CHARACTERIZATION OF SUPEROXIDE DISMUTASE IN ARABIDOPSIS

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Abstract: Superoxide dismutase (SOD: EC 1.15.1.1) from different plant parts of Arabidopsis and paraquat-treated leaves were characterized using polyacrylamide gel electrophoresis. Five different forms of SOD were identified by their sensitivity to cyanide and hydrogen peroxide. The molecular weight of Mn-SOD, Fe-SOD and CuZn-SODs was estimated to be 80KD, 50KD, 35KD, 25KD, 20KD and 15KD, respectively. A similar pattern of Mn-, Fe- and CuZn-SOD was observed in all plant parts. The pIs of the SODs were determined to be 3.8, 4.5, 4.8 and 5.35 by isoelectric focusing. CuZn-SODs contributed the major activity of total SOD. Only the CuZn-SODIII activity showed an apparent increase following the Arabidopsis plants treated with paraquat.

INTRODUCTION

Superoxide dismutase (EC 1.15.1.1) catalyzes the dismutation of superoxide radical (O$_2^-$) to molecular oxygen and hydrogen peroxide, and is considered a major enzymatic defense against O$_2^-$ radicals (Halliwell, 1978). The unstable superoxide anions are formed in biological systems through autoxidations, enzymatic reactions, or leakage from electron transport chains (Elstner, 1987; Fridovich, 1986; Halliwell, 1987); and subsequently these anions cause a deleterious oxidation of lipids, proteins and nucleic acids. Finally, they may seriously disturb normal cell metabolism. SODs distribute ubiquitously among the aerobic organisms and seem to be lacking in the obligate anaerobes (McCord et al., 1971).

Because of the important role of SOD in biological systems, it has stimulated interests in utilizing the enzyme as a privileged object and tool for various studies including protein structure, gene expression, catalytic mechanisms, molecular evolution, cell biology, pharmacology, and medicine (Rotilio, 1986). SOD has also been considered as a biochemical marker for detecting plant stress imposed by ozone injury (Lee and Bennett, 1982), SO$_2$ toxicity (Tanaka and Sugahara, 1980; Tanaka et al., 1982) and oxygen toxicity (Rabinovich and Fridovich, 1983). Studies with “engineered” prokaryotic and eukaryotic systems have been helpful in elucidating the relationship between SOD and stress (Scott et al., 1987; Seto et al., 1990; Tepperman and Dunsmuir, 1990; Pitcher et al., 1991).

SODs are a group of metalloproteins, and three distinct types of SODs containing Mn, Fe or Cu plus Zn as prosthetic metals have been described (Fridovich, 1975; Jackson et al., 1978). Each type of SOD contained multiple forms.

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CuZn-SODs are sensitive to inhibition by CN⁻ and H₂O₂ whereas Mn-SODs and Fe-
SODs are CN⁻-insensitive. Mn-SODs are also insensitive to inhibition by H₂O₂
(Fridovich, 1975). This selective inhibition by CN⁻ and H₂O₂ makes it possible to
distinguish the three types of SOD in the crude homogenates (Droillard et al., 1989).
Studies of the SOD patterns in plants, mammals and fungi indicated that CuZn-
SOD is usually the major type of SOD (Bridges and Salin, 1981; Rotilio, 1986).

*Arabidopsis* is an ideal model plant, because of the small genome size, short life
cycle, small plant size, and the abundant information related to its genetics (Estelle
and Somerville, 1986), biochemistry (Bowman et al., 1988) and molecular biology
(Pang and Meyerowitz, 1987; Somerville, 1989). As a first step to adapt the
*Arabidopsis* system for studying the relationship between SOD and stress and to select
the plant with a high SOD level, characteristics of SOD and its isozyme pattern from
different organs and stages need to be investigated, although some studies regarding
SOD purification and characterization in several plants have been reported
(Kanematsu and Asada, 1989; 1990). In this study, efforts were made to describe the
isozyme pattern of SOD from the normal and paraquat-treated *Arabidopsis*.

**MATERIALS AND METHODS**

The seeds from Columbia wild type of *Arabidopsis thaliana* (L.) Heynh. were
planted in a 1:1:1 mixture of perlite/vermiculite/sphagnum, grown in a growth
chamber at 25/22°C (day/night) with a 12-h photoperiod. The mineral nutrient
solution was supplied regularly (Somerville and Ogren, 1982). Leaves, stems,
flowers and siliques were harvested and weighted after two-month growth. 0.1
g of tissue sample was collected, frozen with liquid nitrogen, and stored at -20°C
in a freezer until use. The tissue was ground in 0.15 M Tris-HCl buffer (pH 7.2)
and centrifuged at 10,000 g for 15 min. The supernatant was used for isozyme
analysis by electrophoresis.

A linear gradient or non-gradient native polyacrylamide gel electrophoresis
(PAGE) was performed with the standard tris-glycine system (Cooper, 1977)
using various gradient acrylamide gel slabs having 1.5 mm thickness. Molecular
weight was determined by using 7-12.5% gradient gel (Lambin and Fine, 1979).
The protein standards were: thyroglobulin, 669,000; ferritin, 440,000; catalase,
232,000; lactate dehydrogenase, 140,000; phosphorylase b, 94,000; albumin, 67,000;
ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100 and
α-lactalbumin, 14,400. After electrophoresis, the SOD activity was identified
by incubating the gel in 2.45 mM nitro blue tetrazolium (NBT) solution for 20 min
in the dark. Subsequently, the gel was washed with distilled water and soaked
in 50 mM sodium-phosphate buffer (pH 7.0) containing 2.8×10⁻⁵ M riboflavin and
2.8×10⁻² M N,N,N',N'-tetramethylethylenediamine (TEMED) until the activity bands
appeared (Beauchamp and Fridovich, 1971). In order to identify the CuZn-SOD,
freshly prepared 5 mM KCN was added to the riboflavin/TEMED/phosphate
buffer staining system. For the hydrogen peroxide treatment, gels were soaked
in 50 mM Na-phosphate buffer (pH 7.0) containing 5 mM hydrogen peroxide for 30 min. After washing with distilled water to remove the H$_2$O$_2$ residue, SOD activity was visualized according to the procedure described previously (Pan and Yau, 1991).

The pI of SOD was determined by isoelectric focusing (IEF). The crude extract of the sample was desalted (Helmerhorst and Stokes, 1980) before IEF. Horizontal IEF was performed on the 5% SERVALYT precoated ultrathin polyacrylamide gels (125 × 125 × 0.15 mm) containing SERVALYT carrier ampholytes (pH 3-10). IEF was carried out at 10°C by the Multiphor II system apparatus. Focusing was conducted at a series voltage of 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1100V with each voltage running for at least 30 min. Samples were applied to paper wicks (4 × 10 mm) which were placed toward the cathod. Electrode buffer was applied to the electrode strips with 0.1 ml/cm. The anode electrode buffer contained 0.33% L-aspartic acid, 0.37% L-glutamic acid while the cathod electrode buffer contained 0.4% arginine, 0.06% L-lysine, and 12% ethylene diamine. After IEF, the gel was stained for SOD activity. Glycan Detection Kit was purchased from Boehringer Mannheim.

For evaluating paraquat (1,1’-dimethyl-4,4’-bipyridinium) effect on SOD activity, one-month-old Arabidopsis plants were sprayed with paraquat solution (0.1-40 ppm) and several leaves were collected after 42 h of treatment. In a similar experiment, the 1.0 ppm paraquat-treated plants were collected after treatment for 1 to 4 days. The collected tissues were ground in 0.15 M Tris-HCl buffer (pH 7.2), centrifuged at 10,000 g, and loaded to a 12.5-15% gradient gel. After electrophoresis, SOD activity staining was performed (Pan and Yau, 1991).

RESULTS AND DISCUSSION

Three major and two minor bands of SOD activity were detected from the leaf extract of Arabidopsis after separation by a linear gradient of native gel (Fig. 1). The upper major band, being insensitive to both cyanide and hydrogen peroxide, was identified as Mn-SOD. The other two major and one minor SOD activity bands that migrated faster were sensitive to cyanide and hydrogen peroxide, indicating that they were CuZn-SODs. These isozymes were named CuZn-SODI, CuZn-SODII, and CuZn-SODIII. One very faint band, which was sensitive to H$_2$O$_2$ inhibition but not inhibited by CN$,^-$, was Fe-DOS (Fig. 1). The SOD isozyme pattern was similar in all plant parts (Fig. 2). Similar results were reported in rice during seed germination and development (Pan and Yau, 1991) and in Ganoelderma tsugae during fruiting body formation (Chao, 1991). The CuZn-SOD activity was predominant in all parts of Arabidopsis. This result is consistent with previous studies in rice (Pan and Yau, 1991) and other higher plants (Bridges and Salin, 1981). When the isozyme activity was expressed on a fresh weight basis, the reproductive organ contained a much higher Fe-SOD activity than those of other vegetative tissues. One form of Fe-SOD was present in Arabidopsis (Fig. 2). This was in agreement with the result from several Cruciferae plants reported previously (Bridges and Salin, 1981). All of them had only one form of Fe-SOD.
Mn-SOD activity, showing the slowest-moving band in gradient gels, indicated that Mn-SOD of Arabidopsis had the highest MW. of SODs. The molecular weights of the Mn-, Fe-SOD, CuZn-SODI, CuZn-SODII and CuZn-SODIII in the leaves of Arabidopsis were determined to be 80KD, 50KD, 35KD, 25KD and 20K, respectively (Fig. 3). The molecular weight of Mn-SOD (80KD) in Arabidopsis was similar to that of Mn-SOD prepared from carnation petals (Droillard and Paulin, 1990) and chicken liver (Weisiger and Fridovich, 1973). Also, this was consistent with that deduced from the nucleotide sequence analysis of a cDNA clone from maize (White and Scandalios, 1988) and yeast (Marres et al., 1985). In addition, the Fe-SOD in the leaves of Arabidopsis with a molecular weight of about 50kD is similar to that of carnation (Droillard and Paulin, 1990) and ginkgo (Duke and Salin, 1985); the MWs of CuZn-SODs of Arabidopsis were in the the range of 20-35KD, which were also reported in rice, fern, tomato, wheat and maize (Yau et al., 1991). In this study, we also found similar characteristics of SODs in the leaf and floral parts of six other plants: Hibiscus rosa-sinensis, Thunbergia aracta, Ixeris laevigata, Mazus faurei, Impatiens balsama and Lantana camara (data not shown).
Fig. 3. Estimation of molecular weights of the SOD isozymes on a 7-12.5% gradient gel.
Lane 1: extract from leaves; lanes 2 and 3 were loaded with the low and high molecular weight standards, respectively; the protein standards were: thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; lactate dehydrogenase, 140,000; phosphorylase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100 and α-lactalbumin, 14,400.

After the crude extract of Arabidopsis was desalted, the SOD activity appeared in four sharp bands on the IEF gel. The pIs of SOD isozymes in Arabidopsis were 3.8, 4.5, 4.8 and 5.35, respectively (Fig. 4). These pIs were comparable to those described in other plants (Duke and Salin, 1985; Kanematsu and Asada, 1989; 1990; Droillard and Paulin, 1990; Yau et al., 1991). We also measured the pIs of SOD from several other plants. Sorghum isozymes had the pIs at pH 3.8, 4.5, and 4.8; and horseradish had the pIs at pH 3.8, 4.2 and 4.4 (data not shown). They all showed the major SOD activity having a pI at pH 3.8. The SODs of Arabidopsis as well as those of rice (Pan and Yau, 1991) and Ganoderma tsugae (Chao, 1991) were also found not to be a glycoprotein.

Paraquat is a potent herbicide that disrupts photosynthetic electron transfer by accepting electrons from photosystem I. And, the paraquat radicals may react with molecular oxygen to produce superoxide radicals and eventually to form the far more damaging hydroxyl radicals. In addition, this herbicide disrupts cellular membranes. Induction of SOD by paraquat was reported with E. coli (Hassan and Fridovich, 1977). Some paraquat resistant biotype plants were shown to be resistant to several oxidative stress, which correlated with an increased level of the enzymes that detoxify active oxygen species (Harper and Harvey, 1978). In
contrast, it was reported that the paraquat-resistant biotype of some weed species was not more tolerant to photoinhibition than the control (Preston et al., 1991). Differential regulations of superoxide dismutases in plants exposed to environmental stress have been demonstrated (Tsang et al., 1991). Attempts were made in this study to observe the SOD activity in the paraquat-treated *Arabidopsis* plants. When paraquat solution (40 ppm) was sprayed to *Arabidopsis* plants, the withered plant showed an increased activity of CuZn-SODIII, but other SOD activity obviously all decreased (Fig. 5A). When sprayed with 10-25 ppm paraquat solution, *Arabidopsis* plants withered severely within one or two days. The older *Arabidopsis* plants showed more susceptibility to paraquat treatment, while the younger plants took more time for withering symptoms to appear. When 1.0 ppm paraquat solution was sprayed to plants for several days, higher CuZn-SODIII activity was shown in the healthy young plants (Fig. 5B), but not observed in the older plants.

In summary, the SOD activity and isozyme pattern were similar in all tissues and organs of *Arabidopsis* and the activity was stable during development. The CuZn-SODIII activity increased in the paraquat-treated plants, while other SOD activity decreased apparently, indicating that CuZn-SODIII was responsive to the environmental stress.

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**Fig. 4.** Isoelectric focusing of *Arabidopsis* superoxide dismutase on Servalyt-precoce pH 3 to 10.

Lane 1: pi standard proteins: from top to bottom, amylglucosidase, pi=3.50; soybean trysin inhibitor, pi=4.55; β-lactoglobulin A, pi=5.2; carbonic anhydrase II, pi=5.4; bovine carionic anhydrase B, pi=5.85; human carionic anhydrase, pi=6.55; horse myoglobin-acidic component, pi=6.85; horse myoglobin-basic component, pi=7.35; lentil lectin-acid component, pi=8.15; lentil lectin-middle component, pi=8.45; lentil lectin-basic component, pi=8.65; trysinogen, pi=9.30. Proteins were stained by coomassie brilliant blue. Lane 2: extract of *Arabidopsis* leaves. Arrows indicat the activity of SOD isozymes.
Fig. 5. The effect of paraquat on the SOD isozymes in Arabidopsis leaves. Electrophoresis was performed on a 12.5-15% gradient gel. A: Plants were sprayed with 40 ppm paraquat for one day. B: plants were sprayed with 1 ppm paraquat for several days. Lane 1: control, lane 2: treated leaves. An amount of extract equivalent to the same amount of fresh weight of leaves was loaded in each lane. Arrows indicate the activity of CuZn-SODIII.

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LITERATURE CITED


阿拉伯芥超氧歧化酶的研究

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摘要

以凝胶電泳法分析阿拉伯芥不同生長階段的葉片、茎、花及以 paraquat 處理過植株之超氧歧化酶 (SOD)。並以 CN⁻ 和 H₂O₂ 對超氧歧化酶不同程度之抑制作用來鑑定其同功酶。阿拉伯芥不同年齡的葉片及不同器官之 SOD 同功酶形式相似，都具有 Mn-SOD, Fe-SOD 和 CuZn-SOD，其分子量分別為 80KD, 50KD, 35KD, 25KD 及 20KD；等電點為 pH 3.8, 4.5, 4.8 及 5.35。CuZn-SOD 爲阿拉伯芥超氧歧化酶中活性最大之一種，不同生長階段的葉片、茎，花之活性雖沒有明顯變化；然而 paraquat 處理過之植株 CuZn-SOD III 活性則明顯增加。