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Inactivation and Degradation of CuZn-SOD by Active Oxygen Species in Wheat Chloroplasts Exposed to Photooxidative Stress

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Changes in CuZn-SOD actvity and content in isolated wheat chloroplasts under the light, and the involvement of protease(s) and/or active oxygen species in this process were studied. Both SOD activity and content decayed with exposure time to photooxidative stress. Ascorbate, a H_2O_2 scavenger, prevented photooxidation-associated inactivation of SOD, while benzoate, a $^{\cdot}OH$ scavenger, prevented SOD degradation. Wheat chloroplasts incubated in the dark did not hydrolyze exogenous or endogenous SOD, either H_2O_2-pretreated or not. Protease inhibitors did not prevent SOD degradation under photooxidative treatment, suggesting that plastid protease(s) did not participate in this process. Purified chloroplast CuZn-SOD was exposed to H_2O_2 and O_2^- or $^{\cdot}OH$-generating systems. O_2^- had no effect on either SOD activity or stability (estimated by native PAGE). H_2O_2 up to 700 μM inhibited SOD in a dose-dependent manner and induced charge/mass changes as seen by native PAGE. $^{\cdot}OH$ also reduced SOD activity by inducing its fragmentation. High levels of active oxygen, as can be generated under strong stress conditions, could directly inactivate and degrade chloroplastic SOD.

Key words: Active oxygens — Chloroplasts — Photooxidative stress — Superoxide dismutase — *Triticum aestivum* — Wheat.

Oxidative stress arises from detrimental effects of active oxygen species, such us H_2O_2 (hydrogen peroxide), O_2^- (superoxide anion radical) and $^{\cdot}OH$ (hydroxyl radical), which react with most cellular components (Cadenas 1989 and references therein). In chloroplasts, O_2^- production increases under conditions where the NADPH/$NADP^+$ ratio is increased and the rate of electron transfer from PSI to O_2 is enhanced (Scandalios 1993). Superoxide dismutases (SODs) play a key role in the antioxidant defense system through the dismutation of O_2^- to H_2O_2 and O_2. SODs are metalloenzymes that occur in three different molecular forms containing Mn, Fe or CuZn as prosthetic metals (Fridovich 1986). The major superoxide dismutase within chloroplasts is accounted by a nuclear-encoded CuZn-SOD (Bowler et al. 1992 and cites therein). Fe- (Asada et al. 1975) and CuZn-SODs (Hodgson and Fridovich 1975) are inhibited by H_2O_2. Therefore the effective action of chloroplastic CuZn-SOD in the antioxidant defense system could depend, at least in part, on the activity of the H_2O_2-scavenging system.

It has been shown that photooxidative stress caused by paraquat strongly modifies SOD activity and chloroplastic CuZn-SOD protein level in leaves (Bowler et al. 1992 and references therein). In agreement with suggestions of Foyer et al. (1994), SOD activity of leaves increased under low-moderate stress. Beyond a certain level of stress, SOD inactivation and degradation began to prevail. We verified this behaviour of SOD in wheat leaf segments (González et al., personal communication). Moreover, when protein synthesis was suppressed by cycloheximide, photooxidative stress caused a progressive decrease in SOD activity and protein levels. It is possible that active oxygens generated under such conditions could inactivate and degrade SOD.

There is strong evidence indicating that in erythrocytes and in *E. coli*, CuZn-SOD is inactivated by active oxygen species generated during oxidative stress. Erythrocytes exposed to O_2^- and H_2O_2 showed a decrease in SOD activity (Salo et al. 1990). In fact, when purified CuZn-SOD was treated with H_2O_2 (Salo et al. 1990) or H_2O_2 and O_2^- (Sinet and Garber 1981), inactivation and fragmentation of the enzyme occurred. Besides, H_2O_2-modified SOD was more prone to proteolytic attack than the untreated enzyme (Davies et al. 1987, Salo et al. 1990). In chloroplasts, a serine-type protease seems to be involved in the rapid hydrolysis of D1 protein (Virgin et al. 1991) and other plastidic polypeptides (Casano et al. 1994) after oxidative modification. However, little information is available regarding the effects of active oxygen species and proteases on plant SODs.

In the present work we studied the possible involvement of active oxygens and of protease(s) on chloroplastic CuZn-SOD activity and degradation. Experiments with isolated chloroplasts showed a role of active oxygen in the inactivation and breakdown of CuZn-SOD. However, enzyme degradation would not be accomplished by plastid proteolytic activity, at least at a rate concordant with SOD

Abbreviations: $^{\cdot}OH$, hydroxyl radical; NEM, *N*-ethyl maleimide; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase; O_2^-, superoxide anion radical; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone.

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breakdown under photooxidative stress. We show that purified CuZn-SOD can be inactivated by H_2O_2 and $^{\cdot}OH$.

Materials and Methods

Plant materials—Wheat (*Triticum aestivum* L. cv. Oasis) plants were sown in vermiculite and grown for 7 d, in a controlled growth chamber at 24°C ± 1°C, under continous white light of 40 W m^{-2}.

Isolation of chloroplast—Intact chloroplasts were prepared from primary wheat leaves according to Mills and Joy (1980) and Kalt-Torres et al. (1984). Briefly, 15 g of leaves were blended with 150 ml of 0.33 M sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 0.15% bovine serum albumin in 50 mM Tris-Cl (pH 7.5), and filtered through 8 layers of cheesecloth. Crude chloroplasts were pelleted by centrifugation at 1,000 × *g* for 3 min and resuspended in the grinding medium without albumin. Intact chloroplasts were separated by centrifugation (10,000 × *g*, 20 min) in a continuous Percoll gradient (40–80%). Intact chloroplasts, with a protein/Chl ratio of 7.5 or more, sedimented as a sharp layer near the bottom. Chloroplasts were rinsed with resuspension medium. Stromal proteins were obtained after osmotic shock according to Casano et al. (1994). All the steps were carried out at 4°C.

Photooxidative treatment on CuZn-SOD in isolated chloroplasts—Photooxidation-induced changes of CuZn-SOD activity and protein content were studied by incubating isolated chloroplasts under different conditions. Thirty μl of intact chloroplasts (7.5 μg protein), suspended in 50 mM Tris-HCl (pH 7.5), 330 mM sorbitol and 2 mM $MgCl_2$ (Medium A) were incubated, for 0 to 4 h at 30°C, either in the dark or under photooxidative light (60 W m^{-2}). In other experiments, intact chloroplasts were incubated in medium A containing either 0 or 5 mM sodium benzoate, 0 or 5 mM sodium ascorbate, for 0 and 1 h under 60 W m^{-2}, at 30°C.

After appropriate incubation times, reactions were stopped with native or SDS-sample buffer [containing 3.5% (v/v) β-mercaptoethanol] and electrophoresed under native (Davies 1971) or denaturing conditions (SDS-PAGE, Laemmli 1970). The remnant CuZn-SOD activty was determined in native gels stained for SOD activity (Beauchamp and Fridovich 1971), and SOD protein content was analyzed by Western blotting of SDS-PAGE using specific antibody and subsequent scanning with a digital image analyzer (Fotodyne Image Analyzer, Fotodyne Inc., U.S.A.).

Proteolytic activity against CuZn-SOD in isolated chloroplasts—In order to detect proteolytic activity against CuZn-SOD in plastids, chloroplasts were incubated with the purified enzyme in a pH range of 5.0 to 8.5. Reaction mixtures contained 3 μg of either native or H_2O_2-pretreated SOD (as substrate) in either 100 mM sodium acetate (from pH 5.0 to 6.0) or 100 mM Tris-maleate (from pH 6.5 to 8.5), plus 2–5 μl of isolated chloroplasts (0.3 μg protein), in a total volume of 30 μl. All the media contained 5 mM $MgCl_2$ and 1 mM β-mercaptoethanol. Pretreatment with H_2O_2 was performed by incubating SOD in the appropriate buffer containing 400 μM H_2O_2 for 30 min at 30°C.

Degradation of endogenous CuZn-SOD was studied by incubating isolated chloroplasts under different conditions. The effect of pH was assayed as described above except that no exogenous CuZn-SOD was added and a larger amount of either control or H_2O_2-pretreated chloroplasts was used (7.5 μg protein). Pretreatment with H_2O_2 was performed by incubating chloroplasts in the appropriate buffer containing 400 μM H_2O_2 for 30 min at 30°C. The influence of incubation time and temperature was determined in 10 μl chloroplasts (7.5 μg protein) incubated with 20 μl of either 100 mM citrate-phosphate pH 5.5 or 100 mM Tris-maleate (pH 7.5) at 20, 30 or 40°C for 0, 1, 2 or 4 h, in the dark. Besides, 30 μl of intact chloroplasts (7.5 μg protein), suspended in Medium A, were incubated with 0 or 2 μl of a protease inhibitors mixture (dissolved in Medium A), for 0 and 3 h under 0 or 60 W m^{-2}, at 30°C. Inhibitors in the reaction medium were: 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mg ml^{-1} pepstatin, 1 mM iodoacetamide, 1 mM *N*-ethyl maleimide (NEM), 5 mM EDTA and 1 mM *o*-phenanthrolin. The effect of each protease inhibitor was also assayed separately in intact chloroplasts suspended in Medium A, incubated for 0 and 3 h at 30°C under illumination.

After appropriate incubation times, reactions were stopped with SDS-sample buffer and subjected to SDS-PAGE. The remnant CuZn-SOD content was analyzed by Western blotting as described.

Active oxygen treatments of purified SOD—Purified wheat CuZn-SOD was exposed to active oxygen species as described below. After the treatments, remnant SOD activity was assayed following the procedure of Stewart and Bewley (1980). In some H_2O_2 treatments, SOD activity was also analyzed through native PAGE, as described. The stability of SOD protein was estimated by native and SDS-PAGE. Proteins were stained with Coomasie Brilliant Blue.

O_2^- treatments: CuZn-SOD (3 units) was incubated in the presence of a O_2^--generating system according to Michelson (1987) with slight modifications. Briefly, this system was composed of 50 μM riboflavine, 0.1 mM EDTA, 50 μM sodium formate, 0.5 unit of bovine catalase (Sigma Co., St. Louis, U.S.A.) and 0 to 0.15 mM NADPH (added just before starting the reactions), in 50 mM Na-K-P_i pH 7.5 (200 μl final volume). O_2^- was generated by exposure for 5 min, at room temperature, to light of 360 nm using two TL 40W/05 (Philips, Wageningen, Netherlands) fluorescent lamps, at 25 cm from the vials, at room temperature. The amount of O_2^- generated under these conditions was calculated according to Auclair and Voisin (1987).

H_2O_2 treatments: CuZn-SOD (6 units) was incubated with 0 to 0.7 mM H_2O_2 in 50 mM Na-K-P_i pH 7.5 containing 0.1 mM EDTA and 0 or 20 mM sodium benzoate (200 μl of final volume), for 30 min at 30°C. Reactions were stopped with 0.5 unit of bovine catalase, previously desalted by Sephadex G-25 filtration.

$^{\cdot}OH$ treatments: CuZn-SOD was exposed to an $^{\cdot}OH$-generating system based on the Fenton reaction by autooxidation of cupric ions, and designed following the system reported by Cohen (1987), with modifications. Six units of SOD were incubated at 30°C in a continuously aerated (3 ml air min^{-1}) reaction mixture containing 50 mM Na-K-P_i pH 7.5, 0.1 mM $CuSO_4$, 0 or 20 mM sodium benzoate and 0 to 5 pulses of 64 nmol ascorbic acid, given at 10 min intervals. This amount of ascorbic acid completely reacted after 10 min, as detected by absorbance at 290 nm (data not shown). The final volume was maintained at 150 μl with water. After 50 min, reactions were stopped by interrupting aeration. Cupric ions, which interfere with the assay of SOD activity, were immediatly removed by chelation with 40 μl of a very dense suspension of iminodiacetic acid-agarose (Sigma Co., St. Louis, U.S.A.) equilibrated with 50 mM Na-K-P_i (pH 7.5), followed by 2 min centrifugation at 14,500 × *g*.

The reaction mixtures were prepared with deionized water and Na-K-P_i was filtered through a column of iminodiacetic acid-agarose before use. Controls with each component of the reaction mixtures were made to rule out interferences with the subsequent assay of SOD activity.

Purification of CuZn-SOD—Chloroplastic CuZn-SOD was

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purified from wheat leaves following the procedure described by Baum et al. (1983), with slight modifications. Briefly, 7-d-old leaves were homogeneized in 50 mM Na-K-P_i (pH 7.0) containing 100 mM NaCl and 1% water-insoluble polyvinylpolypirrolidone, filtered, and centrifuged at 10,000 × *g* for 15 min. The protein precipitate formed at 40 to 90% saturation $(NH_4)_2$ SO_4 was resuspended in 50 mM Na-K-P_i (pH 7.0) containing 100 mM NaCl and dialyzed against the same buffer. Chloroplastic SOD was purified by three chromatographic steps (through Sephadex G-150, DEAE-Sephacell and Sephadex G-75). The procedure yielded 1 mg of enzyme with a specific activity of 15,770 U (mg prot.)$^{-1}$. The molecular mass of the chloroplastic SOD holoenzyme from wheat was approximately 38 kDa (as determined by filtration throught a Sephacryl S-200 column) and the subunit molecular mass was estimated to be 19.5 kDa by SDS-PAGE (data not shown).This molecular weight of the holoenzyme is greater than the determined by Beauchamp and Fridovich (1973) for wheat germ CuZn-SODs. Kanematsu and Asada (1989) reported that the molecular weight of a CuZn-SOD from *Equisetum arvense* was 34000 by gel filtration or 31000 when determined by sedimentation equilibrium. The purified enzyme was inhibited by cyanide indicating it is a CuZn-SOD. The electrophoretic mobility of the purified enzyme, in native gels stained for SOD activity, was coincident with that of the SOD present in isolated chloroplasts, indicating the chloroplastic location of the purified SOD (data not shown).

Antibody against Cu-Zn SOD—Rabbit antisera against chloroplastic Cu-Zn SOD were obtained according to Clausen (1988) and a polyclonal antibody was purified by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography. Exposure to active oxygen could induce modifications on surface amino acid residues of SOD that might affect its immunoreactivity. To test this, control and H_2O_2-treated purified SOD was subjected to native and SDS-PAGE and SOD band(s) were detected by Western blotting and Coomassie Brilliant blue staining. A close correlation between the intensity of band(s) revealed by immunodetection and by protein staining was observed (data not shown), indicated that both the native enzyme as well as SOD modified by active oxygen could be recognized by the antibody with the same efficiency.

All experiments were run in duplicate, and were repeated al least four times.

Results

Photoinactivation of Cu-Zn-SOD in isolated chloroplasts—Changes of CuZn-SOD activity and protein content were followed in isolated intact chloroplasts under high light intensity (photooxidative condition). Incubation in the dark over 4 h did not significantly change either activity (Fig. 1A) or SOD protein (Fig. 1A, B). In contrast, SOD activity progressively decreased in the light (Fig. 1A). SOD protein level sharply decayed during the first h of exposure to light (60% loss), and after 2 and 4 h-incubation, a 70 and 80% decrease was observed, respectively (Fig. 1A, B). Besides, during the first 40–60 min of light exposure activity changes were associated with a decay of the native band of activity and the appearence of new bands of higher electrophoretic mobility (Fig. 2A), showing a similar pattern to that observed when purified CuZn-SOD was treated with H_2O_2 (see below). After 1 h, a marked decrease of the native band of activity was observed, however, higher mobility bands were only barely detectable.

When chloroplasts were incubated in the presence of either ascorbate or benzoate, photodegradation of CuZn-SOD was almost completely suppressed (Fig. 2A, B). However, only ascorbate prevented both activity decay and alterations in the electrophoretic pattern of plastidic SOD (Fig. 2A). These results suggest that active oxygen species, such as H_2O_2 and/or $^{\cdot}OH$, play an important role in the inactivation and degradation of CuZn-SOD in chloroplasts exposed to light.

Wheat chloroplasts do not contain proteolytic activity against CuZn-SOD—Active oxygen species could increase SOD suceptibility to protease-catalyzed degradation. There is evidence indicating that under photooxidative conditions, active oxygen species modify several chloroplastic polypeptides, which, in turn, are rapidly degraded by

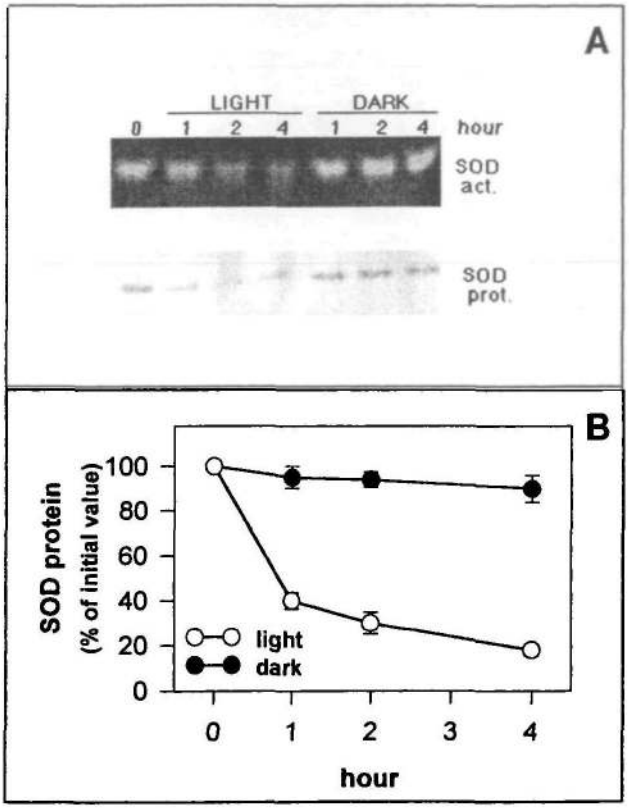

Fig. 1 Effect of photooxidative stress on CuZn-SOD in isolated wheat chloroplasts. Thrity μl of intact chloroplasts (7.5 μg protein), suspended in 50 mM Tris-HCl (pH 7.5), 330 mM sorbitol and 2 mM $MgCl_2$ were incubated in the dark or light (60 W m^{-2}), at 30°C. At indicated times incubations were subjected to native and SDS-PAGE. The remnant CuZn-SOD activity or protein content were estimated by activity staining or Western blotting as described in "Materials and Methods" (A). Western blots were scanned with a digital image analizer and each value in B is the mean ± SE of four independent experiments (only SE values larger than the symbol sizes are represented).

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serine-type (Virgin et al. 1991, Casano et al. 1994) and/or putative metal-type proteases (Desimone et al. 1996). When either native or H_2O_2-pretreated SOD was incubated in a pH range (5.0 to 8.5) with hypotonically ruptured chloroplasts, no significant SOD degradation was detected (Fig. 3A, B, respectively). These results are in agreement with those obtained from autolysis experiments, in which after 3 h incubation of control or H_2O_2-pretreated chloroplasts in the same pH range, no endogenous SOD loss could be observed (Fig. 3C, D, respectively). The lack of significant proteolysis of endogenous SOD was not modified by changing either the time or temperature of the incubation (data not shown). In spite of the absence of proteolytic degradation of CuZn-SOD, at acid pH a clear hydrolysis of several chloroplastic polypeptides was found in gels stained with Coomassie Brilliant Blue (Fig. 3E). On the other hand, we assayed the effect of several protease inhibitors on the loss of SOD protein in isolated chloroplasts incubated in the light. Neither trypsin-like (PMSF and TLCK), metal-type (EDTA and *o*-phenanthroline), aspartyl-type (pepstatin) nor sulfhydryl-type (NEM and iodoacetamide) protease inhibitors, added separately or in a mixture (Table 1), were able to prevent the light-promoted SOD degradation. Taken together these results strongly suggest that chloroplastic CuZn-SOD degradation under photooxidative stress would not be accomplished by plastid proteolytic activity(ies).

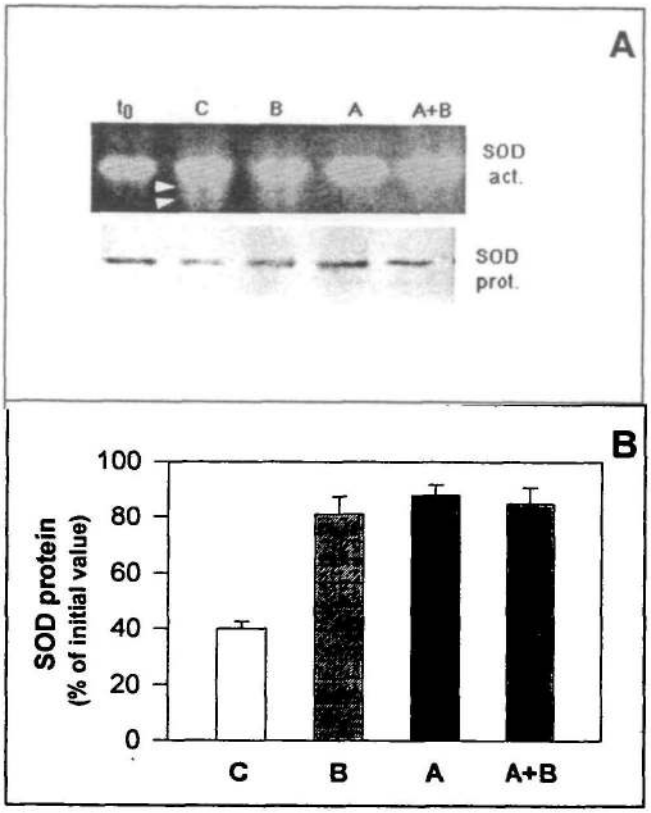

Fig. 2 Effect of ascorbate and benzoate on CuZn-SOD in isolated wheat chloroplasts exposed to photooxidative stress. Thrity μl of intact chloroplasts (7.5 μg protein), suspended in 50 mM Tris-HCl (pH 7.5), 330 mM sorbitol and 2 mM $MgCl_2$ containing either 0 (C) or 5 mM sodium benzoate (B) and/or 0 or 5 mM sodium ascorbate (A), were incubated for 0 and 1 h under 60 W m^{-2}, at 30°C. The remnant CuZn-SOD activity and protein content were estimated by activity staining in native gels or by Western blotting as described in Materials and Methods (A). Western blots were scanned with a digital image analizer and results in B, expressed as percentage of 1 h-incubation respect to 0 h-incubation values, are means ± SE of four independent experiments. White arrows indicate new bands of higher electrophoretic mobility.

Decay of SOD activity and stability by active oxygen species—If photooxidative-promoted CuZn-SOD degradation is not due to a protease-catalyzed hydrolysis, it is possible that active oxygen species could directly inactivate and degrade the enzyme. This proposition was tested by studying the effect of O_2^-, H_2O_2 or $^{\cdot}OH$ on the activity and stability of purified chloroplastic CuZn-SOD.

As shown in Fig. 4A, CuZn-SOD activity was not significantly altered by exposure to O_2^- treatments, even at a O_2^-/protein ratio of 9,700 nmol O_2^- (mg protein)$^{-1}$. No change in the charge/mass ratio of the protein band of wheat SOD was observed by native PAGE (Fig. 5A). SDS-PAGE showed no effect of O_2^- on the total amount of SOD (data not shown). In agreement, Davies (1987) reported that O_2^- did not cause protein aggregation or fragmentation of purified bovine SOD.

When CuZn-SOD was exposed to H_2O_2 for 30 min the remnant activity decayed with the increase in H_2O_2 concen-

Table 1 Effect of protease inhibitors on the light-induced degradation of CuZn-SOD in isolated intact chloroplasts

Inhibitor	Concentration	Inhibition (% of control)
PMSF	1 mM	9
TLCK	0.1 mM	2
Pepstatin	0.1 mg ml^{-1}	nd
Iodoacetamide	1 mM	10
NEM	1 mM	4
EDTA	5 mM	11
o-phenanthrolin	1 mM	6
mixture		9.5

Thirty μl of intact chloroplasts (7.5 μg protein), suspended in isotonic medium, were incubated with or without (control) protease inhibitors 3 h under 60 W m^{-2} at 30°C. Mixture contained all the above cited inhibitors at the indicated concentrations. Reactions were stopped with SDS-sample buffer, subjected to SDS-PAGE and Western blotting employing antibody against CuZn-SOD. Staining intensity was quantified with digital analyzer of images. nd: not detected.

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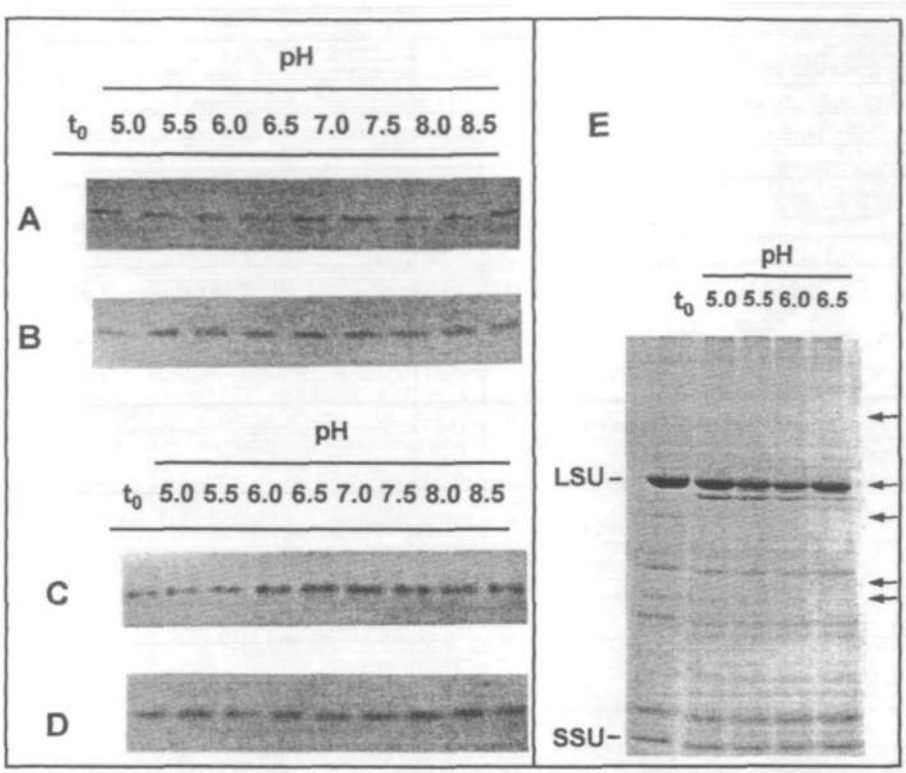

Fig. 3 Effect of pH on the degradation of purified CuZn-SOD by isolated wheat chloroplasts. Three μg of either native (A) or H_2O_2-pretreated SOD (B) dissolved in either 100 mM sodium acetate buffer (from pH 5.0 to 6.0) or 100 mM Tris-maleate buffer (from pH 6.5 to 8.5), were incubated with 2–5 μl of isolated chloroplasts (0.3 μg protein), for 0 (t_0) and 3 h at 30°C in the dark. Pretreatment with H_2O_2 was performed by incubating SOD, at 30°C for 30 min, in the appropriate buffer containing 400 μM H_2O_2. Endogenous CuZn-SOD degradation was assayed by incubating isolated chloroplasts without adding purified enzyme (C and D). Non pretreated (C) or H_2O_2-pretreated chloroplasts (D) (7.5 μg protein in both cases) were incubated in a range of pH. Reactions were stopped with SDS-sample buffer, subjected to SDS-PAGE and the remnant CuZn-SOD content was analyzed by Western blotting. E: Degradation of proteins in isolated wheat H_2O_2-pretreated chloroplasts incubated at acid pH. The experimental procedure was similar to that described in D, except that after SDS-PAGE, polypeptides were stained with Coomassie Brilliant Blue. Arrows indicate some of the rapidly degraded polypeptides.

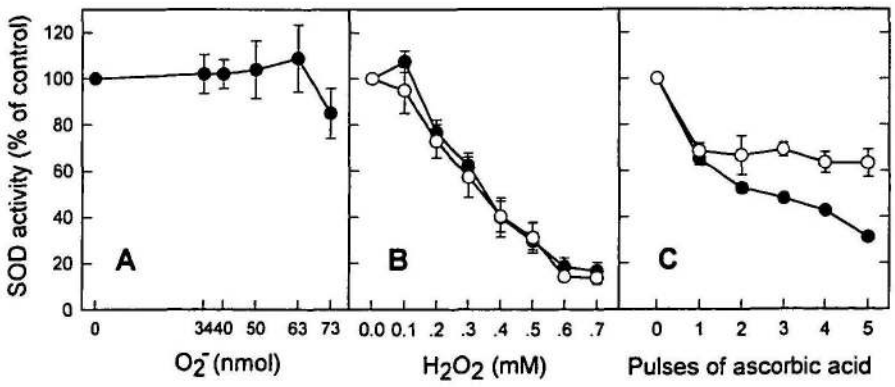

Fig. 4 Effect of active oxygen species on the activity of purified CuZn-SOD. Three units of CuZn-SOD were exposed to a O_2^--generating system (A) and 6 units of enzyme were incubated with either H_2O_2 (B) or an $^{\cdot}OH$-generating system (C) in the presence of 0 (●) or 20 mM Na-benzoate (○). The remnant enzymatic activity was assayed as described in Materials and Methods. Control values were around 15 unit (μg protein)$^{-1}$. Each value is the mean ± SE of at least four independent experiments. For more details see Materials and Methods.

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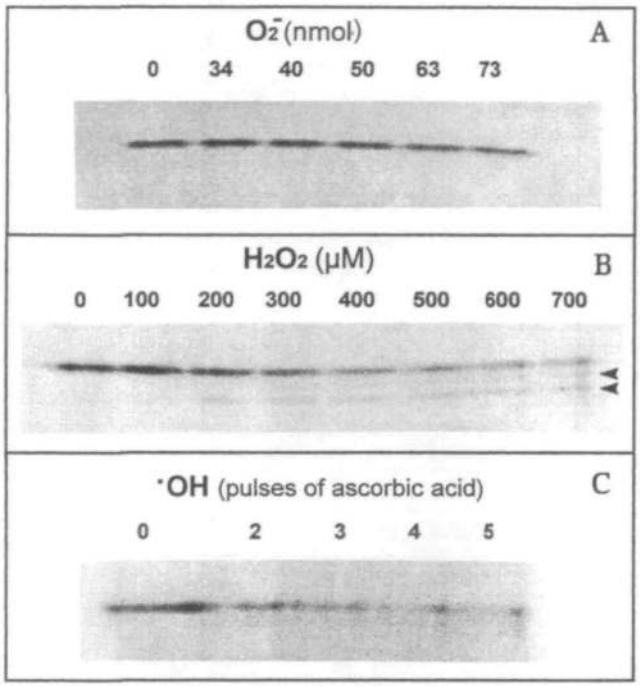

Fig. 5 Effect of active oxygen species on the stability of purified CuZn-SOD of wheat chloroplast. Ten μg of SOD were exposed to H_2O_2 (B), and O_2^- (A) and ·OH-generating system (C), as described in Materials and Methods. Remnant protein was analyzed by native PAGE. Gels were stained with Coomassie Brillant Blue according to standard procedures. Arrows indicate H_2O_2-induced SOD bands.

tration in a dose-dependent manner (Fig. 4B). Some 84% of SOD activity was lost following exposure to 0.7 mM H_2O_2, and I_{50} for H_2O_2 was 355 μM. These results indicate that chloroplastic CuZn-SOD is less sensitive to H_2O_2 than a well characterized cytosolic CuZn-SOD of wheat germ, named SOD I by Beauchamp and Fridovich (1973). Benzoate did not significantly change the slope of the inactivation curve of chloroplastic SOD (Fig. 4B). Physical modifi-

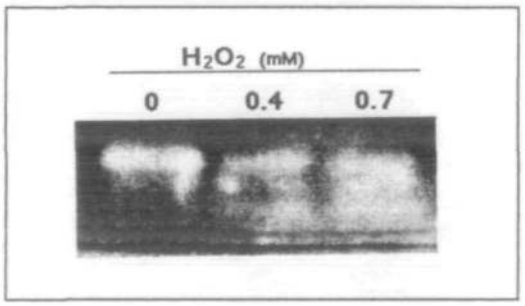

Fig. 6 H_2O_2-modified bands of purified CuZn-SOD retain enzymic activity. Aproximately 2 units of CuZn-SOD were incubated with H_2O_2 at the indicated concentrations. After 30 min at 30°C, samples were subjected to native PAGE and SOD activity was developed as described in Materials and Methods.

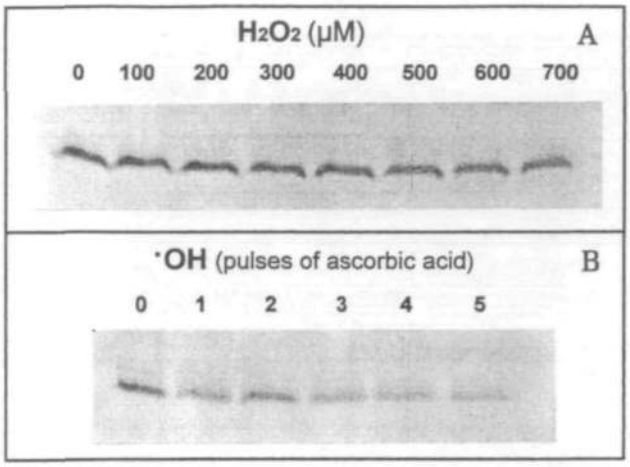

Fig. 7 Breakdown of wheat CuZn-SOD after treatments with H_2O_2 and ·OH radical. Ten μg of SOD were exposed to H_2O_2 (A) and a ·OH-generating system (B), as described in Materials and Methods. Remnant protein was electrophoresed by SDS-PAGE. Gels were stained with Coomassie Brillant Blue according to standard procedures.

cations of CuZn-SOD following H_2O_2 treatments were examined by PAGE under both native and denaturing conditions (Fig. 5B, 7A, respectively). Native PAGE showed a progessive decrease of the native protein and increase in the formation of two to three discreet bands as H_2O_2 increased (Fig. 5B). The increase in the charge/mass ratio of these bands, ranging from 4 to 20% in relation to the native protein, was, however, lower than that observed for bovine SOD (Salo et al. 1990). SDS-PAGE (Fig. 7A) showed only a slight decrease of the SOD monomer in treatments with H_2O_2 higher than 0.4 mM. Therefore, the new bands observed in native PAGE could mainly result from H_2O_2-induced charge modifications rather than from protein fragmentation. Interestingly, the two main bands of higher electrophoretic mobility still conserved SOD activity (Fig. 6).

Wheat SOD was also exposed to ·OH pulses at 10 min intervals. After an initial sharp decay, enzyme activity decreased as the ·OH dose increased to approximately the 30% of the control value (Fig. 4C). Benzoate had no effect on SOD inactivation caused by the first pulse of ·OH. However, this scavenger almost completely prevented oxidative inhibition of the enzyme from the second pulse on. Native PAGE indicated alterations in the native SOD band, specially after 3 to 5 pulses of ·OH (Fig. 5C). In contrast with the H_2O_2 treatments (Fig. 5B), no higher charge/mass discreet bands were observed. SDS-PAGE revealed that 3 to 5 pulses of ·OH induced a progressive loss of SOD monomer (Fig. 7B), suggesting that ·OH-mediated protein fragmentation could, at least partially, account for the degradation of SOD.

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Discussion

Under photooxidative stress, the rate of inactivation and degradation of chloroplastic CuZn-SOD tends to increase with the extent or with the lenght of exposure to the stress. For example, SOD decay was not observed in chloroplasts kept in the dark for 4 h, however, under photooxidative conditions both SOD protein and activity strongly decreased (Fig. 1). This inactivation/degradation process seems to be mediated by active oxygen species.

In animal cells, CuZn-SOD is inactivated and degraded by the combined action of active oxygen species and proteases, which preferentially hydrolyze oxidatively-damaged proteins (Salo et al. 1990, Strack et al. 1996). Chloroplastic CuZn-SOD is not degraded in a similar way. Experimental evidence presented in this paper indicates that wheat chloroplasts do not contain significant protease activity against either native or H_2O_2-pretreated SOD (Fig. 3, Table 1). However, the rate of SOD degradation during the first hour of photooxidative stress would be as high as 30 μg of SOD (mg Chl)$^{-1}$, assuming a plastid SOD content of 40 to 50 μM (Hayakawa et al. 1984), a wheat CuZn-SOD molecular mass of 38 kDa and a chloroplast volume of 35 μl (mg Chl)$^{-1}$ (Asada and Takahashi 1987). In sum, even though we could not completely discard the possible involvement of a plastidic protease in CuZn-SOD breakdown, this proteolytic activity had a marginal effect in the rapid photooxidative-induced degradation of chloroplastic SOD.

Thus, we studied if active oxygen species could act directly in the degradation of chloroplastic SOD. O_2^- radical had no effect on purified wheat CuZn-SOD activity (Fig. 4A). Salo et al. (1990) observed that O_2^- could modify the charge/mass ratio in bovine SOD. However, the electrophoretic pattern of wheat SOD was not altered, even in treatments with high O_2^-/protein ratio (Fig. 5A).

H_2O_2 inhibited wheat CuZn-SOD (Fig. 4B). H_2O_2 can reduce the enzyme-bound Cu^{+2} to Cu^+, which in turn can form Cu^{+2}-$^{\cdot}OH$ with additional H_2O_2. This "in situ" $^{\cdot}OH$ could completely inactivate the enzyme molecule by oxidative modification of histidine residues at the active site (Hodgson and Fridovich 1975). This mechanism could explain the observed H_2O_2-driven inactivation of wheat SOD (Fig. 4B). The lack of effectiveness of benzoate in preventing such inactivation could be due to the fact that it can only scavenge free $^{\cdot}OH$. Besides, H_2O_2 seems to modify amino acid residues other than hystidine at the active site, as suggested by the fact that H_2O_2-induced bands of higher electrophoretic mobility (Fig. 5B) still showed enzymatic activity (Fig. 6).

CuZn-SOD was also inhibited by $^{\cdot}OH$ radicals (Fig. 4C). Benzoate had a biphasic effect suggesting two mechanisms of oxidative damage of CuZn-SOD exposed to a metal-catalized $^{\cdot}OH$-generating system. Initially, Cu^{+2}, added as a part of our $^{\cdot}OH$-generating system, probably bound to residues of His, Pro, Lys, Arg or Cys, which are relatively abundant in all known CuZn-SODs (Baum et al. 1983) thus formed site-specific $^{\cdot}OH$ radicals. This damage is not inhibited by benzoate (Stadtman 1993) and could mainly account for the partial inhibition by the first pulse of ascorbate. From the second pulse on, the generation of free $^{\cdot}OH$ could prevail, since benzoate prevented SOD inhibition. The loss of enzymatic activity observed during the last pulses seemed to be due to the fragmentation of the wheat SOD protein as indicated by native and SDS-PAGE (Fig. 5C, 7B, respectively). In accordance with this idea, Davies (1987) reported that bovine SOD suffered a loss of 74% of the protein staining band in SDS-PAGE after exposure to $^{\cdot}OH$ radicals generated by radiolysis.

The rate of O_2^- and H_2O_2 formation within intact chloroplasts under optimal conditions was estimated to be 240 and 120 μM s^{-1}, respectively (Asada and Takahashi 1987). Antioxidant enzymes, including SOD (Ogawa et al. 1995) and H_2O_2-scavenging enzymes (Miyake and Asada 1992), are preferentially located near the site of O_2^- production. As a consequence, it was estimated that H_2O_2 concentration is less than 8×10^{-7} M (Asada 1992). Under oxidative stress conditions, ascorbate peroxidase activity decrease to a higher extent than SOD activity (Baisak et al. 1994, Del Longo et al. 1993, Luna et al. 1994). Therefore, it is reasonable to assume that, under such conditions, the actual concentration of H_2O_2 could rise several orders of magnitude with respect to that estimated for non-stressed chloroplasts, specially within the localization site of antioxidant enzymes. Besides, when H_2O_2 is not removed by ascorbate peroxidase, the probablility of $^{\cdot}OH$ generation by metal-catalyzed reactions is enhanced (Asada and Takahashi 1987). Micromolar levels of H_2O_2 induced charge/mass alterations on purified CuZn-SOD (Fig. 6) that clearly resembled those observed in photooxidatively stressed chloroplasts (Fig. 2). Ascorbate, that preserves peroxidase activity (Nakano and Asada 1981), greatly reduced inactivation, charge/mass alteration and degradation of CuZn-SOD in wheat chloroplasts (Fig. 2). In contrast, benzoate did not prevent SOD inactivation in chloroplasts but was able to diminish SOD degradation (Fig. 2). We propose that when leaves are exposed to photooxidative stress, the increased concentration of H_2O_2 inactivates plastidic CuZn-SOD. Under such conditions SOD is degraded by a non-enzymatic mechanism involving $^{\cdot}OH$ radicals or species acting like $^{\cdot}OH$.

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