Plant Physiol. (1989) 90, 742–748
0032-0889/89/90/0742/07/$01.00/0

Received for publication August 19, 1988
and in revised form January 25, 1989

Assay and Electrophoresis of Superoxide Dismutase from Red Spruce (*Picea rubens* Sarg.), Loblolly Pine (*Pinus taeda* L.), and Scotch Pine (*Pinus sylvestris* L.)[1]

A Method for Biomonitoring

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ABSTRACT

This paper reports a method for extracting the antioxidant enzyme superoxide dismutase (SOD) from the needles of red spruce (*Picea rubens* Sarg.), loblolly pine (*Pinus taeda* L.), and scotch pine (*Pinus sylvestris* L.) with high efficiency and free from interfering compounds. The extraction employs phosphate buffer with polyvinylpolypyrrolidone and Triton X-100 followed by dialysis overnight. The isozymes of SOD in each species were separated electrophoretically and tested for their sensitivity to KCN and H_2O_2. An isozyme resistant to these inhibitors was found in the spruce but not the pine needles. The isozymes from the spruce needles were examined for individual responses to aging and H_2O_2 inhibition. Four of the five CuZn isozymes in spruce were found to have increased significantly but equally by October of their first year and two of those four isozymes were found to be more sensitive to H_2O_2. The response of the SOD isozymes in loblolly pine seedlings to O_3 was also examined and the isozymes were found to be induced equally. Because the SOD activity in the young pine needles was too low to electrophorese, the SOD activity from the pines in the O_3 experiment had to be partially purified using $CHCl_3$ and ethanol, then concentrated.

SOD,[2] an enzyme catalyzing the reaction of the superoxide radical (O_2^-) with itself to yield H_2O_2 and O_2, is the cell's primary defense against damage by O_2^- (13, 24). O_2^- production can be the result of numerous reactions including the decomposition of O_3 (23) and the oxidation of SO_2 (2). Both of these gases have been implicated in the decline and death of forest trees in North America and Europe (14, 21, 31), a decline that has most severely affected red spruce (*Picea rubens* Sarg.) but has also affected other species (14, 21, 31).

Oxygen radicals have also been postulated to be a major factor in the deleterious effects of aging (16). It has been proposed that as leaves or needles age they are exposed to increasing oxidant stress and, in response, antioxidants might be produced to prolong the life of the tissues (16). There are numerous examples of various antioxidants in plants increasing with age (16, 27, 30).

The following lines of evidence indicate that induction of SOD can be an indicator that O_2^- production is enhanced (19, 24). O_3 damage to Chl and carotenoids in spinach was found to be due to O_2^-, and SOD was initially induced by exposure to O_3 (25). SOD activity in snap beans was found to correlate inversely with the extent of leaf injury from O_3 exposure (17). Moreover, induction of SOD by *N*-(2-[2-oxo-1-imidazolidinyl]ethyl)-*N'*-phenyl urea (EDU) protected against damage from exposure to O_3 (17). Similarly, SO_2 induced SOD in poplar leaves (*Populus euramericana*), and younger leaves with higher SOD activity were more resistant to SO_2 (30). Finally, SOD also inhibited the accumulation of H_2O_2 in spinach leaves treated with SO_2 (29).

Thus, a simple assay for SOD applicable to many tree species would be of value in determining oxidant exposure and enzyme responses. Endogenous phenolics represent a major obstacle to developing assays for enzymes in conifers (4, 18). There are also problems with extraction efficiency and low mol wt reductants interfering with the assay (13). Two methods for assaying needles for SOD activity have been reported (9, 28), but we found them inadequate to extract the SOD activity in the conifer species tested.

Another index of a plant's response to stress could be found in the response of individual isozymes of SOD such as the response in maize to paraquat, in peas and tobacco to nutrients, and in rice to salinity (24). In each instance the isozymes varied independently (24). In maize, the isozymes were shown to be from independent genes (19). The isozymes of CuZnSOD from wheat germ, which are different gene products, have shown different sensitivities to H_2O_2 (5). Since significant levels of H_2O_2 have been found in cloud and

[1] This research was supported by funds provided by the Northeastern Forest Experiment Station's Spruce-Fir Research Cooperative and the Southern Commercial Forest Research Cooperative within the joint U.S. Environmental Protection Agency-U.S. Department of Agriculture Forest Service Forest Response Program. The Forest Response Program is part of the National Acid Precipitation Assessment Program. This paper has not been subject to EPA or Forest Service peer review and should not be construed to represent the policies of either agency.

[2] Abbreviations: SOD, superoxide dismutase; CuZnSOD, copper- and zinc-containing superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; FeSOD, iron-containing superoxide dismutase; NBT, nitro blue tetrazolium; PVPP, polyvinylpolypyrrolidone.

rainwater (15), the sensitivity of SOD to H_2O_2 could have important consequences for the survival of plants exposed for long periods of time to both H_2O_2 and superoxide-generating pollutants.

The isozymes of SOD are classed according to the metal at the active site: cooper and zinc (CuZnSOD), manganese (MnSOD), or iron (FeSOD). Of these, the FeSOD is rarely found in plants (8) and MnSOD usually represents only 3 to 5% of the total activity in plants (24). The three classes can be distinguished by their sensitivity to inhibitors since only the CuZnSOD is inhibited by cyanide, whereas both CuZnSOD and FeSOD are inhibited by H_2O_2, and MnSOD is insensitive to both inhibitors (13, 24).

In this paper, techniques for extracting and assaying SOD activity from red spruce needles and pine needles are presented. The isozymes were separated electrophoretically and compared. In addition, the responses of the red spruce isozymes to both seasonal changes and H_2O_2, and the response of loblolly pine isozymes to O_3, were examined to determine if individual isozymes of SOD in each species would respond differently.

MATERIALS AND METHODS

Materials

Red Spruce seedlings (*Picea rubens* Sarg.) were supplied by the U.S. Forest Service. The barerooted seedlings were potted and grown in the Duke greenhouse. Additional spruce needles were supplied by the Boyce Thompson Institute, Ithaca, NY. These were placed in vials as they were harvested and immediately immersed in liquid N_2. The needles were shipped to Duke in liquid N_2 and stored at −70°C until analyzed. In addition, needles from a red spruce that had been transplanted to Durham, NC, from the Virginia mountains (Grayson County) were used to obtain fresh needles from another population.

Lobolly pine needles (*Pinus taeda* L.) were obtained from mature trees growing in the Duke University Forest area and were analyzed fresh. The scotch pines (*Pinus sylvestris* L.) were located 19 km from the laboratory, and the needles were frozen in liquid N_2 after cutting, then stored at −70°C. Additional lobolly pine needles were obtained from seedlings growing in open top chambers in Duke Forest, and stored at −70°C.

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except for some of the xanthine oxidase which was obtained from U.S. Biochemical (Cleveland, OH).

SOD Extraction

Needles were homogenized by grinding with a Polytron 10A generator (Brinkman, Westbury, NY) at high speed for 20 s, stopping every 5 s to clear debris from the blades. The exact composition of the buffer and other details of the extraction used in each experiment will be discussed in "Results." Where indicated, the samples were dialyzed overnight against 2 L of pH 7.8, 0.05 M potassium phosphate. The dialysis buffer was changed twice at 6 h intervals. To estimate the recovery, an aliquot (30 μL) of a solution of bovine erythrocyte SOD (3.5 mg/mL) was added to the homogenizing buffer before homogenizing. The recovery of MnSOD from pine needles was estimated by adding an aliquot (50 μL) of a solution (2 mg/mL) of *Escherichia coli* MnSOD (gift of W. Beyer) to the homogenizing buffer before homogenizing.

Assays

SOD was assayed by the method of McCord and Fridovich (20) and the method of Beauchamp and Fridovich (6) with xanthine and xanthine oxidase as the superoxide generator as noted in the table and figure legends. Activity was calculated as described by Asada *et al.* (3). PAGE was by the method of Beauchamp and Fridovich (6). The gels were 6 × 85 mm and were scanned at 560 nm using a Shimadzu (Kyoto, Japan) UV-260 spectrophotometer. The ends of the gels were removed before scanning because the cuvette would not hold the entire gel. The scans are inverted in the figures so the bands appear as peaks even though they represent decreases in absorbance.

H_2O_2 Reaction

For the experiment to measure the relative H_2O_2 sensitivities of the spruce SOD isozymes, H_2O_2 and KCN (to inhibit peroxidase) were added to a dialyzed extract (pH 7.8) of the needles to a final concentration of 10 and 1 mM, respectively. Aliquots were taken at 0.5 h intervals and quenched by adding 34,000 units of catalase (Sigma c-100). This catalase preparation was found to be free of SOD. The samples were dialyzed overnight to remove the KCN, then assayed for SOD activity and electrophoresed to separate the isozymes.

O_3 Exposure

The loblolly pine seedlings for the O_3 treatment were grown in open-top chambers 3 m in diameter by 3 m tall in Durham County, NC. The seedlings were planted in the ground a year before the treatment was started. Air was blown into the lower part of the chambers through charcoal filters. The O_3 concentration in the treated chambers was maintained at approximately 3 times the O_3 concentration outside the chambers 24 h a day using an O_3 generator and computer controlled monitor. The ambient seasonal average for O_3 was 48 nL/L. The O_3 in the control chambers averaged 20 nL/L but varied from 9 to 62 nL/L. The O_3 exposure for the treated seedlings averaged 129 nL/L and varied from 33 to 230 nL/L. The needles were harvested 3 months after the start of the O_3 exposure.

The dialyzed extracts from the needles were partially purified by extraction with ethanol and chloroform (20) because the control samples had to be concentrated so much that other compounds present interfered with the electrophoresis. The samples were concentrated using Amicon Centriprep 10 concentrators (Danvers, MA). The samples from the control needles required a 20-fold concentration, whereas the O_3 treated samples required only a 6-fold concentration.

RESULTS

Spruce SOD Assay and Electrophoresis

As has been reported by Castillo *et al.* (9), an extract can be prepared from spruce needles that does not reduce NBT at volumes equivalent to one unit of SOD activity. This method showed high levels of activity in the assay, but no bands of activity were seen on polyacrylamide gels. In our experiments, the needles were homogenized in 0.05 M Mes (pH 6.5), 0.01 M NaCl, 0.005 M $MgCl_2$ (4), then centrifuged 1.5 min at approximately 13,000*g* with a micro-centrifuge. Addition of PVP-40 gave artifactual activity similar to that reported by Schulz (28). In our experiments, this was due to the inhibition of the oxidation of xanthine. The rate of change in absorbance at 295 nm decreased from a control rate of 0.017/min to a rate of 0.014/min when 2 μL of the supernatant containing 1% (w/v) PVP-40 were added to a 3 mL reaction mix.

To eliminate these problems, we developed a method of homogenizing in 0.05 M potassium phosphate (pH 7.8), 0.1 mM EDTA, and 4% (w/v) PVPP followed by dialysis overnight. However, if the homogenate was centrifuged and only the supernatant dialyzed (Table I), less than 40 units/g wet weight were recovered. A much higher recovery of activity was obtained by dialyzing the whole homogenate and then centrifuging (Table I). On polyacrylamide gels this extract showed 6 achromatic bands (Fig. 1a) with the slowest migrating band being resistant to both 5 mM KCN (Fig. 1b) and 20 mM H_2O_2 (Fig. 1c). This indicates that band 6 is MnSOD and the other bands, which were inhibited by KCN, are Cu-ZnSOD. The cyanide-resistant activity was assayed as being about 8% of the total activity (Table I), but was variable enough that band 6 was not always detectable on the gels. Whether or not band 6 was seen seemed to depend on the seedling used, the extraction procedure used, and whether the needles were fresh or frozen (see following paragraph). The cyanide-resistant activity was also more labile than the cyanide-sensitive activity. In an extract stored at 4°C after dialyzing, band 6 was no longer detectable on the gels the second day after homogenizing, whereas the other bands remained for 2 weeks at 75% of the initial activity. While this extraction procedure recovered an apparently high level of activity, spiking with bovine erythrocyte SOD indicated only about half the activity (47%) was being recovered (Table I). It was later found that the extraction efficiency could be improved by adding 0.3% (w/v) Triton X-100 to the homogenizing buffer (26). As shown in Table I, adding Triton X-100 doubled the recovered activity from both dialyzed samples to essentially 100%, and eliminated the need to dialyze the whole homogenate.

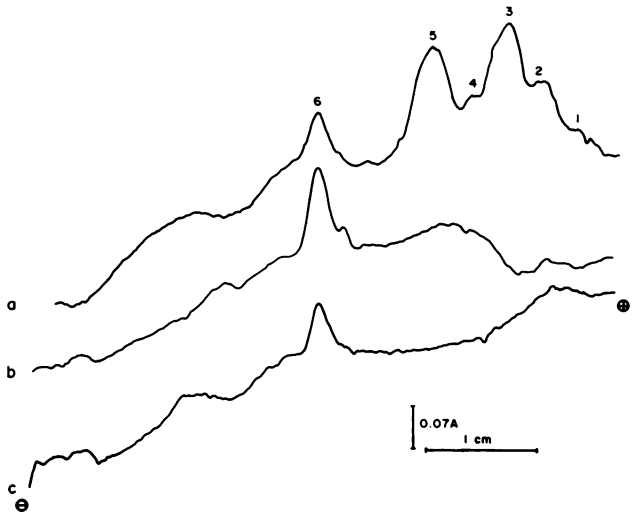

Figure 1. Isozymes of superoxide dismutase from red spruce extracted without Triton X-100. The activity was extracted as described for Table I, dialyzing the whole homogenate without Triton X-100. Five units of activity were loaded on each gel. The gels were stained photochemically with NBT (6) and scanned at 560 nm. The traces are (a) a gel stained without any inhibitor treatment, (b) a gel soaked in 5 mM KCN for 1 h before staining, (c) a gel soaked in 20 mM H_2O_2 for 1 h before staining.

Table I. *Extraction of Superoxide Dismutase Activity from Red Spruce Needles (Picea rubens Sarg.)*

Spruce needles (0.3 g) were homogenized in 6 mL of 0.05 M potassium phosphate (pH 7.8), 0.1 mM EDTA, 4% (w/v) PVPP with or without 0.3% (w/v) Triton X-100. The homogenates were either centrifuged (10 min, 12,000*g*, 2°C) and the supernatant dialyzed, or the homogenate dialyzed and then centrifuged. Cyanide resistant activity was measured in the presence of 5 mM KCN. To estimate the recovery, an aliquot (30 μL) of a solution of bovine erythrocyte SOD (3.5 mg/mL) was added to the homogenizing buffer before homogenizing. Activity was determined using the Cyt *c* assay (20).

Experimental Condition	Activity Recovered	Activity
	units/g fr wt (SD)	%
Without Triton X-100		
Supernatant dialyzed	39 (4)	
Homogenate dialyzed	209 (9)	
Recovery		47
KCN resistance		7.5
With Triton X-100		
Supernatant dialyzed	412 (23)	
Homogenate dialyzed	428 (35)	
Recovery		125

To determine the effect of Triton X-100 on the extraction of the individual SOD isozymes from fresh needles (Fig. 2), needles from the mountain grown spruce were used. The isozyme composition remained the same (Fig. 2) except that the buffer with Triton X-100 was somewhat more efficient at extracting the band 6 isozyme. Band 6 does not appear in the scan of the gel when the SOD was extracted without Triton X-100 (Fig. 2a). Band 6 does appear on the scan when Triton X-100 was used (Fig. 2b) but was not visually detectable. The band was visible in the gel when the activity loaded on the gel was doubled to 10 units (Fig. 2c). After the needles were frozen at −70°C, band 6 was no longer detected on the scan even when 10 units of activity were loaded on the gel. Comparison of Figures 1 and 2 show that there is no difference in the number of SOD isozymes from the two seedling sources. The electropherograms of the SOD isozymes in the needles from the Boyce Thompson Institute were also indistinguishable from the electropherograms from the other spruce needle populations. When a spruce sample that had been spiked

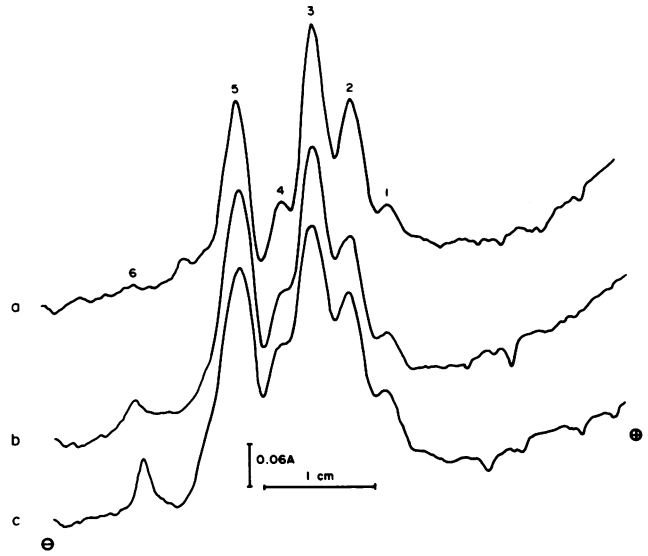

Figure 2. Comparison of superoxide dismutase isozymes from red spruce extracted without or with Triton X-100. Conditions were the same as Figure 1 except for the addition of 0.3% (w/v) Triton X-100 to the homogenizing buffer. The traces show (a) activity extracted without Triton X-100, 5 units of activity loaded; (b) activity extracted with Triton X-100, 5 units of activity loaded; (c) activity extracted with Triton X-100, 10 units of activity loaded.

Table II. *Extraction of Superoxide Dismutase Activity from Loblolly (Pinus taeda L.) and Scotch (Pinus sylvestris L.) Pine Needles*

Conditions were the same as for Table I except that the PVPP was increased to 5% (w/v). The recovery of MnSOD was estimated by adding an aliquot (50 μL) of a solution of *E. coli* MnSOD (2 mg/mL) to the homogenizing buffer before homogenizing.

Experimental Condition	Activity Recovered	Activity
	units/g fr wt (sd)	%
Without Triton X-100		
Loblolly pine	<26	
Scotch pine	344 (20)	
Loblolly recovery		0
With Triton X-100		
Loblolly pine	338 (52)	
Scotch pine	944 (19)	
Loblolly recovery		82
Loblolly KCN resistance		6
MnSOD (*E. coli*) recovery		77

before homogenizing with bovine erythrocyte SOD was electrophoresed, no unexpected bands were detected.

Pine SOD Assay and Electrophoresis

Assaying loblolly pine needles for SOD activity proved to be more difficult (Table II). An extract prepared in the Mes buffer was quite efficient at reducing Cyt *c* and NBT. A loblolly needle extract from an acetone powder, prepared as described by Schulz (28) for scotch pine, showed very little activity but did interfere with the assays. The extraction procedure with PVPP, but without Triton X-100 described for red spruce, showed no activity when used with loblolly pine needles, but extracted activity nearly equal to that of the spruce from scotch pine needles (Table II). This may help to explain why the acetone powder method (28) worked for the scotch pine but not the loblolly pine. However, adding Triton X-100 to the homogenizing buffer significantly increased the level of SOD activity extracted from both scotch and loblolly pine (Table II). The extraction efficiency was improved to better than 80% and the samples were free from compounds that interfered with the assay. Although the assay indicated that 6% of the activity was cyanide resistant (Table II), no cyanide or H_2O_2 resistant bands appeared on polyacrylamide gels. However, when *Escherichia coli* MnSOD was added to the homogenizing buffer, 77% of the activity (Table II) was recovered and KCN/H_2O_2 resistant activity was seen on polyacrylamide gels.

Electrophoresis of the extracts from loblolly pine needles showed 6 achromatic bands (Fig. 3a) all of which were cyanide sensitive. Even when 10 units of activity were loaded on the gels, no cyanide resistant bands were seen. The isozyme profile from the extracts of scotch pine needles is shown for comparison (Fig. 3b). The four bands shown were all cyanide sensitive and corresponded very closely to the cyanide sensitive bands reported by Schulz (28). The cyanide insensitive bands reported by Schulz (28) were never observed, even when 12 units of activity were loaded on the gels. The area of increased absorbance near the top of the gel corresponded to dark green bands and were probably Chl binding proteins.

Spruce SOD Isozyme Response to Aging

During an experiment with red spruce, it was observed that the SOD activity in the needles increased during the fall of their first year (Fig. 4). While there was a slight change in the fresh weight/dry weight ratio from August (ratio = 2.62 ± 0.02) to October (ratio = 2.28 ± 0.04), the increase in SOD activity was much larger than could be accounted for by the decrease in the fresh weight/dry weight ratio. To determine how the individual isozymes in spruce varied over time,

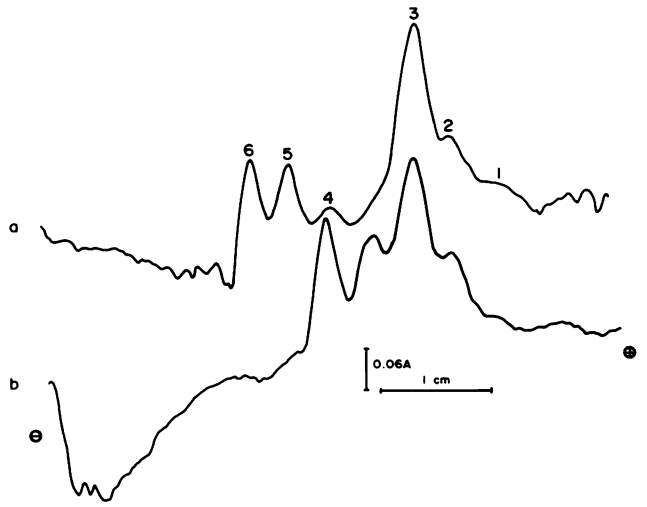

Figure 3. Isozymes of superoxide dismutase from needles of (A) loblolly pine and (B) scotch pine. The activity was extracted as described for Table II using Triton X-100 and electrophoresed as for Figure 1. Six units of activity were loaded on each gel.

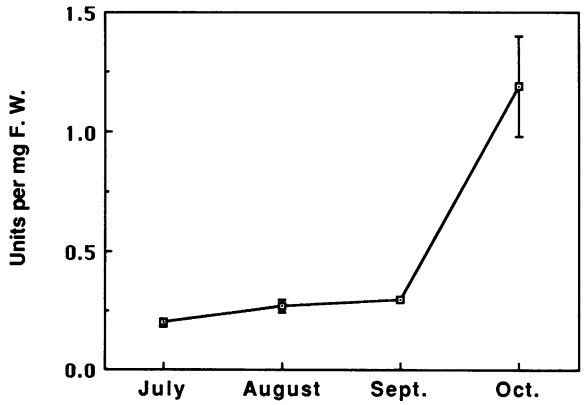

Figure 4. Superoxide dismutase activity in first year (1986) spruce needles. The needles were harvested from spruce seedlings growing in open-top chambers at the Boyce Thompson Institute, Ithaca, NY. For the assay, the needles (0.3 g) were homogenized in 6 mL of 0.05 M Mes (pH 6.5), 0.01 M NaCl, and 0.005 M $MgCl_2$, centrifuged in a microcentrifuge (1.5 min) and assayed using the xanthine/xanthine oxidase reduction of NBT (6).

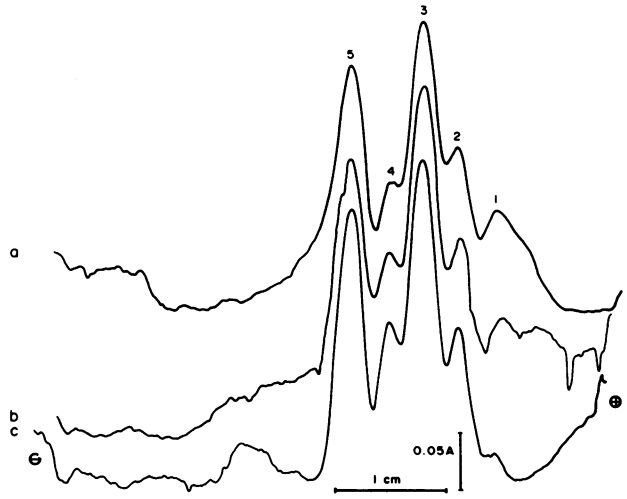

Figure 5. Isozymes of superoxide dismutase in red spruce needles before and after the fall increase in activity. Extraction and electrophoresis were as for Figure 1. The needles were from the same seedlings as Figure 4. Five units of activity were loaded on each gel; adjusting the dilution to keep the volume loaded constant. (a) August, (b) October, (c) 1986 needles taken fresh from a seedling at Duke University in May 1987.

extracts of these spruce needles were electrophoresed (Fig. 5). Bands 2 through 5 were found to increase uniformly from August (Fig. 5a) to October (Fig. 5b). However, band 1, in October, was a smaller fraction of the total activity than it had been in August. For comparison, fresh needles from the same needle generation (1986) but a different spruce population were harvested when they were a year old (May 1987), extracted, and electrophoresed. The isozyme pattern (Fig. 5c) is the same as the pattern for the needles from October, except that band 1 constitutes an even lower fraction of the total activity. By the assay of McCord and Fridovich (20), the SOD activity in the needles was 54 units/g wet weight (August), 185 units/g wet weight (October), and 705 units/g wet weight (May). One month old needles (110 units/g) and 1 year old needles (377 units/g) were taken simultaneously from a single seedling in the Duke greenhouse, extracted and, electrophoresed. The results confirmed the results shown in Figure 5.

Spruce SOD Isozyme Inhibition by H_2O_2

The cuprozinc isozymes of SOD are known to be irreversibly inhibited by H_2O_2, and different isozymes from the same organism can have different sensitivities to H_2O_2 (5). Red spruce extracts incubated with 10 mM H_2O_2 demonstrated a time dependent loss of SOD activity (Fig. 6). In 1.5 h, 58% of the activity was lost. The individual isozymes were very different in their sensitivity to the H_2O_2 (Fig. 7). Isozyme 5 was almost completely gone in the first half-hour (Fig. 7b) and isozyme 4 was greatly diminished (about 35% in 1.5 h, Fig. 7d). The other three isozymes were relatively unaffected.

O_3 Induction of Pine SOD Isozymes

All of the SOD isozymes observed in loblolly pine were induced equally by the O_3 treatment (Fig. 8). The control needles (Fig. 8a) had an SOD activity of 38 units/g wet weight (mean O_3: 20 nL/L). The O_3 treated needles (Fig. 8, b and c) had an activity of 114 units/g wet weight (mean O_3: 129 nL/L). The isozyme pattern of a treated sample that was concentrated without the ethanol/chloroform extraction demonstrated that the extraction did not affect the isozymes (Fig. 8c). It should also be noted that the isozyme pattern in Figure 8 is the same as that for the needles in Figure 3a even though the needles for 3a were a year old and had an activity of 550 units/g wet weight. The needles for Figure 8 were only about 3 months old.

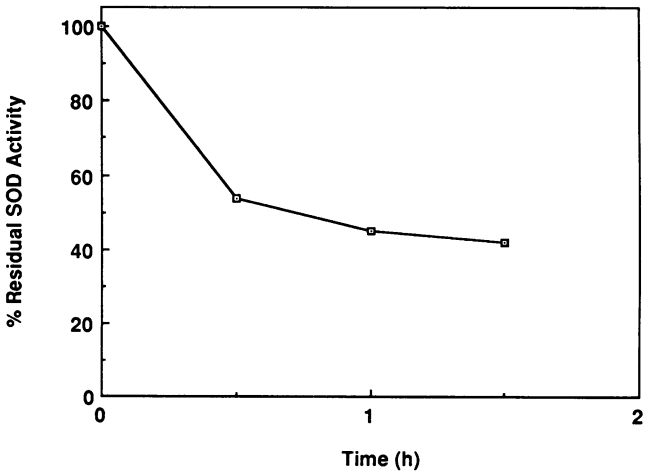

Figure 6. Rate of inhibition of red spruce SOD by H_2O_2. SOD was extracted from red spruce needles as described for Table I without Triton X-100. H_2O_2 and KCN were added to final concentrations of 10 and 1 mM, and reacted for 1.5 h at room temperature (pH 7.8 phosphate buffer). Aliquots were taken at 0.5 h intervals and the reaction quenched with catalase. The KCN was dialyzed out and the samples assayed for activity (20).

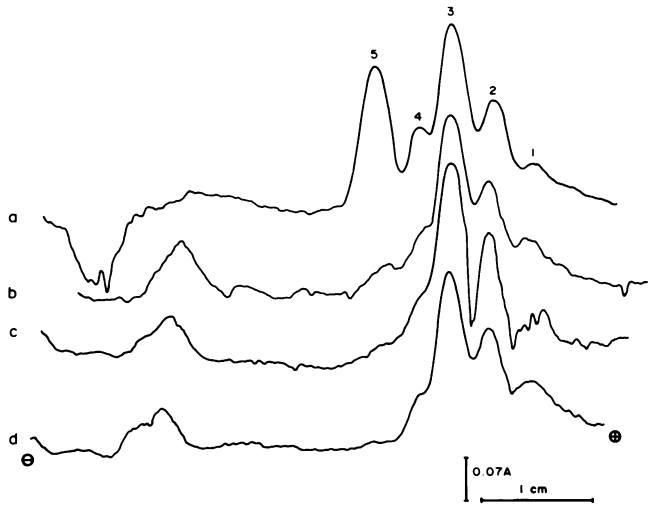

Figure 7. Isozymes of SOD at each time point during the H_2O_2 reaction. Aliquots of each time point (Fig. 6) were electrophoresed and stained as for Figure 1. Five units of activity were added to gel (a), and an equal volume added to each of the other gels. (a) 0 h, (b) 0.5 h, (c) 1.0 h, (d) 1.5 h.

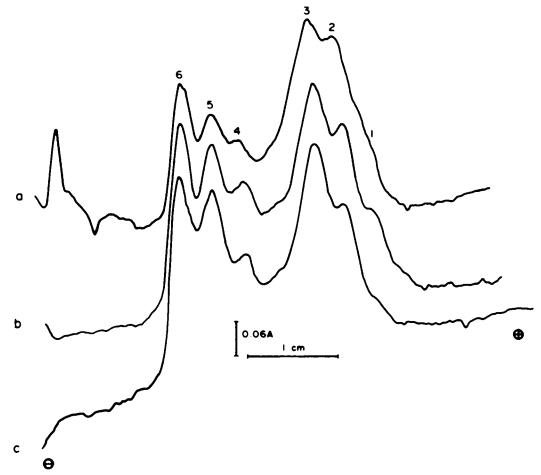

Figure 8. Isozymes of SOD in the needles of O_3 treated loblolly pine seedlings. The extraction and electrophoresis were the same as Figure 3 except two of the samples were extracted with ethanol and chloroform before the electrophoresis. All three were concentrated using Amicon Centiprep 10 concentrators. Six units of activity were loaded on each gel, keeping the volume nearly constant by adjusting the concentration. (a), Control; (b), O_3 treated; (c), O_3 treated but not extracted with ethanol/chloroform.

DISCUSSION

With the increasing problem of forest loss due to air pollutants, some of which can generate O_2^-, there is a need to be able to measure the SOD activity in conifers. We recommend extraction of the SOD activity from red spruce with 0.05 M potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.3% (w/v) Triton X-100, and 4% (w/v) PVPP, followed by dialysis. The concentrations of Triton and PVPP will need to be optimized for different species. This procedure, using Triton X-100 and PVPP, provides a method that gives high yields of SOD activity, free of interferences, and is relatively simple to use. The method has allowed us to examine the response of the different SOD isozymes in red spruce to aging and H_2O_2 exposure, and the response of the loblolly pine SOD isozymes to chronic O_3 exposure. The method is potentially applicable to a wide range of conifers that may not be amenable to being assayed using previously reported methods. Since different isozymes are specific to different organelles (19, 24), this approach might be also used to assay for organelle specific stress.

A change in activity in trees with age or time of year has been reported previously both for SOD (27, 30) and other antioxidant enzymes (12, 27). Kunert and Ederer (16) have previously suggested that increasing damage from oxygen radicals might be the mechanism of aging and, in order to extend the life of the tissues, that antioxidants in leaves and needles might increase as the tissues age. Our observation that SOD activity increased as the needles aged is consistent with this hypothesis. Interestingly, four of the isozymes in the red spruce needles increased equally as the needles aged, a result that differs from the observations in peas, rice, maize, and tobacco (24). This variation in response might be due to mechanistic differences, *i.e.* that nutrient and age-related changes in the levels of SOD activity might not be directly mediated by O_2^- as has been proposed for SOD induction by paraquat (24). The variation in response might also be indicative of a difference in genetic regulation between the species of plants. The maize SOD isozymes are different gene products (19), and the nonuniform responses of the SOD isozymes from spruce to H_2O_2 suggest that spruce CuZnSOD isozymes are different gene products. This is supported indirectly by the observation that the two CuZnSOD isozymes in wheat germ, which also varied in sensitivity to H_2O_2, were shown by their amino acid compositions to be different gene products (5).

The peroxide inhibition of the SOD isozymes is a phenomenon that needs to be investigated further *in vivo*. It is conceivable that H_2O_2 in clouds and rainwater (15) could deplete the needles of SOD, making them more sensitive to pollutants which generate O_2^-. Other synergistic effects have been reported (7, 22). While the concentration of H_2O_2 used here was 100 to 400 times that found in clouds and rain (15), clouds can enshroud the trees on a mountaintop for many hours (14), thus increasing the chance of damage. However, trees have enzymes to eliminate H_2O_2 (27), so the extent of damage would depend on the efficiency of these enzymes. Since different isozymes of SOD are localized in different cell compartments (24), the extent of damage would also depend on how accessible the most sensitive isozymes are to atmospheric pollutants.

Loblolly pine exposed to chronically elevated O_3 conditions showed an increase in SOD activity that is probably a response to O_2^- generated from the O_3 (23). This is consistent with other observations that O_3 and SO_2, either singly or in combination, cause an induction of antioxidant activity in forest trees and supports the theory that air pollution stress is mediated by toxic oxygen species (9, 22, 27). All of the pine SOD isozymes increased equally in response to O_3, a pattern not found in the maize response to paraquat (19). This difference may be attributable to differences between paraquat

and O_3, such as transport and subcellular distribution, although these toxicants both generate O_2^- (19, 23). However, it is also possible that this variation in isozyme response between conifers and maize is the result of a difference in gene expression. In maize, the four SOD isozymes are the products of four unlinked genes (19), but the pine SOD isozymes might be the product of a single gene and differ because of posttranslational modification, as has been suggested for bovine and human erythrocyte SOD (10, 11) and horseradish peroxidase (1). Additionally, the pine SOD isozymes may have been induced at different rates, and differences would have been seen if the pine needles had been collected closer to the beginning of the O_3 exposure. A comparison with the results in peas and rice is more difficult because nutrient and aging effects might not be mediated by O_2^-.

In summary, the use of Triton X-100 and PVPP facilitates the extraction of SOD activity from the conifer species tested with a high efficiency and free from interfering reductants or phenolics. This method may work well enough for conifer species to allow interspecies as well intraspecies comparisons while monitoring for responses to increasing O_2^- injury. We have successfully used the method to examine the effects of aging, H_2O_2, and O_3 on both the total SOD activity and the activity of individual SOD isozymes in either spruce or pine seedlings.

ACKNOWLEDGMENTS

The authors would like to thank the Boyce Thompson Institute, particularly Dr. Leonard Weinstein, Dr. Ruth Alscher, and Mr. Jonathan Cumming, for assisting with this project. We thank the Spruce-Fir Research Cooperative, Northeast Forest Experiment Station, particularly Dr. Gerard Hertel, Program Manager, for project coordination, and the Southern Commercial Forest Research Cooperative for supporting the research on pine seedlings. We would also like to thank Dr. Wayne Beyer, Dr. Irwin Fridovich, and Dr. James Siedow for their advice and the loan of equipment, and Mr. Edward Fendick for computer assistance.

LITERATURE CITED

1. **Aibara S, Yamashita H, Mori E, Kato M, Morita Y** (1982) Isolation and characterization of five neutral isoenzymes of horseradish peroxidase. J Biochem **92:** 531–539
2. **Asada K** (1980) Formation and scavenging of superoxide in chloroplasts, with relation to injury by sulfur dioxide. Res Rep Natl Inst Environ Study Jpn **11:** 165–179
3. **Asada K, Takahashi M, Nagate M** (1974) Assay and inhibitors of spinach superoxide dismutase. Agric Biol Chem **38:** 471–473
4. **Beadle CL, Hart JW, Jarvis OG** (1983) The extraction and activity of carboxylases: Sitka spruce and some other conifers. Photosynthetica **17:** 321–333
5. **Beauchamp CO, Fridovich I** (1973) Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta **317:** 50–64
6. **Beauchamp CO, Fridovich I** (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:** 276–287
7. **Bender J, Jager H-J, Seufert G, Arndt U** (1986) Untersuchungen zur Einzelund Kominationswirkung von SO_2 und O_3 auf den Stoffwechsel von Waldbaumen in Opentop-Kammern. Angew Botanik **60:** 461–479
8. **Bridges SM, Salin ML** (1981) Distribution of iron-containing superoxide dismutases in vascular plants. Plant Physiol **68:** 275–278
9. **Castillo FJ, Miller PR, Greppin H** (1987) Extracellular biochemical markers of photochemical oxidant air pollution damage to Norway spruce. Experientia **43:** 111–120
10. **Civalleri L, Pini C, Rigo A, Federico R, Calabrese L, Rotilio G** (1982) Isolation and preliminary characterization of electrophoretic variants of copper, zinc superoxide dismutase. Mol Cell Biochem **47:** 3–9
11. **Costi N** (1978) Electromorphs of human Cu,ZnSOD are charge isomers. Biochem Genet **16:** 739–742
12. **Esterbauer H, Grill D** (1978) Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. Plant Physiol **61:** 119–121
13. **Fridovich I** (1986) Superoxide dismutases. *In* A Meister, ed, Advances in Enzymology and Related Areas of Molecular Biology, Vol 58. John Wiley and Sons, New York, pp 61–97
14. **Johnson AH** (1983) Red spruce decline in the northeastern U.S.: Hypotheses regarding the role of acid rain. J Air Pollut Control Assoc **33:** 1049–1054
15. **Kelly TJ, Daum PH, Schwartz SE** (1985) Measurement of peroxides in cloudwater and rain. J Geophys Res **90:** 7861–7871
16. **Kunert KJ, Ederer M** (1985) Leaf ageing and lipid peroxidation: the role of the antioxidants vitamin C and E. Physiol Plant **65:** 85–88
17. **Lee EH, Bennett JH** (1982) Superoxide dismutase: a possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L.) Plant Physiol **69:** 1444–1449
18. **Loomis WD** (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol **31:** 528–544
19. **Matters GL, Scandalios JG** (1986) Effect of the free radical-generating herbicide paraquat on the expression of the superoxide dismutase (SOD) genes in maize. Biochim Biophys Acta **882:** 29–38
20. **McCord JM, Fridovich I** (1969) Superoxide dismutase: an enzymic function of erythrocuprein. J Biol Chem **244:** 6049–6055
21. **McLaughlin SB** (1985) Effects of air pollution on forests. A critical review. J Air Pollut Control Assoc **35:** 512–534
22. **Melhorn H, Seufert G, Schmidt A, Kunert KJ** (1986) Effect of SO_2 and O_3 on production of antioxidants in conifers. Plant Physiol **82:** 336–338
23. **Peleg M** (1976) The chemistry of ozone in the treatment of water. Water Res **10:** 361–365
24. **Rabinowitch HD, Fridovich I** (1983) Superoxide radicals, superoxide dismutases and oxygen toxicity in plants. Photochem Photobiol **37:** 679–690
25. **Sakaki T, Kondo N, Sughara K** (1983) Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: Role of active oxygens. Physiol Plant **59:** 28–34
26. **Salin ML, Bridges SM** (1982) Isolation and characterization of an iron-containing superoxide dismutase from water lily, *Nuphar luteum*. Plant Physiol **69:** 161–165
27. **Schulz H** (1986) Biochemische and faktoranalytische Untersuchungen zur Interpretation von SO_2-Indikationen Nadeln von *Pinus sylvestris*. Biochem Physiol Pflanzen **181:** 241–256
28. **Schulz H** (1983) Aktivitatsbestimmung der Superoxid-Dismutase-Isoenzyme von *Pinus sylvestris* Nadeln im Polyacrylamidgel. Biochem Physiol Pflanzen **178:** 249–261
29. **Tanaka K, Kondo N, Sugahara K** (1982) Accumulation of hydrogen peroxide in chloroplasts of SO_2-fumigated spinach leaves. Plant Cell Physiol **23:** 999–1007
30. **Tanaka K, Sugahara K** (1980) Role of superoxide dismutase in defense against SO_2 toxicity and an increase in superoxide dismutase activity with SO_2 fumigation. Plant Cell Physiol **21:** 601–611
31. **Tomlinson GH II** (1983) Air pollutants and forest decline. Environ Sci Technol **17:** 246A–256A