식물생명공학회지 제34권 제4호
J Plant Biotechnol
Vol. 34, No. 4, 355-361 (2007)

# Purification and Partial Characterization of a Peroxidase from Perilla Callus

**Yeon Jae Hur, Han Gil Lee, Gaosheng Hu, Won Bok Chung, Soon Jae Jeong, Young Byong Yi, Jaesung Nam, Young Soo Chung, Jai Heon Lee, and Doh Hoon Kim***

*College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea*

ABSTRACT Cotyledons of perilla6 were cultured on MS medium containing 0.5 mg/ℓ NAA and 0.5 mg/ℓ BA for 7 weeks. The activity of perilla peroxidase was observed to increase following culture stages as assessed by peroxidase assay. A peroxidase (POD) was purified from perilla tissue cultured on MS medium for 7 weeks. The peroxidase was purified using ion exchange and gel filtration chromatography. The perilla peroxidase had a molecular mass of 30 kDa by SDS-PAGE. We showed that the N-terminal amino acid sequence of this protein shared 67% identity with the tea peroxidase. As indicated by SDS-PAGE, the banding pattern of the 30 kDa polypeptide present in total soluble protein from perilla tissue was increased following culture stages. Immunoblot analysis indicated that perilla peroxidase protein appeared after 3 weeks of perilla tissue culture, and continued to increase with extended duration of tissue culture for at least 7 weeks.

## Introduction

Peroxidases (EC 1.11.1.7) are ubiquitous plant enzymes that are widely distributed in the plant kingdom and can be found in vacuoles, tonoplasts, plasmalemma, and inside and outside of the cell wall (Adam et al. 1999). Peroxidases have been known to exist as multiple isoforms that play a known role in several biological functions in plants. (Gaspar 1986). Most higher plants possess a large number of peroxidase isoenzymes, which are encoded by multigene families. Each plant species is known to encode approximately 8 to 15 peroxidase families. Among them, APXs (ascorbate peroxidases) and GPXs (guaiacol peroxidases) are two important peroxidase isoforms. The role of chloroplast APX is particularly well documented (Chen and Asada 1989). Chloroplast APX is so well recognized that several Calvin cycle enzymes are readily inactivated if the hydrogen peroxide concentration is not maintained at a low level through the catalytic activity of APX (Kaiser 1979). GPXs are characterized by their broad specificity in response to an electron donor, and they utilize guaiacol and pyrogallol as electron donors in assays of their activity. There are significant reactivity differences between APX- and guaiacol-type peroxidases. The biochemical characteristics of plant peroxidases have been suggested, including polymerization of lignin and suberin monomers (Hiraga et al. 2001). The functions of plant peroxidases are correlated with the response to wound healing and pathogen attack, salt stress tolerance, development, catabolism of auxin, senescence, somatic embryogenesis, and scavenging of damaging hydrogen peroxide from the cell (Cordewener et al. 1991; Klotz and Lagrimini 1996). Peroxidases are capable of oxidizing a broad variety of organic compounds, including phenols, aromatic amines, indoles and sulfonates, by using hydrogen peroxide as the oxidant (Adam et al. 1999, Veitch and Smith 2001). In the peroxidase reaction, hydrogen peroxide accepts two electrons during oxidation of the organic compound, and the products of the reaction are water and a free radical. Because of their broad substrate specificity, the

*Corresponding author Tel 051-200-5680 Fax 051-200-7505
E-mail: dhkim@dau.ac.kr

patterns of their temporal and spatial expression and their responses to environmental stimuli, the functions of many plant peroxidases have been difficult to define. Different isoperoxidases may be more or less involved in various aspects of organogenesis, such as organ initiation, development, differentiation, and dedifferentiation.

An increase in peroxidase activity in response to several stress conditions is well documented. However, it is still not clear whether peroxidase isoenzymes are responsible for scavenging $H_2O_2$ in plant cells (Asada 1992). Despite the interest in peroxidases and their various roles during plant cell growth, little is known about the functions of their isozymes. The functions of peroxidase isozymes are especially unknown in perilla. In this study, we describe the isolation, purification, and characterization of a peroxidase from perilla callus over the duration of tissue culture.

## Materials and Methods

### Enzyme Assay and Electrophoresis

Materials: The cotyledons of perilla were excised from 7-day-old seedlings within approximately 0.5 cm from where the first leaf emerged. The explants were placed on MS medium containing vitamins, 3% sucrose, 0.5 mg/ℓ NAA and 0.5 mg/ℓ BA. Fresh perilla callus were obtained from tissue culture and the callus were used for peroxidase purification. Fresh prepared samples were frozen and stored at -70℃ until use.

Crude extract: Perilla callus were removed from frozen storage and homogenized at 4℃ using phosphate buffer pH 7.0. The extract was centrifuged, and the supernatant was used for further purification.

Protein precipitation: Protein precipitation was carried out using ammonium sulfate. This was performed at an initial saturation level of 35% followed by centrifugation, and then the saturation level was increased to 80% followed by centrifugation. The precipitate from the 80% ammonium sulfate solution was redissolved in 0.05 M Tris-HCl pH 7.8, dialyzed overnight against the same buffer and used in the purification steps.

Anion exchange chromatography: A 2.5 × 50 cm Bio-Rad column packed to a height of 45 cm with DEAE-Cellulose was equilibrated with 0.05 M Tris-HCl buffer pH 7.8. Perilla callus extract was loaded onto the column and washed with equilibrating buffer using an 86 mL/h flow rate. The retained protein was eluted at the same flow rate using a linear 1 L gradient of 0.1 M NaCl in the above buffer. Fractions of 6.5 mL were collected, the absorbance was read at 280 nm and POD activity was measured. All chromatographic steps were performed at temperatures of 4℃.

Gel filtration chromatography: Fractions from the DEAE-Cellulose column eluted during washing with equilibrating buffer that showed POD activity were combined. These fractions were concentrated by ultrafiltration using Millipore stirred ultrafiltration cells with 10 kDa molecular weight cutoff membranes (Millipore) and dialyzed against 0.04 M sodium phosphate buffer pH 6.0. The sample was loaded into a 2.5 × 50 cm Bio-Rad column packed to a 45 cm height with Sephacryl S-200, which was previously equilibrated with 0.04 M phosphate buffer pH 6.0. The sample was then washed with equilibrating buffer using a 49 mL/h flow rate. Fractions of 6.5 mL were collected.

### Protein and Peroxidase Activity Determination

Protein was quantified using the dye-binding method of Bradford with bovine serum albumin (BSA) as a standard. During the purification process, protein was measured by absorbance at 280 nm. POD activity was determined by monitoring the timecourse of the change in absorbance at 420 nm upon oxidation of the substrate catalyzed by the enzyme. Guaiacol (Sigma Chemical Co.) was used as substrate. The final reaction mixture contained 50 mM guaiacol, 50 $\mu$L of enzyme, 10 mM $H_2O_2$, and 50 mM Tris-acetate buffer pH 6.0 in a total volume of 1.5 mL (Lu and Whitaker 1974). The assay was performed at 25℃ using a UV-spectrophotometer connected to a temperature

controller. The absorbance increase at 420 nm was monitored for up to 3 min, and the slope of the linear portion of the curve was used to determine activity. Enzyme activity was calculated using an extinction coefficient of 25.5 $mM^{-1}$ $cm^{-1}$ for tetraguaiacol. One unit of enzyme activity was defined as the amount of guaiacol consumed in 1 min.

### Enzyme Characterization

The purity and molecular weight of the different enzyme fractions were analyzed using SDS-PAGE under reducing conditions. This was conducted using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gels were prepared according to the method of Bollag et al. (1996) with some modifications. The stacking gel had 4% T and 2.6% C, whereas the separating gel had 12% T and 2.6% C. %T refers to the total acrylamide content (w/v), whereas %C is the ratio of crosslinking reagent (bisacrylamide) to acrylamide monomer (w/w). All gels were run at a constant current (10 mA/plate). The following molecular weight markers used for electrophoresis were obtained from Bio-Rad: galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa). Gels were stained with Coomassie blue.

### N-Terminal Amino Acid Sequencing

For protein sequencing, the protein was electroblotted onto polyvinyliadene difluoride (PVDF) membrane (ProBlott™, Applied Biosystems) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer and 10% methanol at 50 volt for 45 min at room temperature. Protein samples on PVDF membrane were detected by staining with amido black staining solution (0.1% amido black 10B in 1% acetic acid/40% methanol). The protein band was excised from the membrane and then analyzed by the Procise Protein sequencing system (PE Applied Biosystems).

### Antibody Preparation and Immunoblotting

Purified sample was mixed with an equal volume of Freund's complete adjuvant (a total of 200 μl, Sigma) and injected into Balb/c mice. Two successive injections of antigens mixed with equal volumes of Freund's incomplete adjuvant (a total of 200 μl) were administered at 1-week intervals beginning 1 week after the first injection. Blood was collected 3 days after the last injection with antigens alone and centrifuged at 13,000 rpm for 5 min. The supernatant antibodies were stored at -70℃ until use. Electrophoretic transfers of proteins to nitrocellulose and immunoblot analysis were performed as previously described. (Towbin et al. 1979).

## Results and Discussion

### Peroxidase Activity

In order to understand the molecular events during callus formation, we investigated the activity of POD during different stages of callus formation in perilla (Fig. 1). Enzyme activity was measured after cotyledons of perilla were cultured on MS medium supplemented with 0.5 mg/ℓ of BA and NAA for 0, 3, 5 and 7 weeks, respectively. Our results demonstrated that the activity of POD gradually increased during the 0-7 week culture period. This result suggested that increased POD activity was associated with callus formation in perilla.

Peroxidase plays a role in the metabolism of hormones in plant. Additionally, peroxidases are among those enzymes that modify properties of the cell wall. This occurs because some

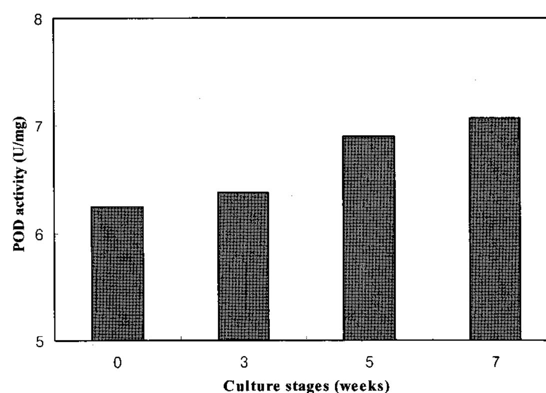


**Figure 1.** Changes in peroxidase activity extracted from perilla tissues cultured on MS medium at different culture stages. Enzyme activity was determined at 0, 3, 5 and 7 weeks after culture of perilla cotyledon.

of them are targeted to the cell wall and may catalyze the cross-linking of extensins and pectins. It is also accepted that the cross-linking of extracellular matrix proteins may be a protective mechanism in eukaryotic cells to cope with a variety of stress conditions (Bradley et al. 1992, Gaspar 1986). Thus, accumulation of $H_2O_2$ from an elicitor induced oxidative burst drives the peroxidase-mediated cross-linking of structural proteins in the cell wall. An extensive body of literature suggests that the involvement of certain peroxidases could be important during plant development and differentiation. Organ initiation and development in plant explants involves the promotion of meristematic activity and maintenance. Therefore, peroxidases that may be crucial for several important biological roles are not sufficiently understood.

## Purification of Peroxidase

The crude extract was precipitated with different concentrations of ammonium sulfate. The precipitate formed between 35% and 80% ammonium sulfate saturation recovered most peroxidase activity. Ammonium sulfate precipitation helped to improve peroxidase purification and concentrate the crude extract. This was done because defined peaks could not be obtained. After anion exchange chromatography, peroxidase was distributed into two peaks the first and second of which eluted with the salt gradient (Fig. 2A). Bound protein with activity fractions was eluted at 1 M NaCl in phosphate buffer pH 7.8. The fractions eluted with the salt gradient were pooled, concentrated, and further purified by gel filtration chromatography. Fig. 2A shows that peroxidase activities are mainly distributed in two peaks. When there is a charge asymmetry present, however, localization of charge clusters on the protein surface may sometimes allow its adsorption at or beyond its isoelectric point, regardless of the mobile-phase pH (Kopaciewicz et al. 1983). Hydrophobic interactions may partly contribute to this effect (Scopes 1994).

The retained pooled fractions were applied to a Sephacryl S-200 column. The elution profile (Fig. 2B) showed mainly one peak with peroxidase activity, which was labeled SP. Peak SP was a nonretained fraction, and this fraction had peroxidase activity. Gel filtration chromatography separated out contaminants. Wang and Luh (1983) reported *Rz* values for soluble and ionically bound asparagus peroxidases of 0.25 and 0.59, respectively, after ammonium sulfate precipitation and chromatographic steps with Sephacryl S-200. Anion exchange chromatography greatly improved the specific activity and purification factor.

## Peroxidase Characterization

The purified fractions were assayed for purity and molecular

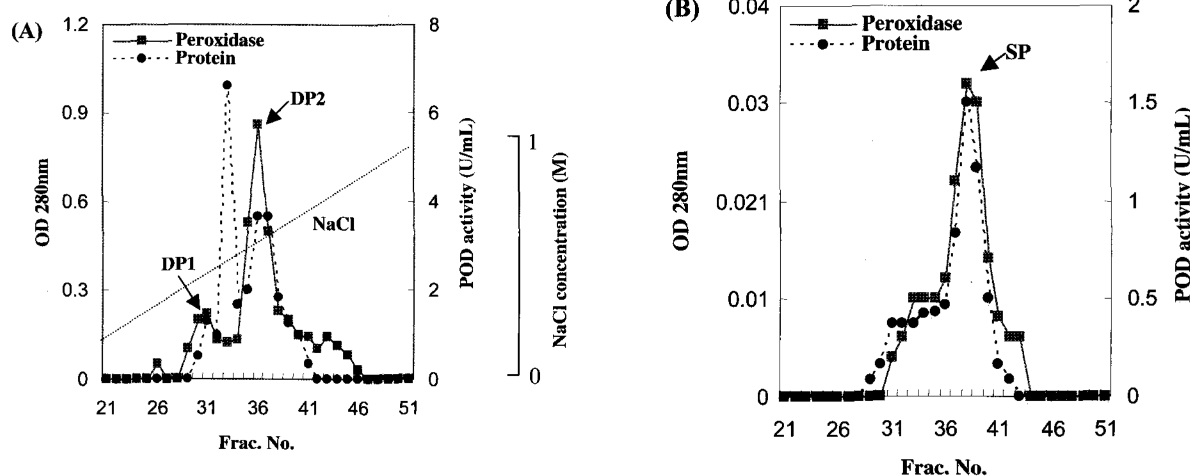


**Figure 2.** DEAE-cellulose (A) and Sephacryl S-200 (B) chromatography of perilla callus peroxidase after ammonium sulfate precipitation. The columns were equilibrated with 50 mM potassium phosphate buffer (pH 6.1). DP1, DP2 and SP are peroxidase activity peaks. Absorbance was measured at 280 nm, and peroxidase activity was assayed as described in the text.

weight using SDS-PAGE (Fig. 3). Fractions 38 and 39 were homogeneous as the Coomassie blue stained gel showed only one band for each fraction (Fig. 3, lanes 3 and 4). The molecular weights of the denatured peroxidases were 30 kDa. The molecular weight determined by SDS-PAGE in the presence of 2-mercaptoethanol was found to be very similar to that obtained under non-reducing conditions, indicating that fractions 38 and 39 contained single polypeptide proteins. According to Vamos-Vigiazo (1981), the molecular weight of peroxidases from a variety of fruits and vegetables ranges from 30 to 54 kDa, which is in agreement with the results obtained here. The molecular weights reported here are lower than those found for wheat germ peroxidase (35 kDa; Converso and Ferna´ndez, 1995), green asparagus peroxidase (34 kDa; Wang and Luh 1983), opuntia peroxidase (58 kDa; Padiglia et al. 1995) and pepper fruit acidic peroxidase (50 kDa; Pomar et al., 1997)

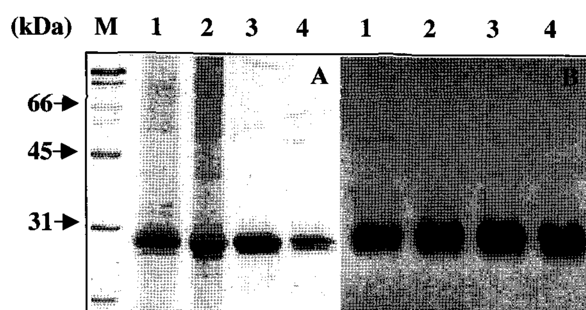


**Figure 3.** Electrophoretic pattern on polyacrylamide gels from protein and peroxidase peak activity fractions obtained by Sephacryl S-200. M, Molecular weight standard. Lane 1, Crude extract; Lane 2, Precipitate of 80% ammonium sulfate; Lane 3-4, fraction No. 38-39. Native-PAGE was performed with a 10% separating gel and 4% stacking gel in the Tris-glycine buffer system (A). The gel was stained to determine peroxidase activity (B).

## Amino Acid Sequence of the N-Terminal Region

The purified protein (SP) was electroblotted onto PVDF membrane and then the N-terminal amino acid region was sequenced. Figure 4 shows the N-terminal amino acid sequence data for 15 residues of perilla peroxidase, and how it compares with data available for peroxidases from other plants. The 15 amino acid sequences of the N-terminus were aligned with the sequences of peroxidases from tea, pea and spinach (Tanaka et al. 1991). The amino acid sequence of perilla peroxidase showed 67% identity to tea peroxidase, and low identity to other plant peroxidases. A multiple sequence alignment of the amino acid sequence demonstrated that Serine, Phenylalanine and Isoleucine residues of perilla peroxidase are highly conserved with other plant peroxidase sequences.

## Immunoblot Analysis

In order to further characterize the expression of protein during perilla tissue culture, the purified protein (SP) was electroeluted from the SDS-PAGE gel and used to produce an antibody in mice. Expression of protein during perilla tissue culture was confirmed by SDS-PAGE gel (Fig. 5A). The results showed that the banding pattern of the 30 kDa polypeptide from total soluble protein of perilla callus was increased following culture stages. Immunoblot analysis to detect the presence of perilla peroxidase was positive for the 30 kDa polypeptide after 3 to 7 weeks of perilla tissue culture, but negative for the perilla cotyledon protein (Fig. 5B). Immunoblot analysis indicated that the perilla peroxidase protein appeared after 3 weeks of perilla tissue culture, and continued to increase with extended duration of tissue culture

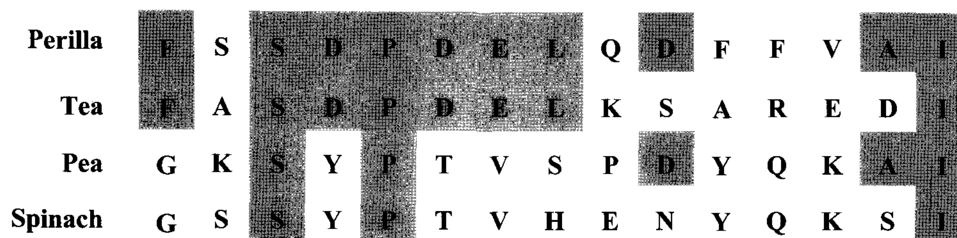


**Figure 4.** Alignment of the amino acid sequence of perilla peroxidase with corresponding sequences from the database. Highly conserved amino acids are shaded in black.

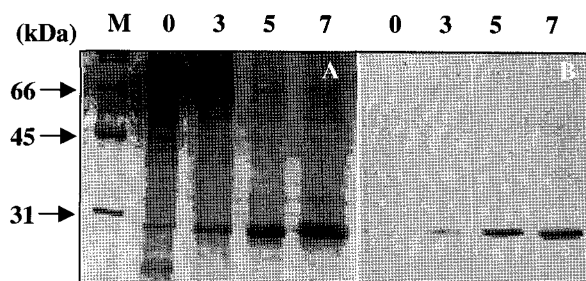


**Figure 5.** Immunoblot analysis of protein components from soluble protein isolated from perilla callus. Antibody was prepared from the purified single-band 30KD polypeptide (SP), which showed peroxidase activity. The same amount of protein (10$\mu$g) was applied to each lane for SDS-PAGE (A) and subsequent immunoblotting (B). The gel was electroblotted onto a nitrocellulose membrane and reacted with alkaline phosphatase- conjugated goat anti-mouse IgG. M, Molecular weight standard; 0-7, Perilla tissues cultured on MS medium for 0 to 7 weeks.

for at least 7 weeks. Peroxidase activity may play a major role in plant tissue necrosis and subsequent cell death during in vitro regeneration via somatic organogenesis in conifers (Wei and Ronald 2005). Cell death is correlated with elevated levels of peroxides. Polyphenols can be oxidized by air, peroxidase or polyphenoloxidase. Peroxidase and polyphenoloxidase have been associated with mechanical injury as well as many environmental stress responses such as radiation, desiccation, drought, wounding, extreme temperature and pathogen attack (Gadea et al. 1999, Tang 2000).

## Acknowledgements

This Paper was supported by the Dong-A University Research Fund in 2005.

## References

Adam W, Lazarus M, Saha-Moller CR, Weichold O, Hoch U, Haring D, Schreier P (1999) Biotransformations with peroxidases. AdV Biochem Eng Biotechnol 63: 73-108

Asada K (1992) Ascorbate peroxidase a hydrogen peroxide scavenging enzyme in plants. Physiol Plant 85: 235-241

Bollag DM., Michael D, Edelstein SJ (1996) Isoelectricfocusing. In Protein Methods. Wiley-Liss Publishing: New York

Bradley DJ, Kjellbom P and Lamb CJ (1992) Elicitor and wound induced oxidative cross linking of a praline rich plant cell wall protein: A novel, rapid defense response. Cell 70: 21-30

Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30: 987-998

Converso DA, Ferna´ndez ME (1995) Peroxidase isozymes from wheat germ: purification and propertie. Phytochemistry 40: 1341-1346

Cordewener JO, Booij H, Van der Zandt H, Van Engelen F,.Van Kammen A, de Vries S (1991) Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta 184: 478-486

Gadea J, Conejero V, Vera P (1999) Developmental regulation of a cytosolic ascorbate peroxidase gene from tomato plants. Mol Gen Genet 262: 212-219

Gaspar T (1986) In Molecular and Physiological Aspects of Plant Peroxidases (Greppin H, Penel C and Gaspar T, eds) University of Geneva, Geneva 455-468

Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. Plant Cell Physiol 42: 462-468

Kaiser WM (1979) Reersible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated intact chloroplasts by hydrogen peroxide. Planata 145: 377-382

Klotz KL, Lagrimini LM (1996) Phytohormone control of the tobacco anionic peroxidase promoter. Plant Mol Biol 31: 565-573

Kopaciewicz W, Rounds MA, Fausnaugh J, Regnier FE (1983) Retention model for high-performance ion-exchange chromatography. J Chromatogr 266: 3-21

Lu AT, Whitaker JR (1974) Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. J Food Sci 39: 1173-1178

Pomar F, Bernal MA, Merino F (1997) Purification, characterization and kinetic properties of pepper fruit acidic peroxidase. Phytochemistry 46: 1313-1317

Scopes RK (1994) Protein Purification. Principles and Practice; Springer-Verlag: New York 158.

Tanaka K, Takeuchi E, Kubo A, Sakaki T, Haraguchi K and Kawamura Y (1991) Two immunologically different isozymes of ascorbate peroxidase from spinach leaves. Arch Biochem Biophys 286: 371-375

Tang W (2000) Peroxidase activity of desiccation-tolerant loblolly

pine somatic embryos. In Vitro Cell Dev Biol Plant 36: 488-491 dehydration

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer to proteins from polyacrylamide gels to nitrocellulose sheets : Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354

Vamos-Vigiazo L (1981) Polyphenol oxidase and peroxidase in fruit and vegetables. Crit Rev Food Sci Nutr 45: 49-127

Veitch NC, Smith AT (2001) Horseradish peroxidase. AdV Inorg Chem. 51: 107-162.

Wang Z, Luh BS (1983) Characterization of soluble and bound peroxidase in green asparagus. J Food Sci 48: 1412-1417

Wei Tang, Ronald JN (2005) Peroxidase and catalase activities are involved in direct adventitious shoot formation induced by thidiazuron in eastern white pine (*Pinus strobus* L.) zygotic embryos. Plant Physiology and Biochemistry 43: 760-769

(Received October 26, 2007; Accepted Novermber 26, 2007)