Original paper

Enzymatic Browning and Polyphenol Oxidase of Mung Bean Sprout during Cold Storage

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Received February 14, 2018 ; Accepted March 18, 2018

Mung bean sprouts turn brown during cold storage. Here, we cloned polyphenol oxidase (PPO) mRNA, and then examined its expression and active form to clarify the mechanism of this browning. A PPO cDNA encoding 592 amino acids showed high homology to PPO genes of Fabaceae plants and had highly conserved motifs, including the active site and transit peptide to the plastid thylakoid. Expression of PPO mRNA was almost constant. The translated PPO protein was considered to migrate into the thylakoid, but the active PPO was mainly present in the cytosol or soluble fraction, and its molecular mass was 31 kDa, smaller than the translated protein. The membrane structure of mung bean sprouts is heavily disrupted during cold storage. These results showed that PPO transported into the thylakoid is solubilized and decomposed to its active form, which oxidizes phenolic compounds in the cytosol to turn sprouts brown during cold storage.

Keywords: mung bean sprout, enzymatic browning, polyphenol oxidase

Introduction

From the standpoint of food processing and shelf life, enzymatic browning is categorized into two types; immediate and delayed. In the former, browning happens in a short time. A typical example of this type is the browning of apples. When an apple is crushed or cut, the juice or surface turns brown immediately or within a few minutes. In this type of enzymatic browning, sufficient amounts of both polyphenol oxidase (PPO; EC. 1.10.3.1) and its substrate polyphenols exist in separate compartments of intact cells. The enzymatic reaction starts when this compartmentation is disrupted by physical damage such as crushing or cutting. On the other hand, a typical example of delayed enzymatic browning is that of cut lettuce during cold storage. When lettuce leaves are crushed, the juice does not turn brown. However, the cut edges of lettuce gradually turn brown over the course of several days of storage. In this type of browning, substrate polyphenols are present at low level in intact leaves, but after the leaves are cut, polyphenols are synthesized as a response to injury. Synthesized polyphenol compounds are then successively

oxidized by PPO to turn brown. In either case, the browning or discoloration of fresh vegetables, fruits, and minimally processed fresh products during food processing or storage is often a limiting factor of consumer acceptance.

Mung bean sprouts are a major bean sprout consumed in eastern Asian countries including China, Korea and Japan. However, it is highly perishable, with a shelf life limited by both its appearance, easily turning brown or dark in color during cold storage, and microbiological qualities. As mung bean sprouts contain an active PPO protein (Takeuchi *et al.*, 1992) and phenolic compounds (Strack *et al.*, 1985), the discoloration of mung bean sprouts is an example of enzymatic browning, but there have been few reports on this phenomenon. We previously showed that the heat-shock treatment of mung bean sprouts inhibited browning by repressing the induction of phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) activity, a key biosynthetic enzyme for polyphenols or phenolics (Nishimura *et al.*, 2012). Furthermore, we cloned PAL cDNA and showed that PAL activity increased with induction of mRNA (Sameshima *et al.*, 2016). These results confirmed that

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the browning or discoloration of mung bean sprouts is a delayed type of enzymatic browning. However, there is a definite difference in the browning between cut lettuce and mung bean sprouts. In cut lettuce, PAL is induced as a response to injury, cutting or physical damage. Compartmentation is disrupted by cutting. On the other hand, the browning of mung bean sprouts happens without cutting or crushing. The aim of this study was to clarify the mechanisms of enzymatic browning of mung bean sprouts during cold storage. Here, we cloned the PPO cDNA of mung bean sprouts and demonstrated that PPO mainly existed in soluble fraction or cytosol as an active form in mung bean sprouts, which was considered to oxidize phenolic substrates leaked from the vacuole.

Materials and Methods

Materials Mung bean (*Vigna radiata*) sprout specimens were purchased from a retail shop in Tokyo between 2014 and 2016, and were used for experiments without further storage.

Cloning of PPO About 2 g of mung bean sprout specimens was frozen using liquid nitrogen and pulverized with a pestle, then total RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. cDNA was obtained from the RNA extract using the PrimeScript RT Reagent Kit (Takara Bio, Ohtsu, Japan). In a preliminary experiment, more than 20 combinations of PPO sequences of plants, such as *Trifolium pratense* (Forward (F): 5'-TAGCCTTTGGTGCTCCAGTG-3', Reverse (R): 5'-GCCATCAGGAGCATCCCAAT-3'), *Medicago sativa* (F: 5'-TTACCTCGTTTGACCAACGG-3', R: 5'-AGTGGGTAGGCTAGCTGTGA-3'), *Chaenomeles speciosa* (F: 5'-AAGCCTATAGCCCCACCAGA-3', R: 5'-GTTGTGGACTTGAGCTCGA-3'), *Triticum aestivum* (F: 5'-GAAGACGTTGCTGTTCCCTGG-3', R: 5'-ATCTCGATTCCCTCCACGAC-3'), *Solanum tuberosum* (F: 5'-GGTATGCGTTTTCCCTGCCAT-3', R: 5'-CTCCTGTCGCTTTCCATTCG-3'), and *Phaseolus vulgaris* (F1: 5'-TTGCCAATGCCACCTTC-3' or F2: 5'-TCCTGATGCCGATGGAA-3', R1: 5'-TGGGAAACTCCCGAAGC-3' or R2: 5'-CACGTGCTGCACCAACA-3'), were used for polymerase chain reaction (PCR). PCR products (about 500 bp) were obtained from four combinations of primers (*Trifolium pratense* F/*Medicago sativa* R, *Medicago sativa* F/*Medicago sativa* R, *Chaenomeles speciosa* F/*Solanum tuberosum* R, and *Phaseolus vulgaris* F2/R2). Among these combinations, only the PCR product (530 bp) obtained using the primer pair *Phaseolus vulgaris* F2/R2 could be sequenced, and it showed a high similarity with the PPO of *Vigna angularis* (XM 017580096.1) and *Glycine max* (XM 003545834.3). The 3'-terminal was determined by the rapid amplification of cDNA ends (RACE) method using PolyT primer, 5'-GGCCACGCGTCGACTAGTACTTTTTTTT-3' and a PCR primer (F: 5'-ATCTCAACCAGCCCAACAAC-3' R:

5'-GGCCACGCGTCGACTAGTAC-3'). The 5'-terminal was determined by the PCR method (F (*Phaseolus vulgaris*): 5'-ATGGCTTCTATCTCTTATCTTTCTTTTG-3'), R (sequenced here): 5'-GTTGTTGGGCTGGTTGAGAT-3'). The obtained PCR product was sequenced, and the 1,957 bp sequence was submitted to DDBJ (accession number, LC100016).

Modeling of mung bean PPO and preparation of anti-PPO antibody Plausible structures of mung bean PPO were depicted using Swiss-Model Workspace (Arnold *et al.*, 2006), for which *Vitis vinifera* PPO (2p3x.1.A; Virador *et al.*, 2010) and *Coreopsis grandiflora* PPO (aurone synthase, 4z12.1A; Molitor *et al.*, 2016) were used as templates. Considering the predicted structure, rabbit anti-PPO antibody was prepared using KLH protein conjugated with a polypeptide of mung bean PPO cloned here (C-L(373)GGNRRDFTSDSWL(386)) by an S-S bond (Eurofins Genomics, Tokyo, Japan). Antiserum was purified using an antigen peptide-conjugated affinity column (Eurofins Genomics).

Real-time quantitative PCR (qPCR) of PAL qPCR of PPO was conducted using an Applied Biosystems 7300 Real Time PCR System (Life Technologies, Tokyo, Japan). Two pairs of PPO (PPO1; F1: 5'-CCACTTTGTTTCCTGGCAAT-3' and R1: 5'-GTTGTTGGGCTGGTTGAGAT-3', and PPO2; F2: 5'-AACCTCGATGATCCTGATGC-3' and R2: 5'-ATTGCCAAGGAACAAAGTGG-3') were used as PPO detection primers. The product sizes were 125 and 115 bp, respectively. As a quantification control, three primer pairs derived from 18S rRNA of mung bean (M27797), actin mRNA of *Vigna radiata* (AF143208), and putative beta-tubulin mRNA of *Vigna radiata* (AY220546) were compared: 18S rRNA-F: 5'-TTGTATCCATCCAGGCCTTC-3', 18S rRNA-R: 5'-CCTAAAGAACCGTCCCAACA-3' (product size: 119 bp); actin-F: 5'-GGAATTGGAAACTGCCAAGA-3', actin-R: 5'-ATGGATGGCTGGAACAGAAC-3' (product size: 123 bp); tubulin-F: 5'-TACACTGGGGAAGGAATGGA-3', tubulin-R: 5'-CTCGGCATACTGGTCATCCT-3' (product size: 150 bp).

Enzyme extraction and assay The procedure of Shin *et al.* (1997) was adapted for the extraction of PPO with some modifications. In a cold room (4–6 °C), mung bean sprouts (ca. 100 g) wrapped in aluminum foil (Nippaku foil; Mitsubishi Aluminum, Tokyo, Japan) to avoid light and stored for 0–6 days were homogenized in 100 mL of an isotonic solution (pH 7.5) containing 20 mM tris (hydroxymethyl) aminomethane, 100 mM KCl, 10 mM MgCl₂, 200 mM sucrose, and 5 mM ascorbic acid for 10 s 3 times with a mixer (TM836; TESKOM, Tokyo, Japan). Each homogenate was filtered through four layers of cotton gauze. Part of the obtained filtrate was dialyzed against 100 mM Na phosphate buffer (pH 6.0) and was used as the total homogenate. The other filtrate was further separated into precipitate and supernatant fractions by centrifugation at 6,000 × g for 5 min. The supernatant fraction was separated into <30 %, 30–80 %, and 80 %> saturated ammonium sulfate

fractions, before 30-80 % saturated ammonium sulfate fraction was dialyzed against 100 mM sodium phosphate buffer (pH 6.0) and used as the cytosol or soluble fraction. The precipitate fraction was washed with the isotonic solution, and then crushed in 10 mM sodium phosphate buffer (pH 6.0) using a sonicator (Bronson 1510J-MTH; Emerson Japan, Tokyo, Japan) and a vortex mixer. The homogenate was centrifuged at $20,000 \times g$ for 20 min, before the supernatant was used as the plastid or insoluble fraction. In a simplified method, total homogenate was separated into the precipitate and supernatant fractions by centrifugation at $6,000 \times g$ for 5 min. The supernatant was dialyzed against the phosphate buffer and was used as the soluble fraction or cytosol, while the precipitate suspended in the phosphate buffer was dialyzed against the phosphate buffer and used as the plastid or insoluble fraction.

PPO activity was measured by the spectrophotometric method at 325 nm to detect the decrease in 5-caffeoyl-L-quinic acid (5-*O*-(3,4-dihydroxycinnamoyl)-L-quinic acid (chlorogenic acid; CQA) as the substrate (Fujita *et al.*, 1991). The reaction solution (3.0 mL) consisting of 100 mM sodium phosphate (pH 6.0), 0.5 mM CQA, and enzyme extract was incubated at 30 °C for about 10 min, and then the initial rate of decrease was estimated. A decrease in 1.0 μ mole CQA per min was defined as 1 unit.

For estimation of optimal pH or pH stability of the enzyme, 100 g of mung bean sprouts was homogenized in 100 mL of 10 mM sodium phosphate buffer (pH 6.0 or 8.0), respectively, and the homogenate was then filtered through four layers of cotton gauze. The obtained extract was mixed with 100 mM sodium acetate buffer (pH 4.0-6.0) or 100 mM sodium phosphate buffer (pH 5.0-8.5), before each PPO activity was measured for estimation of optimal pH. For estimation of pH stability, the pH of the extract was adjusted to pH 4-9 with 4 M acetic acid or 2 M NaOH, and left at 6 °C for 24 h, before each PPO activity was measured at pH 6.0.

SDS-PAGE and western blotting Mung bean sprouts stored for 0-6 days at 4-6 °C in the dark were homogenized in 100 mL of reverse-osmosis water. Each homogenate was filtered through four layers of cotton gauze. The obtained filtrate was used as a crude extract for PPO. Each extract was heated or not heated at 110 °C for 5 min in sample buffer containing SDS and 2-mercaptoethanol or containing SDS, respectively, before being separated by 10.0 % SDS-PAGE. For detection of PPO activity, non-heated samples were used, and gels were incubated in a 100 mM sodium phosphate buffer (pH 6.0) containing 1 mM CQA and 1 mM (+)-catechin for 15 min.

For western blotting analyses, proteins were transferred from the gel onto a nitrocellulose membrane (BioTrace NT, Pall, NY) at 3 mA/cm² for 45 min using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA), which was washed with PBS containing 0.05 % Tween 20 (PBST) and blocked with 3 % skim milk in PBST. The

membrane was incubated for 1 h with the rabbit anti-PPO antibody described the above (Eurofins Genomics, Tokyo, Japan) that had been diluted to 1: 5,000 (v/v) in PBST, before being incubated for 1 h with goat anti-rabbit peroxidase (Funakoshi, Tokyo, Japan) that had been diluted to 1: 10,000 (v/v) in PBST. Immuno-reactive bands were stained by using an EzWestLumi plus (ATTO, Tokyo, Japan), before being scanned by LAS-4000 (Fujifilm, Tokyo, Japan).

Estimation of injuries of membranes or cells Mung bean sprout samples (40 g) were stored at 4-6 °C in the dark for 0, 2, 4 and 6 days, before being immersed in reverse-osmosis water at 6 °C for 3 or 24 h. The electroconductivities and UV spectra of the immersion solution were measured at 25 °C using SS-71 (Horiba, Kyoto, Japan) and U-3310 (HITACHI, Tokyo, Japan), respectively. The samples were then autoclaved at 121 °C for 20 min, and the values of autoclaved samples were set to 100 %. Cell injuries (%) were estimated as described by Naveed *et al.* (2016).

$$[1 - (1 - T_1/T_2)/(1 - C_1/C_2)] \times 100 \quad \dots\dots\text{Eq. 1}$$

where, C_1 is electroconductivity at storage day 0 before autoclaving, C_2 is electroconductivity at day 0 after autoclaving, T_1 is electroconductivity at storage day 2, 4, or 6 before autoclaving, and T_2 is electroconductivity at day 2, 4, or 6 after autoclaving.

Results and Discussion

Cloning of PPO mRNA from mung bean sprout A nucleotide sequence of 1,957 bp was obtained, which included an initiation codon ATG, a termination codon TGA, and an open reading frame (1,776 bp) encoding 592 amino acids (Fig. 1). The molecular mass was calculated as 66,718 kDa. The amino acid sequence contained the highly conserved active sites or copper binding domains (CuA and CuB; Virador *et al.*, 2010), and showed high homology to PPO genes of Fabaceae plants (Table 1). These results indicated that the cloned gene was a mung bean PPO. Recently, another group also isolated mung bean PPO (XM 014661987). Two PPOs showed very high homology (99 %).

The translated protein of this PPO had about 9.7 kDa of a transit peptide to plastid thylakoid, similarly as other PPOs (Fig. 2). This result also supports the notion that this clone was PPO. Thus, the mung bean PPO is likely transported to the plastid after translation, like other PPOs. The molecular mass of this mature PPO was calculated to be about 57 kDa. Similarity analysis of three-dimensional structure suggested that this PPO had a unique shielding region on the C-terminal side like aurone synthase, a type of PPO (Moritole *et al.*, 2016), although PPO is usually active without limited proteolysis of the C-terminal side (Hunt *et al.*, 1993; Haruta *et al.*, 1998 ; Tsurutani *et al.*, 2002; Nishimura *et al.*, 2003). A plausible structure of an active mung bean PPO after a transit peptide

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1  MASISYLSFV  SPVNNFCPNS  ITLPSSFHPF  PQFQYPKVTR↓ VRCSGNKNNE  50
51 TPKASEAEVA  DIVGNRRKVL  VGLGGLCGAV  SLNNSPFAFA↓ APISPPDLST  100
101 CGPPDLAEGV  KPTNCCPPSS  KIIDFKFPPS  NQPLRVRPAA  HLVTDDYLAK  150
151 YKKALDLMKK  LPSKDPRNFMCuA QOANVHCAYC DGAYHQVGFP DLDLQVHNSW  200
201 LFFPYHRWYL  YFYERILGSL  INDPTFALPF  WNWDAPGGMK  LPSIYAQRQS  250
251 PLYDPLRNPN  HQPPTLVDLD  FNLDDPDADG  NISSNLTIMY  RQDVSNGKTP  300
301 TLFLGNAYRA  GDEPDPGGGSCuB VENVPHGPVH GWTDGLNQPN NENMGTFYSA  350
351 ARDPIFYSHH  SNIDRLWSVW  KTLGGNRRDF  TDSDWLES GF  LFYDENKNLV  400
401 RVKVKDCLDT  RKLGYVYQDV  DIPWLN SKPT  LRKPRARNVA  LAQNFGVGAA  450
451 HAAETSRSVK  FPLVLDSVVR  TRVKRPNKSR  SKEEKEEEEE  VLVIEEIEFD  500
501 RNTPVKFDVF  INEDDTQIR  PDNTEFAGSF  VSVPHTHKHR  NKNIKTCLRL  550
551 GLTDLLEELE  AEDDDSVMT  LVPRYGKGRV  TIRNIKIELI  AD  600

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Fig. 1. Amino acid sequence of PPO of mung bean sprout. Plausible active sites (CuA and CuB), and N- and C-terminal cleavage sites are underlined, and marked with down and up arrows, respectively.

Table 1. Sequence similarity of the PPO gene among several Fabaceae plants.

Fabaceae plants	Accession number	Similarity (%)	
		Nucleotide	Amino acid
<i>Vigna radiata</i> (mung bean)	LC10016 (cloned here)	100 (1957 bp)	100 (592 AA)
<i>Vigna radiata</i> (mung bean)	XM 014661987	99	99
<i>Vigna angularis</i> (azuki bean)	XM 017580096	95	93
<i>Phaseolus vulgaris</i>	XM 007148138	88	84
<i>Glycin max</i> (soybean)	XM 003545834	88	84
<i>Cajanus cajan</i> (pigeon pea)	XM 020366071	85	79
<i>Arachis ipaensis</i>	XM 016326600	78	74

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A. 1 MASISYLSFV  SPVNNFCPNS  I-TLPSS--F  HPFPQFQYPK  -----VT  RVRCS  44
B. 1 MASISHLSFV  SSVNNFCPNS  I-TLPSSS-L  HPFQQFQSPK  -----IT  RVRCS  45
C. 1 MASISYLSFV  SSVNNVSPNS  I-TPPSS--L  HPFSQFQSAK  TRKPKH NHIP  RVTCT  52
D. 1 MASIPHLSFV  STVNNFSPYS  T-TPPSS--L  HPFSQFQSAK  YKKPKH HHIP  RVTCT  52
E. 1 MASMS-APLV  TSATSIIPTT  F-LSPFSQKY  HRISSFGNPR  -----HSNLQ  AVSCK  49
F. 1 MTSLS-PPVV  TTPTAPNPDT  KPLSPFSQNN  SQVSLLT KPK  -----RSLGR  EVSCN  50

45 GNKN-----N  ETPKASEAEV  -----ADIVG  NRRKVLVGLG  GLCGAVS-LN  NS-PFAFA  90
46 GNKN-----N  ETPKASEEEV  -----ADIVG  NRRKVLVGLG  GLCGAVT-LN  NN-PFAFA  91
53 GNPV-----H  QTPNPSEEQL  -----PHIVE  NRRNVLLGLG  GLCGAVT-LN  NN-PFAFA  98
53 GNQN-----N  PTPNPSEGEF  -----PHIVG  HRRSVLFGLG  GFCGAST-LN  NNNPFAFA  99
50 ATNNS-DQN  KNPSTSSNDH  DHENPSPVNL  DRRNVLLIGL  SLYGGVAGLG  SD-PFAVA  105
51 ATNN-----  -----DQFDQAQSKL  DRRNVLLGLG  GLYG-VAGMV  TD-PRGFG  90

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Fig. 2. Plausible transit sequences to plastid thylakoid among plant PPOs. A, *Vigna radiata* (cloned here); B, *Vigna angularis* (XP 017435585); C *Phaseolus vulgaris* (XP 007148200); D *Glycine soja* (KHN08552); E, *Malus domestica* (AGU01537); F *Malus domestica* (BAA21676). X, stromal peptidase processing site; Y, thylakoid peptidase processing site. A twin arginine site specific to thylakoid is boxed.

and a shielding region on the C-terminal side were removed was depicted using Swiss-Model Workspace (Arnold *et al.*, 2006) and an active grape PPO (Virador *et al.*, 2010) as shown in Fig. 3. Histidine residues (H176, H197 and H206 of CuA,

and H326, H330 and H360 of CuB in Fig. 1) were involved in the coordination of two copper ions like other PPOs (Virador *et al.*, 2010).

PPO expression during cold storage of mung bean sprout

Fig. 3. Predicted structure of mung bean PPO (A91-L425) using an active *Vitis vinifera* PPO (2p3X.1.A) as a template. Six histidine residues coordinated to a ligand Cu_2O depicted by three circles are shown. A shaded part from L373 to L386 was a polypeptide used for preparing anti-PPO antibody.

In a preliminary experiment, we compared threshold cycle (C_t) values of 18S rRNA, actin, tubulin, PPO1, and PPO2. The combination of actin and PPO2 was selected for quantification, because they formed a single product, the C_t value of actin was the closest to that of PPOs, and the change in the delta C_t value between actin and PPO2 by dilution was the least (data not shown).

PPO expression of mung bean sprouts was then examined during cold storage using qPCR (Fig. 4). The data of PAL (Sameshima *et al.*, 2017) was plotted at the same time. The expression of PAL mRNA was induced at day 2, while that of PPO mRNA was almost constant. Clear induction of PPO mRNA was not apparent during cold storage. This result suggests that PPO activity remained almost constant during cold storage (Nishimura *et al.*, 2012).

Subcellular localization of PPO The sequence data described the above showed that mung bean PPO is transported to the plastid thylakoid after translation. We therefore examined the PPO activities of total homogenate, plastid or insoluble fraction, and cytosol or soluble fraction of mung bean sprouts during cold storage. In a preliminary experiment, two preparation methods for these fractions were examined. As definite differences were not apparent (data not shown), a more simplified method was used to examine the distribution of PPO in mung bean sprouts during cold storage. PPO activity was mainly detected in the cytosol or soluble fraction during cold storage (Fig. 5). The activity in the cytosol or soluble fractions accounted for about 90 % of total activities, while that of the plastid or insoluble fractions only accounted for about 10 %.

Fig. 4. Expression of PPO and PAL mRNAs during cold storage. Mung bean sprouts were stored at 4–6 °C. Each mRNA was determined by qPCR using actin as an internal standard gene, and the expression at day 0 was set to 1.0. Different letters showed a significant difference against the value at day 0 ($n=3$).

Clear differences between enzyme activities per sample and specific activities were not apparent during cold storage.

Next, we examined the molecular species of PPO by SDS-PAGE. After electrophoresis, the gel was stained with a mixture of CQA and catechin. As a result, a band clearly turning brown, which showed PPO activity, was apparent in all samples during cold storage (Fig. 6-A). The molecular mass of this band was estimated about 31 kDa.

For western blotting analyses, anti-mung bean sprout PPO antibody was prepared. An antigen polypeptide was designed considering a plausible three-directional structure of the PPO, as shown in Fig. 3. The prepared antibody inhibited PPO

Fig. 5. Subcellular localization of PPO activity during cold storage. PPO activities of total homogenate, soluble fraction, and plastid of mung bean sprouts stored for 0-6 days at 4-6 °C were measured as described in the material and method section. Activities per sample weight (A) and specific activities (B) of PPO were shown. Different letters showed a significant difference among the same fraction ($p < 0.05$; $n=3$).

Fig. 6. PPO activities analyzed by SDS-PAGE (A), and western blotting analyses of PPO proteins (B). Mung bean sprouts were stored for 0-6 days at 4-6 °C. Total homogenate of the sprout was applied to SDS-PAGE without heating (A) and with heating (B).

activity of crude extract of mung bean sprouts (data not shown). The same homogenates used for detecting PPO activities on SDS-PAGE were completely denatured by heating, before being applied for western blotting analyses. As a result, two bands at about 47 kDa and 31 kDa were apparent (Fig. 6-B) in all samples during cold storage. The later band having a smaller molecular weight corresponded to that showing PPO activity, while the former band having a larger molecular weight did not show PPO activity. These results suggested that limited proteolysis occurred for the formation of active PPO in mung bean sprout. From the similarity to grape PPOs (Sarry *et al.*, 2004 and Virador *et al.*, 2010), a plausible C-terminal cleavage site was marked in Figs. 1 and 7A. The

sequence of RKLGYVYQDV DIPWLNSKP showing high homology to the C-terminal of grape PPO and following several basic amino acid residues was a tryptic cleavage site. Figure 7 shows a plausible cleavage site comprising 38 kDa of an active PPO, and a model of the active PPO in which an active site of copper ions is apparent. We will need to examine whether the bands at 47 kDa and 31 kDa were the mature PPO (calculated to be 57 kDa) and the active PPO (calculated to be 38 kDa), respectively. In general, C-terminal cleavage is not required for plant PPOs to be activated (Hunt *et al.*, 1993; Haruta *et al.*, 1998 ; Tsurutani *et al.*, 2002; Nishimura *et al.*, 2003). This unique proteolysis of mung bean PPO may become a new target for regulating the browning of mung bean sprouts,

Fig. 7. Modeling of a latent mung bean PPO (A) using *Coreopsis grandiflora* PPO (4z12.1A) and an active PPO (B) in which a C-terminal side was removed using *Vitis vinifera* PPO (2p3X.1.A) as templates, respectively. A plausible cleavage site of the C-terminal side was shown in A. In the top panel of B, an active center of Cu_2O was apparent. A1 in this model was A91 in that of Fig. 3.

Fig. 8. Cell or membrane injuries of mung bean sprouts during cold storage. Mung bean sprouts were stored for 0-6 days at 4-6 °C, before each sample was immersed in water and its electroconductivity and absorbance at 270 nm and 325 nm were measured (A). Cell injury (B) was calculated as described in the method. Different letters showed a significant difference among the same measurement items ($p < 0.05$; $n=3$).

although we do not understand the physiological significance of this activation by C-terminal cleavage.

Injuries to membranes or cells were then estimated. After storage of mung bean sprouts, the sample was immersed in water. If membranes or cells are injured during cold storage, various electrolytes and contents should leak into water, leading to an increase in the electroconductivity and UV absorbance of the immersion solution. As shown in Fig. 8-A, the electroconductivity and absorbance at 270 nm and 325 nm increased during cold storage. Absorbance at 270 nm was an absorption maximum of the immersion solution. The

absorbance at 325 nm was another absorption maximum of the immersion solution, and corresponded to the absorption maximum of the major polyphenol in mung bean sprouts, *trans*-caffeoyltartaric acid, which is a good substrate for mung bean sprout PPO, and it increased during cold storage (Sameshima *et al.*, 2016). Figure 8-B clearly shows that the cells or membranes of mung bean sprouts were heavily injured during cold storage. These observations suggest that membrane permeability was increased, or cell compartmentation was gradually disrupted during cold storage, and that substrates of PPO, phenolics, were eluted from vacuoles to cytosol. The

Fig. 9. Optimal pH (A) and pH stability (B) of mung bean sprout PPO. PPO activities of crude extract of mung bean sprouts were measured in 100 mM Na acetate buffer (pH 4-6) and 100 mM Na phosphate buffer (pH 5-8.5). The pH of crude extract of mung bean sprouts (0 h) was adjusted to pH 4-9 and left at 6 °C for 24 h, before each PPO activity was measured at pH 6.0 (n=3).

Fig. 10. Plausible schema of enzymatic browning of mung bean sprout during cold storage. The top panel shows mung bean sprout before storage. An active PPO is mainly present in cytosol, and phenolic compounds synthesized by PAL induction leak into cytosol during cold storage, which causes enzymatic browning.

optimal and stable pH of the PPO and the pH of cytosol or homogenate is another important factor for enzyme action or browning. Figure 9 showed that the optimal pH of mung bean PPO was 5-6 and that the enzyme was stable at pH >5. The pH of mung bean sprout was 6.0-6.3 during cold storage. These results suggest the active PPO was stable and could oxidize phenolics in cytosol.

In general, PPO is present in plastids (Murata *et al.*, 1997),

while its substrates, phenolics, are present in vacuoles (Yamaki, 1994). Disruption of this compartmentation leads to the interaction between phenolics and oxidative enzymes, and then to enzymatic browning. The present study clearly showed that an active PPO in mung bean sprout was present in the soluble fraction or cytosol, and that electrolytes and phenolics leak out from cells during cold storage. These observations showed why mung bean sprouts gradually turn brown during cold storage

without cutting.

In conclusion, we cloned PPO mRNA of mung bean sprouts and showed that its expression was almost constant during cold storage, which corresponded to the constant PPO activities during cold storage (Nishimura *et al.*, 2012), but that the major active form PPO was present in cytosol and its molecular mass was smaller than the thylakoidal protein. On the other hand, membrane systems of mung bean sprouts are gradually injured during cold storage. These results indicated that phenolic compounds synthesized by the induction of PLA during cold storage (Sameshima *et al.*, 2016) were gradually oxidized by the active PPO present in cytosol, which resulted in browning in tissues (Fig. 10). It will be necessary to examine subcellular localization, molecular species, and activity of PPO before cold storage or during sprouting.

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