Superoxide Dismutase Activity in Needles of Norwegian Spruce Trees (Picea abies L.)

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ABSTRACT

The activity of superoxide dismutase was investigated in needles of spruce trees. To obtain maximum activity, needles were homogenized in the presence of Triton X-100 and polyvinylpyrrolidone. Superoxide dismutase activity was measured in dialyzed extracts with a modified epinephrine assay (HP Misra, I Fridovich, J Biol Chem 247: 3170–3175) at pH 10.2. The extracts contained 70 to 120 units of superoxide dismutase per milligram protein. One unit of superoxide dismutase was completely inhibited in the presence of 20 micromolar NaCN. On native polyacrylamide gels three electromorphs were visualized after staining for activity. All three species were sensitive to CN⁻ and H₂O₂ and were therefore assumed to be Cu/Zn-superoxide dismutases. Superoxide dismutase activity was dependent on the age of the needles and declined by approximately 25% within 3 to 4 years.

Superoxide radicals are generated during the metabolism of aerobic organisms, either as end products of enzymatic reactions or as accidental side products of cellular redox reactions (11). These radicals are a potential source of injury because they may act directly on biological targets or indirectly by initiating the production of highly reactive oxygen species such as O₂ or OH⁻ via H₂O₂ and the Haber Weiss reaction (11, 12, 26). The first line of defense against oxygen toxicity is, therefore, the removal of superoxide radicals before the formation of other harmful products can occur. Superoxide dismutases are considered as the major enzymic defense present in cells (11). They scavenge O₂⁻ with a rate close to the diffusion limit (17) by the reaction (18):

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2. \]

Superoxide dismutases have been discovered so far in all aerobic organisms (12). The enzymes contain Cu/Zn, Mn, or Fe in their catalytic center (12). The Mn enzyme was detected in mitochondria of animals (12) and plants (2, 14) and in glyoxysomes (7). The Fe-superoxide dismutases are frequently observed in bacteria, but only rarely in higher plants (12). The plant enzyme was found to be associated with chloroplasts (25, 26). Most abundant in plants are Cu/Zn-superoxide dismutases, which were characterized by a broad pH-opti-

\[ \text{mum between pH 7 and 10 (10) and an inhibition by cyanide and hydrogen peroxide (1, 13, 23, 29). The major part of the activity of this species was found in chloroplasts (11, 26) with a distinct portion associated with the thylakoid membranes (14).} \]

In all species so far examined, the activity of superoxide dismutase was found to be dependent on the age and developmental stage of the tissue analyzed (20). Young leaves contained higher superoxide dismutase activity than mature and senescing leaves (20). Data on developmentally determined changes in superoxide dismutase activity in leaves of conifers are, however, lacking. Since these leaves can persist for many vegetation periods, they may have a special demand for detoxifying harmful oxygen species and may undergo changes in superoxide dismutase activity different to that of leaves from deciduous plants. Therefore, the objective of the present experiments was to investigate the activity of superoxide dismutase in spruce needles of different age. A method designed for the determination of superoxide dismutase activity in crude extracts of spruce was, however, not available. The necessity of adaptation and of carefully controlled conditions in superoxide dismutase assays has recently been emphasized by Beyer and Fridovich (4). As needles of spruce contain high amounts of components, e.g. ascorbate, glutathione (22), which interfere in superoxide dismutase assays, extraction and assaying procedure (after Misra and Fridovich, 19) for superoxide dismutase from spruce had first to be optimized.

MATERIALS AND METHODS

Plant Material

Experiments were performed with needles from Norway spruce trees (Picea abies [L.]). The trees showed no symptoms of injury. Six-year-old spruce trees were obtained from a tree nursery. One- to 2-year-old needles from these trees were used for the optimization of the superoxide dismutase assay. Needles from 100- to 150-year-old, single standing trees located at the westside slope of Katzenstein Mountain near Garmisch-Partenkirchen (F.R.G.) were used for the characterization of the superoxide dismutase species. Superoxide dismutase activity in needles of different age was investigated in 36-year-old trees growing at the east side of a spruce forest near Bad Münstereifel (F.R.G.). The needles were sampled according to the procedure of Weidner and Kraus (27). The samples were stored under liquid nitrogen.

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Extraction of Spruce Needles

Needles were powdered under liquid nitrogen in a precooled mortar. The powder (1 g) was transferred into a homogenizer (Potter-Elvejem) containing 20 mL of cold extraction buffer (1 g insoluble PVP, 0.5% [v/v] Triton X-100, 100 mM KH₂PO₄/K₂HPO₄, pH 7.8) prepared the previous day and stored at 4°C. The powder was homogenized for 1 min and spun down for 20 min at 48,000g. The supernatant was clarified by a second centrifugation step (10 min, 12,000g). The volume of the supernatant was determined and an aliquot (usually 3 mL) was dialyzed in the dark against a total of 200 volumes of phosphate buffer (20 mM KH₂PO₄/K₂HPO₄, pH 7.8) with four changes of buffer during 1 d. The dialyzed extract could be stored frozen at −20°C for at least 30 weeks without significant loss of superoxide dismutase activity. After thawing and before the determination of the activity of superoxide dismutase, the extract was clarified by centrifugation (10 min, 5,000g).

Assay of Superoxide Dismutase Activity in Extracts

Superoxide dismutase activity in extracts of spruce needles was determined by a modification of the protocol of Misra and Fridovich (19): at alkaline pH, O₂⁻ serves as chain propagation species for the autoxidation of epinephrine to adrenochrome. Superoxide dismutase competes with this reaction, thus decelerating the adrenochrome formation. One unit of superoxide dismutase is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%.

The modified assay contained: 800 μL of buffer I (62.5 mM Na₂CO₃/NaHCO₃ [pH 10.2], 125 μM EDTA), 10 μL catalase (1.3 units bovine liver catalase/mL), and 10 μL epinephrine (10.5–11.0 mg epinephrine in 2 mL 0.1 N HCl), varying amounts of dialyzed spruce extract (up to 180 μL). The final volume of 1 mL was adjusted by addition of buffer II (20 mM KH₂PO₄/K₂HPO₄ [pH 7.8], 0.5% [v/v] Triton X-100). The epinephrine solution was adjusted to cause a change in absorbance of 0.021 to 0.024/min at 480 nm in the controls. The assay was performed in thermostated cuvettes at 30°C. The changes of absorption in controls and different dilutions of spruce extract were recorded by a spectrophotometer (Uvikon 710). For the determination of superoxide dismutase activity each assay was repeated for five volumes of extract and five controls three to four times. To calculate units of superoxide dismutase in the extracts, data were transformed as described for other superoxide dismutase assays (1, 4): 1 unit = V_{control}/V_{extract} − 1, where V is the slope of the change in absorbance in the absence and V in the presence of spruce extract. The transformation resulted in a linear correlation between superoxide dismutase activity and the amount of extract used in the assay. Correlation and regression analysis were performed as described by Sachs (24).

Assay of Superoxide Dismutase Activity on Gels

Crude extracts diluted with sample buffer (1:1) (30 mM Tris/HCl [pH 6.8], 25% glycerol, 0.005% bromphenol purple) were loaded onto native polyacrylamide gels, 3% stacking gel, 7.5% separating gel. After electrophoresis, superoxide dismutase activity containing bands were visualized as described by Beauchamp and Fridovich (3). For selective inhibition of superoxide dismutase, the gels were preincubated for 30 min in phosphate buffer containing either 1 mM NaCN or 1 mM H₂O₂ at ambient temperature.

Chl and Protein Determination

The Chl content of the extract was determined by the method of Jeffrey and Humphrey (15). The protein content was measured as described by Bradford (6). Bovine serum albumine, used as a protein standard, was diluted with phosphate buffer (20 mM KH₂PO₄/K₂HPO₄ [pH 7.8], and 0.5% [v/v] Triton X-100).

Reagents

Enzymes, bovine serum albumin, and epinephrine (grade II) were purchased from Sigma. The water was of Milli Q quality.

RESULTS

Optimization of the Extraction and Measuring Conditions for Superoxide Dismutase from Spruce Needles

To retain the activity of several spruce enzymes in extracts, it was shown to be essential to include phenol scavengers during the homogenization step (27, 28). Therefore, the needles were routinely extracted in the presence of insoluble PVP (5% [w/v]). To obtain maximum activity of superoxide dismutase in extracts of spruce, increasing amounts of the detergent Triton X-100 were present during the homogenization. Figure 1 shows that the ratio of 5% (w/v) needle powder and 0.5% (v/v) Triton X-100 during the extraction procedure yielded optimal activity. Higher amounts of deter-
gert decreased the enzyme activity insignificantly. In the absence of detergent, only 25% of the maximal activity of superoxide dismutase was obtained. A low yield of enzymic activity from spruce in the absence of detergent was also observed for glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme supposed to be water soluble (28). Therefore, a low activity of superoxide dismutase after detergent-free extraction does not necessarily indicate that a major portion of the enzyme is membrane-associated and/or not released from cellular compartments.

A comparison of the activity of commercial horseradish superoxide dismutase with a typical spruce extract is given in Figure 2. Increasing amounts of enzyme exhibited increasing inhibition of adrenochrome formation with a saturation level of 90% for the commercial enzyme and of 80% for spruce extracts. For horseradish superoxide dismutase the failure to reach 100% inhibition was attributed to alternative oxidative pathways (4, 12, 19). Apparently, in crude extracts from spruce superoxide radicals were removed by alternative reactions to a higher extent than in commercial horseradish superoxide dismutase preparations. This was not due to the presence of low mol wt components, as spruce extracts were dialyzed prior to the enzyme assay. Extensive dialysis was crucial, because low mol wt components present in spruce needles in high concentrations are known to interact with superoxide radicals, e.g. ascorbic acid, reduced glutathione (16), and reduced NAD(P)⁺ (5).

Peroxidases at high concentrations can disturb the superoxide dismutase assay used in the present investigation in a way leading to an underestimation of activity. At pH 8, a pH-value often employed in other superoxide dismutase assays, spruce peroxidases were still active to about 30 to 50% of their maximum activity. At pH 10.2, however, they exhibited only 1% of their maximum activity (W Schürmann, personal communication). Moreover, catalase was included in the assay to remove hydrogen peroxide, the substrate of peroxidases. Thus, interference by peroxidases was negligible. Inclusion of catalase also prevented H₂O₂ accumulation, thereby preventing product inhibition of Cu/Zn-superoxide dismutases. It also improved the signal-to-noise ratio of the test by removing hydrogen peroxide contamination of the water.

Characterization of the Spruce Superoxide Dismutases

To obtain information on the superoxide dismutase species, present in spruce extracts, we investigated the sensitivity of the superoxide dismutase activity toward cyanide. The cyanide concentrations indicated in Figure 3 were added to the enzyme assays and to the controls. One unit of superoxide dismutase was inhibited by 2 μM NaCN to 50%. Complete release of adrenochrome formation was observed in the presence of 20 μM NaCN. 83% (±17%) of the activity survived a treatment with CHCl₃/ethanol (3:5 [v/v]) (8), inhibiting preferentially Fe- and Mn-containing superoxide dismutases (not documented). Therefore, it appeared that at pH 10.2 predominantly Cu/Zn-superoxide dismutases contributed to the measured enzyme activity. However, a putative Mn-superoxide dismutase may have been inhibited to about 60 to 90% at pH 10, as observed for the enzymes from Escherichia coli and from chicken liver (10). Under these conditions, Mn-superoxide dismutases may not be detected with the assay applied.

To find out whether manganese or iron containing superoxide dismutases also play a role in oxygen detoxification in spruce needles, crude extracts were subjected to gel electrophoresis. After staining for activity at pH 7.8, three bands of different electrophoretic mobility were detected (Fig. 4): one major band with mean mobility (Rₑ = 0.50), one minor band with high mobility (Rₑ = 0.57), and another weak band of low mobility (Rₑ = 0.27). The differences in electrophoretic mobility seemed to indicate mainly differences in electric charge rather than in mol wt, since the electromorphs copurified after gel permeation (not shown). The detection limit of
Superoxide dismutase activity in the gel was approximately 0.3 units as determined by the epinephrine assay (Fig. 4, lane b). All electromorphs were sensitive to cyanide as well as to hydrogen peroxide (Fig. 4, lanes c and d). Therefore, we concluded that the enzymic capacity to detoxify superoxide radicals in spruce needles was mainly, if not entirely, due to the activity of Cu/Zn-superoxide dismutases.

Superoxide Dismutase Activity in Dependence on the Age of the Needles

The activity of superoxide dismutase was investigated in four needle generations. Samples were taken from the same trees twice, once in winter (February) and once in summer (June). Table I shows that the activity of superoxide dismutase was highest in 1-year-old needles and then declined at about 25% in 3- to 4-year-old needles. Regression analysis of the data (24) revealed that this decline was coupled to the age of the needles with computed F-values of 3.61 (June) and 4.34 (February) and P = 0.05. In addition, we measured Chl and protein content in the extracts obtained from the needles (Table II). The Chl content increased by about 50% from the first to the second needle generation and was slightly enhanced in the summer as compared to the winter. The protein content of needles of different age showed no significant changes, but was higher in winter than in summer. A decline in superoxide dismutase activity was observed in older needles, regardless of whether Chl, protein content, or fresh weight was chosen as a basis. Superoxide dismutase activity was slightly higher in summer, but showed the same characteristic decline with increasing age of the needles as observed in winter.

DISCUSSION

In the present investigation the extraction procedure for superoxide dismutase from needles of spruce was optimized by inclusion of 0.5% of the nonionic detergent Triton X-100 in the extraction buffer. Dialysis eliminated redox components of low mol wt as ascorbic acid and glutathione, which are involved in nonenzymic detoxification of toxic oxygen species (12, 21). These components are abundant in cells and interact with superoxide radicals (16, 22). Therefore, they can interfere with assays for superoxide dismutase which rely on the participation of $O_2^{-}$ as the chain propagating species. The activity of superoxide dismutase was determined with a modified epinephrine assay at pH 10.2 (19). As observed in the original assay, 90% inhibition of the adrenochrome formation was achieved with a commercially available superoxide dismutase. The maximal inhibition obtained with spruce extracts was 80%. Apparently, extracts from spruce needles still contain components interfering with the assay. It has been concluded that the major disadvantage of the epinephrine assay was the need to determine superoxide dismutase activity at the unphysiological pH value of 10.2 (12). However, at this pH-value, interferences by peroxidases present in crude extracts of spruce or by spontaneous dismutation of $O_2^{-}$ were avoided. Superoxide dismutase activity in extracts of spruce needles varied between 70 and 120 units/mg protein depending on needle age and season. Crude extracts of spruce contained three electromorphs of superoxide dismutase, which were inhibited by $H_2O_2$ as well as by CN$^-$. This observation

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### Table I. Activity of Superoxide Dismutase in Needles of Increasing Age

<table>
<thead>
<tr>
<th>Needle Age</th>
<th>Superoxide Dismutase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer</td>
</tr>
<tr>
<td>years</td>
<td>units/g fresh weight</td>
</tr>
<tr>
<td>1</td>
<td>1389 (258)*</td>
</tr>
<tr>
<td>2</td>
<td>1238 (68)</td>
</tr>
<tr>
<td>3</td>
<td>1157 (97)</td>
</tr>
<tr>
<td>4</td>
<td>1063 (120)</td>
</tr>
</tbody>
</table>

* Standard error; n = 4.

### Table II. Protein/Fresh Weight and Chl/Fresh Weight-Ratios in Needles of Increasing Age

<table>
<thead>
<tr>
<th>Needle Age</th>
<th>Protein</th>
<th>Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer</td>
<td>Winter</td>
</tr>
<tr>
<td>years</td>
<td>mg/g fr wt</td>
<td>µg/g fr wt</td>
</tr>
<tr>
<td>1</td>
<td>11.9 (0.9)*</td>
<td>13.2 (0.6)</td>
</tr>
<tr>
<td>2</td>
<td>12.6 (0.8)</td>
<td>13.2 (0.8)</td>
</tr>
<tr>
<td>3</td>
<td>12.5 (0.6)</td>
<td>13.6 (1.7)</td>
</tr>
<tr>
<td>4</td>
<td>11.2 (0.9)</td>
<td>12.8 (0.5)</td>
</tr>
</tbody>
</table>

* Standard error; n = 4.
indicates that the activity of superoxide dismutase in needles of spruce was mainly due to Cu/Zn-species. Most higher plants contain, however, two different isozymes with Mn or Cu/Zn, respectively, in their catalytic center. The Mn-enzyme is known to contribute in some plants only to 3 to 5% of the total activity (11). Therefore, a rather low activity of a Mn-enzyme may have not been detected in the present investigation.

Asada et al. (1) reported that two units of Cu/Zn superoxide dismutase from spinach were completely inhibited in the xanthine-xanthine oxidase/Cyt c-system at pH 7.8 by 1 mM cyanide. Yseaert-Vanneste and Vanneste (29) showed, however, that the sensitivity of superoxide dismutase toward cyanide was strongly dependent on the pH-value in the assay and increased approximately 10 times, if the pH in the assay was increased from pH 7.8 to 10.0. At pH 10, 1.12 units of superoxide dismutase from bovine erythrocytes were already inhibited by approximately 65% in the presence of 20 μM cyanide (29). This cyanide concentration completely inhibited 1 unit of spruce superoxide dismutase under our assay conditions, pH 10.2. Whether or not superoxide dismutase from spruce was actually more sensitive toward cyanide remained conceivable, because the investigations compared here were performed with different test systems displaying different sensitivity for superoxide dismutase activity (cf. Table 5, ref. 4).

Cu/Zn-superoxide dismutases of plants were reported to be preferentially associated with chloroplasts (11). It has been assumed that one of the physiological roles of this enzyme was the detoxification of superoxide radicals, produced in vivo when molecular oxygen serves as electron acceptor instead of NADP⁺ (26). It was therefore tempting to speculate that the superoxide dismutase activity measured in extracts from spruce needles was mainly due to chloroplastic enzymes. The production of O₂⁻ under excess light energy and its detoxification via superoxide dismutase was expected to be of higher impact in needles containing higher amounts of chlorophyll. We observed, however, that the activity of superoxide dismutase decreased by approximately 25% within 3 to 4 years with increasing age of the needles, while the Chl content of the needles initially increased, then remained constant. In that respect, the observed decline in superoxide dismutase activity was surprising. This result may be explained by a shift of the detoxification system in older needles toward nonenzymic removal of O₂⁻ (9) or may hint to additional functions of superoxide dismutase in younger needles (i.e. regulation of lignification [20]).

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LITERATURE CITED