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Hydroxyl Radical-Generating Function of Horseradish Cu,Zn-Superoxide Dismutase

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Cu,Zn-superoxide dismutase (SOD) was purified from horseradish by using Mono Q and Superose 12 FPLC column chromatography. The native molecular mass of the purified enzyme was approximately 33 kDa, as determined by gel filtration. The subunit molecular weight, as estimated by SDS-PAGE, was 16 kDa. These results indicated that the native enzyme is a homodimer. We investigated the free radical-generating function of horseradish Cu,Zn-SOD by using a chromogen, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) which reacts with ·OH radicals to form $ABTS^{+\cdot}$. The formation of $ABTS^{+\cdot}$ was required for both active Cu,Zn-SOD and H_2O_2. The optimal pH for the free radical-generating activity of this enzyme was 6.0–8.0, and it retained about 40% of its maximum activity when exposed at 40°C for 15 min. A neutral scavenger, ethanol, inhibited the $ABTS^{+\cdot}$ formation by horseradish Cu,Zn-SOD more effectively than that by the mammalian enzyme. These results suggest that the active channel of horseradish enzyme is slightly larger than that of the mammalian enzyme.

Keywords: Copper,Zinc-superoxide dismutase, Horseradish, Hydrogen peroxide, Hydroxyl radicals.

Introduction

Superoxide dismutases (SOD) (EC1.15.1.1) protect cells and tissues from the damaging effects of oxidative stress by removing $O_2^{-\cdot}$. Three types of SOD have been identified, containing either copper and zinc (Cu,Zn), manganese (Mn), or iron (Fe) as prosthetic metals in the reaction center (Asada and Takahashi, 1987). These different metalloenzymes can be distinguished by selective inhibition: Cu,Zn-SODs are inhibited by cyanide, Fe and Cu,Zn-SODs are inhibited by H_2O_2, whereas Mn-SODs are resistant to both inhibitors (Fridovich, 1986). Fe-SOD and Mn-SOD show extensive primary sequence and structural homology but have little homology with the Cu,Zn-SOD enzyme. Mn-SODs occur in mitochondria, in chloroplast bound to the thylakoids, and in glyoxysomes. Fe-SODs are frequently found in prokaryotes, but only rarely in higher plants. Cu,Zn-SODs are the major isozymes in plants and have been localized in the soluble chloroplast fraction and in the cytosol (Jackson *et al.*, 1978; Kawiatowski and Kaniguga, 1986; Wingsle *et al.*, 1991).

In addition to the usual SOD activity, mammalian Cu,Zn-SODs are known to exhibit anion binding capacity, inactivation by its own reaction product H_2O_2, and a peroxidative function (Yim *et al.*, 1990; Kim and Kang, 1997). Previously, a series of studies analyzed the molecular mechanism of the inactivation process of Cu,Zn-SOD by H_2O_2 (Border and Fridovich, 1985; Vuillaume, 1987; Salo *et al.*, 1990) and generation of the free hydroxyl radical has been a matter of great concern. If hydroxyl radical is generated in biological systems, it could react with the SOD molecule and other molecules in the vicinity of its generation sites. Although SODs have been extensively investigated in higher plants, the free radical-generating function of plant Cu,Zn-SOD has not been reported.

In this study, we investigated the free radical-generating function of horseradish Cu,Zn-SOD. For this purpose, we first purified horseradish Cu,Zn-SOD and studied the properties of this enzyme. Its activity was measured by using a chromogen, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacts with ·OH radicals to form $ABTS^{+\cdot}$. We also investigated the effect of pH, temperature, and radical scavengers on the free radical-generating function of this enzyme.

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Materials and Methods

Purification of horseradish Cu,Zn-SOD Cu,Zn-SOD was purified as described by the modified method of Kang *et al.* (1997). The commercially available enzyme was purified to electrophoretic homogeneity by a chromatographic procedure using Mono Q and Superose 12. Briefly, 1 mg of SOD preparation from horseradish (Sigma, St. Louis, USA) was dissolved in 2.5 mM potassium phosphate (pH 7.8), 0.1 mM EDTA (buffer 1), followed by dialysis against the same buffer. This enzyme solution was applied to Mono Q (Pharmacia, Uppsala, Sweden) column equilibrated with buffer 1. After washing with 5 vol of buffer 1, bound proteins were eluted with a linear gradient (2.5 to 50 mM) of potassium phosphate. Active fractions were concentrated to 0.5 ml by an Amicon YM-10 ultrafilter. The retentate was applied to Superose 12 (Pharmacia) column equilibrated with 10 mM potassium phosphate (pH 7.8), 0.1 mM EDTA. The enzyme was eluted with the same buffer and active fractions were pooled and concentrated by the Amicon YM-10 ultrafilter. All solutions were treated with Chelex 100 resin (Bio-Rad, Hercules, USA).

Measurement of the dismutation activity of Cu,Zn-SOD The dismutation activity of SOD was measured by monitoring the inhibition of ferricytochrome *c* reduction by the xanthine/xanthine oxidase reaction described by McCord and Fridovich (1969). The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA in a cuvette at 25°C. The reaction mixture contained 10 μM ferricytochrome *c*, 50 μM xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome *c* at 550 nm of 0.025 absorbance unit per min. Under these defined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome *c* by 50% (to a rate of 0.0125 absorbance unit per min) was defined as 1 unit of activity. Protein concentration was determined by the method of Murphy and Kies (1960).

Measurement of the free radical-generating activity of Cu,Zn-SOD The free radical-generating activity of Cu,Zn-SOD was measured by using a chromogen, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS). On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, $ABTS^{+\cdot}$, which is conveniently followed at λ_{max} at 415 nm (ε_{415} of $3.6 \times 10^4\ M^{-1}\ cm^{-1}$) (Childs and Bardsley, 1975). The assay mixture contained 0.1 mM EDTA, 50 mM ABTS, 10 mM H_2O_2, and 5 μM horseradish Cu,Zn-SOD in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.6. The reaction was initiated by addition of hydrogen peroxide, and the increase in absorbance at 415 nm was measured by a uv/vis-spectrophotometer (Shimadzu, UV-1601). All assay solutions were bubbled with a 5% CO_2, and 95% N_2 gas mixture. Air was not used to balance the 5% CO_2 gas composition because molecular oxygen is capable of scavenging free radicals (Yim *et al.*, 1993).

Results

Purification and characterization of horseradish Cu,Zn-SOD The pure horseradish SOD was obtained by using a combination of Mono Q (Fig. 1A) and Superose 12 (Fig. 1B) FPLC column chromatography. The dismutation activity of horseradish SOD was strongly inhibited by 1 mM KCN or 1 mM H_2O_2 (data not shown). These results indicate that the purified horseradish SOD belong to the Cu,Zn-SOD. The specific dismutation activity of horseradish Cu,Zn-SOD preparation, as measured by monitoring the inhibition of ferricytochrome *c* reduction by the xanthine/xanthine oxidase reaction (McCord and Fridovich, 1969), was 4344 ± 36 U/mg.

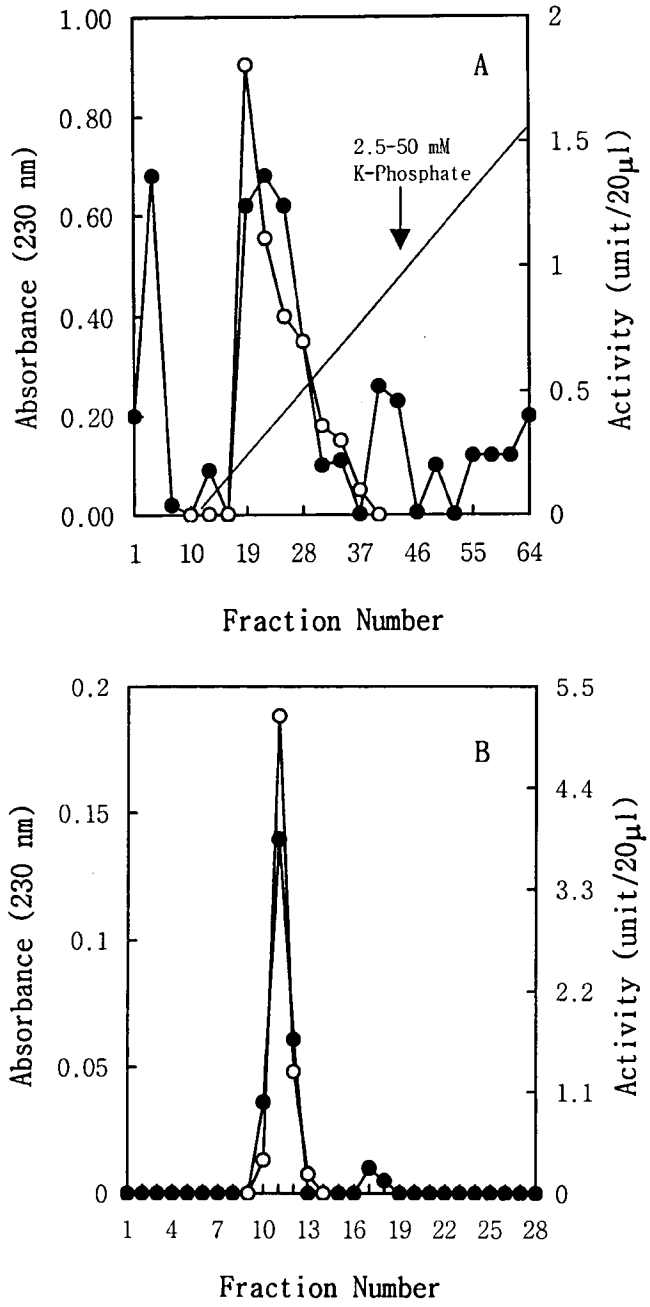

Fig. 1. Chromatographic purification of horseradish Cu,Zn-SOD. (A) Mono Q FPLC column chromatography. (B) Superose 12 FPLC column chromatography. A_{230} (●); Activity (○).

On SDS-PAGE, horseradish Cu,Zn-SOD appeared as a single band, indicating that the preparation was homogenous (Fig. 2). The molecular mass of horseradish Cu,Zn-SOD was determined by gel filtration on FPLC and found to be approximately 33 kDa (Fig. 3). The subunit size of the enzyme was determined by SDS-PAGE to be 16 kDa.

Free radical-generating activity of Cu,Zn-SOD We investigated the free radical-generating function of horseradish Cu,Zn-SOD. The peroxidative reaction has shown that ABTS reacts with ·OH radicals and produces $ABTS^{+\cdot}$ according to the following reaction.

$$ABTS + \cdot OH \rightarrow ABTS^{+\cdot} + OH^{-}$$

Results obtained from an optical spectrum, depicted in Fig. 4, demonstrate this function. Spectrum (a) originates from $ABTS^{+\cdot}$ generated in the solution containing 0.1 mM EDTA, 50 μM ABTS, 10 mM H_2O_2, and 5 μM horseradish Cu,Zn-SOD in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.6. The heat-inactivated enzymes and free copper ions could hardly catalyze the formation of $ABTS^{+\cdot}$ (spectrums b and c), indicating that active enzyme was required. In our study, the formation of ·OH radical adducts of ABTS was monitored, while in the inactivation study the remaining dismutation activity of Cu,Zn-SOD was measured (data

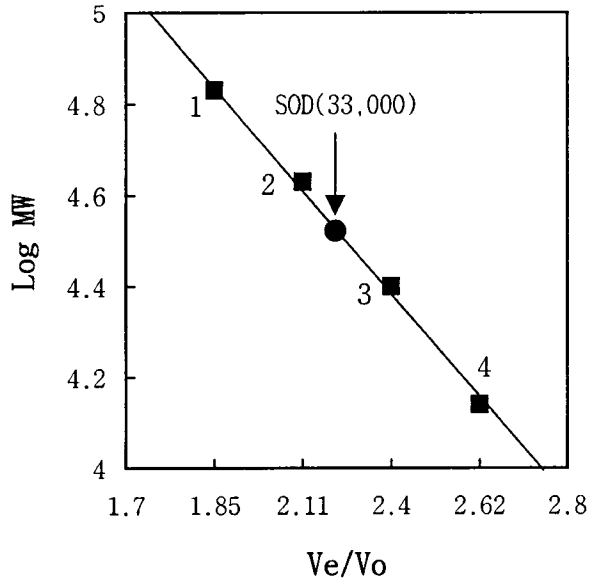

Fig. 3. Molecular mass determination of horseradish Cu,Zn-SOD by gel filtration on FPLC using Superose 12 column. Protein samples were loaded onto Superose 12 FPLC column pre-equilibrated with 10 mM potassium phosphate (pH 7.8) buffer containing 0.1 mM EDTA. Proteins were eluted with the same buffer. Native molecular mass markers: 1, BSA (67 kD); 2, ovalbumin (43 kD); 3, chymotrypsin (25 kD); 4, ribonuclease (13.7 kD).

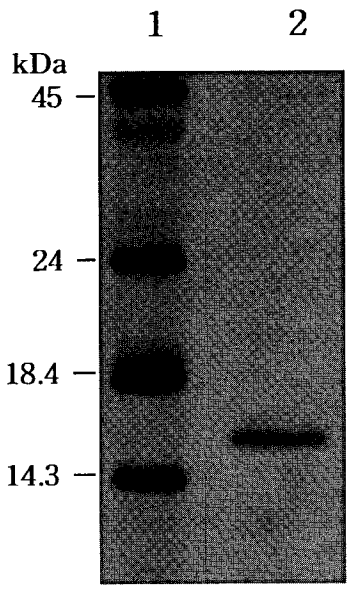

Fig. 2. SDS-PAGE of the purified horseradish Cu,Zn-SOD. Lane 1, Molecular weight calibration proteins (from top to bottom: ovalbumin; trypsinogen; a-lactoglobulin; lysozyme). Lane 2, the purified horseradish Cu,Zn-SOD. Protein samples (9 μl) were mixed with 3 μl of reducing buffer containing 8% SDS, 40% glycerol, 0.25% bromophenol blue, and 20% β-mercaptoethanol and then heated at 100°C for 10 min. After chilling on ice, the mixtures were deposited on the gel and run at constant voltage of 100 V.

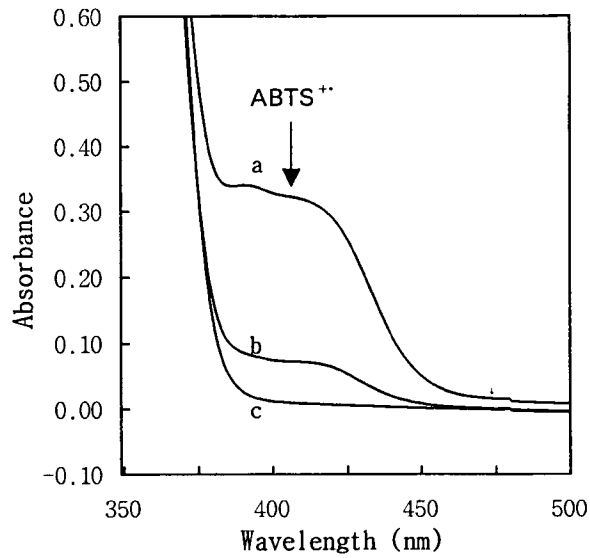

Fig. 4. Electronic absorption spectra of the $ABTS^{+\cdot}$ formed in solutions containing H_2O_2 and horseradish human Cu,Zn-SOD. The spectra were obtained with a solution containing 50 μM ABTS, 10 mM H_2O_2, 0.1 mM EDTA in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.6. In addition, the reaction mixture contained 5 μM Cu,Zn-SOD (spectrum a), 5 μM heat-inactivated Cu,Zn-SOD (spectrum b), and 10 μM $CuSO_4$ (spectrum c). The spectra were recorded at 3 min after initiating the reaction with H_2O_2.

not shown). These results may indicate that the rate of the ·OH radical generation by Cu,Zn-SOD is much faster than that of the loss of the dismutation activity, suggesting that some ·OH radicals are released from the metal site.

Effect of pH and temperature on the free radical-generating activity of Cu,Zn-SOD The free radical-generating activity of horseradish Cu,Zn-SOD was observed with the optimum pH range from 6.0 to 8.0 (Fig. 5). It has been reported that the optimum pH range of mammalian Cu,Zn-SOD was pH 7.6–9.5 (Yim *et al.*, 1996; Kim and Kang, 1997). Our data indicate that the enzyme catalyzed the formation of ·OH radical in more acidic conditions.

Next, we examined the enzyme's thermal stability. The free radical-generating activity retained about 40% of the maximum activity when exposed at 40°C for 15 min (Fig. 6). This data was similar to the result obtained from human Cu,Zn-SOD (Kim and Kang, 1997).

Effect of radical scavengers on the free radical-generating activity Cu,Zn-SOD A radical scavenger, azide anion, strongly inhibited the formation of $ABTS^{+\cdot}$ (Fig. 7, curve c) whereas ethanol slightly inhibited the reaction (Fig. 7, curve b). These results indicated that an anionic scavenger, azide, had easy access inside charged active channels of the enzyme and could react with the ·OH radicals, whereas neutral alcohol stayed outside the active channel and could hardly intercept the newly-formed ·OH radicals.

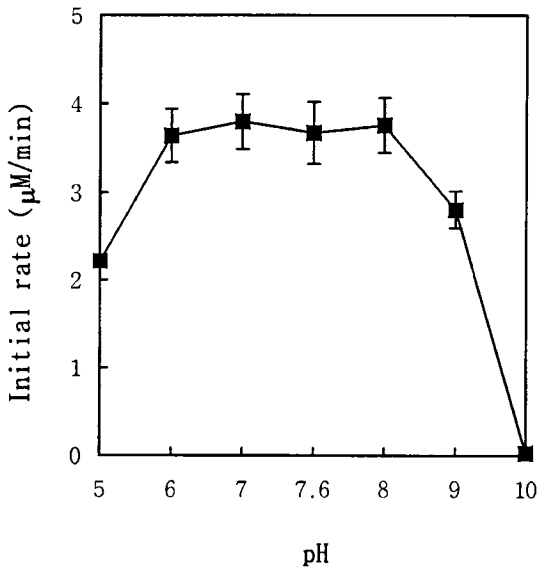

Fig. 5. Effects of pH on the initial rates of hydroxyl radical formation by horseradish Cu,Zn-SOD. The reaction mixtures contained 5 μM horseradish Cu,Zn-SOD, 50 μM ABTS, 10 mM H_2O_2, and 0.1 mM EDTA in 23.5 mM sodium citrate (pH 5.0) or potassium phosphate (pH 6.0–7.0) or $NaHCO_3/CO_2$ (pH 7.6) or $Na_2CO_3/NaHCO_3$ (pH 8.0–10.0) buffer.

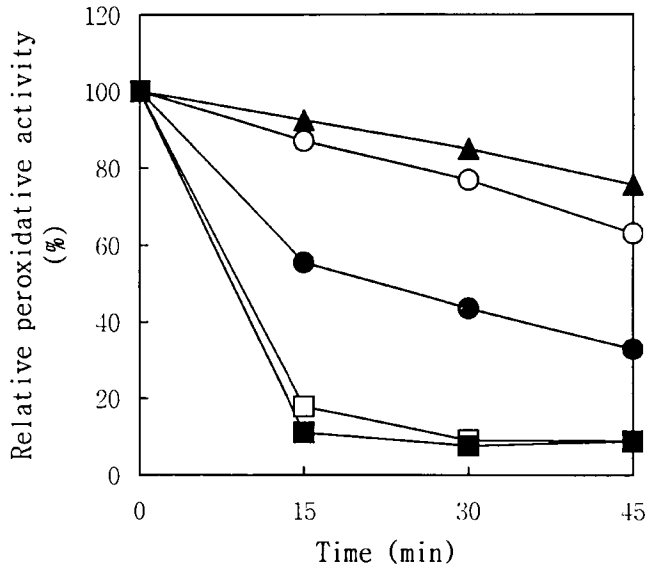

Fig. 6. The thermal stability of horseradish Cu,Zn-SOD for the free radical-generating activity. Enzyme was placed in heating blocks at 15°C (▲), room temperature (○), 40°C (●), 60°C (□), and 80°C (■), with indicated heating time. The reaction mixtures contained 5 μM horseradish Cu,Zn-SOD, 50 μM ABTS, 10 mM H_2O_2, and 0.1 mM EDTA in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.6.

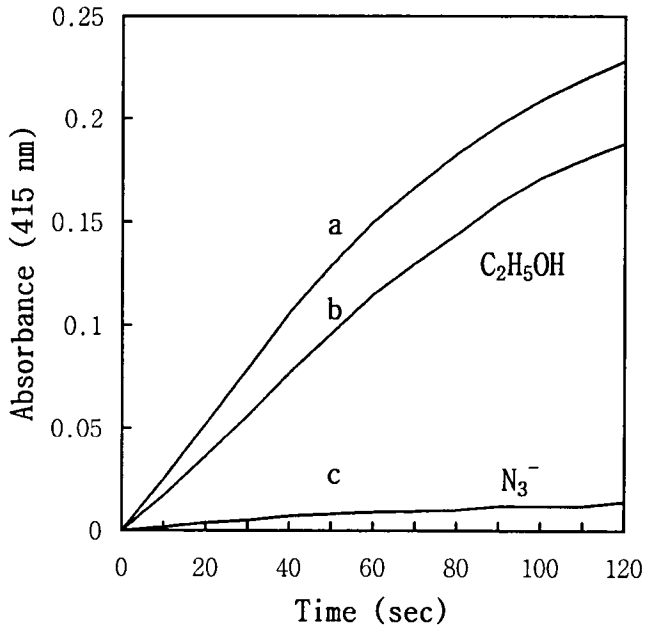

Fig. 7. Effects of radical scavengers on the formation of $ABTS^{+\cdot}$ catalyzed by the horseradish Cu,Zn-SOD. Time course of $ABTS^{+\cdot}$ formation obtained with the reaction mixtures containing 5 μM horseradish Cu,Zn-SOD, 50 μM ABTS, 10 mM H_2O_2, and 0.1 mM EDTA in 23.5 mM $NaHCO_3/CO_2$ buffer at pH 7.6, either in the absence of radical scavenger (curve a), in the presence of 0.1 M ethanol (curve b), or in the presence of 0.1 M azide (curve c).

Discussion

In this study, we obtained the pure horseradish Cu,Zn-SOD through Mono Q and Superose 12 FPLC column chromatography. The specific dismutation activity was 4344 ± 36 U/mg. This value is in the lower end when compared to isolated Cu,Zn-SODs from other plant sources (Kanematsu and Asada, 1970; Baum and Scandalios, 1981; Asada and Takahashi, 1987). This difference may have derived from the method used for determining protein concentration. The purified enzyme consists of two identical subunits, the molecular mass of which was estimated to be about 16 kDa on SDS-PAGE. Most plant Cu,Zn-SODs isolated so far, with few exceptions, are homodimers having a molecular mass of 30 to 33 kDa (Kanematsu and Asada, 1990; Wingsle *et al.*, 1991; Kroniger *et al.*, 1992). It has been reported that the chloroplastic Cu,Zn-SOD had a subunit molecular weight of 20 kDa and the cytosolic enzyme had a subunit molecular weight of 16 kDa (Wingsle *et al.*, 1991; Kroniger *et al.*, 1992). Our results indicate that the purified horseradish Cu,Zn-SOD belonged to the cytosolic form.

It has previously been shown that mammalian Cu,Zn-SODs have a free radical-generating function, which catalyzed the formation of ·OH radicals with H_2O_2 as a substrate (Yim *et al.*, 1990; Kim and Kang, 1997). However, the free radical-generating function of plant Cu,Zn-SOD had not been reported until now. In this study, the free radical-generating activity of horseradish Cu,Zn-SOD was measured by using a chromogen, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacts with ·OH radicals to form $ABTS^{+\cdot}$. Our result showed that the enzyme catalyzed the free radical-generation in the presence of H_2O_2 (Fig. 4). The initial rate of $ABTS^{+\cdot}$ formation (4.17 μM/min) obtained in our study was larger than the value obtained in the mammalian enzyme study (2.77 μM/min) (Kim and Kang, 1997) implying that horseradish enzyme is a better catalyst for ·OH radical production than mammalian enzyme.

In animal cells, the production of $O_2^{-\cdot}$ is much larger as the concentration of oxygen increases during exposure to hyperoxia, or when the respirator chain becomes inhibited leading to an increased concentration of reducing equivalents (Turrens *et al.*, 1991). Yim *et al.* (1993) have mentioned that at least 0.1 mM/min H_2O_2 will be produced continuously under physiological conditions and at a much higher rate in adverse conditions such as hyperoxia or ischemia reperfusion. It has been reported that the K_m value of human Cu,Zn-SOD for H_2O_2 was 9.3 mM (Kim and Kang, 1997). Thus, the free radical-generating function of human Cu,Zn-SOD using H_2O_2 as a substrate will be operative *in vivo*. Recently, we have shown that this function as a free radical generator may be responsible in part for the deleterious effects observed by the Cu,Zn-SOD and H_2O_2 systems (Kang and Kim, 1997a; 1997b). It has been suggested that the gain-of-function of the familial amyotrophic lateral sclerosis (FALS) mutant Cu,Zn-SODs may be partly responsible for the development of FALS symptoms due to biological damage resulting from elevated levels of ·OH radicals (Yim *et al.*, 1996; 1997). Hence, this hydroxyl radical-generating function, in part, provides an explanation for the association of the Cu,Zn-SOD with deleterious effects on cells.

Although the free radical-generating function of mammalian Cu,Zn-SOD may be associated with deleterious effects observed by the oxidative damage, it is not known what are the roles of this function in plant cells. It has been reported that with plant Cu,Zn-SOD, the hydrogen peroxide is produced via catalytic disproportionation of the superoxide generated by NAD(P)H oxidase, and associated with lignification (Ogawa *et al.*, 1997). However, VanToai and Bolles (1991) reported that the postanoxic injury of soybean roots was associated with an increased superoxide anions production capacity coupled with a reduced superoxide dismutase activity. If hydroxyl radicals are generated by plant Cu,Zn-SOD in abnormal conditions (at a high concentration of H_2O_2), it could damage the SOD molecule and then could induce the reduction of SOD dismutation activity. Therefore, the free radical-generating function of plant Cu,Zn-SOD may also contribute to the deleterious effects observed by the Cu,Zn-SOD and H_2O_2 system.

X-ray crystallographic studies (Getzoff *et al.*, 1983; 1992) revealed that the active channel of mammalian Cu,Zn-SOD is positively-charged with amino acid residues, with Arg-141 inside and Lys-120 and Lys-134 at the top of the channel. These amino acid residues are responsible for the electrostatic guidance of the anionic substrate $O_2^{-\cdot}$ to the active site to yield the superoxide dismutation. Other small anions, such as cyanide, azide, halide, and phosphate, are also known to have easy access inside the channel and to bind to the active site (Rigo *et al.*, 1977; Van Camp *et al.*, 1982; Mata de Freitas and Valentine, 1984). Although the crystallographic structure of plant Cu,Zn-SOD has not been reported, the unique nature of this active channel was in accordance with our experimental results. A radical scavenger, azide anion, strongly inhibited the formation of $ABTS^{+\cdot}$ (Fig. 7, curve c), whereas ethanol slightly inhibited the reaction (Fig. 7, curve b). The inhibitory rate of azide on the formation of ·OH radicals was in agreement with the reported rate obtained in the study of mammalian enzymes (Kang and Kim, 1997a; 1997b). However, the inhibitory rate of ethanol was much higher than the rate obtained in the mammalian enzyme study (Kang and Kim, 1997a; 1997b). Our data indicate that ethanol has easier access inside active channels of horseradish than of mammalian enzyme. These results suggest that the active channel of horseradish enzyme is slightly larger than that of the mammalian enzyme, thus making it more accessible to H_2O_2.

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