Phyton (Horn, Austria)	**Vol. 35**	Fasc. 2	233–242	28. 12. 1995

Activity of Superoxide Dismutase and Malondialdehyde Content in Lichens along an Altitude Profile

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With 4 Figures

Received January 17, 1995

Accepted March 26, 1995

Key words: Malondialdehyde, superoxide dismutase, altitude, lichens.

Summary

SCHLEE D., KANDZIA R., TINTEMANN H. & TÜRK R. 1995. Activity of superoxide dismutase and malondialdehyde content in lichens along an altitude profile. – Phyton (Horn, Austria) 35 (2): 233–242, 4 figures. – English with German summary.

The occurrence of the superoxide dismutases and the malondialdehyde content in the lichens *Hypogymnia physodes* and *Pseudevernia furfuracea* collected from different altitudes in the Central Eastern Alps (altitude runs from 1080 m up to 1940 m) were determined. Both specific SOD activity and MDA content were much higher in *P. furfuracea* than in *H. physodes*. Maxima were determined in *Pseudevernia furfuracea* at 1270 m and at higher altitudes (1800–1940 m). In crude extracts six SOD isozymes have been characterized by native PAGE and identified as Cu/Zn-, Mn- and Fe-SODs by inhibition studies. A dominant Cu/Zn-SOD (SOD 6 with low mobility on the gels) contributed 60 to 70 % of the total activity found on the gels. By comparison, SOD activity was determined in *Cetraria islandica* and *Cladonia verticillaris*. The role of SOD in detoxification and adaptation mechanisms to environmental stress factors, particularly to ozone, is discussed.

Zusammenfassung

SCHLEE D., KANDZIA R., TINTEMANN H. & TÜRK R. 1995. Aktivität der Superoxiddismutase und der Malondialdehyd-Gehalt in Flechten entlang eines Höhenprofils. –

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Phyton (Horn, Austria) 35 (2): 233-242, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Das Vorkommen der Superoxiddismutasen und der Malondialdehyd-Gehalt der Flechten *Pseudevernia furfuracea* und *Hypogymnia physodes*, gesammelt in verschiedenen Höhen der östlichen Zentralalpen (1080 m–1940 m Seehöhe), wurden bestimmt. Sowohl die spezifische Aktivität der SOD als auch der MDA-Gehalt war in *Pseudevernia furfuracea* höher als in *Hypogymnia physodes*. Maxima ergaben sich in *Pseudevernia furfuracea* in 1270 m Seehöhe und in höheren Lagen (1800 m–1940 m). Im Rohextrakt wurden mindestens sechs SOD-Isoenzyme mittels PAGE nachgewiesen und durch Hemmungsstudien als Cu/Zn-, Mn- und Fe-SOD identifiziert. Eine dominante Cu/Zn-SOD (SOD 6 mit der geringsten Mobilität im Gel) stellt 60–70 % der im Gel nachgewiesenen Gesamtaktivität dar. Zum Vergleich wurden die SOD-Aktivitäten in *Cetraria islandica* und *Cladonia verticillaris* bestimmt. Es wird die Rolle der SOD hinsichtlich Detoxifikation und Adaptation an Umweltstreß, insbesondere Ozoneinfluß, diskutiert.

1. Introduction

Potentially toxic oxygen species occur in all aerobic organisms. They are ubiquitously generated in many biological oxidations as products of enzymic reactions and of cellular redox reactions (ASADA & TAKAHASHI 1987, BANNISTER & al. 1987). The toxicity has been attributed to their interaction with other cellular components, particularly lipids and pigments. Unfavorable environmental conditions can enhance the production of such toxic oxygen radicals, e.g. superoxide anions ($O_2^{\cdot -}$) in plant cells (BOWLER & al. 1989, ELSTNER & al. 1988). Many xenobiotics and air pollutants also manifested their toxic effect through oxidative stress caused by active oxygen species and other free radicals. In many cases this is also accompanied by altered functional organization of biomembranes caused by lipid peroxidations (FRIDOVICH 1986 a, KAKKAR & al. 1992).

Oxidative damage is prevented normally by an "antioxidative system" in the cells. In this protective mechanism superoxide dismutases (EC 1.15.1.1) are essential constituents (ASADA 1988, FRIDOVICH 1986 b, SCANDALIOS 1993). These enzymes represent a class of metalloproteins which catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Three types of SOD can be distinguished according to their prosthetic metall cofactor (BANNISTER & al. 1987): copper/zinc-, manganese- and iron-SOD. Cu/Zn-SODs are major isoforms in plants (KRÖNIGER & al. 1992, WINGSLE & al. 1991, 1992).

The gaseous air pollutants sulfur dioxide and photooxidants with ozone as the main component are discussed as possible parameters in forest ('novel forest decline') and also lichens decline ('lichen desert'). Air pollution measurements at the altitude profile 'Zillertal' (Austria) (BOLHAR-NORDENKAMPF 1989) show that the SO_2 concentrations are very low in higher altitudes. In such regions ozone ist the most prominent air pollu-

tant. Therefore, these areas are called "clean air regions". Due to the occurrence of generally elevated ozone concentrations at the timberline the risk of air injuries is increased, presumably only in combination with other stress factors, e.g. climatic conditions (SMIDT 1989).

Ozone as a very agressive photochemical oxidant causes destructive damage to plants such as depression of photosynthesis and breakdown of photosynthetic pigments and lipids. Ozone molecules themselves act as a strong oxidizing agent on plants causing lipid peroxidation and pigment cooxidation. And further, ozone toxicity involves the catalysis of free radical formation and the accumulation of active oxygen species, which cause oxidative damage to plants.

Lichens are useful bioindicators of pollution because they can absorb and accumulate different toxic compounds (e.g. heavy metals, radionuclides) and have different sensitivities to air pollutants such as SO_2 and ozone (see SCHLEE 1977, 1992, TÜRK 1992, BARTHOLMESS & al. 1994 for references). Only few studies have been published concerning the influence of ozone on lichens. Species-specific ozone damaged both photosynthesis and biont ultrastructure (EVERSMAN & SIGAL 1987). Differences in photosynthetic activity were noted in lichens collected from different elevations in the Venezuelan Anden (LARCHER & VARESCHI 1988) and in the Central Eastern Alps (Austria) (TÜRK 1981, 1983). Thiol content in lichens increased as a function of altitude. Thiol compounds may protect plants from the formation of oxygen radicals (GUTTENBERGER & al. 1991). In the very sensitive lichen *Pseudevernia furfuracea* the glutathion content varied also at different altitudes. The highest content was measured in samples collected at 1400 m above sea level (KRANNER & al. 1992).

The purpose of this investigation was to examine the activity of SOD participating in the detoxification of active oxygen species and the amount of malondialdehyde as a breakdown product of the lipid peroxidation in relation to an altitude profile (Central Alp, Austria) in the lichen species *Hypogymnia physodes* and *Pseudevernia furfuracea*.

Abbreviations: BHT: butylated hydroxytoluene; MDA: malondialdehyde; NBT: nitroblue tetrazoliumchloride; PMS: phenazine methosulfate; SOD: superoxide dismutase; TMP: tetramethoxypropane

2. Material and Methods

2.1. Plant Materials

Samples of *Pseudevernia furfuracea* (L.) ZOPF var. furfuracea, *Hypogymnia physodes* (L.) NYL. and *Cetraria islandica* (L.) ACH., were collected along an altitude profile from 1080 m up to 1940 m above sea level in Salzburg, Lungau, St. Michael – Katschberg – Aineck (Austria). *Cladonia verticillaris* (RADDI) FR. originated from Sta. Rita, Paruiba, Brazil. After air drying for some days the lichen material was stored at –25° C.

2.2. Methods

2.2.1. Extraction

Samples were frozen in liquid nitrogen, pulverized and stored at –70° C. 125 mg lichen powder was suspended in 2 ml of ice-cold extraction buffer (0.05 M KH_2PO_4/NaOH, pH 7.8, 1 mM Na_2EDTA, 5 mM mercaptoethanol, 1 % insoluble polyvinylpyrrolidone, and 0.2 % Triton X-100) and homogenized by ultra turrax (Janke & Kunkel, Staufen, Germany) for 1 min at 13500 rpm in an ice bath. The homogenate was centrifuged at 13000 x g for 40 min at 4° C, and the supernatant was clarified by filtration. The filtrate was used for the enzyme determination.

2.2.2. SOD Assay

SOD activity was measured by inhibition of the formazan formation according to the method (xanthine / xanthine oxidase test) of BEAUCHAMP & FRIDOVICH 1971.

The reaction mixture contained the following solutions: 2.25 ml of 0.05 M Tris/maleate buffer, pH 8.3, including 0.15 mM Na_2EDTA, 0.2 ml nitroblue tetrazolium chloride (3 mg per 10 ml buffer), 0.1 ml xanthine oxidase solution (SERVA, Heidelberg), and 0.1 ml xanthine solution (23 mg xanthine, 0.3 ml 1 N NaOH, 1.7 ml aqua bidest; immediatly for use 1:20 with aqua bidest dilute). An aliquote of the crude extract (diluted with phosphate buffer, pH 7.8, according to 0.2–1 units of SOD) was added. The reaction was started by addition of an aliquote of the xanthine oxidase solution [diluted in cold aqua bidest. according to $\Delta E_{560} \cdot \text{min}^{-1} = 0.06$ for the basic reaction (V)]. After incubation for 1–2 min at 25° C the activity was followed for 5 min at 560 nm in a Shimadzu UV 160A photometer.

One unit of SOD is defined as the amount of enzyme which inhibits the formazan formation to 50 %. The unit is equal to (V/v) – 1, where V and v are the reduction rates in the absence and presence of the crude enzyme, respectively (ASADA & al. 1974, MCCORD & FRIDOVICH 1969).

Inhibitor studies were performed with 2 mM KCN in order to distinguish between SOD isozymes.

2.2.3. Protein Determination

The protein content was measured with the Folin reagent according to LOWRY & al. 1951 and BSA as standard.

2.2.4. Electrophoresis

Native disc-gel electrophoresis was performed on crude protein according to a modified procedure of MAURER 1971.

Samples were separated on 7.5 % polyacrylamide gels at 4° C for 5 hrs at 25 mA resp. for 16 hrs at 10 mA in 50 mM Tris and 384 mM glycine adjusted pH 8.3 with HCl. The SOD activity was localized on these gels using the *in situ* staining techniques with NBT, PMS and NADH according to a modified method of NISHIKIMI & al. 1972.

Inhibitor studies with 2 mM KCN or 5 mM H_2O_2 were performed directly on gels. The three classes can be distinguished by their sensitivity to inhibitors. Thus, the Cu/Zn-SOD is inhibited by cyanide and H_2O_2 and Fe-SOD by H_2O_2; Mn-SOD is insensitiv to both reagents (FRIDOVICH 1986 b).

The densitometric determination of SOD activities on the gels was carried out using a video-densitometer ('Basys' system, Biotec Fischer, Reiskirchen, Germany).

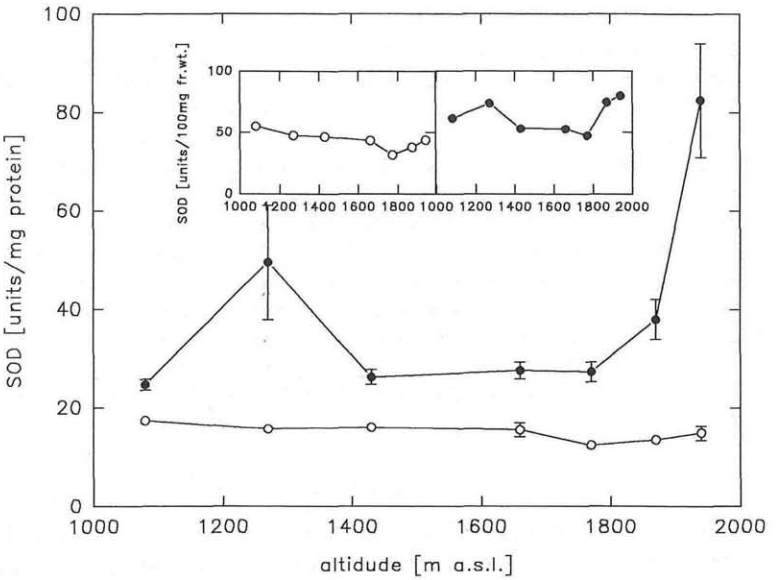

Fig. 1: SOD activity (units per mg proteins or per 100 mg fr. wt.) of *Pseudevernia furfuracea* (●) and *Hypogymnia physodes* (○) thalli growing along a verticale profile in the Central Eastern Alps (Austria, Lungau, Katschberg) in altitudes from 1080 to 1940 m a.s.l.

Bars = standard deviations (n = 3). Inset = SOD units per fr. wt.

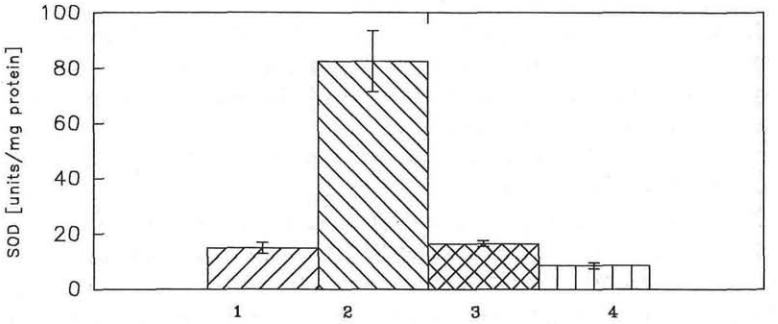

Fig. 2: SOD activity (units per mg proteins) of different lichen species collected from Central Eastern Alps (Austria, Lungau, Katschberg) at 1940 m a.s.l. (*Hypogymnia physodes, Pseudevernia furfuracea, Cetraria islandica*) and in Brazil (*Cladonia verticillaris*).

Bars = standard deviations (n = 3); 1 = *H.* physodes; 2 = *P. furfuracea;* 3 = *C. islandica;* 4 = *Cl. verticillaris.*

2.2.5. Determination of Malondialdehyde

500 mg lichen powder were extracted in 6 ml buffer consisting 5.6 ml 0.5 M glycine buffer, pH 3.3, 0.4 ml 10 % insoluble polyvinylpyrrolidone and 0.08 ml 10 % Triton X-100 using an ultra turrax homogenizer (3 min at 13500 rpm in an ice bath). After centrifugation of the homogenate at 13000 × g for 30 min at 4° C and filtration, the clarified filtrate was used for determination. The mixture contained the following, freshly prepared solutions: 0.375 ml thiobarbiturate (0.5 % in 0.025 M NaOH, 0.025 ml 10 mM $FeCl_3 \cdot 6\ H_2O$, 0.02 ml BHT (0.28 % in ethanol) and 0.5 ml of the filtrate. The mixture was heated for 15 min at 95° C. After rapid cooling at 4° C in an ice bath, 0.25 ml TCA (35 %) and 0.5 ml chloroform were added and, following vortex mixing for 15 s, the extract was centrifuged for 10 min at 5000 × g to ensure complete separation into two phases. The absorption of the supernatant was determined at 532 nm using a Hitachi U-1100 spectrophotometer. The MDA concentration was calculated with tetramethoxy-propane (Aldrich Chemie, Steinheim, Germany) as standard.

3. Results and Discussion

Lichens react very species-specifically to unfavorable environmental conditions and they show different responses in metabolic reactions in relation to pollutant stress. As can be seen from figure 1 SOD activity is significantly higher in *Pseudevernia furfuracea* than in *Hypogymnia physodes*. An increase in the activity of SOD in *P. furfuracea* was observed along the altitude profile at 1270 m and at 1800 up to 1940 m above sea level. This higher activity might be induced as a response to multiple stress conditions, particularly to ozone stress, which may occur at these altitudes. On the other hand, *H. physodes* is probably more resistant to ozone (Nash & Sigal 1979). The SOD activity was not altered in dependency of the altitude in this lichen species (fig. 1).

In comparison with *P. furfuracea* an *H. physodes* the SOD activity was also determined in *Cetraria islandica*, a terrestrial species, at the same location and altitude (1940 m) and in a dry-adapted *Cladonia verticillaris* collected from different locations in Brazil. In *C. islandica* the SOD activity was similar to that found in *H. physodes*. Contrarily, in *Cl. verticillaris* there is a significant low SOD level (fig. 2).

An increase in the activity of SOD was observed in *Picea abies* (Castillo & al. 1987, Jung & al. unpublished) and *Pinus taeda* (Tandy & al. 1989) exposed to ozone. On the contrary, in seedlings of *Pinus sylvestris* SOD activity was not altered after ozone exposure, and in spinach a decrease in SOD activity was found due to ozone stress (Wingsle & al. 1991, 1992). Thus the effects of ozone on SOD activity of plants appear to be highly dependent on the specific type of organism and on the specific exposition conditions including the concentration range.

In by Sephadex G200 concentrated protein extract of *P. furfuracea* six SOD isozymes have been detected by native PAGE. The isozymes were

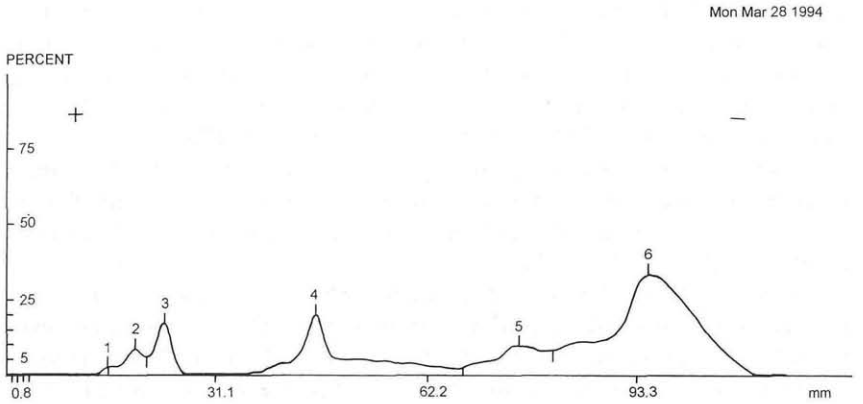

Fig. 3: Profile of SOD isozymes in *Pseudevernia furfuracea* from Central Eastern Alps collected at 1940 m a.s.l. (Austria, Lungau, Katschberg).
Protein samples were concentrated by Sephadex G200 treatment (500 mg lichen powder in 2 ml buffer; 60 µl of crude extract) and separated on non-denaturing 7,5 % acrylamide gel (16 hr at 10 mA and 4° C). The gel was stained for SOD activity and evaluated using a video-densitometer (Biotec Fischer, Reiskirchen, Germany).

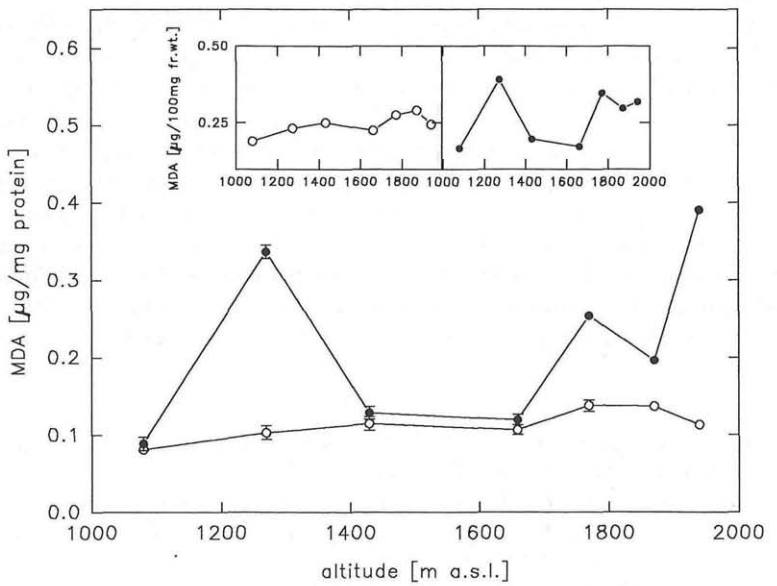

Fig. 4: Malondialdehyde content related to total soluble protein or to fr. wt. in *Pseudevernia furfuracea* (●) and *Hypopgymnia physodes* (○) thalli growing along an altitude profile (from 1080 to 1940 m a.s.l.) in the Central Eastern Alps (Austria, Lungau, Katschberg). Bars = standard deviations (n = 3). Inset = MDA content per fr. wt.

named after their relative migration in gels. SODs 1–3 (high mobility) have been identified as Fe-SODs. SODs 4–5 are presumably Mn-SODs. SOD band 6 (low mobility) was inhibited by 1 mM cyanide (data not shown) and has been identified as Cu/Zn-SOD. This isozyme contributed to two thirds (60–70 %) of the total activity found on the gels (fig. 3).

The SOD isozyme pattern in *H. physodes* was very similar. The major isozyme SOD 6 as Cu/Zn-SOD was inhibited by 1 mM cyanide and further by 1 mM H_2O_2. In both lichen species this pattern was not significantly altered in relation to the altitude.

Figure 4 shows the amount of malondialdehyde measurable in *P. furfuracea* and *H. physodes*, expressed on the basis of protein content or fresh weight. Corresponding to SOD activity (fig. 1) MDA concentration is lower in the ozone resistant *H. physodes* than in *P. furfuracea* suggesting an increased level of lipid peroxidation in the latter. Maxima were determined at 1270, 1770 and 1940 m along the altitude profile. In *C. islandica* (at 1940 m) and *Cl. verticillaris* the MDA content is very low (data not shown).

An increase in the concentration of glutathione was observed in lichens at 1400 m (KRANNER & al. 1992). The altitude dependency of the thiol content in lichens was similar to that found in spruces (GRILL & al. 1988, 1990) and can be an essential constituent of a protective 'antioxidative system' in the organisms.

We suggest that in lichen species, in the same way, the multiple forms of SOD, particularly a dominant Cu/Zn-SOD, participate in the responses of detoxification and adaptation mechanisms to multiple environmental conditions in relation to the altitude-dependent stress factors. SOD is known to be involved in various repair mechanisms by scavenging free oxygen radicals (WINGSLE & al. 1992, SCANDALIOS 1993). The enhanced SOD activity in *P. furfuracea* may be a reaction of the lichen to detoxify the ozone taken up from the air along an altitude profile. On the other hand, in *H. physodes*, a more resistant lichen to ozone, both specific SOD activity and MDA content as a measure of the lipid peroxidation are very low and they were not altered in dependency to the altitude.

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