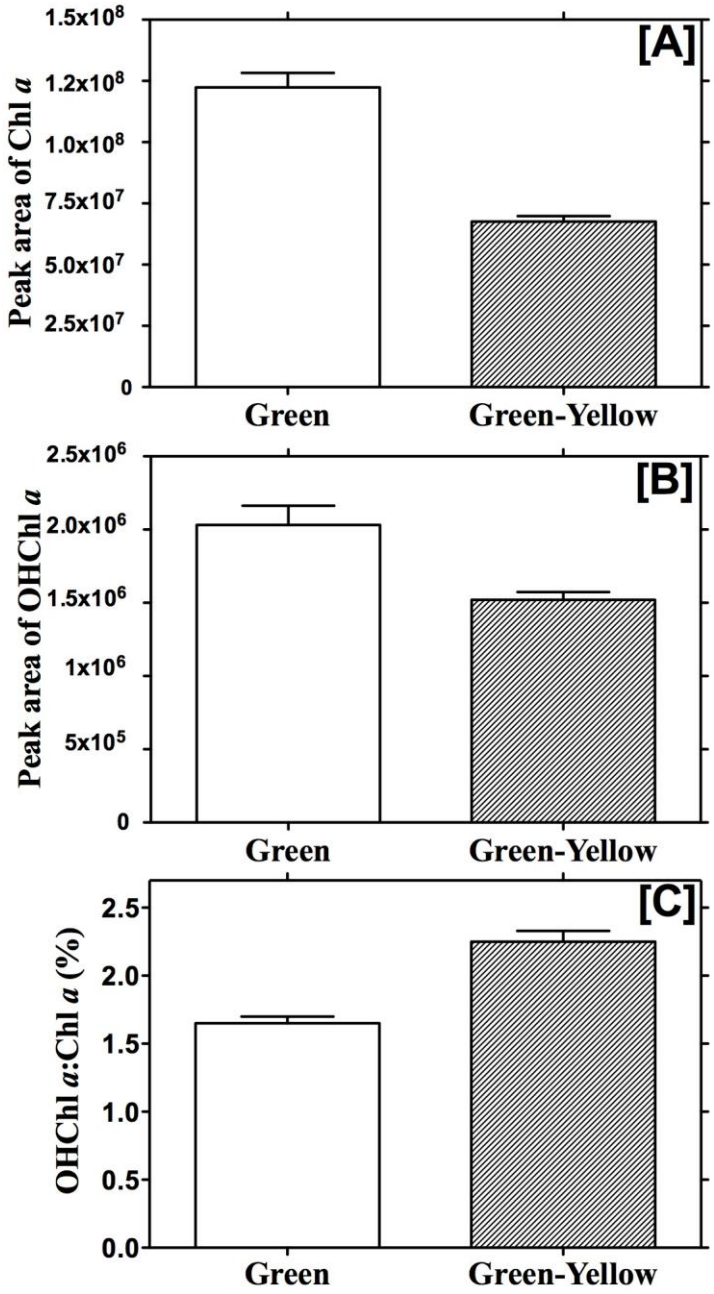

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710 Fig. 5