Effect of Cadmium on Oxidative Stress and Activities of Antioxidant Enzymes in Tomato Seedlings

Cho, Un-Haing* and In-Taek Kim
Department of Biology, Changwon National University
Changwon City, Kyungsangnamdo, 641-773, Korea

ABSTRACT : Leaves of two-week old seedlings of tomato (*Lycopersicon esculentum*) were treated with various concentrations (0~100 M) of $CdCl_2$ for up to 9 days and subsequent growth of seedlings, symptoms of oxidative stress and isozyme activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) were investigated. Compared with the non-treated control, Cd exposure decreased biomass but increased Cd accumulation, hydrogen peroxide production and lipid peroxidation as malondialdehyde (MDA) formation in leaves and roots. Further studies on the developmental changes of isozyme activities showed that Fe-SOD, Cu/Zn-SOD and one of three APX isozymes decreased and CAT and one of four POX isozymes increased in leaves, whereas Fe-SOD, one of three POX isozymes and two of four APX isozymes decreased and CAT increased in roots, showing different expression of isozymes in leaves and roots with Cd exposure level and time. Based on our results, we suggest that the reduction of seedling growth by Cd exposure is the oxidative stress resulting from the over production of H_2O_2 and the insufficient activities of antioxidant enzymes particularly involved in the scavenging of H_2O_2. Further, the decreased activities of SOD and APX isozymes of chloroplast origin, the increased activities of CAT and POX and high H_2O_2 contents with Cd exposure might indicate that Cd-induced oxidative stress starts outside chloroplast.

Key words : Antioxidant enzymes, Cd Hydrogen peroxide, Oxidative stress

INTRODUCTION

Cadmium (Cd) is one of the most toxic metals in the present environment and is easily taken up by roots and translocated to different plant parts (Baker *et al.* 1994). The high Cd accumulation generally causes growth inhibition and even plant death due to the reduction of enzyme activity (Ouariti *et al.* 1997, Van Assche and Clijsters 1990), photosynthesis (Lee *et al.* 1976), transpiration (Barcelo and Poschenrieder 1990) and nutrient uptake (Sanita di Toppi and Gabbrielli 1999).

One of the biochemical changes occurring in plants subjected to various environmental stress conditions is the production of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide, singlet oxygen and hydroxyl radicals ($^{\cdot}OH$) (Iturbe-Ormaetxe *et al.* 1998, Cho and Park 2000). The ROS have a role in lipid peroxidation, membrane damage and consequently in plant senescence (Fridovich 1986, Thompson *et al.* 1987), and antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POX) and catalases (CAT) are involved in the scavenging of ROS (Asada 1992, Foyer 1993). SOD is a metalloprotein that catalyzes the dismutation of superoxide to H_2O_2 and molecular oxygen (Allen 1995). SOD enzymes are classified according to their metal cofactor and their subcellular localization. The predominant forms are a mitochondrial Mn-SOD, a cytosolic Cu/Zn-SOD, a chloroplastic Cu/Zn-SOD, and a chloroplastic Fe-SOD. Various antioxidant enzymes such as CAT and POX eliminate H_2O_2. CAT found predominantly in peroxisomes dismutase H_2O_2 into H_2O and O_2, whereas POX decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Sudhakar *et al.* 2001). Ascorbate peroxidase (APX) is primarily located in both chloroplasts and cytosol and eliminates peroxides by converting ascorbic acid to dehydroascorbate (Asada 1992). As a member of the ascorbic acid- glutathione cycle, APX is one of the most important enzymes playing a crucial role in eliminating toxic H_2O_2 from plant cells (Foyer *et al.* 1994).

The objective of present study is to investigate whether the Cd-induced phytotoxicity expressed as growth inhibition is mediated by the altered expression of antioxidant enzymes particularly involved in the scavenging of H_2O_2 and subsequent oxidative stress using tomato seedlings, which have been used as a research model to better understand metal uptake and metal-induced phytotoxicity (Ma-

*Corresponding author; Phone: 82-55-279-7445, e-mail: uhcho@sarim.changwon.ac.kr

zhoudi *et al.* 1997, Cho and Park 2000). Physiological and biochemical analyses including activities and isozyme analysis of various antioxidant enzymes will provide new insights into the processes of Cd toxicity in plants. This information is also pre-required to understand the uptake, persistence and distribution of Cd in plants. Further, the resistant plants capable of storing or detoxifying Cd and the susceptible plants might be used in the phytoremediation of Cd-contaminated environment and as a contamination indicator, respectively.

MATERIALS AND METHODS

Plant culture and treatments

Seedlings of tomato (*Lycopersicon esculentum* Mill cv. *Seokwang*) were germinated in pots containing perlite: vermiculite (1:1) mixture in a controlled environment chamber at 25℃ with 12 h of light (250μM $m^{-2}s^{-2}$) and 70~80% humidity. Two week-old seedlings were transferred to aerated nutrient solutions containing half-strength Hoagland solution (Hoagland and Arnon 1938) with 0, 10, 30, 50 and 100μM Cd (as $CdCl_2$). The solution was changed every 3 d. Plants collected from each treatment after 3, 6 and 9 days were dried for 48 h at 70℃ and weighed for biomass and Cd determination. For measurements of lipid peroxidation and isozyme analyses, fresh samples were weighed and used.

Cd content

Leaves were washed twice in deionized water, and the roots of intact plants were washed with ice-cold 5 mM $CaCl_2$ solution for 10 min to displace extracellular Cd (Rauser 1987). The plant material was dried for 48 h at 70℃, weighed and 1 g ground into fine powder before wet ashing in 5 ml of $HClO_4$: HNO_3 (1:4) solution for 24 h at 25℃. Cd was determined directly by atomic absorption spectrophotometry (Varian 200AA equipped with SIPS, Autralia) using an air-acetylene flame and Cd hollow-cathode lamp.

Analyses of H_2O_2 and lipid peroxidation

The H_2O_2 contents in leaves or roots were measured according to the modified method of KI/starch assay (Ros-Barcelo *et al.* 2002, Olson and Varner 1993). Five hundred milligrams of fresh leaves or roots were ground with 5 ml of the KI/starch reagent(Ros-Barcelo 1998) and incubated for 16 h at 25℃. The absorbance of the incubation medium was then measured at 572 nm before and after addition of 1 mg of ascorbic acid. Absorbance differences were proportional to the H_2O_2 concentration in the 10~50μM range and were used to estimate the H_2O_2 contents in extracts.

The level of lipid peroxidation in the leaves and roots was determined as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction described by Dhindsa *et al.* (1987). The concentration of MDA was calculated based on A_{532}-$A6_{00}$ (=155 mM^{-1} cm^{-1}).

Analyses of SOD, POX, CAT and APX isozymes

All leaf samples except for APX activity were ground with liquid nitrogen and homogenized with extraction buffer containing 10 mM potassium phosphate buffer (pH 7.8), 0.5% triton X-100 and 1.0% polyvynylpyrrolidone. For APX, leaves were homogenized and extracted according to the method of Lee and Lee (2000). The extracts were centrifuged at 12,000×g for 15 min, and protein concentration of the supernatant was determined using the Bradford dye-binding assay (Bradford 1976) with bovine serum albumin as a standard protein.

For SOD, the protein samples (50μg per lane) were separated by native PAGE on a separating gel of 12% (w/v) polyacrylamide with 5% (w/v) stacking gel in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine. After incubation for 30 min in 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM KCN (inhibitor of the Cu/Zn-SOD) or 2 mM H_2O_2 (inhibitor of the Fe-SOD and Cu/Zn-SOD) (Lee and Lee 2000), the gels were stained for 30 min in the dark using a 1:1 mixture of (a) 0.06 mM riboflavin and 0.651% (w/v) TEMED, and (b) 2.5 mM nitroblue tetrazolium (NBT), both in 50 mM phosphate buffer at pH 7.8 and developed for 20 min under light conditions (McKersie *et al.* 2000). After staining, the gels were photographed with a digital camera (Nikon Coolpix-990, Japan). For POX, CAT and APX, the protein samples (50μg per lane) were separated by native PAGE on a separating gels of 10% (w/v) polyacrylamide with 5% stacking gel in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine. For POX, the band formation due to the formation of oxidized amino-ethyl-carbazole during H_2O_2 breakdown by peroxidase was detected and photographed immediately (Manchenko 1994). Isozymes of APX were identified with the method described by Lee and Lee (2000) except the use of 12.5 mM NBT for 60 min instead of 2.45 mM for 10~20 min at the last procedure. For CAT, the gels separated by native PAGE were incubated in a staining solution described by Manchenko (1994).

Statistics

The data are the means±SE of three independent replicates. The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The mean differences were compared utilizing Duncans multiple range tests.

RESULTS AND DISCUSSION

Growth response and Cd accumulation

The effect of Cd on seedling growth expressed as dry weight and Cd accumulation is shown in Table 1. Compared with the non-treated control, Cd exposure with over 30μM induced a significant reduction of dry weight measured on day-9 after Cd treatment. The seedlings accumulated substantial amounts of Cd in the leaves and roots, and the accumulation increased concurrently with the treatments applied. The Cd content in leaves for 9 d was approximately 32% of that in roots. Therefore, roots accumulate much higher Cd than leaves. The consistent increase of Cd accumulation in leaves with prolonged treatments indicates that high Cd content in the root is not a limiting factor for the translocation of absorbed Cd during Cd exposure period investigated. The high Cd retention in roots might be due to cross-linking of Cd to carboxyl groups of the cell wall (Barcelo and Poschenrieder 1990) and /or to an interaction with thiol residues of soluble proteins of phytochelatins (Leita *et al.*, 1993). In leaves, Cd accumulation may be driven by active transpiration and the stem behaves as a cation exchange column towards the top of the plant (Hardiman and Jacoby 1984).

H_2O_2 production and lipid peroxidation

To know whether oxidative stress is involved in the reduction of seedling growth, H_2O_2 contents and lipid peroxidation in leaves and roots were investigated (Fig. 1). Subjecting tomato seedlings to over 30μM Cd for 6 days significantly increased the level of H_2O_2 in comparison with control plants. Although the mechanism of Cd-induced H_2O_2 formation is not known at present, heavy metals are known to be involved in many ways in the production of ROS (Halliwell and Gutterridge 1984), and the H_2O_2 accumulation caused by Cd exposure may occur in a manner similar to that in plants stressed (Prasad *et al.* 1994). It is conceivable to suppose that a decrease of enzymic and non-enzymic free radical scavengers caused by heavy metals (De Vos *et al.* 1993) may also contribute to the shift in the balance of free-radical metabolism towards H_2O_2 accumulation.

Measuring lipid peroxidation that is usually monitored by measuring MDA as the decomposed polyunsaturated fatty acids could assess oxidative damage (Dhindsa *et al.* 1976). MDA formation in seedling exposed to Cd was higher than in non-treated seedlings. Exposure to over 50μM Cd on day-6 and over 10μM Cd on day-9 caused lipid peroxidation to increase in both leaves and roots. A consistent increase in MDA level measured on day-6 and day-9 paralleled an increase of H_2O_2 levels.

The mechanism of lipid peroxidation induced by Cd exposure is not clearly understood. However, heavy metals are involved in many ways in the production of reactive oxygen species that induce peroxidation of membrane lipids (Halliwell and Gutteridge 1984). Since Cd is not an active transition metal as Cu is (De Vos *et al.* 1993), it may not directly generate toxic oxygen species (Ouariti *et al.* 1997). However, Cd enhance lipoxygenase activity (Somashekaraiah *et al.* 1992), and the products of the lipoxygenase reaction, mainly peroxy, alkoxy and hydroxyl radicals, are themselves reactive and result in further membrane lipid deterioration leading to membrane permeability (De Vos *et al.* 1991). H_2O_2 and O_2^- may interact in the presence of metal ions or metal chelates to produce highly reactive hydroxyl radicals ($^{\cdot}OH$). The increased H_2O_2 or other ROS production might be involved in the lipid peroxidation. Further, since Cd exposure has increased MDA formation, the observed changes in the biomass of tomato seedlings might at least

Table 1. Dry weights of seedlings and Cd contents in leaves and roots of seedlings exposed to various concentrations of Cd for up to 9 days. Values are means SE of three independent replicates. Values in a column followed by the same letter are not significantly different at the 0.05 levels according to Duncans multiple range tests.

Cd μ(M)	Dry weight (mg/plant)	Cd (μg/g dry weight)	
		Leaf	Root
0	33.752±2.43a	0.06±0.05a	0.08±0.06a
10	32.40±3.09a	1 6.69±1.08a	165.44±5.86a
30	29.10±0.20b	49.45±6.01c	213.5±28.00c
50	28.10±2.03b	83.93±2.60d	322.83±30.92d
100	18.18±0.86c	142.67±7.14e	441.97±51.06e

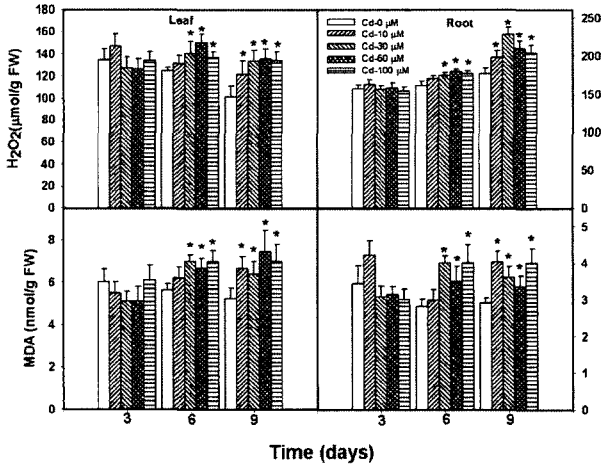

Fig. 1. Changes in H_2O_2 levels and lipid peroxidation in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are means±SE of three independent replicates. *Significant at the 0.05 levels compared with non-treated control according to *F*-test.

be due to the tissue or cell damage as indicated by lipid peroxidation. Destruction of lipid components of membrane by lipid peroxidation causes membrane impairment and leakage (Thompson *et al.* 1987).

Isozyme activities of SOD, POX, CAT and APX

Cd exposure also altered the activities of antioxidant enzymes including SOD, CAT, POX and APX (Figs. 2~5). Compared with the non-treated control, the isozyme activities of antioxidant enzymes particularly involved in the scavenging of ROS and H_2O_2 were selectively modified with Cd exposure.

Using KCN to inhibit Cu/Zn-SOD or H_2O_2 to inactivate both Cu/Zn-SOD and Fe-SOD, three types of SOD isozymes (Mn-SOD, Fe-SOD and Cu/Zn-SOD) were identified (Fig. 2). When the activity of each isozyme of SOD was analyzed on native PAGE gels by comparing the intensity of SOD activity in the leaf and root extracts relative to a known standard (*E. coli* Fe-SOD), Cd application caused a decrease of both Fe-SOD and Cu/Zn-SOD in leaves and Fe-SOD in roots. Although the origins of Cu/Zn-SOD and Fe-SOD observed on activity gel are not known, the low activities of both isozymes in root might indicate that the isozymes were originated mostly from chloroplasts. In plants, Cu/Zn-SOD is an isoform with the highest induction level in response to various stimuli (Mittler and Zilinskas 1994, Kaminaka *et al.* 1999), and the chloroplastic CuZn-SOD could contribute to O_2^- elimination in the chloroplast (Polle 1997). The Mn-SOD activity detected in this experiment was not superior to the activities of Fe-SOD and Cu/Zn-SOD. Similar results have been observed in some plant species (Bowler *et al.* 1992). The lower activity might indicate that mitochondria where Mn-SOD isoforms could be related to O_2^- generation by the electron transport chain (Borsani *et al.* 2001) was less affected by Cd exposure.

Meanwhile, the decrease of SOD activity might be due to the damage of chloroplast and plastid or metabolic alterations connected with oxidative stress including lipid peroxidation, and both Fe-SOD and Cu/Zn-SOD might be the enzymes mainly affected by Cd exposure. In conclusion, the enhancement of H_2O_2 with Cd treatments (Table 1) could not be ascribed to expression of SOD.

Among four POX isozymes (POX-1, 2, 3 and 4) in leaves and three isozymes (POX-1, 2 and 3) in roots identified on the activity gels, POX-2 and POX-3 increased in leaves but POX-2 decreased in roots with Cd exposure (Fig. 2). Although the increased H_2O_2 was presumed to contribute to the overall increase of POX activity, the roles of POX isozymes were not known at this time. However, the high expression of POX-2 in both leaves and roots might indicate that the isozyme was originated mostly from outside chloroplast. The relative distribution of peroxidase might be involved in the removal of H_2O_2 as well as lignification of tissues (Otter and Polle 1994). Although the peroxidase reaction is unspecific towards the type of stress (Radotic *et al.* 2000), the capacity to synthesize peroxidases in leaves was used as a parameter for monitoring and mapping the defense of pollution (Keller 1974).

Cd exposure also increased CAT activity in both leaves and roots (Fig. 3), and the increase might be due to the increased H_2O_2 contents. Since CAT is found predominantly in peroxisomes (Sudhakar *et al.* 2001), the increased H_2O_2 was presumably originated from outside chloroplast and supported that oxidative stress might start outside chloroplast.

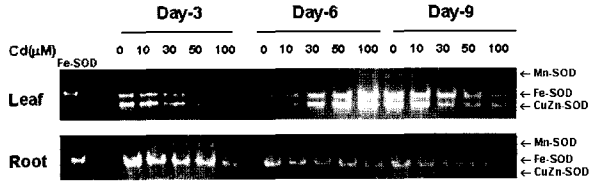

Fig. 2. Native PAGE of leaf and root extracts (50μg protein per lane) after SOD activity staining. Electrophoresis was conducted on a separating gel of 12% (w/v) polyacrylamide with 5% stacking gel in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine at 4C, 20 mA for 2 h. After PAGE, the gel was stained for 30 min in the dark using a 1:1 mixture of (a) 0.06 mM riboflavin and 0.651% (w/v) TEMED, and (b) 2.5 mM nitroblue tetrazolium (NBT), both in 50 mM phosphate buffer at pH 7.8 and developed for 20 min under light conditions. Isozyme activities are shown relative to 2 U of E. coli Fe-SOD on the same native PAGE gel.

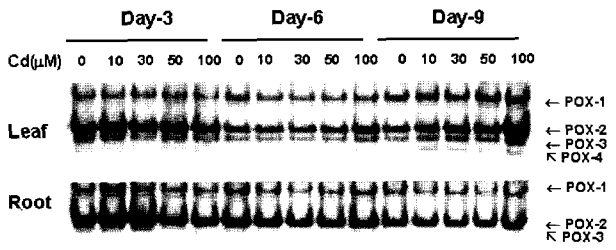

Fig.3. Native PAGE of leaf and root extracts (50μg protein per lane) after POX activity staining. Electrophoresis was conducted on a separating gel of 10% (w/v) polyacrylamide with 5% stacking gel in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine at 4C, 30 mA for 1.5 h. After PAGE, the gel was incubated in a staining solution containing 100 ml of 50 mM sodium acetate (pH 5.0), 50 mg of 3-amino-9-ethyl-carbazole and 0.75 ml of 3% H_2O_2 without light at room temperature until red-brown bands appeared, rinsed with water and fixed in 50% glycerol.

As a member of the ascorbic acid-glutathione cycle, APX is one of the most important enzymes playing a crucial role in eliminating toxic H_2O_2 from plant cells (Asada 1992, Foyer *et al.* 1994). When APX isozymes in leaves and roots were analyzed on the activity gel (Fig. 4), two isozymes (APX-2 and 3) among three isozymes (APX-1, 2 and 3) observed in leaves and two isozymes (APX-1 and 2) among four isozymes (APX-1, 2, 3 and 4) observed in roots decreased with Cd exposure except on day-6 in leaves. Further, high activity in leaves but low activity of APX-3 in roots implied that the isozyme was originated from chloroplast. The decreased activities of APX isozymes might be related to the localization of APX-2 and -3 in chloroplast or plastid where the decreased activity of Fe-SOD and CuZn-SOD, an indicative of the decreased production of H_2O_2, were observed (Fig. 2). Meanwhile, catalase is more accessibility to Cd-induced H_2O_2 outside chloroplast.

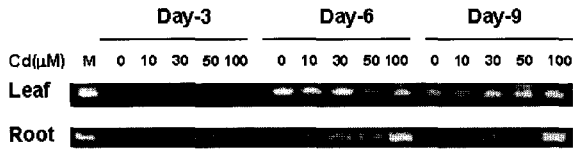

Fig. 4. Native PAGE of leaf and root extracts (50μg protein per lane) after CAT activity staining. Electrophoresis was conducted on a separating gel of 10% (w/v) polyacrylamide with 5% stacking gel in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine at 4C, 30 mA for 2 h. After PAGE, the gel was incubated in a solution containing 0.03% H_2O_2 for 15 min, rinsed with water and incubated in a 1:1 mixture of 2% potassium ferricyanide and 2% ferric chloride for 5 min. Activities are shown relative to 2 U of CAT from cow peroxisome on the same native PAGE gel. M : CAT from cow peroxisome.

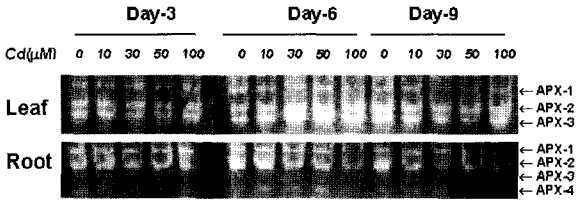

Fig. 5. Native PAGE of leaf and root extracts (50μg protein per lane) after APX activity staining. After pre-run of the gel in a tank buffer for 30 min, samples were loaded and electrophoresis was conducted on a separating gel of 10% (w/v) polyacrylamide with 5% stacking gel in a tank buffer containing 25 mM Tris (pH 8.3), 2 mM ascorbic acid and 192 mM glycine at 4C, 30 mA for 1.5 h. After PAGE, the gel equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min was incubated in a solution containing 50 mM sodium phosphate (pH 7.0), 4 mM ascorbic acid and 2 mM H_2O_2 for 20 min. The gel was washed in the electrode buffer for 1 min and was submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 20 min with gentle agitation

Changes of isozyme activities observed in this research indicate that over production of H_2O_2 to induce oxidative stress might first occur outside chloroplast and the activities of antioxidant enzymes might not be sufficient to remove ROS including H_2O_2. Cd exposure might enhance ROS production and disturb cytosolic metabolism (Sanita di Toppi and Gabbrielli 1999) before further damage to chloroplast and mitochondria. ROS reactions have been suggested to play an important role in the degradation process of membrane (Thompson *et al.* 1987). Since SOD located in various compartments is a major scavenger of O_2^- radicals and catalyzes the disproportionate of two O_2^- radicals to H_2O_2 and O_2 (Salin 1987), the observed decrease in SOD activity after Cd exposure could decrease the ability of the seedlings to scavenge O_2^- radicals that is highly reactive, forming hydroperoxides with enes and dienes (Salin 1987, Halliwell and Gutteridge 1989). Meanwhile, high H_2O_2 content but insufficient expression of scavenging enzymes with Cd exposure might induce oxidative stress first in cytosol. The increased lipid peroxidation is probably due to the harmful effect of over production of H_2O_2 or its poisonous ROS derivatives (Bowler *et al.* 1992). Excessive levels of ROS result in damage to the cell organelles including photosynthetic or respiratory apparatus, ultimately leading to severe cellular damage and chlorosis of leaves. H_2O_2 itself is a powerful inhibitor of metabolism including carbon fixation (Kaiser 1976). Further, oxidation-reduction of metal ions by H_2O_2 and O_2^- through Haber-Weiss reaction produces the most toxic hydroxyl radical (·OH) (Imlay and Linn 1988, Halliwell and Gutteridge 1989).

However, further researches on the specific distributions, identification and roles of the isozymes and the potential for ROS production in different organelles are required to explain the distinct regulatory mechanisms of antioxidant enzymes.

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