Effect of calcium and zinc on subcellular distribution, activity and thermosensitivity of superoxide dismutase in *Mnium affine*

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Abstract

In the leafstem moss *Mnium affine* two superoxide dismutase (SOD) isoforms were found in chloroplasts and two in mitochondria. Four other isozymes were probably cytosolic and two of them had high activity and thermostability and were very sensitive to H_2O_2. On the other hand, one of the mitochondrial isoenzymes was very sensitive to high temperature. The activity and thermosensitivity of SOD was considerably dependent on calcium and zinc ions. The effect was different for the individual isoforms and related to their subcellular distribution. Calcium ions predominantly activated and stabilized one cytosolic and the mitochondrial SODs, while zinc ions influenced one chloroplastic and two cytosolic SODs.

Additional key words: chloroplastic isoform, cytosolic isoform, metal ions, mitochondrial isoform, moss, polyacrylamide gel electrophoresis.

Introduction

The peculiarities of aerobic metabolism led to the appearance of an effective defense system against activated oxygen in the early stages of evolution of plants (Asada *et al.* 1980). Superoxide dismutase (SOD) catalyzes the conversion of the superoxide radicals ($O_2^{\cdot-}$) to hydrogen peroxide, which can be removed by catalase or peroxidases. These three enzymes are responsible for the level of the $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ in plant tissues, and therefore for resistance to environmental conditions leading to oxidative stress (Bowler *et al.* 1992).

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Abbreviations: BSA - bovine serum albumin; EDTA - ethylenediaminetetraacetic acid; NBT - nitroblue tetrazolium; PAGE - polyacrylamide gel electrophoresis; SOD - superoxide dismutase; TEMED - N,N,N',N'-tetramethylethylendiamine.

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Three types of SOD have been found in various plant species, depending on the prosthetic metals: Cu,Zn-, Mn- and Fe-containing enzymes. It is supposed that Cu,Zn-SOD appeared during the last stages of evolution of green photosynthetic algae (Kanematsu and Asada 1989a), and that very soon thereafter the two different types of Cu,Zn-SOD evolved. This enzyme is located mainly in chloroplasts, but is also found in cytosol and apoplast (Hayakawa *et al.* 1984, Kwiatowski and Kaniuga 1986, Kanematsu and Asada 1989a, Herouart *et al.* 1994, Ogawa *et al.* 1997). Mn-SOD from *Zea mays*, *Nicotiana plumbaginifolia*, *etc.*, has been found in mitochondria (White and Scandalios 1989, Bowler *et al.* 1989).

The mosses, although lacking many of the specialized structures of higher plants are able to overcome severe environmental conditions. Information about SOD from mosses and its role in survival in extreme environmental conditions is scarce. Stress induced changes in enzyme activity depended on drought tolerance in *Tortula ruralis* and *Cratoneuron filicinum* (Dhindsa and Matowe 1981). SOD activity was completely preserved for twelve months in desiccated moss *Mnium affine*, and significantly increased during the rehydration period (Christov and Bakardjieva 1998a).

In this paper we report activity, subcellular distribution, thermosensitivity, effect of calcium and zinc and isoenzyme forms of SOD from leafstem moss *Mnium affine*.

Materials and methods

The mosses (*Mnium affine*) were collected from their natural habitat during June August at Mount Vitosha near Sofia at an altitude of 900 - 1000 m. They were maintained in the soil they were growing in for 2 - 3 weeks in the laboratory, and then fully grown leaves were used for the experiments. All operations for preparation of enzyme extract were performed at 4 °C. The leaves were homogenized in 0.1 M Tris-HCl buffer, pH 7.8, containing 0.1 mM etylenediaminetetraacetic acid (EDTA) and 1 % polyclar. After 20 min, the homogenate was filtered through 4 layers of muslin and centrifuged for 30 min at 15 000 *g*. The supernatant was dialyzed for 24 h against half strength extraction buffer without polyclar, centrifuged for 20 min at 15 000 *g*, and the supernatant used in the experiments. The quantity of protein in the enzyme extracts was determined by the method of Bradford (1976).

The activity of SOD was assayed by the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). The photochemical procedure was chosen as being more independent of the presence of different contaminants. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 10 mM methionine, 0.053 mM NBT, 0.0053 mM riboflavin. The reaction was started by switching on the light and was allowed to run for 7 min. The absorbance at 560 nm was measured. One enzyme unit was taken to be the volume of extract corresponding to 50 % inhibition of the reaction carried out without addition of enzyme. The effect of elevated temperatures (40, 50, 60, 70, 80, and 90 °C) was measured after heating the enzyme extract at the respective temperatures for 1 min in water bath, followed by cooling to room temperature.

SOD isozymes were separated by electrophoresis on native 7.5 % polyacrylamide gel according to Beauchamp and Fridovich (1971). The gels were first soaked in solution of 0.2 mM NBT for 20 min, then in 0.028 mM riboflavin and 0.025 mM N,N,N',N'-tetramethyletylendiamine for 20 min, and illuminated in tubes containing 5 mM K-phosphate at pH 7.8. The stained gels were scanned with a densitometer.

The effect of calcium and zinc ions was studied using $CaSO_4$ and $ZnSO_4$ which was added to the enzyme extract with a final concentration of 5 mM. After a 15 min incubation and temperature treatment SOD activity was measured. The effect of the calcium or zinc ions on the different SOD isoenzymes was examined by soaking the gels for 15 min in 5 mM sulfate solution of the respective ion, washing the gels with distilled water and finally staining for SOD isoenzymes visualization. The effect of high temperature on the SOD isoenzymes was examined after ion treatment, incubating the gels for 90 s in a water bath at the respective temperature followed by cooling and staining.

Subcellular fractionation was performed according Boutry *et al.* (1987) with some modifications. Fresh leaves (5 g) were homogenized in 5 cm^3 50 mM Tris-HCl buffer, pH 7.8, containing 0.33 M sucrose, 0.2 % bovine serum albumin (BSA), 0.1 % ascorbic acid and 0.05% β-mercaptoethanol. After filtration through four layers of muslin homogenate was centrifuged at 4 000 *g* for 60 s. The pellet (crude chloroplast fraction) was resuspended in 2 cm^3 suspension medium (0.4 M manitol, 10 mM K_2HPO_4, pH 7.2, 0.1 % BSA) layered onto two step Percoll gradient (4 cm^3 80 % Percoll, 0.1 % BSA, 5 cm^3 40 % Percoll, 0.1 % BSA) and centrifuged for 10 min at 16 500 *g* in a *Beckman* centrifuge with a *SW41* rotor. The interface which contained purified chloroplasts was recovered. The supernatant from the first centrifugation was further centrifuged at 15 000 *g* for 15 min. The pellet (crude mitochondrial fraction) was resuspended in 2 cm^3 of suspension medium, layered onto three step Percoll gradient (3 cm^3 40 % Percoll, 0.1 % BSA, 3 cm^3 21 % Percoll, 0.1 % BSA, 2 cm^3 11 % Percoll, 0.1% BSA) and centrifuged for 30 min at 25 000 *g*. The interface (40 % - 21 % Percoll) which contained purified mitochondria was recovered. The organelles obtained were lysed with addition of Triton X-100 in final concentration 0.01 % and after 30 min the homogenates were centrifuged 20 min at 10 000 *g*. Supernatants were used for electrophoresis.

Results

SOD activity from *Mnium affine* gradually decreased up to 60 °C and was strongly inhibited above this temperature. At room temperature and in the interval between 30 - 50 °C an activating effect of calcium was observed. In contrast, zinc ions slightly inhibited SOD activity in that temperature interval. At 70 °C both metal ions increased the thermostability of the enzyme (about 20 %). This stabilizing effect was preserved at 80 °C only in the presence of zinc ions (Fig. 1).

Electrophoretic separation, using native PAGE, showed 8 SOD isoforms in *Mnium affine*. Four of them were major bands with great mobility and high activity (Fig. 2*a*). Two of the fast moving and highly active isoenzymes were very sensitive to 5 mM

H_2O_2 (Fig. 2) and most probably they are Fe-containing forms. The activity of these two isoforms were influenced by the physiological state of mosses and the environmental conditions (unpublished results).

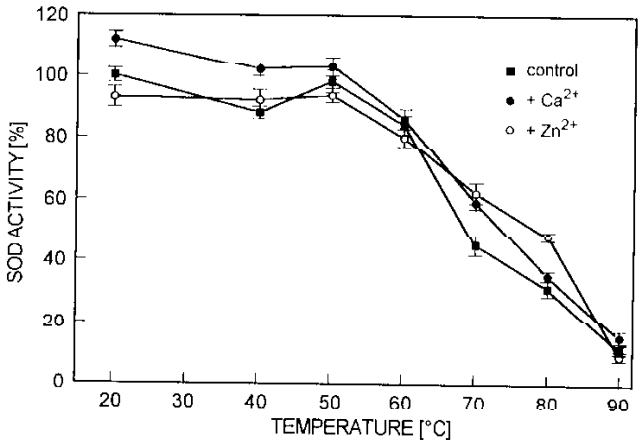

Fig. 1. Changes in the activity of SOD from *Mnium affine* depending on temperature and *in vitro* addition of calcium and zinc ions.

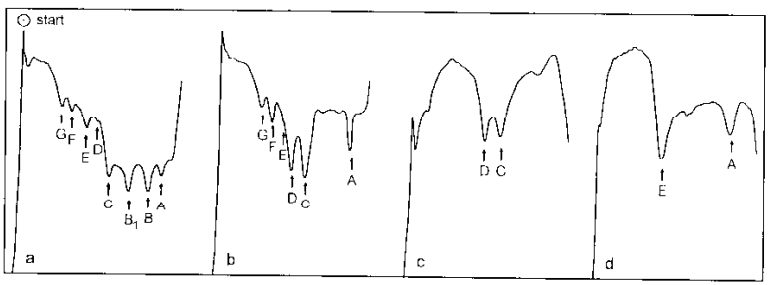

Fig. 2. Isoenzyme pattern of SOD from *Mnium affine* in a crude enzyme extract (*a*), after 5 mM H_2O_2 treatment for 15 min after electrophoresis and before staining (*b*), mitochondrial fraction (*c*), and chloroplast fraction (*d*).

The subcellular distribution of SOD isoforms from *Mnium affine* was traced (Fig. 2*c*,*d*) using extracts from purified mitochondrial and chloroplast fractions and applying native PAGE. Two SOD isoforms were found in mitochondrial fraction (Fig. 2*c*). According to their R_f values they probably coincided with the bands *C* and *D* in the crude extract (Fig. 2*a*). Also two SOD isoforms were found in chloroplast fraction - most probably corresponding to the bands *A* and *E* of the crude extract.

After short-term (90 s) increase in temperature (50 °C) the activity of the fast moving bands (*B* and B_1 - which are most probably cytosolic Fe-containing

isoenzymes) was increased (Fig. 3*b*). They were very stable and did not change their activity even at 70 °C (Fig. 3*c*), while the activity of band C (most probably mitochondrial isoenzyme) was significantly decreased. On the other hand, the band marked as *D* (also mitochondrial isoenzyme) showed clear activation (Fig. 3*c*) in this

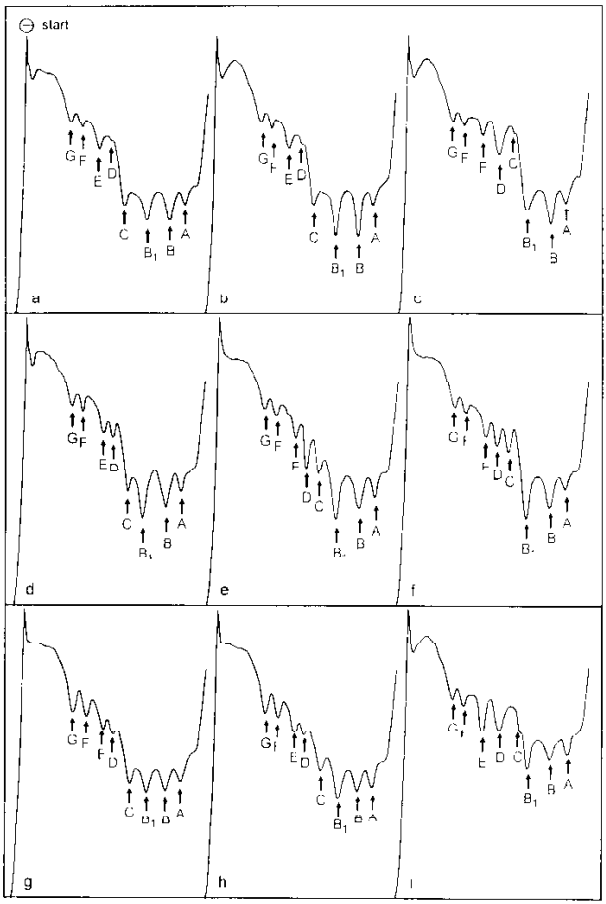

Fig. 3. Effect high temperature treatment (*a*, *b*, *c* - 20, 50 and 70 °C, respectively) and *in vitro* addition of 5 mM calcium (*d*, *e*, *f*) or 5 mM zinc (*g*, *h*, *i*) ions at the same temperatures on different SOD isoenzymes from *Mnium affine*.

case. The addition of calcium ions changed the activity of the SOD band *D* at 20 °C (Fig. 3*d*) and more clearly at 50 °C (Fig. 3*e*). At 70 °C (Fig. 3*f*) calcium ions increased the thermostability of the other mitochondrial SOD isoenzyme, band *C* and isoenzyme marked as band B_1. Incubation of the enzyme with zinc ions (Fig. 3*g*) led to a slight decrease in SOD activity of the fast moving bands and to increased activity

of the slow moving isoenzymes. The thermostability of the fast moving SOD isoforms (bands *B*, *B1* and *C*) is decreased by zinc, but the activity of the SOD bands marked as *F* and *G* (most probably cytosolic SODs) and *E* (chloroplast isoform) was additionally increased. From the obtained results it is evident that these two ions can activate and thermostabilize some SOD isoforms.

Discussion

Almost all characterized Cu,Zn-SODs and Mn-SODs are found to be dimers or tetramers, and their activity strongly depends on the stability of this tertiary structure. It is presumed that their heat sensitivity is connected with denaturation of subunit structure (Kanematsu and Asada 1989b). That is why, although the temperature treatment above 50 °C has no physiological relevance, it is important for the tracing of eventual interaction between metal ions and enzyme protein.

Our results showed that activity and thermosensitivity of SOD from moss *Mnium affine* is considerably dependent on calcium and zinc ions. The effect is different for the individual isoforms and is related to their subcellular distribution. The individual isoforms of SOD showed also different sensitivity to high temperature - part of them being very stable thus ensuring SOD function at extreme situations. Very sensitive to high temperature was one of the mitochondrial isoenzymes (*band C*), as we also established for the mitochondrial SOD isoform from *Medicago rigidula* (Christov and Bakardjieva 1998b). A difference between the two plants, however, is the presence of a second SOD isoform in mitochondria of *Mnium affine*, which is very stable and even activated after high temperature treatment. This fact might be connected with the higher ability of the moss to survive stress (Christov and Bakardjieva 1998a). In our experiments the activating and stabilizing effect of calcium and zinc ions on different SOD isoforms was confirmed. In the case of *M. rigidula* the most profound effect of calcium ions was observed on mitochondrial SOD (Christov and Bakardjieva 1998b). The same tendency was also observed in *Mnium affine*. Calcium ions in both plant species probably activate some cytosolic isoforms. It is interesting to note that one of the chloroplast SOD of *Mnium affine* (*band E*) was activated and stabilized by zinc ions. The same tendency was also found for one of the mitochondrial SOD isoforms from *M. rigidula* (Christov and Bakardjieva 1998b). The second chloroplast SOD (*band G*), which was not considerably influenced by temperature and metal ions, was found to be important in the surviving of mosses after desiccation (Christov and Bakardjieva 1998a). In *Mnium affine* zinc ions also activated some most probably cytosolic SODs, different from those influenced by calcium.

The specific reaction of individual isoforms towards metal ions and temperature may be thought off as a manifestation of a very important mechanism for regulating enzyme function. Based on our results for different plant species: green alga *Scenedesmus acutus* (Bakardjieva *et al.* 1994), moss *Mnium affine*, *Medicago rigidula* (Christov and Bakardjieva 1998a) and other higher plant species

(unpublished data) we propose that the discussed mechanisms appeared early in plant evolution.

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