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Effect of calcium and zinc on the activity and thermostability of superoxide dismutase

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Abstract

The effect of calcium and zinc ions on superoxide dismutase (SOD) from four plant species (*Taxus baccata*, *Pinus sylvestris*, *Medicago rigidula*, and *Zea mays*) was followed at three temperatures: optimal (20 °C), increased (50 °C), and high, inhibiting temperature (70 - 80 °C). At 20 and 50 °C *in vitro* added calcium increases SOD activity, but the degree was different for the plants investigated. The effect of zinc ions at the same temperatures varied in the investigated plants from activation to inhibition. An inhibiting effect of high temperature on SOD activity was diminished in the presence of calcium or zinc ions. It was shown that calcium and zinc ions can increase activity and thermostabilize different SOD isoforms.

Additional key words: *Medicago rigidula*, *Pinus sylvestris*, SOD activation, *Taxus baccata*, *Zea mays*.

Introduction

The toxic superoxide radical is formed in many metabolic reactions in plant cell, and superoxide dismutase (SOD) plays a crucial role in the maintenance of its low cellular concentration. Thus an increased SOD activity is essential in oxidative stress tolerance (Bowler *et al.* 1992, McKersie *et al.* 1991, Slooten *et al.* 1995). However, oxidative damage is evident under stress when the production rate of $O_2^{\cdot}$ exceed the scavenging ability of the cell (Navari-Izzo *et al.* 1997, Casano *et al.* 1997). That is why the elucidation of the mechanisms of regulation of SOD activity and factors influencing SOD stability is of great importance.

Well known is the role of calcium ions for peroxidase, POD (Xu and van Huystee 1993) and of zinc ions for Cu,Zn SOD activity. The aim of our study was to elucidate the role of Ca^{2+} and Zn^{2+} as effectors of SOD isolated from yew, Scots pine, alfalfa, and maize. We continue our investigations carried out with some lower plants *Scenedesmus acutus* (Bakardjieva *et al.* 1994), *Mnium affine* (Christov and Bakardjieva 1998, 1999), and *Polypodium vulgare*. The same plant species were also used in our previous experiments, clarifying the effect of Ca^{2+} and other metal ions on POD and catalase (unpublished). All together we could receive information on the role of these ions on the major participants in the defence complex against the activated oxygen species.

Materials and methods

Samples from yew (*Taxus baccata* L.), Scots pine (*Pinus sylvestris* L.) and alfalfa (*Medicago rigidula* L.) were collected from their natural habitat during June - August on Mount Vitosha near Sofia, and were acclimated for

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Abbreviations: EDTA - ethylenediaminetetraacetic acid, POD - peroxidase, SOD - superoxide dismutase, Triton - t-octylphenoxy-polyethoxyethanol.

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2 d to conditions equal for all plants (temperature of 22 °C , relative humidity of 60 % and 14-h photoperiod with irradiance of 350 μmol m^{-2} s^{-1}. Maize (*Zea mays* L.) plants were grown in nutrient solution for two weeks at the same conditions.

All operations for preparing the enzyme extracts were performed at 4 °C. Fresh and fully grown leaves were homogenized in 0.1 M Tris-HCl buffer, pH 7.8, containing 0.1 mM EDTA, and 0.2 % Triton X100 in the case of conifers or 1 % polyclar in the case of maize. After 30 min the homogenates were filtered through 4 layers of gauze and centrifuged for 30 min at 15 000 *g*. The supernatant was dialyzed for 24 h against half-strength extraction buffer and centrifuged for 20 min at 15 000 *g*. Further enzyme extract was purified by gel filtration using *Sephadex G-25*. The protein content was determined by the method of Bradford (1976).

SOD activity was assayed according to McCord and Fridovich (1969). The reaction mixture contained 50 mM KH_2PO_4, 0.1 mM EDTA, 0.05 mM xanthine, 0.01 mM ferricytochrome *c*, and xanthine oxidase. Amount of xanthine oxidase was chosen to cause change in absorbance 0.025 units per min at 550 nm (*UV-VIS 1601, Shimadzu*, Tokyo, Japan) in samples without SOD. One unit of SOD is defined as the amount of the enzyme that inhibits by 50 % the control rate. The effect of the elevated temperatures was examined by heating the enzyme extract for 60 s in water bath, cooling it to room temperature and immediately measuring SOD activity. SOD was separated by electrophoresis on non-denaturating 7.5 % polyacrylamide gel and different SOD bands were localized according to Beauchamp and Fridovich (1971).

The effect of Ca^{2+} and Zn^{2+} was studied using calcium or zinc sulphates with 5 mM final concentration in enzyme extract. The effect of the metal ions on the different electrophoretically separated SODs was examined soaking the gels after electrophoresis for 10 min in 5 mM solution of the respective ion sulphate and after washing three times in distilled H_2O, the gels were stained for SOD visualization. The effect of high temperature on the SOD isoenzymes was studied incubating the gels for 90 s in water bath before staining and after soaking in ions solution.

The results presented are the means of least two independent experiments for conifers and three independent experiments for maize and alfalfa.

Results

The changes in SOD activity caused by increased temperatures were species specific (Fig. 1). On the basis of these data two increased temperatures were chosen at which the effects of Ca^{2+} and Zn^{2+} on the activity and thermostability of SOD were investigated: one at which activity was increased, or not markedly changed (50 °C for SOD from *T. baccata*, *P. sylvestris* and *Z. mays* and 60 °C for SOD from *M. rigidula*), and other with well expressed inhibiting effect (70 °C for SOD from *T. baccata* and *M. rigidula* and 80 °C for SOD from *P. sylvestris* and *Z. mays*).

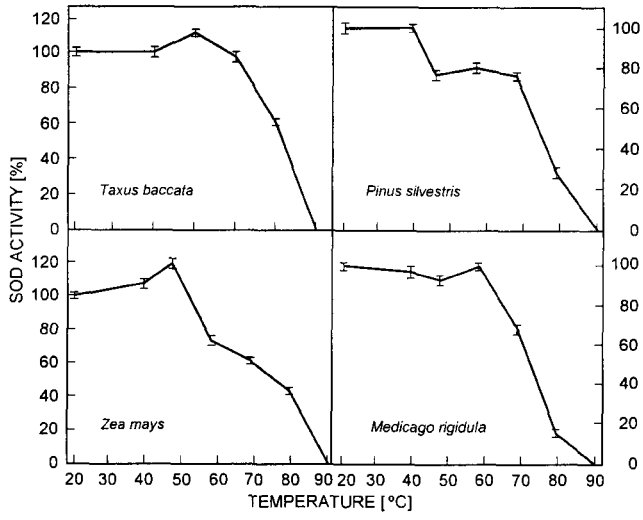

Fig. 1. SOD activities from *Taxus baccata*, *Pinus sylvestris*, *Zea mays* and *Medicago rigidula* after short term treatment (60 s) at different temperatures. The activity of SOD at 20 °C is accepted as 100 %. Means ± SE ($n = 6$ for *T. baccata* and *P. sylvestris*, $n = 9$ for *Zea mays* and *Medicago rigidula*).

After addition of Ca^{2+}, an increase in activity of SOD extracted from all plants was established at 20 °C (Fig. 2). The effect of Zn^{2+} varied from activation in *P. sylvestris*, to small inhibition in *T. baccata* and maize. A positive effect of Ca^{2+} on SOD activity was also found in *P. sylvestris* at 50 °C and *M. rigidula* at 60 °C. At these temperatures, an increased activity of SOD caused by Zn^{2+} was also observed, and in *M. rigidula* the effect of Zn^{2+} was greater than that of Ca^{2+}. In *T. baccata* the activating effect of Ca^{2+} was well expressed at 20 °C, while slight stabilizing effect at 70 °C was found. In the other investigated plants Ca^{2+} considerably increased the thermostability of SOD. The effect of Zn^{2+} on SOD thermostability was similar with the exception of maize.

These effects of calcium were confirmed in experiments with commercial purified Cu,Zn-SOD (*SERVA*, Heidelberg, Germany) (Fig. 2E). Ca^{2+} increased SOD activity at 20 °C and completely alleviate inhibiting effect of high temperature (2 min at 70 °C). All these data proved a significant role of Ca^{2+} for SOD function and thermostability.

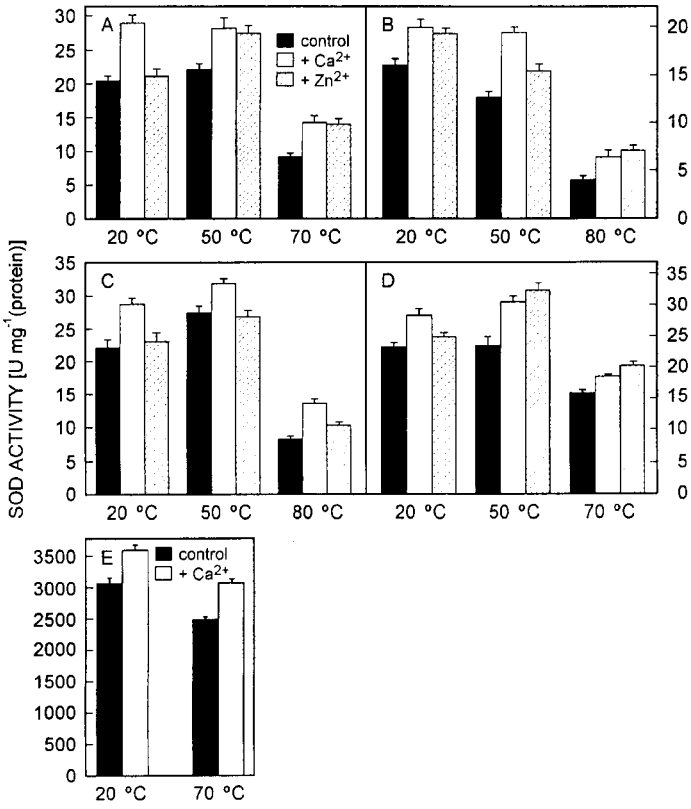

Fig. 2. Effect of Ca^{2+} and Zn^{2+} on SOD activity at different temperatures: *A* - SOD from *Taxus baccata*, *B* - SOD from *Pinus sylvestris*, *C* - SOD from *Zea mays*, *D* - SOD from *Medicago rigidula*, and *E* - commercial Cu,Zn-SOD. Means ± SE ($n = 6$ in *A,B*; $n = 9$ in *C,D,E*).

Further we examined the effect of Ca^{2+} and Zn^{2+} on the individual SOD isoforms. SOD from *T. baccata* was electrophoretically separated into seven bands (Fig. 3*A*), four of them being fast moving and highly active. At 70 °C the activity of all SOD isoforms was inhibited. At 20 °C the Ca^{2+} increased activity of some slow moving bands (E, F, G). At 50 °C this effect was preserved and even enhanced. At 70 °C the stabilizing effect of Ca^{2+} on band F was clearly demonstrated and even some activation was observed. Zn^{2+} decreased activity of the band F at 20 °C, but at 50 °C the other slow moving bands (E and G) were stabilized. At 70 °C a clear stabilizing effect of Zn^{2+}on band B was found, while the activity of the other bands was slightly decreased.

Electrophoretic separation of SOD from *P. sylvestris* showed five fast moving bands (Fig. 3*B*). At 50 °C their activity were decreased and to a greater extent at 80 °C. At 20 and 50 °C Ca^{2+} increased activity of bands C and E. The greater stability of SOD from *P. sylvestris* at 80 °C in the presence of Ca^{2+} was connected with well expressed thermostabilization of band C. The effect of Zn^{2+} was similar to that of Ca^{2+}, especially at 80 °C.

Eight SOD bands were found in maize (Fig. 4*A*). Bands A, B, F, and G were clearly activated after

treatment with 50 °C, while the high temperature (80 °C) inhibited mainly the fast moving isoforms. At 20 °C a well expressed activation of SOD bands A and B by Ca^{2+} was observed. At 50 °C the effect of Ca^{2+} on bands A and B was preserved and band F was also activated. But at 80 °C Ca^{2+} stabilized only band A. In this case the addition of Zn^{2+} had no effect.

Eight SOD bands were found in alfalfa (Fig. 4*B*). The increased temperatures caused a visible inhibition of band D at 60 and 70 °C, while the activity of band H at these two temperatures was fully preserved. At 20 °C in the presence of Ca^{2+} SOD bands C and D were activated. At 60 °C such effect was observed for bands C and H, while band D was partially stabilized. At 20 °C the presence of Zn^{2+} resulted in clear activation of band H and C. At 60 °C this effect was also observed and at 70 °C the activity of band C was fully preserved.

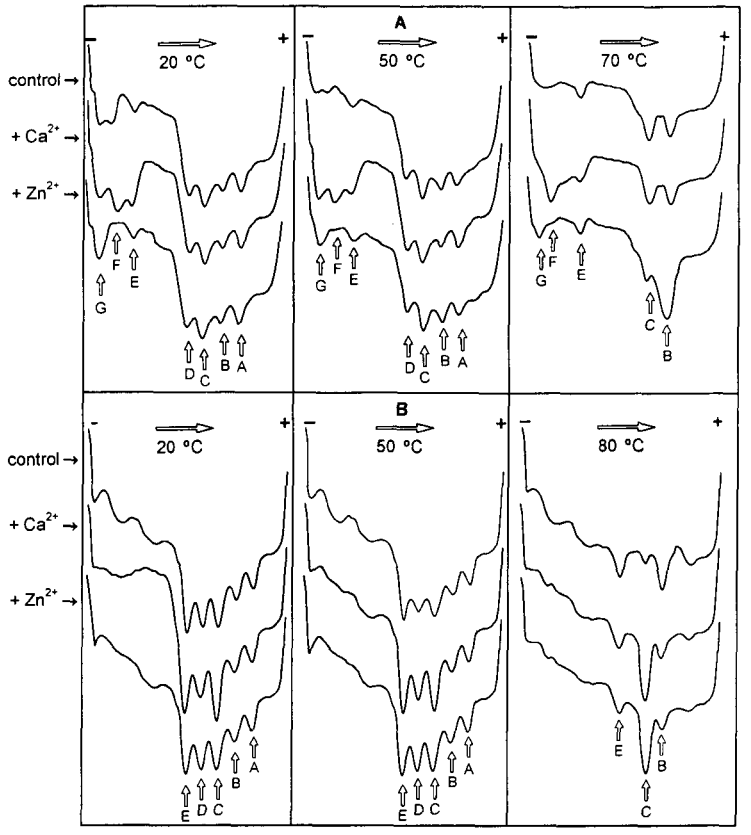

Fig. 3. The effect of Ca^{2+} and Zn^{2+} on electrophoretic pattern of SOD from *Taxus baccata* (*A*) at 20, 50 and 70 °C and *Pinus sylvestris* (*B*) at 20, 50, and 80 °C. The protein content in all gels was 80 μg. SOD bands were visualized according to Beauchamp and Fridovich (1971). All gels were scanned at equal sensitivity of the densitometer.

After subcellular fractionation bands A and H were found in the chloroplast fraction, bands D and G in the mitochondrial fraction and the rest in the cytosol (Christov and Bakardjieva 1998). A strong activation effect of Ca^{2+} was demonstrated on a mitochondrial SOD (here marked as D) and the main cytosolic SOD (band C). Zn^{2+} activated chloroplastic SODs, especially band H.

Discussion

Our investigations showed a clear positive effect of Ca^{2+} and Zn^{2+} on SOD activity and thermostability. The degree of activation was different for SODs of different sources, but sufficient to be of physiological importance. In comparison with Zn^{2+}, effect of Ca^{2+} at 20 °C was more clearly expressed. At slightly increased temperatures the positive effect of both ions was even better expressed. At inhibiting temperatures (70 - 80 °C) these two ions increased SOD thermostability. It has been supposed (Kanematsu and Asada 1989) that the heat denaturation of some SODs is dependent on their subunit structure. Thus the observed thermostabilization of some SOD isoforms

in presence of the metal ions might be due to the stabilization of their subunit structure. The stabilizing effect of Ca^{2+} was also confirmed with commercial SOD.

Our experimental results indicated that the individual SOD isoforms react differently to added Ca^{2+} or Zn^{2+}. A similar reaction to high temperature and *in vitro* addition of Ca^{2+} and Zn^{2+} was found for the different SODs from *Scenedesmus acutus* (Bakardjieva *et al.* 1994). However, in *Mnium affine* Ca^{2+} predominantly activated and thermostabilized the mitochondrial SODs and one cytosolic SOD, while Zn^{2+} activated and increased thermostability of the chloroplastic SODs (Christov and Bakardjieva 1999) similarly as in *M. rigidula* (Christov and Bakardjieva 1998).

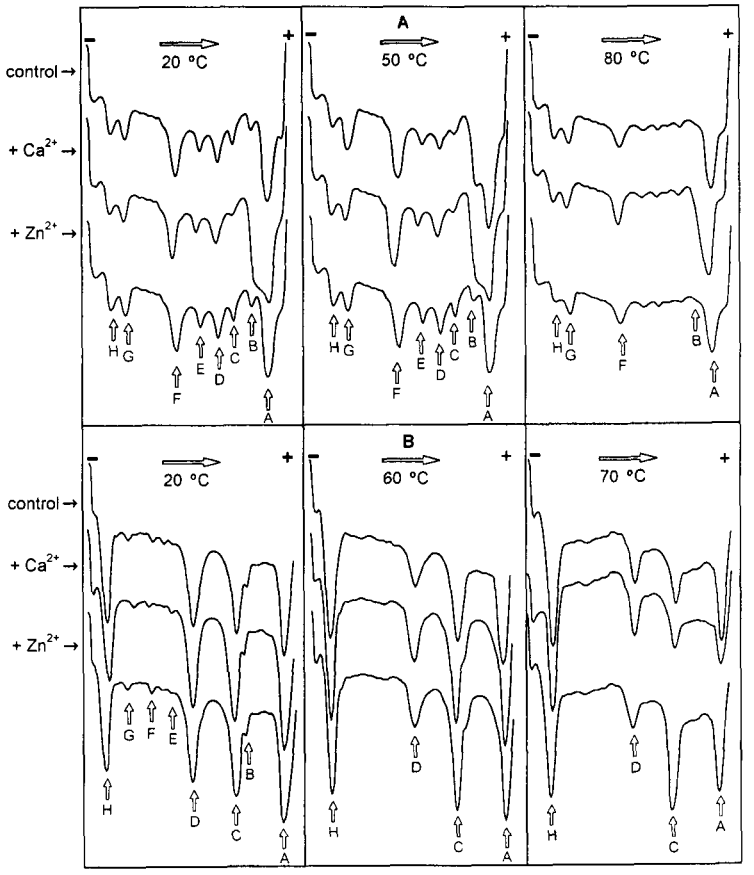

Fig. 4. The effect of Ca^{2+} and Zn^{2+} on electrophoretic pattern of SOD from *Zea mays* (*A*) at 20, 50 and 80 °C and *Medicago rigidula* (*B*) at 20, 60, and 70 °C. The protein content in all gels is 70 μg. SOD bands were visualized according to Beauchamp and Fridovich (1971). All gels were scanned at equal sensitivity of the densitometer.

These data proved that the changes in calcium concentration might influence activity and thermostability of some SOD isoforms in plants. A similar role of Ca^{2+} was also found for POD (Bakardjieva *et al.* 1996). It may be suggested that calcium plays a definite role for the defense enzyme complex in plants. Zinc ions also influence SOD activity and thermostability, but in most cases other SOD isoforms than Ca^{2+}. An elevated Zn^{2+} concentration caused in some cases a inhibition of POD and especially of catalase activity (Bakardjieva *et al.* 1994, 1996). That is why it may be presumed that Ca^{2+} plays more important role than Zn^{2+} in regulating the defence system in plants.

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