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The isozymes of superoxide dismutase in rice

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Abstract. Superoxide dismutase (SOD: EC 1.15.1.1) in rice from different physiological stages and different lines were characterized using electrophoresis in polyacrylamide gels. Three types of SOD were identified by their sensitivity to cyanide and hydrogen peroxide. Similar multiple superoxide dismutase patterns were observed in different organs of rice and at different stages during seed germination or seed development. Two Mn-SODs and four CuZn-SODs of rice were present. Most of the SOD isozymes were found to have pI between pH 5.2 and 5.8 by isoelectrofocusing. The CuZn-SODs contributed the major activity of total SOD and showed apparent changes in activity at different physiological stages. Moreover, some additional SODs, proposed to be Fe-SOD, were present in the reproductive organ of rice. SOD isozymes did not show significant variation in the ten lines of rice.

Key words: Isozyme; Rice; Superoxide dismutase.

Introduction

Superoxide dismutases (EC 1.15.1.1) appear to be ubiquitous among oxygen-metabolizing organisms and to be lacking in the obligate anaerobes (McCord *et al.*, 1971). They catalyze the dismutation of the superoxide radical (O_2^-) to molecular oxygen and hydrogen peroxide and are considered as the major enzymatic defense against O_2^- radicals (Halliwell, 1978). Unstable superoxide anions are formed in biological systems through autoxidations, enzymatic reactions, and leakage from membrane electron transport chains (Elstner, 1987; Fridovich, 1986; Halliwell, 1987) and can cause deleterious oxidations of lipids, proteins and nucleic acids. Therefore, they can seriously disturb normal cell metabolism. SODs are a group of metalloenzymes (Fridovich, 1975; Jackson *et al.*, 1978). Three distinct essential types of SODs containing either Mn, Fe or Cu plus Zn as prosthetic metals have been described. Each type of SOD has multiple forms. CuZn-SODs are sensitive to CN^- and H_2O_2, whereas Fe-SODs are sensitive to H_2O_2 but insensitive to CN^-, and Mn-SODs are insensitive to CN^- and H_2O_2 (Fridovich, 1975). This selective inhibition by CN^- and H_2O_2 makes it possible to distinguish the three types of SODs in the crude homogenates (Droillard *et al.*, 1989).

The SOD pattern in various plants has been described (Bridges and Salin, 1981). In plants, as well as in mammalian and fungal cells, CuZn-SOD is a major SOD (Rotilio, 1986), although plants contain Mn- and Fe-SOD in addition to CuZn-SOD. Fe-SOD was previously thought to be exclusively restricted to prokaryotic organisms and some eukaryotic algae but has also been found in several species of higher plants (Almansa *et al.*, 1989; Bridges and Salin, 1981; Kwiatowski *et al.*, 1985). The important role of superoxide and SOD in the biological system led to the ultilization of the enzyme as a privileged object and tool for studies ranging from protein structure to gene expression, from catalytic mechanisms to molecular evolution, from cell biology to pharmacology and medicine (Rotilio, 1986). Although some studies regarding SOD purification and characterization in rice have been reprted (Kanematsu

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and Asada, 1989; 1990), there has been no report concerning the SOD isozyme pattern of rice at the different developmental stages. In this report, we describe the isozyme pattern of SOD at different physiological stages in ten lines of rice. This report is the first to describe the presence of Fe-SOD in the reproductive organs of rice.

Materials and Methods

Chemicals

Nitro blue tetrazolium (NBT), xanthine, and xanthine oxidase were purchased from the SIGMA Chemical Company. Riboflavin and tetramethylenediamine (TEMED) were purchased from the SERVA Chemical Company.

Experimental Materials

Rice seeds (Oryza sativa cv. Tainon 67) were germinated and grown in a growth chamber at 25/22°C (day/night) with a 12 h photoperiod, and were harvested at intervals after water absorption. Rice grains were harvested at various days after pollination from the local field, frozen with liquid nitrogen, and then stored at -20°C in a freezer until use. Leaves (leaf dimension: 150 × 4 mm) were taken from 21-day-old rice plants. Different lines of rice seeds were gifts of the Taiwan Agricutural Research Institute.

Electrophoresis and SOD Activity Stain

Analytical disc polyacrylamide gel electrophoresis (PAGE) was performed with the modified procedure of Gabriel (1971). Electrophoresis was conducted in a 1.5 mm 10% acrylamide slab gel in standard tris-glycine buffer, pH 8.3. An appropriate amount of sample was applied, and electrophoresis from cathode to anode continued for ca. 1.5 h at 150 V. Immediately after electrophoresis, the SOD activity was identified by incubating the gel first in a 2.45 mM NBT solution for 25 min, and then soaking in a 50 mM Na-phosphate buffer (PH 7.8) containing 2.8×10^{-5} M riboflavin and 2.8×10^{-2} M TEMED for another 25 min. Light is necessary for photoreaction in the SOD activity staining. To identify CuZn-SODs, 2 mM KCN was included in the riboflavin solution for activity staining. For hydrogen peroxide treatment, the gels were first soaked in 3 mM hydrogen peroxide in a 50 mM Na-phosphate buffer (PH 7.8) containing 0.5 mM EDTA for 30 min, followed by SOD activity staining (Beauchamp and Fridovich, 1971).

Isoelectric Focusing

Horizontal isoelectrofocusing was performed on the 5% SERVALYTE Precote ultrathin polyacrylamide gels (Size: 125 × 0.15 mm) containing SERVALYT carrier ampholytes (pH 3-10). IEF was carried out at 10°C with the Multiphor II system apparatus (LKB Bromma, Sweden). During operation, the application of several drops of n-decane under the Precote as a heat exchange liquid was necessary. Focusing was carried out at a constant power of 7W and a limiting voltage of 1700 volts was used for 1.5 h. Samples were applied to paper wicks (4×10 mm; Pharmacia LKB) which were placed in the middle of the electrodes. The electrode buffer was applied to the electrode strips. The anode buffer buffer contained 0.33% L-aspartic acid and 0.37% L-glutamic acid, and the cathode electrode buffer contained 0.4% arginine, 0.06% L-lysine and 12% ethylene diamine. After electrofocusing, the gel was stained for SOD activity. The pI of SOD can be determined from the standard pI present on the same gel.

SOD Assay

SOD activity in extracts of rice was based on the indirect spectrophotometric modified method of Forman and Fridovich (1973). One unit of SOD was defined as the amount of enzyme which inhibited NBT reduction by 50%.

Results and Discussion

The extracts of rice seedlings revealed five bands of SOD activity on native PAGE gel described above (Fig. 1C). The four bands of SOD activity were sensitive to both cyanide and hygrogen peroxide, indicating that they are CuZn-SODs. The isozymes of CuZn-SOD are referred to as CuZn-SODs I, II, III and IV in the order from the cathode (Fig. 1C). The uppermost band was insensitive to both cyanide and hydrogen peroxide, being identified as Mn-SOD I, and another similar one was fast-moving on the gel and named Mn-SOD II (Fig. 1A, 1B). For identification of the SOD pattern in different organs of 7-day-old rice seedlings, we divided the rice seedlings into three divisions: shoot, embryo and root. From Fig. 1 we can find that CuZn-SOD III,

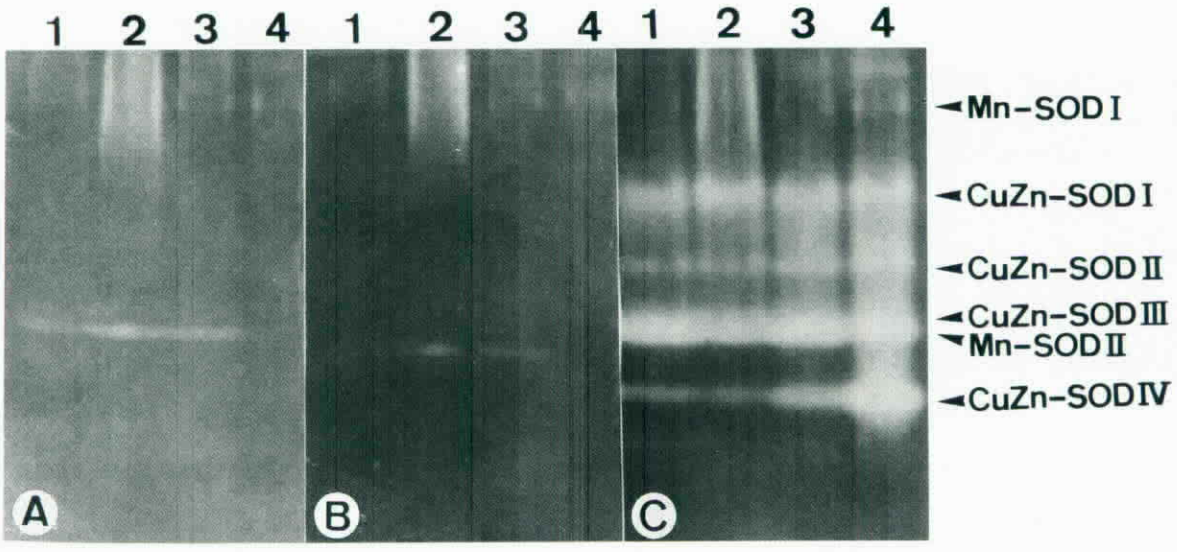

Fig. 1. The SOD isozyme activity extracted from the different organs of rice seedlings. A: the gel was treated with 2 mM KCN; B: treated with 3 mM H_2O_2; C: the control. Lanes 1-3, show the samples taken respectively from the various organs of 7-day-old seedlings: root, endosperm, and shoot; lane 4, the leaves taken from 21-day-old rice plants. Each sample contained the same amount of fresh weight.

which comigrated with Mn-SOD II on this gel, contributed the major activity of SOD in root, endosperm and shoot. The crude leaf extract showed nearly the same SOD pattern as that of the root, endosperm and shoot. However, the leaf showed much higher SOD activity of CuZn-SOD IV (Fig. 1C) on the basis of per fresh weight, which was also observed in the leaves of arabidopsis (data not shown).

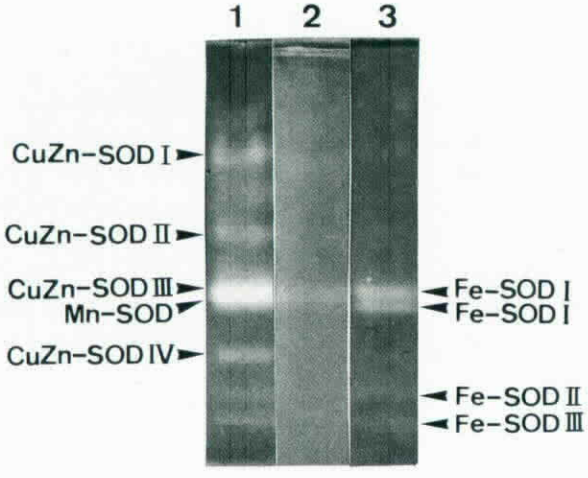

Fig. 2. The SOD isozyme activity extracted from the rice flowers. Lane 1, the control; lane 2, gel treated with 3 mM H_2O_2; lane 3, gel treated with 2 mM KCN.

Interestingly, the flowers contained several additional activity bands of SODs which have not been observed in other parts of rice plants. These SODs, possibly having low molecular weight, moved fast on the gels. These additional SODs were identified as Fe-SODs which were CN^--insentive and H_2O_2-sensitive (Fig. 2). This was the first report to show SODs in the reproductive organ of rice. Although Fe-SOD was previously considered to be not very common in higher plants, Fe-SOD in rice flowers (Fig. 2) and in carnation petals (Droillard *et al.*, 1989) or in Brassica leaves has been reported to be present in both mitochondria and peroxisomes (Droillard and Paulin, 1990).

Furthermore, in order to investigate the possible qualitative and quantitative changes of SODs during rice seed germination and seed development, SOD activity and the identification of SOD isozymes of crude rice extracts taken from different physiological stages were studied. Although the apparent total SOD activity in rice seeds after various days of germination showed no significant difference (data not shown). CuZn-SOD I contributed the highest activity in rice seeds. This CuZn-SOD I activity decreased gradually during seed germination (Fig. 3A), but CuZn-SOD III activity increased inversely.

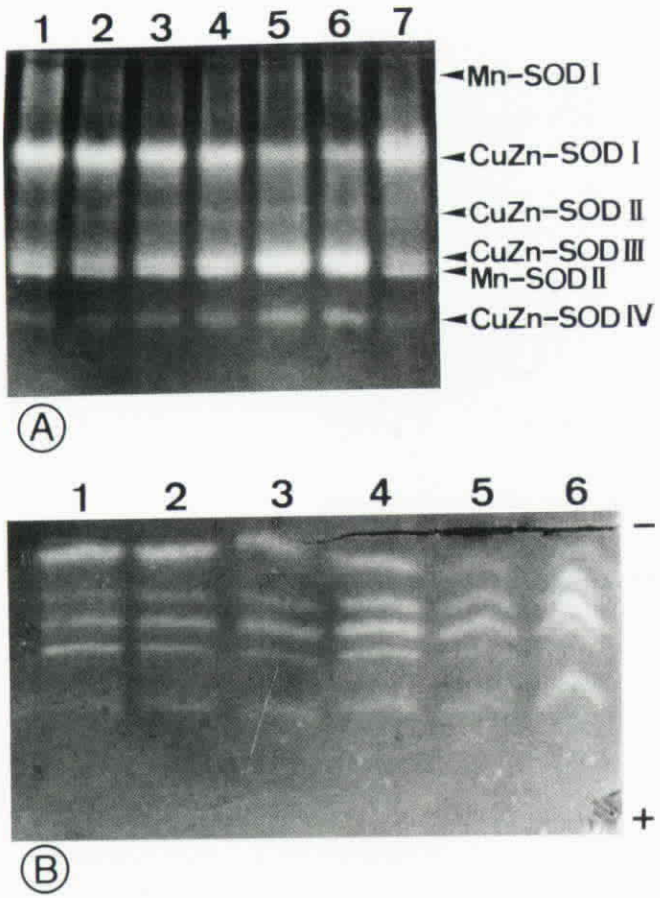

Fig. 3. The SOD isozyme activity extracted from rice after seed germination. Lanes 1–6, show the samples taken at 1, 2, 3, 4, 6, and 8 days, respectively, after water absorption; lane 7, extract made from dry seeds. Each sample contained the same amount of grain. A: electrophoresis was run on the 10% PAGE gel; B: electrophoresis was run on the IEF gel, pH 3–10.

Similar SOD patterns in the various stages of rice seedlings during germination were revealed either by native PAGE or IEF (Fig. 3B). However, we did not succeed in identifying the specific type of SOD on the IEF. Most of the SOD isozymes were found to have pI between pH 5.2–5.8. The pIs of the SOD of rice are comparable to those described for other plants (Duke and Salin, 1985; Kanematsu and Asada, 1989; 1990; Droillard and Paulin, 1990). The SOD of rice was indicated not to be a glycoprotein by the DIG Glucan Differentiation kit supplied by the Boehringer Mannheim (data not shown). The SOD patterns of rice grains during seed development did not change obviously, similar to the case after seed germination. This means that the SOD belongs to a house-keeping enzyme in rice, which acts as a defensive agent against oxygen toxicity. However, the CuZn−SOD I activity increased gradually during seed development (Fig. 4) and reached its highest level in the mature dry seeds; the reverse phenomenon was observed in the germination stage. Although slight changes in some SODs were observed, CuZn-SOD I was the only one to show apparent changes during the development of rice. However, the physiological signifi-

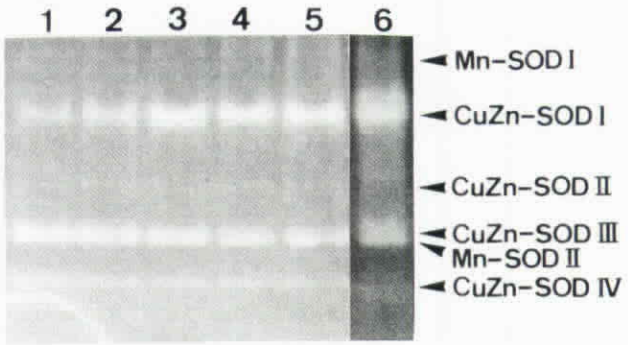

Fig. 4. The SOD isozyme activity extracted from the rice grains during seed development. Lanes 1–5, show the samples taken at 7, 14, 21, 30, and 35 days, respectively, after pollination; lane 6, extract made from dry seeds. Each sample contained the same amount of grain.

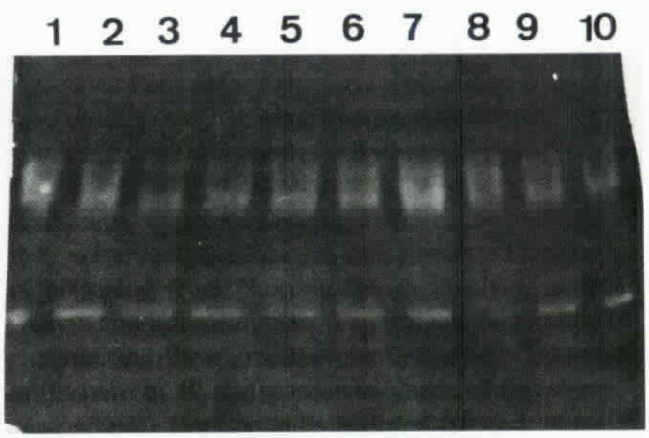

Fig. 5. The SOD isozyme activity in ten varieties of rice grains. Lane 1, Tainon 67; lane 2, Taichung 65; lane 3, Taichung 1; lane 4, Tainon 20; lane 5, Tainon 2; lane 6, Taichung 70; lane 7, Pegonil; lane 8, Mira; lane 9, PE-116; lane 10, E-1. Each sample contained the same amount of grain.

cance is not clear at this time.

Environmental stress can cause severe disturbance of cellular metabolism, which can possibly upset the balance of oxygen free radical production. Therefore, high constitutive levels or high induced levels of antioxidants in a plant cell are suggested to provide resistance to a particular stress (Spychalla and Desborough, 1990). SOD is one of the tissue antioxidants for protection against the potentially cytotoxic forms of activated oxygen. Accordingly, we examined the SOD pattern and enzyme activity of 10 lines of rice. In spite of the fact that there were distinct differences in the amylose content and the seed weight in the ten lines of rice seeds (Pan and Su, 1991), the same patterns and activity of SOD isozymes were present with the exception of Mira, which had a lower CuZn-SOD III activity level (Fig. 5). Further studies are necessary to examine the relationship between SOD activity and resistance to environmental stress.

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水稻超氧歧化酶同功異構酶的研究

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以膠體電泳法分析水稻不同生理階段及十種不同品系之超氧歧化酶(SOD)。並以 CN^-和 H_2O_2 對超氧歧化酶同功異構酶不同程度之抑制作用來鑑定其同功異構酶。水稻發芽階段之不同器官及種子形成時之 SOD 同功異構酶形式相似，都具有二種 Mn-SOD 和四種 CuZn-SOD，多數 SOD 同功異構酶之等電點介於 pH 5.2-5.8 之間。CuZn-SOD 爲水稻超氧歧化酶同功異構酶中活性最大之一群，其在不同生理階段中活性有明顯變化。水稻花器另含有 Fe-SOD。十種不同品系之超氧歧化酶同功異構酶形式並無明顯不同。