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Purification of Two Superoxide Dismutase Isozymes and Their Subcellular Localization in Needles and Roots of Norway Spruce (*Picea abies* L.) Trees[1]

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ABSTRACT

Two isozymes of superoxide dismutase (SOD; EC 1.15.1.1) were purified from Norway spruce (*Picea abies* L.) needles to apparent electrophoretic homogeneity. Purification factors were 354 for SOD I and 265 for SOD II. The native molecular mass of both purified enzymes was approximately 33 kD, as determined by gel filtration. The subunit molecular weights, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, were 20,000 for SOD I and 16,000 for SOD II in the presence of 2-mercaptoethanol, and 15,800 and 15,000, respectively, in its absence. These results indicate that the native enzymes were homodimers whose subunits contained intrachain disulfide bonds. Isoelectric points determined by nondenaturing isoelectric focusing were 4.5 and 5.5 for SOD I and II, respectively. NH_2-terminal sequence analysis of the first 22 to 23 amino acids revealed 70 to 75% sequence identity with chloroplastic CuZn SODs from other plant species for SOD I, and 75% sequence identity with the cytosolic CuZn SOD from Scots pine for SOD II. SOD I was the major activity in needles and it was associated with chloroplasts. SOD II activity was dominant in roots.

Aerobic organisms must cope with potentially toxic oxygen species generated as products of enzymic reactions or as accidental side products of cellular redox reactions (2). During photosynthesis, superoxide radicals can be produced at a steady-state rate of 3.5×10^{-9} mol mg^{-1} Chl s^{-1}, even under low light intensities (12). Unfavorable environmental conditions such as low temperatures, desiccation stress, and air pollutants can enhance the production of toxic oxygen species in plant cells (7). Oxidative damage is prevented by a protective system of antioxidants and enzymes. In this defense system, SODs[2] (EC 1.15.1.1) are essential constituents (8). They catalyze the disproportionation of superoxide radicals (19) at a rate close to the diffusion limit (rate constant $K_{SOD} = 2 \times 10^9$ M^{-1} s^{-2} [12]) and, thus, prevent the oxidation of cellular components by the radical itself or by active oxygen species, which can be derived from $O_2^{\cdot -}$ by chain reactions (7).

Three types of SOD have been identified, containing either copper and zinc (CuZn), manganese (Mn), or iron (Fe) as prosthetic metals in the reaction center (2). These different metalloenzymes can be distinguished by selective inhibition: CuZn SODs are inhibited by cyanide, Fe and CuZn SODs are inactivated by H_2O_2, whereas the Mn SODs are resistant to both inhibitors (9). Fe SOD and Mn SOD show extensive primary sequence and structural homology but have little homology with the CuZn enzyme (2). Mn SODs occur in mitochondria (9, 13), in chloroplasts bound to the thylakoids (11), and in glyoxysomes (6). Fe SODs are frequently found in prokaryotes, but only rarely in higher plants (9). CuZn SODs are the major isozymes in plants and have been localized in the soluble chloroplast fraction and in the cytosol (13, 17, 28).

Although SODs have been extensively investigated in higher plants, little information with respect to characterization and subcellular localization is available for evolutionarily old plants such as gymnosperms. Two CuZn SOD isozymes from Scots pine needles have been purified, characterized, and found to be localized in the cytosol and chloroplasts (28).

In extracts of Norway spruce needles, one minor and two major CuZn SOD isozymes have been identified by native PAGE (22). In the present article, we describe the purification, characterization, and localization of the two major CuZn SOD isozymes in needles and roots of Norway spruce.

MATERIALS AND METHODS

Plant Materials

One to three-year-old needles were obtained from approximately 50- to 150-year-old Norway spruce (*Picea abies* L., Karst.) trees. Roots were harvested from 3-year-old potted Norway spruce plants.

Extraction of Roots

Washed roots were powdered under liquid nitrogen. Further extraction of root powder (2 g) was performed as previously described for needles (22).

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[2] Abbreviations: SOD, superoxide dismutase; CHES, cyclohexylaminoethanesulfonic acid; ME, 2-mercaptoethanol.

Isolation of Chloroplasts

Isolation of chloroplasts was modified according to Gegenheimer (10) and White (27). Branches of mature trees harvested in November were kept in the dark at 4°C for 4 d. Needles (100 g) were homogenized in a Waring Blendor (model CB-6) for 30 s under liquid nitrogen. All further operations were performed at 0 to 4°C. The frozen needle powder was suspended in 350 mL of extraction buffer containing 0.3 M sorbitol, 50 mM Hepes, pH 6.7, 2 mM EDTA, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.1% (w/v) BSA. The homogenate was filtered through eight layers of Miracloth (Calbiochem) (crude extract) and centrifuged at 6000*g* for 1 min. The pellet was suspended in extraction buffer. Ten milliliters of this suspension was layered on a step gradient of Percoll (Pharmacia) that consisted of 15 mL of 90% (v/v) Percoll and 12.5 mL of 40% (v/v) Percoll in extraction buffer. The gradient was centrifuged in a swinging bucket rotor at 4000*g* for 15 min (brake on 70%). The green band found at the interface of the two Percoll layers was collected, diluted with 10 volumes of extraction buffer, and pelleted by centrifugation at 4000*g* for 30 s. The pellet was washed once and resuspended in extraction buffer. Chloroplasts were lysed by addition of 1 volume of lysis buffer (10 mM KH_2PO_4/K_2HPO_4, pH 7.8; 2% [v/v] Triton X-100). After stirring the lysis mixture for 30 min, the sample was centrifuged (15 min, 10,000*g*). The supernatant fluid was desalted on a small column of Sephadex G-25 (PD-10, Pharmacia) equilibrated with 100 mM KH_2PO_4/K_2HPO_4, pH 7.8, and used for the determination of enzyme activities. For the determination of enzymic activities in the crude extract, an appropriate amount of lysis buffers was added.

Purification of SOD Isozymes

The purification was performed in five steps as described below and summarized in Table I. Chromatographic procedures were performed by fast protein liquid chromatography (Pharmacia) at room temperature. All other purification steps were performed at 0 to 4°C. Desalting of samples was always performed by gel filtration on Sephadex G-25 columns (PD-10), which had been equilibrated with the buffer indicated.

Crude Extract

Needles (100 g) were frozen in liquid nitrogen and homogenized in a Waring Blendor (4 × 30 s at maximum speed) under liquid nitrogen. After addition of 1 L extraction buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.8, 4% [w/v] insoluble PVP, 0.5% [v/v] Triton X-100), the homogenate was stirred for 30 min and filtered through three layers of Miracloth (Calbiochem) to remove needle residues. The filtrate was centrifuged (10,000*g*, 20 min).

$(NH_4)_2SO_4$ Fractionation

Solid $(NH_4)_2SO_4$ was slowly added to the supernatant fluid to achieve 55% saturation at 4°C. After stirring for 45 min, the precipitate was removed by centrifugation (10,000*g*, 30 min). The supernatant fraction, which contained the SOD activity, was brought to 75% saturation with $(NH_4)_2SO_4$ and stirred for 45 min. After centrifugation at 10,000*g* for 30 min, the pellet containing the SOD activity was dissolved in 10 mL of CHES buffer (10 mM CHES, pH 9.0). This buffer was also used in the following steps. The sample was desalted with CHES buffer to remove $(NH_4)_2SO_4$.

Anion-Exchange Chromatography

The sample was adsorbed onto a Mono Q HR 5/5 Column (0.5 × 5 cm) (Pharmacia) equilibrated with CHES buffer. The column was washed with buffer until the baseline was stable (UV detection, 280 nm). Proteins were eluted in 20 mL of a linear salt gradient (0–0.3 M NaCl) in CHES buffer at a flow rate of 0.8 mL/min, and 1-mL fractions were collected. Fractions containing SOD activity were pooled, desalted with CHES buffer, and concentrated by lyophilization.

Gel Filtration

The lyophilized sample was dissolved in CHES buffer (800 μL) and applied in 200-μL aliquots to a Superose 12 HR 10/30-Column (Pharmacia) equilibrated with CHES buffer containing 0.1 M NaCl. The flow rate was 0.5 mL/min and fractions of 0.5 mL were collected. Active fractions were combined and desalted with CHES buffer.

Table I. *Summary of the Protocol for Purifying SOD I and SOD II from 100 g of Spruce Needles*

Purification Step	Total Activity	Specific Activity	Yield	Purification
	units	*units mg protein^{-1}*	%	*-fold*
1) Crude extract	91,000	113	100	1
2) 55–75% $(NH_4)_2SO_4$ precipitation	78,000	1,700	85	15
3) FPLC (Mono Q)	37,000	7,000	41	62
4) FPLC (Superose 12)	14,000	12,200	15	108
5) FPLC (Mono Q)				
SOD I	3,500	40,000	4	354
SOD II	2,000	30,000	2	265

Anion-Exchange Chromatography

The sample (10 mL) was applied to a second Mono Q HR 5/5 column equilibrated with CHES buffer. The column was washed with CHES buffer until the baseline was stable. The bound SODs were eluted with a linear gradient from 60 to 160 mM NaCl in CHES buffer in a total volume of 40 mL. The flow rate was 0.8 mL/min and 1-mL fractions were collected. The SOD activity eluted in two distinct peaks (Fig. 1). The purified enzymes were stored, after desalting with CHES buffer, at −20°C. They were found to be stable for up to 10 months.

Molecular Mass Determination

The molecular mass of the native isozymes was determined by gel filtration on fast protein liquid chromatography with a Superose 12 HR 10/30 column equilibrated with 50 mM KH_2PO_4/K_2HPO_4, pH 7.8, containing 0.1 M NaCl. The flow rate was 0.5 mL/min. BSA (67 kD), ovalbumin (45 kD), and ribonuclease I (13.7 kD) were used as molecular mass markers.

Sequencing

The purified native enzymes were desalted on a Sephadex G-25 column (PD-10) with water (Milli Q, Millipore Waters, FRG), lyophilized, and then used for sequencing of the amino-terminal region by sequential Edman degradation (Applied Biosystems gas phase protein-peptide sequencer).

Electrophoresis

Electrophoresis was performed at 15°C on a Phast System with precast gels (Pharmacia). Desalted samples were used for native PAGE on 20% polyacrylamide gels and for non-denaturating isoelectric focusing in a pH gradient from 3 to 9. For SDS-PAGE on 8 to 25% polyacrylamide gradient gels, samples were boiled for 5 min with 2% (w/v) SDS in the presence or in the absence of 5% (v/v) ME. Protein bands on the gels were stained with silver (4). SOD activity staining was performed as described by Beauchamp and Fridovich (3). The gels were scanned by an Ultro Scan XL Laser Densitometer (Pharmacia).

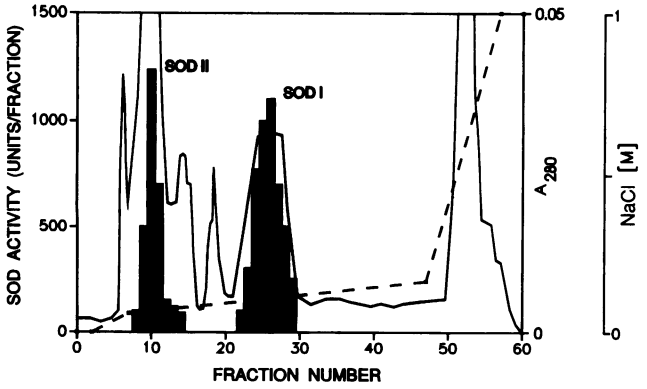

Figure 1. Separation of two SOD isozymes on a shallow (60–160 mM) NaCl gradient by anion-exchange chromatography. The black bars indicate SOD activity per fraction. The broken line represents the NaCl gradient, and the solid line the absorbance at 280 nm. Further details are described in "Materials and Methods" (purification, step 5).

Determination of Enzyme Activities

SOD activity was measured with a modified epinephrine assay as described previously (20, 22). One unit of SOD is defined as the amount of enzyme that inhibits the epinephrine assay by 50%. Activities of glucose 6-P isomerase (EC 5.3.1.9) and NADP-glyceraldehyde 3-P dehydrogenase (EC 1.2.1.13) were determined according to standard protocols (26, 29).

Protein and Chl determinations

The protein content was measured with the bicinchoninic acid reagent (Pierce, München, FRG) and BSA as standard. The Chl content was measured in 80% (v/v) acetone (18).

RESULTS

Purification of SOD I and SOD II

Of the three SOD activities present in spruce needle extracts (22), only the two major activities were detected on minigels, whereas the minor SOD activity was generally below the detection limit (Fig. 2, lane 5). The two major SOD isozymes were named after their relative migration in native gels, SOD I (high mobility) and SOD II (low mobility) (Fig. 2, lanes 2 and 1, respectively). SOD I contributed to about two-thirds of the total activity found on these gels (cf. Fig. 4C). Both enzymes copurified during $(NH_4)_2SO_4$ fractionation, anion exchange chromatography (salt gradient from 0 to 0.3 M NaCl in 20 mL), and gel filtration (Table I). Separation of the two isozymes was achieved by anion-exchange chromatography when a shallow, 60 to 160 mM salt gradient was employed similar to that described by Wingsle et al. (28) (Fig. 1). SOD I was purified approximately 354-fold to a specific activity of 40,000 units mg^{-1} protein and SOD II approximately 265-fold to a specific activity of 30,000 units mg^{-1} protein (Table I).

Each isozyme gave a major protein band in native PAGE, which corresponded to the location of SOD activity (Fig. 2). The location of the purified enzymes in the gel was identical to the activity bands from crude extracts, indicating that the enzymes were not grossly modified during the purification procedure (Fig. 2, lane 5). SDS-PAGE confirmed that both isozymes had been purified to apparent electrophoretic homogeneity, because only one major protein band was detected when purified samples were applied (Fig. 3).

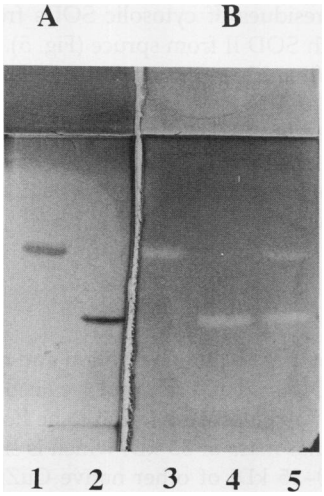

Figure 2. Native PAGE of purified SOD isozymes from spruce needles. A, Silver stain: 1, SOD II (about 0.2 μg); 2, SOD I (about 0.2 μg). B, Activity stain: 3, SOD II; 4, SOD I; 5, crude extract (step 1) concentrated by $(NH_4)_2SO_4$ precipitation (30–90% saturation). SOD activity corresponding to about 5 units was applied to each lane.

Characterization of SOD I and SOD II

The molecular mass of both native SOD isozymes was 33 kD as determined by gel filtration. To determine the subunit mol wts, the purified SOD isozymes were denatured and subjected to SDS-PAGE. One single protein band was detected for each isozyme corresponding to a mol wt of 15,800 for SOD I and 15,000 for SOD II (Fig. 3, lanes c and d, respectively). When the purified enzymes were boiled in the presence of ME, SDS-PAGE showed that the subunit mol wts were shifted to 20,000 for SOD I (lane b) and 16,000 for SOD II (lane e). These results suggest that SOD I and SOD II were homodimers that contained intrachain disulfide bridges.

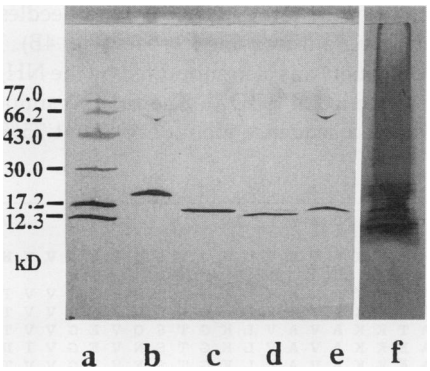

Figure 3. SDS-PAGE of the purified SOD isozymes from spruce needles. Lanes: a, Molecular mass calibration proteins (kD) from top to bottom: ovotransferrin; BSA; ovalbumin; carbonic anhydrase; myoglobin; Cyt c; b, SOD I + ME; c, SOD I; d, SOD II; e, SOD II + ME; f, crude extract. ME treatment is described in "Materials and Methods."

Table II. *Distribution of Cytosolic and Chloroplastic Enzyme Activities in the Crude Extract and Purified Chloroplast Fraction*

Preparation of chloroplasts and enzyme assays were performed with 100 g of needles as described under "Materials and Methods." Total enzymic activities and Chl in crude extract were used to calculate relative recoveries in the chloroplast fraction.

Parameter	Total Amount		Recovery in Chloroplasts
	Crude extract	Chloroplast	
			% *of crude extract*
Chl (mg)	3.48	0.53	15
Glucose 6-P isomerase (nkat)	832	5	0.6
NADP-Glyceraldehyde 3-P dehydrogenase (nkat)	143	4.4	3.1
SOD (units)	3400	260	7.6

Isoelectric focusing of the native enzymes revealed a pI value (isoelectric point) of 4.5 for SOD I and 5.5 for SOD II. Both purified SODs were inhibited by 1 mM H_2O_2 and 1 mM cyanide (data not shown), as found previously in crude needle extracts (22).

Localization of SOD Isozymes

To investigate the subcellular localization of the SOD isozymes, chloroplasts were isolated from mature spruce needles. Because the cell walls of spruce needles have high mechanical strength, and most chloroplasts contain starch grains, even after extended dark incubation, the yield of chloroplasts was low. Only 0.5% of the total Chl originally present in the mature needles (0.98 mg Chl/g fresh weight) was recovered in the final chloroplast fraction (Table II). The chloroplast fraction contained 20% of the specific glyceraldehyde 3-P dehydrogenase activity (nkat mg Chl^{-1}), which is a chloroplast stromal marker enzyme, and 50% of the specific SOD activity (units mg Chl^{-1}) as compared to the crude extract (Table II). The percent activity of glucose 6-P isomerase found in the chloroplast fraction, when compared to the recovery of the stromal marker enzyme, would correspond to a cytoplasmic contamination of about 20% if its activity was entirely localized in the cytosol. However, the contamination was probably lower because some glucose 6-P isomerase activity is likely localized in the chloroplasts, as observed for spinach (23).

When SOD activity associated with the chloroplast fraction was analyzed by native PAGE, only SOD I was detected (Fig. 4A). In root extracts, SOD II was the dominant isozyme, whereas SOD I accounted for less than 15% of the total SOD activity (Fig. 4B). These results indicate that SOD I was predominately localized in chloroplasts and SOD II was in the cytosol of spruce cells.

To corroborate this finding, the N-terminal amino acid

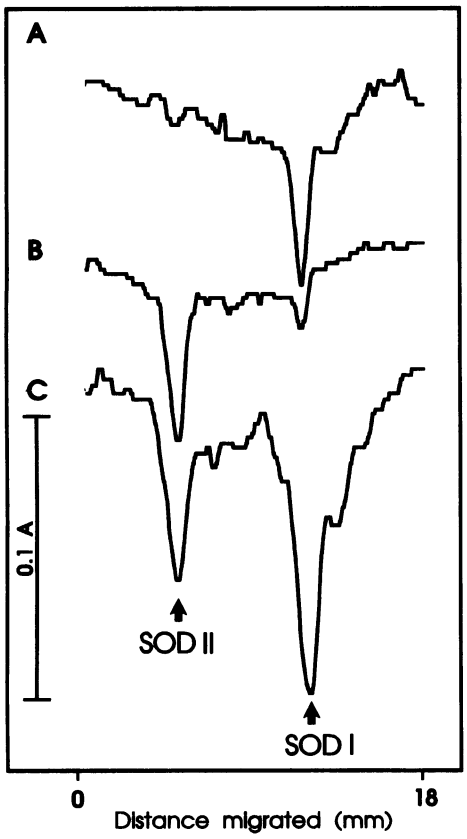

Figure 4. Occurrence of SOD I and SOD II in the soluble fraction of isolated chloroplasts (A) and in crude extracts from roots (B) and spruce needles (C). Samples were concentrated by $(NH_4)_2SO_4$ precipitation (30–90% saturation), desalted, and subjected to native PAGE. The gel was stained for SOD activity and scanned at 633 nm.

sequences of SOD I and SOD II were determined and compared to cytosolic and chloroplastic SOD sequences from other plant species. Out of 23 amino acid residues identified for SOD I, 17 or 16 residues were identical with chloroplastic SODs from other plant species, including evolutionarily related and distant plants such as pine and spinach (Fig. 5). In contrast, only 10 or less amino acid residues of SOD I were identical with residues of cytosolic SODs from other plant species and with SOD II from spruce (Fig. 5).

Among the first 22 amino acids of SOD II, 21 residues were identified and 16 were identical with the cytosolic SOD from Scots pine (Fig. 5). The number of identical amino acid residues between spruce SOD II and the cytosolic SODs from spinach and maize was considerably lower (9 and 11 residues, respectively, Fig. 5).

DISCUSSION

In the present article, the purification and characterization of two major SOD isozymes from spruce needles is described. The two native isozymes were found to be homodimers with a native molecular mass of 33 kD, which is similar to molecular masses (30–35 kD) of other native CuZn SODs (1, 14, 15, 28). The subunit mol wts of 15,800 (SOD I) and 15,000 (SOD II) were also in the range of those found in other plant species (1, 14, 15, 28) (Fig. 3).

When SOD subunits were completely reduced by ME, their mol wts shifted upward to 20,000 and 16,000 for SOD I and SOD II, respectively (Fig. 3). This suggests that the subunits of both isozymes contained intrachain disulfide bonds. Similar mobility shifts in the presence of ME were observed for CuZn SODs purified from rice and spinach (14, 15), but surprisingly not for Scots pine (28), which is evolutionarily closely related to Norway spruce. However, it is noteworthy that the chloroplastic isozyme from pine had a subunit mol wt of 20,400 (28), which is similar to the subunit mol wt of reduced SOD I. The cytosolic isozyme from pine showed a subunit mol wt (16,500) similar to reduced SOD II from spruce. It is possible that data on pine SOD refered to reduced enzyme subunits as well, because Wingsle et al. (28) used ME in their homogenization buffer.

In leaves, a minor fraction of CuZn SOD activity is localized in the cytosol, whereas a major portion of the activity is present in the chloroplast (2, 14, 17). In spruce, SOD II was probably localized in the cytosol, because it was not found in the chloroplast fraction (Fig. 4A). It contributed only to about one-third of the total SOD activity in needles (Fig. 4C) and was the dominant isozyme in roots (Fig. 4B). The likely cytosolic localization was also supported by the NH_2-terminal amino acid sequence of SOD II. The first 22 amino acids of SOD II revealed a sequence identity of about 75% with the

Figure 5. Amino acid sequences of the amino terminal regions of spruce SOD I and II in comparison with those of chloroplastic and cytosolic CuZn SODs from other plants. The figures in parentheses refer to the corresponding reference. –, Unidentified residue.

Chloroplast CuZn SOD	5	10	15	20
Spruce SOD I (this work)	- T K K A V V V L W G I S Q V E G V V N L L Q E			
			Y	
Petunia (25)	A T K K A V A V L K G T S N V E G V V T L T Q D			
Tomato (21)	A T K K A V A V L K G N S N V E G V V T L S Q D			
Rice I (15)	A T K K A V A V L K G T S Q V E G V V T L T Q D			
Horsetail (15)	A E K K A V A V L K G T S N V E G V I N L F Q E			
Pea (24)	A A K K A V A V L K G T S E V E G V V T L T Q D			
Spinach II (16)	A T K K A V A V L K G T S N V E G V V T L T Q E			
Pine III (28)	A A K K A V A V L K G D S Q V E G V V T L S Q E			
Cytosol CuZn SOD				
Maize II (5)	V K A V A V L A G T D - V K G T I F F S Q E			
Spinach I (15)	G K A V V V L S S N E G V V G T V V F A Q E			
Pine I (28)	G L L K A V V V L N G A A - V K G V V Q F T D G			
			Y	
Spruce SOD II (this work)	S P L K A V A V L T G A - D V K G V V Q F T			

cytosolic SOD from Scots pine, and only about 50% with chloroplastic and cytosolic SODs from other plant species (Fig. 5). Still, the homology between the chloroplast and cytosol SOD sequence family was high because about 30% of the residues were replaced by conservative substitutions.

SOD I from spruce was found in the soluble fraction of chloroplasts (Fig. 4A). In addition, NH_2-terminal sequence analysis of 23 amino acid residues of SOD I revealed 70 to 75% sequence identity with the chloroplast sequence family (Fig. 5). A high sequence identity in the amino-terminal region of the chloroplastic CuZn SODs in plants of great evolutionary distance, such as spruce and spinach, indicates a slow rate of mutation for this CuZn SOD isozyme as compared to numerous other plant proteins. This supports the suggestion of Kanematsu and Asada (15) that mutations decreasing the activity of chloroplastic CuZn SOD are probably lethal for plants.

Asada and Takahashi (2) speculated that a high production of $O_2\cdot^-$ radicals in chloroplasts during photosynthesis necessitates a high level of SOD activity to prevent oxidative damage to chloroplastic components. Hodgson and Raison (12) found a production rate of $O_2\cdot^-$ of 12.5 μmol $\cdot$ mg^{-1} Chl $\cdot$ h^{-1} at relatively low light intensities (350 μmol photon $m^{-2} \cdot s^{-1}$). This corresponds to a production rate of v = 100 μM $\cdot$ s^{-1}, assuming that the specific chloroplastic volume is 35 μL $\cdot$ mg^{-1} Chl (2). In the absence of SOD or other scavengers for $O_2\cdot^-$, the steady-state concentration of $O_2\cdot^-$ in the chloroplast would amount to 15 μM (with $v = k_s \cdot [O_2^-]^2$, and the spontaneous dismutation rate of $O_2\cdot^-$ at pH 7, $k_s = 4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [2]). The present results estimate a chloroplastic SOD concentration of about 10 μM using the specific SOD I activity (40,000 units $\cdot$ mg protein^{-1}) (Table I), the chloroplastic SOD activity (490 units $\cdot$ mg Chl^{-1}), the mol wt of SOD I (40,000), and the specific volume of chloroplasts (2). If SOD is present at this level and other scavengers are not significantly involved in the removal of $O_2\cdot^-$ in chloroplasts, the steady-state concentration of $O_2\cdot^-$ is estimated at 5 nM (with $v = k_{SOD} \cdot [O_2^-] \cdot [SOD]$ and $k_{SOD} = 2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ [2]). Thus, SOD I possibly decreases the level of $O_2\cdot^-$ 3000-fold. This estimation indicates that SOD I provides effective protection against the accumulation of $O_2\cdot^-$ in chloroplasts of the healthy spruce needles used in the present investigation. The subcellular localization of the two SOD isozymes provides a means for investigating organelle-specific oxidative stress in spruce.

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