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 (Rabinowich 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 H_2O_2 wherase Mn-SODs and Fe-SODs are CN^--insensitive. Mn-SODs are also insensitive to inhibition by H_2O_2 (Fridovich, 1975). This selective inhibition by CN^- and H_2O_2 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 electroiphoresis.

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_2O_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 CuZu-SODI, CuZn-SODII, and CuZn-SODIII. One very faint band, which was sensitive to H_2O_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 *Ganoderma 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.
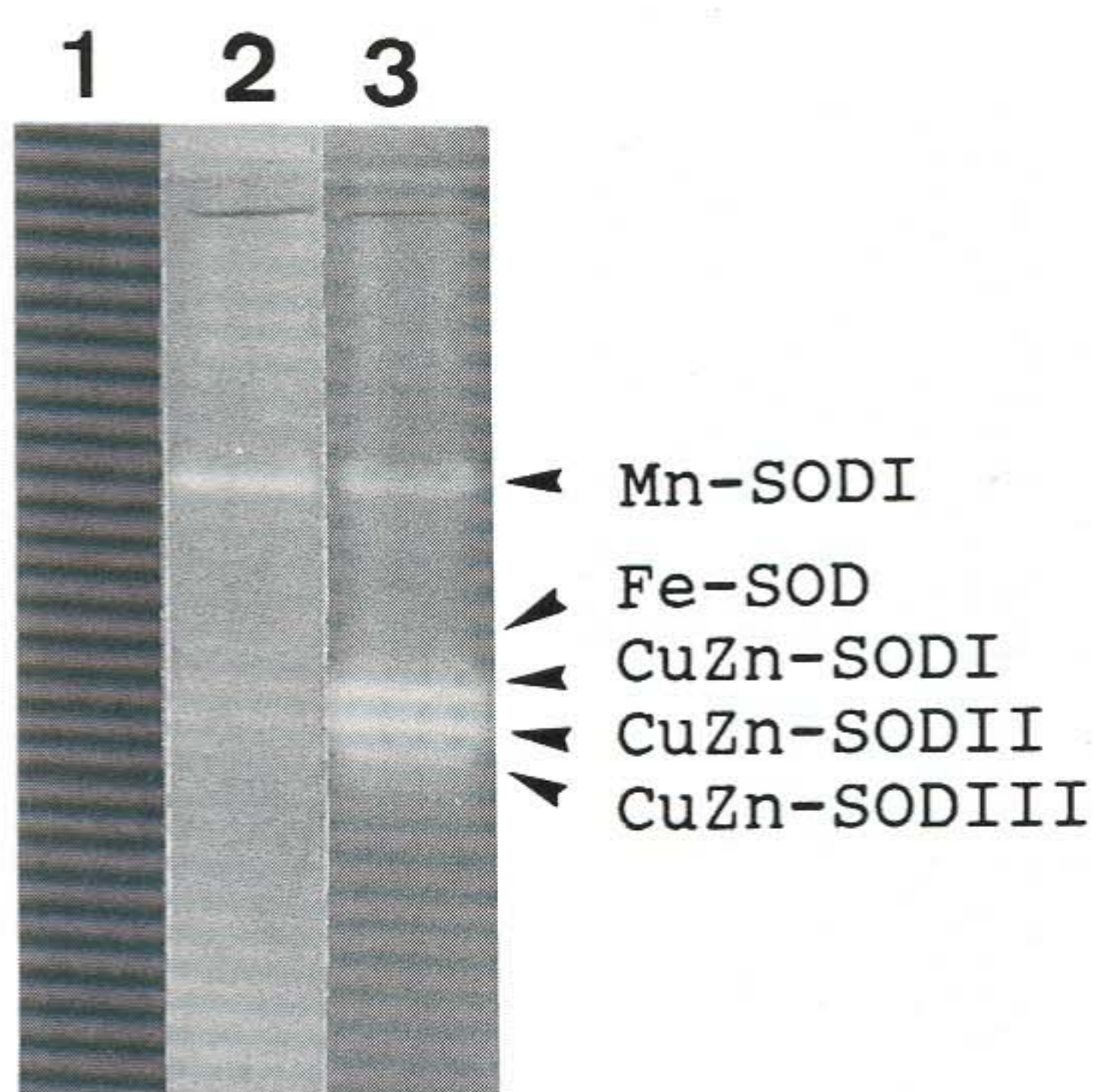
Fig. 1. The SOD isozymes in the leaves of *Arabidopsis*.
After electrophoresis on the 12.5-15% gradient gel, the gel was treated with 5mM $H_2 O_2$ (lane 1) or 5mM KCN (lane 2). Lane 3 was the control. Each lane was loaded with an amount of extract equivalent to the same amount of fresh weight of tissue.
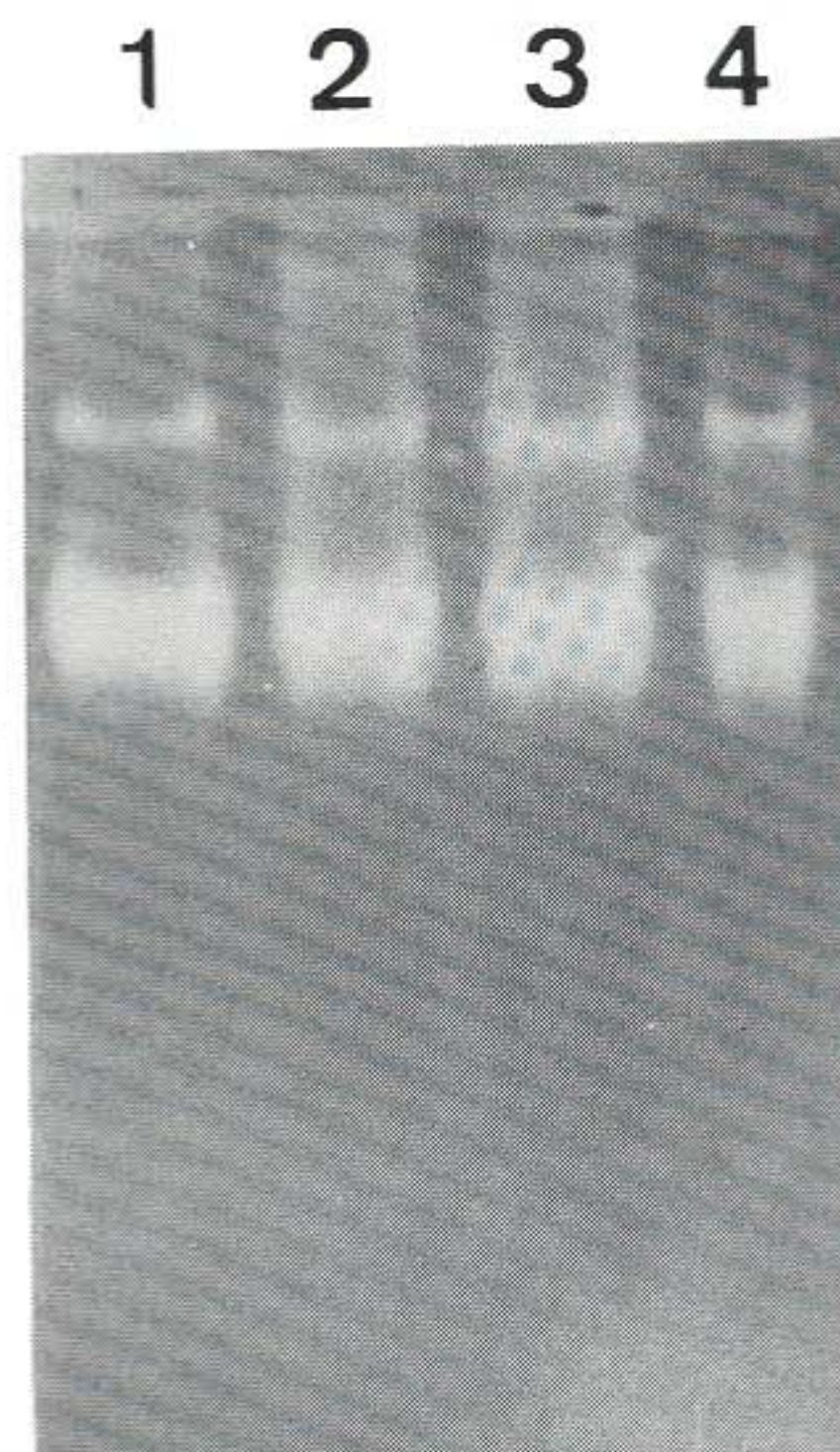

Fig. 2. The SOD isozyme activity in the various tissues of *Arabidopsis*. Electrophoresis was performed on the 15-30% gradient gel. Lane 1: floral parts; lane 2: siliques; lane 3: leaves; lane 4: stems. Each lane was loaded with an amount of extract equivalent to the same amount of fresh weight of tissue.

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 arecta*, *Ixeris laevigata*, *Mazus faurei*, *Impatiens balsama* and *Lantana camara* (data not shown).
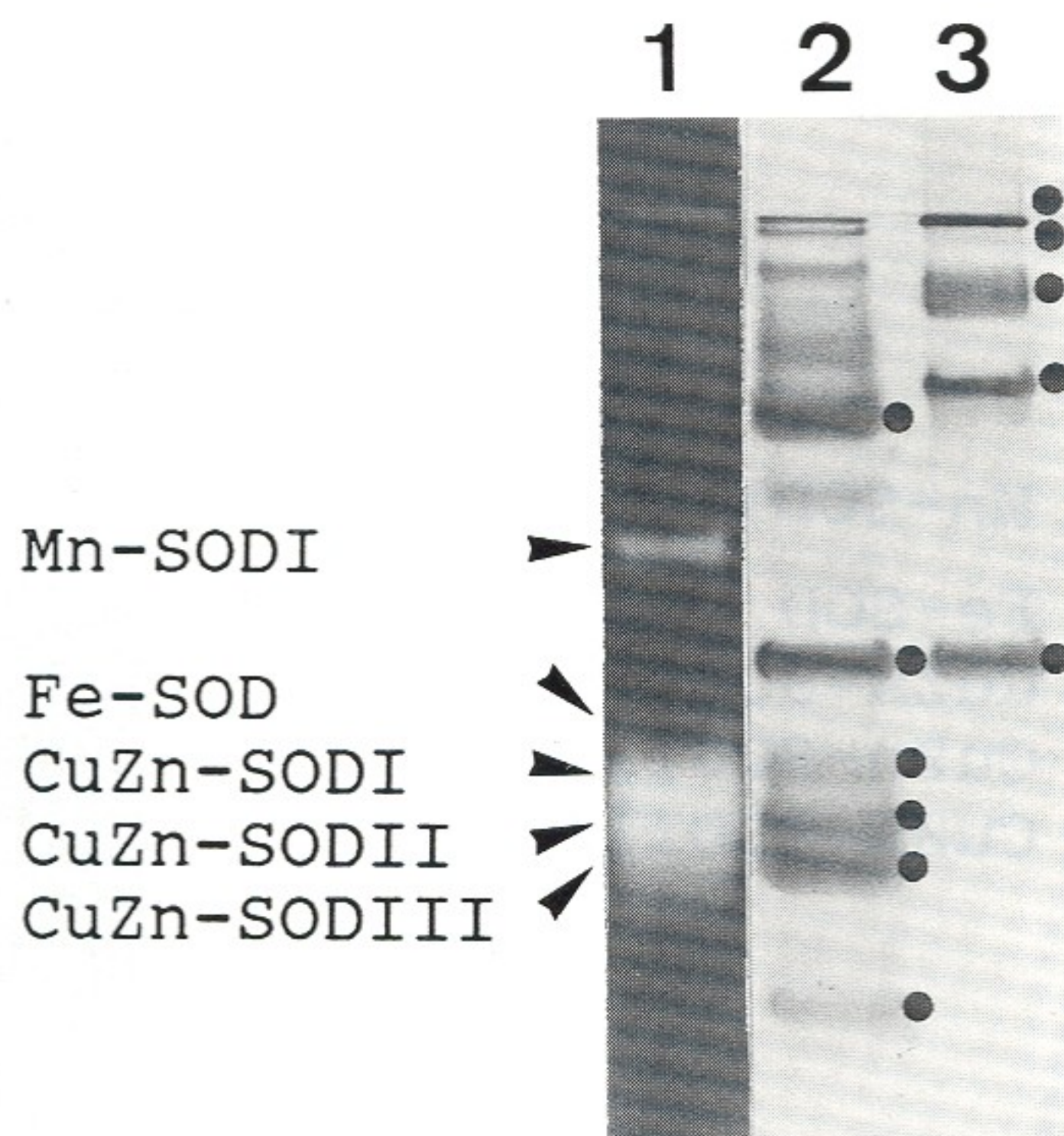
Fig. 3. Estimation of molecular weights of the SOD isozymes on a 7-12.5%
gradient gel.
Lane 1: extract from leaves; lanes 2 and 3 were loaded with the low
and high molecular weight standards, respectively; the protein standards
were: thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; lactate
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 activty 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 paraguat 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
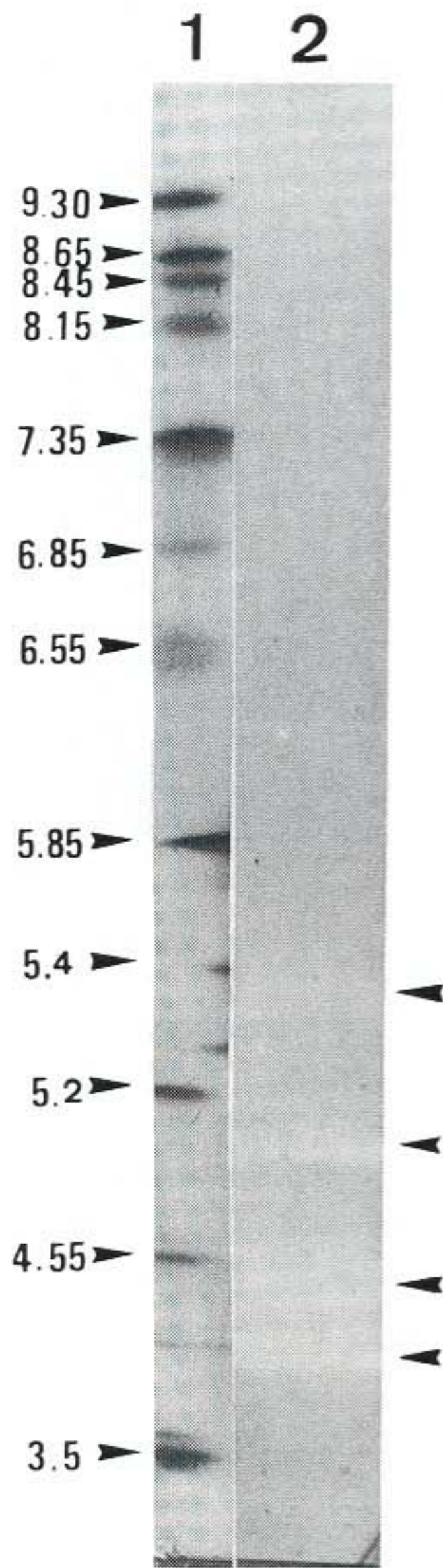the enzymes that detoxify active oxygen species (Harper and Harvey, 1978). In

Fig. 4. Isoelectric focusing of *Arabidopsis* superoxide dismutase on Servalyt-precote pH 3 to 10.
Lane 1: pI standard proteins: from top to bottom, amyloglucosidase, pI=3.50; soybean trysin inhibitor, pI=4.55; β-lactoglobulin A, pI=5.2; carbonic anhydrase II, pI=5.4; bovine carbonic anhydrase B, pI=5.85; human carbonic 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 indicate the activity of SOD isozymes.

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 symptons 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.
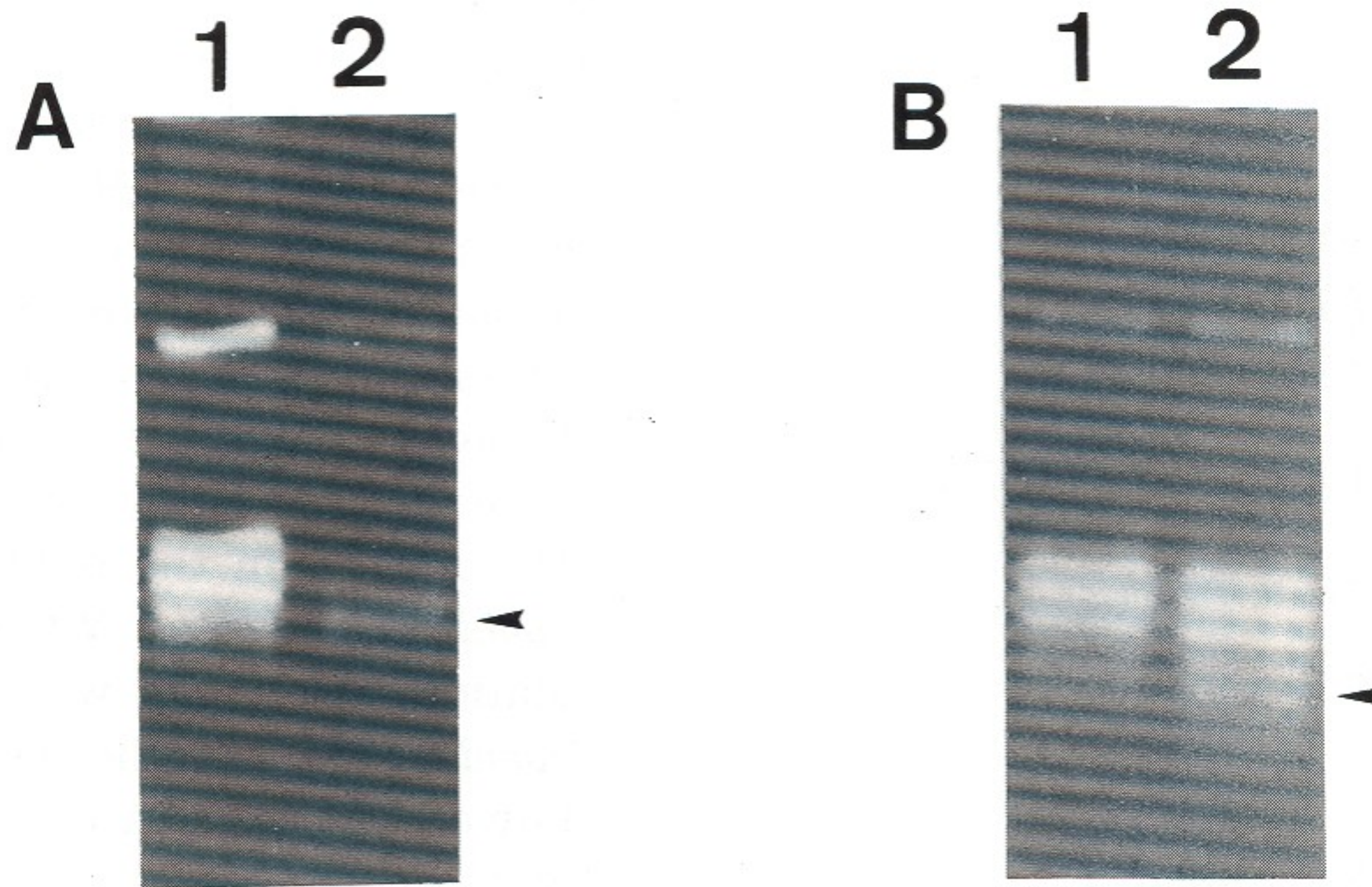
Fig. 5. The effect of paraquat on the SOD isozymes in *Arabidopsis* leaves. Electrophoresis was performed on a 12.5-15% gradient gel. A: Plants were sprayed with 40 ppm paraquat for one day. B: plants were sprayed with 1 ppm paraquat for several days. Lane 1: control, lane 2: treated leaves. An amount of extract equivalent to the same amount of fresh weight of leaves was loaded in each lane. Arrows indicat the activity of CuZn-SODIII.

ACKNOWLEDGEMENTS

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阿拉伯芥超氧歧化酶的研究

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摘　　要

以膠體電泳法分析阿拉伯芥不同生長階段的葉片，莖，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 為阿拉伯芥超氧歧化酶中活性最大之一羣，不同生長階段的葉片，莖，花之活性雖沒有明顯變化；然而 paraquat 處理過之植株 CuZn-SOD III 活性却明顯增加。