Isolation, Purification, and Subcellular Localization of Isozymes of Superoxide Dismutase from Scots Pine (Pinus sylvestris L.) Needles

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ABSTRACT

Two of four isozymes of superoxide dismutase (SOD) (EC 1.15.1.1) were purified from Scots pine (Pinus sylvestris L.) needles. One form was cytosolic (SOD-1) and the other was associated with chloroplasts (SOD-3). The holoenzyme molecular masses was estimated at approximately 35 kilodaltons by gel filtration. The subunit molecular weight of the dimeric enzymes was estimated to 15.5 kilodaltons (SOD-1) and 20.4 kilodaltons (SOD-3) on sodium dodecyl sulfate-polyacrylamide gels. The NH2-terminal sequence of the pine enzymes showed similarities to other purified superoxide dismutases located in the corresponding compartment. The cytosolic form revealed two additional amino acids at position 1 and 2 at the NH2-terminal. Both forms were cyanide- and hydrogen-peroxide-sensitive and SOD-3 was found to contain approximately one copper atom per subunit, indicating that they belong to the cupro-zinc SODs. The isoelectric point was 4.9 and 4.5 for SOD-1 and SOD-3, respectively.

SO2 than older leaves with comparably lower activity (27). Tanaka et al. (28) showed that paraquat-resistant tobacco with more than doubled SOD activity, but no enhanced levels of glutathione reductase or ascorbate peroxidase activity, was less sensitive to SO2 exposure than were control plants.

"Waldsterben" was first noticed among conifers, and seasonal variation of the activity of SOD in spruce and pine growing in areas exposed to air pollutants has been examined (14). However, there are several different isozymes of SOD in conifers that might react differently to air pollution stress as indicated by Schultz (25) and Tandy et al. (28a). These authors presented electrophoretic data of isozymes of SOD from pine and spruce species.

Three types of SODs have been found in plants and they are classified according to the metal at the catalytic center, copper and zinc (CuZn), manganese (Mn) or iron (Fe) (9). They can be distinguished by their sensitivity to inhibitors. Thus, the CuZn SOD is inhibited by cyanide and H2O2, and Fe SOD by H2O2; Mn SOD is insensitive to both reagents.

The most abundant SODs in plants are the CuZn SODs, which are found mainly in the cytosol and chloroplasts. Mn SOD is usually localized within the mitochondrial matrix (2) but has also been isolated from chloroplast thylakoids (13). The Fe SODs are found in three families of higher plants Ginkgoaceae, Nymphaeaceae and Cruciferae (7) and the subcellular localization is unclear.

In this report we describe the isolation, purification, NH2-terminal amino acid sequence, and intracellular localization of two isozymes of SOD in Scots pine needles. This work is a part of our research on effects of air pollutants on physiological processes in Scots pine.

MATERIAL AND METHODS

Isolation of Protoplasts

Pine seeds (Pinus sylvestris L.) were germinated and grown in darkness for 3 weeks and thereafter transferred to a controlled environment cabinet for 4 d with: 18 h photoperiod with a PFD of 300 μmol m-2 s-1 (Osram HQI 400W); day temperature 25°C; night temperature 15°C; RH 70 to 80%. Etiolated seedlings were used to minimize compounds that interfered with the protoplast preparation. The needles were harvested and cut into small pieces and kept on ice in tissue wash medium (0.5 m sorbitol, 1 mM CaCl2, 1% PVP-25, 1...
mm ascorbate, 5 mM Hepes, pH 7.2). The needle pieces were then gently vacuum-infiltrated in fresh tissue wash medium. After transfer to 50 mL digestion medium (0.5 mM sorbitol, 2% cellulase [Cellulysin, Calbiochem], 0.5% pectinase [Macerase, Calbiochem], 0.2% PVP-40, 0.2% BSA, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM ascorbate, 10 mM Mes-KOH, pH 5.5) the vacuum-infiltration was repeated before the leaf pieces were incubated at 28°C for 2.5 h. The digestion medium was removed and the protoplasts were released by shaking the leaf pieces in 15 mL wash medium: (0.6 M sucrose, 5% dextran [mol wt 4000, w/v], 1 mM MgCl₂, 1 mM ascorbate, 5 mM Hepes, pH 7.0). The suspended protoplasts were transferred to a centrifuge tube with narrow neck and overlayed with 5 mL wash medium without dextran (0.6 M sucrose, 1 mM MgCl₂, 1 mM ascorbate, 5 mM Hepes, pH 7.0) and finally 1 mL (0.4 M sucrose, 0.1 M sorbitol, 1 mM MgCl₂, 5 mM Hepes, pH 7.0). After centrifugation for 5 min at 500g in a swing-out rotor, purified protoplasts were collected from the upper interface. The recovery was 80 to 100 µg Chl from approximately 5 g of needles. The photosynthetic activity of the isolated protoplasts were assayed polarographically (Hansatech, UK) at saturating CO₂ concentration.

Fractionation of Protoplasts

Protoplasts were diluted with break medium: (0.25 M sucrose, 0.25 M sorbitol, 10 mM KCl, 0.5 mM MgCl₂, 0.06% BSA, 0.2% PVP-40, 1 mM ascorbate, 10 mM Hepes, pH 7.2) and passed three times through a 20 µm nylon net mounted on a plastic syringe. The complete breakage of protoplasts was verified by examination in a light microscope. The extract was centrifuged at 400g for 4 min in a swing-out rotor. The resulting pellet was dark green and the supernatant light green. The supernatant was removed and further centrifuged at 11000g for 4 min. After this centrifugation a small green pellet and an almost clear supernatant were obtained. The pellets from the two centrifugations were suspended in break medium.

Assays of Enzymes

Enzyme activities coupled to oxidation or reduction of pyridine nucleotides (NAD, NADP) were measured spectrophotometrically at 340 nm. Fumarase (EC 4.2.1.2) was used as a marker enzyme for mitochondria and measured according to Hatch (12). PEP carboxylase (EC 4.1.1.31) was used as marker for the cytosol and assayed according to Gardeström and Edwards (10). NADP dependent triosephosphate dehydrogenase (NAPD-TPD; EC 1.2.1.13) was assayed according to Winter et al. (30) and used as a chloroplast marker. SOD was determined spectrophotometrically at 250 nm with the direct KO₃ assay described by Marklund (18). One unit is defined as the activity that brings about a decay in O₂⁻ concentration at a rate of 0.1 s⁻¹ in 3 mL of buffer. Staining for SOD activity in gels was performed as described previously by Beauchamp and Fridovich (5).

Enzyme Purification

Current and 1-year-old needles (500 g) from 15-year-old Scots pine trees were collected from field-grown trees in January. All operations were carried out at 0 to 4°C except for the analyses on HPLC, which were performed at 20°C. Six concomitant steps were used in the purification procedure as described below.

Step 1

Extraction

The needles were homogenized with an ultra-turrax (Janke & Kunkel, GmbH, FRG) at maximum speed for 2 min in 5 L of 50 mM Tris-SO₄ (pH 7.5), containing 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% PVP 360 (soluble, w/w).

Step 2

Ammonium Sulfate Fractionation

The homogenate was filtered through a Büchner funnel to remove needle residues and brought to 35% saturation with solid ammonium sulfate. After stirring for 30 min, the precipitate was removed by centrifugation at 5000g for 25 min. The supernatant was brought to 90% saturation with additional ammonium sulfate and stirred for 1 h. The precipitate was collected by centrifugation at 5000g for 35 min, and dissolved in 125 mL of 10 mM Tris-SO₄ (pH 7.0), containing 10 mM 2-mercaptoethanol, 1 mM MgCl₂, 0.2 mM ascorbate, and 1 mM EDTA.

Step 3

Ion-Exchange Chromatography on Diethylaminoethyl Cellulose DEAE-52

The sample was dialyzed against 3 L of the previously described Tris-SO₄ buffer and applied to a column (3 × 24 cm) of DEAE-52 (Whatman, Springfield Mill, UK), equilibrated with the same buffer. The column was washed with 175 mL of the buffer at a flowrate of 3 mL h⁻¹ cm⁻². Under these conditions, SOD-1 and SOD-3 did not bind to the column and were detected in the void volume. The eluent was brought to 90% saturation with solid ammonium sulphate and the precipitate was collected by centrifugation at 15,000g for 20 min.

Step 4

G-150 Sephadex Gel Filtration

The pellet from the previous step was dissolved in 10 mL of 10 mM sodium phosphate (pH 7.0), containing 1 mM EDTA and applied to a G-150 Sephadex column (2.5 × 90 cm). The elution rate was 5 mL h⁻¹ cm⁻².

Step 5

Phenyl-Superose HR 5/5 Chromatography

Active fractions from step 4 were concentrated with a macroosolute concentrator (Amincon, UPH-43) having a cut-
off filter of 10 kD. Ammonium sulphate was added to the sample to raise the concentration to 1.7 M. Samples (0.2–1mL) were applied to a hydrophobic interaction column (Phenyl-Superose HR 5/5, LKB Pharmacia, Sweden) equilibrated with the previously described phosphate buffer containing 1.7 M ammonium sulphate. The column was washed with 3 mL of buffer and eluted with a 23 mL linear gradient of 1.7 to 0 M ammonium sulphate in the buffer. The solvents were delivered at a flowrate of 0.5 mL/min by HPLC dual pumps (LKB Pharmacia, Sweden).

**Step 6**

**Ion-Exchange Chromatography on Diethylaminoethyl Cellulose TSK-DEAE 5PW**

Active fractions from step 5 were combined and gel-filtrated with a G-25 column, equilibrated with 2.5 mm sodium phosphate (pH 7.0), containing 1 mm EDTA. Samples (200μL) were applied to a TSK DEAE-5PW column (LKB Pharmacia, Sweden) and the solvents were delivered at a flowrate of 0.5 mL/min using the HPLC system described above. The column was washed with 15 mL of the phosphate buffer, and then eluted with a 20 mL linear gradient of 2.5 to 40 mm sodium phosphate (pH 7.0), containing 1 mm EDTA. The activity eluted in two distinct peaks. The purified enzymes was stored at −80°C.

**Metal Analysis**

The metal content of SOD-3 was determined using a Zeeman 3030 atomic absorption spectrophotometer, HGA-600 graphite tube atomiser and a PR-100 printer (Perkin Elmer, Norwalk, CT). The enzyme was dialyzed prior to analysis against 10 mm Tris-SO₄ (pH 8.0), which had been passed through a chellex 100 column (Bio-Rad, Richmond, CA) to remove metals.

**Electrophoresis**

Native-PAGE, SDS-PAGE, and IEF were performed at 15°C using a Phast system with appropriate gels and buffer strips (LKB Pharmacia, Sweden). For native-PAGE, 8 to 25% gradient polyacrylamide gels were run for 200 V h. SDS-PAGE was carried out on 10 to 15% gradient polyacrylamide gels for 60 V h. Samples were solubilized at 100°C for 5 min in 10 mm Tris-HCl (pH 7.4), containing 5% (w/w) 2-mercaptoethanol, 2.5 (w/w) % SDS, and 1 mm EDTA. PhastGel IEF 3 to 9 were used for IEF. Proteins were visualized by silver-staining.

**Enzyme Molecular Mass Determination**

The molecular mass of SOD-1 and SOD-3 were determined by gel filtration using HPLC with two columns in series, TSK-300 and Superose Hr 12 (LKB Pharmacia, Sweden), equilibrated with 50 mm sodium phosphate (pH 7.0), containing 0.15 M NaCl. Ovalbumin (45 kD), chymotrypsinogen (25 kD), and Cyt c (12.4 kD) were used as molecular mass markers. In addition, SOD from bovine liver was used as an internal standard.

**Con A-Sepharose Chromatography**

Samples from step 2 in the purification procedure (see above) and purified samples of SOD-1 and SOD-3 were loaded onto a Con A-Sepharose column (LKB Pharmacia, 0.7 × 2.5 cm), equilibrated with 50 mm sodium phosphate (pH 7.0), containing 0.15 M NaCl. The elution rate was 30 mL h⁻¹ cm⁻².

**Protein Determination**

Protein content was estimated from the absorbance at 280 nm, assuming that A = 1 corresponds to 1 mg of protein per mL. Purified samples of SOD-1 and SOD-3 were hydrolyzed, using 4 N-methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole as a catalyst, according to Simpson et al. (26) and analyzed for amino acids by HPLC (20).

**Sequencing**

About 200 pmol of SOD-1 and SOD-3 were determined for their NH₂-terminal sequences by sequential degradation using the Edman-method (model 477 A protein/peptide sequencer coupled with the on-line model 120 A PTH Analyser, Applied Biosystems, Foster City, CA).

**RESULTS**

**Isozyme Pattern**

When a crude extract from pine needles (cf. step 2, “Materials and Methods”), seeds, etiolated seedlings, or a pine protoplast fraction was examined by active staining on polyacrylamide gels, four electrophoretic forms of SOD activity appeared (Figs. 1, 2a). These forms was designated SOD-1,
SOD-2, SOD-3, and SOD-4 in order of their migration to the anode and in accordance with the recommendation of the Subcommittee on Multiple Molecular Forms of Enzymes of the IUPAC-IUB (1971). Roots showed reduced activity of SOD-3 and SOD-4 (Fig. 1). When 3 mM NaCN or 3 mM H$_2$O$_2$ was included in the active staining assay these bands disappeared (Fig. 1, lanes 5 and 6) indicating that they belong to the CuZn SODs.

Subcellular Distribution

Protoplasts obtained from pine seedlings showed CO$_2$-dependent oxygen evolution when incubated in assay medium with 5 mM KHCO$_3$ in a PFD of 500 μmol m$^{-2}$ s$^{-1}$. The initial rate after 1 to 2 min in light was about 40 μmol O$_2$ mg Chl$^{-1}$ h$^{-1}$, then the rate decreased considerably, but after about 10 min the oxygen evolution was 30 to 40 μmol O$_2$ mg Chl$^{-1}$ h$^{-1}$. After differential centrifugation of the protoplast extract the distribution of marker enzyme activities for the chloroplast (NADP-TPD), mitochondria (fumarase), and cytosolic fraction (PEPcase) showed good correlation with the corresponding fraction (Table I). Values of 65% recovered activity, or higher, were found for all marker enzymes in the correct compartment. About 13% of the chloroplast marker (NADP-TPD) was found in the cytosolic fraction suggesting a low breakage of chloroplasts (Table I). The same order of contamination between fractions has also been reported when wheat leaf protoplasts were fractionated (11). SOD-1 and SOD-2 activities, determined by active staining on PAGE under non-denaturing conditions, was found in the cytosolic fraction (Fig. 2b) and SOD-3 in the chloroplastic fraction (Fig. 2c). We thus conclude that SOD-1 and SOD-2 are cytosolic enzymes and SOD-3 is a chloroplastic enzyme.

Purity of the Isozyme Preparation

The procedure used to purify SOD from Scots pine resulted in two isozymes, SOD-1 and SOD-3, having specific activities of 221,000 and 239,000 units (mg protein)$^{-1}$, respectively (Table II). These values correspond to 5,740 and 6,218 units (mg protein)$^{-1}$, respectively, in the McCord and Fridovich xanthine oxidase/cytochrome c assay (18). The SOD-1 and SOD-3 preparation gave single protein bands on native-PAGE gels, which also coincided with SOD activity (Fig. 3, A and B). These bands comigrated with SOD-1 and SOD-3 from crude extracts on nondenaturing gels (cf. Fig. 2). This indicated that no degradation of the enzymes occurred during the purification procedure. On SDS-PAGE, SOD-1 and SOD-3 appeared as single bands, indicating that the preparation was homogeneous (Fig. 4).

Con A-Sepharose Chromatography

SOD-1 and SOD-3 did not bind to Con A-Sepharose. This suggests that no neutral carbohydrates were present in the enzymes.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovered Activity</th>
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<tr>
<td></td>
<td>Purity Activity</td>
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<tr>
<td></td>
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<td>11,000g supernatant</td>
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<td>Recovery %</td>
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Table I. Distribution of Marker Enzyme Activities in Fractions Obtained from Pine Protoplasts by Differential Centrifugation

The total activity (μmol (mg Chl)$^{-1}$ h$^{-1}$) was: PEPcarboxylase (PEPcase) 22, Fumarase 32, NADP-triosephosphate dehydrogenase (NADP-TPD) 69. The values are the mean from two fractionation experiments.
Metal Content

The copper content in SOD-3 corresponded to a protein molecular weight of approximately 18,300 per copper atom. The well-known Zn contamination of samples caused problems. No manganese or iron could be detected in any of the analysed samples.

Molecular Mass Determination by Gel Chromatography

The SOD-1 and SOD-3 eluted at a position that corresponded to a molecular mass of approximately 35 kD (Fig. 5). SOD from bovine liver eluted at the same position as the pine enzymes.

NH₂-Terminal Sequence

The NH₂-terminal amino acid sequence for the cytosolic (SOD-1) and chloroplastic (SOD-3) isozymes was determined up to 24 residues (Fig. 6). Positions 4, 5, 6, 8, 9, 15, and 17 are highly conserved compared to SODs from both the cytosol and the chloroplast (Fig. 6). In addition SOD-1 was conserved in position 21 compared to the other sources of the cytosolic enzyme. The cytosol SOD from Scots pine showed 41 and 43% homology to the NH₂-terminal sequence (residue 3–24) from maize (8) and spinach (17), respectively. However, two additional amino acids, glycine and leucine were detected at position 1 and 2 of the cytosolic SOD from Scots pine. Thus, this isozyme was more equal to the chloroplastic forms listed here (Fig. 6). The homology of the NH₂-terminal sequence (residue 1–23) between Scots pine and pea (23) and spinach stromal (15) enzyme was very high, 87 and 83%, respectively.

Isoelectric Point

pI values of SOD-1 and SOD-3, determined by isoelectric focusing, were 4.9 and 4.5, respectively (Fig. 7).

### DISCUSSION

The coding sequence (cDNA) from cytosolic superoxide dismutase has been isolated, cloned, and sequenced in maize (8) and tomato (21). The cDNA for the chloroplast-located SOD was cloned and sequenced from tomato by Perl-Treves et al. (21) and also from pea and petunia (23, 29). The cDNAs coding for the mitochondria-located Mn SOD were recently isolated and sequenced from tobacco (6). SODs related to the same subcellular compartment were shown to be highly conserved. There is, however, a lack of information in particular from evolutionarily old plants such as gymnosperms. Here we describe the purification to homogeneity of two major isozymes of SOD from Scots pine. These were designated SOD-1 and SOD-3 from their migration on PAGE (Fig. 1). In addition two minor bands, SOD-2 and SOD-4, were also detected.

All of these forms of SODs were sensitive to CN⁻ and H₂O₂. SOD-3 was found to contain approximately 1 Cu per subunit molecule but no Mn or Fe could be detected. The results indicate that the four SODs detected on PAGE from Scots pine belong to the CuZn SODs. However, when extracts from mature needles were analyzed, a CN⁻-insensitive activity was detected and this suggests that also a Mn SOD is present in pine needles. The activity of this SOD corresponded to approximately 1 to 4% of the total activity. This activity could not be confirmed by a visualized band when SOD was stained for activity in the presence of 3 mM CN⁻ or 3 mM H₂O₂. A report by Shultz (24) described nine different isozymes from Scots pine needles, detected by gel-scanning of active stained gels; four were CuZn SODs and five Mn SODs. In the Shultz investigation, a acetone-extraction procedure was used, whereas we and Tandy et al. (28a) used a procedure without acetone and showed that Scots pine contained only four isozyme bands, which all were cyanide and H₂O₂-sensitive. However, other conifers as red spruce and loblolly pine contain...
Figure 4. SDS-PAGE of SOD-1 and SOD-3 from Scots pine needles. Molecular weight calibration proteins (Pharmacia, Sweden): 1, phosphorylase b (94,000); 2, albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, trypsin inhibitor (20,100); 6, α-lactalbumin (14,400).

Figure 5. Molecular mass determination of SOD-1 and SOD-3 by gel filtration on HPLC using two columns in series, TSK-300 and Superose Hr 12. Ovalbumin (45 kD), chymotrypsinogen (25 kD), and Cyt c (12.4 kD) were used as molecular mass markers. The values of triplet runs of standard proteins are given as mean ± SD. SOD-1, SOD-3, and bovine liver SOD values are the mean of two experiments.

Figure 6. Amino acid sequences of the amino-terminal of chloroplast and cytosol isozymes of CuZn SOD from pine and their alignments with those from other plants. Shaded boxes shows amino acids which are shared by the cytosol and the chloroplast and white boxes shows sequences that are shared by either the cytosol or the chloroplast. —, Unidentified residue; a, maize SOD-2 (8); b, spinach I (16); c, pine 1 (this work); d, pea (23); e, spinach II (17); f, pine 3 (this work).

The molecular masses of SOD-1 and SOD-3 were determined by gel filtration on HPLC and found to be approximately 35 kD for both. The subunit size of the two enzymes was determined by SDS-PAGE to 16.5 (SOD-1) and 20.4 (SOD-3) kD, respectively. The same results were obtained in presence or absence of 5 mM β-mercaptoethanol indicating that no intrachain disulfide bonds were present. Why SOD-3 showed an apparently lower molecular mass than expected from the masses of the subunits is not known. Most CuZn SODs isolated so far, with few exceptions, are homodimers having a molecular mass of 30 to 33 kD. Rice was found to...
The isozymes of SOD-1 to SOD-4 did not bind to Con A-Sepharose indicating that no neutral carbohydrates were present. Baum and Scandalios (4) examined three isozymes from maize for their carbohydrate content. They found a very low content of carbohydrates, but concluded that it could be due to background interferences of the sample.

The NH₂-terminal (1–24) from SOD-1 and SOD-3 from pine showed great similarities to published sequences from other plant sources. For the chloroplastic isozyme from Scots pine, the homology was higher when compared to chloroplastic isozymes of SOD from other plant sources than to the cytosolic isozyme from Scots pine itself. The stromal SOD-3 showed 87% and 83% homology to the same isozyme from pea and spinach, respectively. However, the cytosolic SOD showed lower, about 45% homology, when compared to other cytosolic-type SODs (Fig. 6). These findings were also consistent when up to 50 residues of different chloroplast-type and cytosolic-type of SODs were compared (16). The cytosolic SOD from Scots pine showed, compared to all other forms of plant cytosol SODs reported, one unique difference in composition at its NH₂-terminal with two additional amino acids at position 1 and 2 (Fig. 6).

The finding that the chloroplast isozyme showed high and the cytosolic isozyme showed low homology compared with other plant sources do also fit in the theory of evolutionary mutation rate of these isozymes proposed by Kanematsu and Asada (16). Their argument was that the higher rate of production of superoxide radicals during illumination (3) in the chloroplast lowered the acceptable rates of mutation. Furthermore, the two (or more) CuZn SODs that have been found in the cytosol allow mutation changes to be accommodated more easily than in the unique chloroplast CuZn SOD.

**ACKNOWLEDGMENTS**

We are greatly indebted to Torgny Nåsholm for carrying out the amino acid analysis.

**LITERATURE CITED**

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