

# Composition and Properties of Hydrogen Peroxide Decomposing Systems in Extracellular and Total Extracts from Needles of Norway Spruce (*Picea abies* L., Karst.)<sup>1</sup>

Andrea Polle\*, Krisanu Chakrabarti<sup>2</sup>, Wolfgang Schürmann<sup>3</sup>, and Heinz Rennenberg

Fraunhofer Institut für Atmosphärische Umweltforschung, Kreuzteckbahnstr. 19, D-8100 Garmisch-Partenkirchen, Federal Republic of Germany

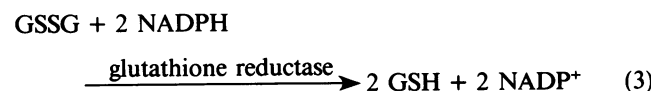
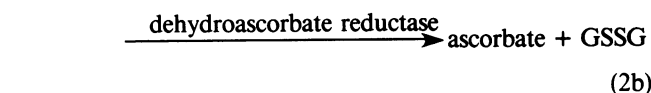
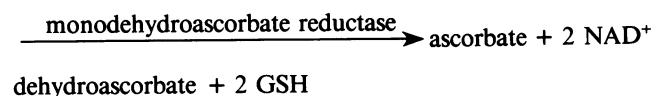
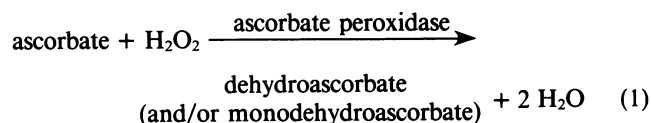
## ABSTRACT

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging systems of spruce (*Picea abies*) needles were investigated in both extracts obtained from the extracellular space and extracts of total needles. As assessed by the lack of activity of symplastic marker enzymes, the extracellular washing fluid was free from intracellular contaminations. In the extracellular washing fluid ascorbate, glutathione, cysteine, and high specific activities of guaiacol peroxidases were observed. Guaiacol peroxidases in the extracellular washing fluid and needle homogenates had the same catalytic properties, i.e. temperature optimum at 50°C, pH optimum in the range of pH 5 to 6 and low affinity for guaiacol (apparent  $K_m = 40$  millimolar) and H<sub>2</sub>O<sub>2</sub> (apparent  $K_m = 1\text{--}3$  millimolar). Needle homogenates contained ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, glutathione reductase, and catalase, but not glutathione peroxidase activity. None of these activities was detected in the extracellular washing fluid. Ascorbate and glutathione related enzymes were freeze sensitive; ascorbate peroxidase was labile in the absence of ascorbate. The significance of extracellular antioxidants for the detoxification of injurious oxygen species is discussed.

Plants are exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) from both external and internal sources. In the environment, H<sub>2</sub>O<sub>2</sub> is photochemically produced in the atmosphere and is found in concentrations up to 100 μM in rain, clouds, and fog (15). On the cellular level, H<sub>2</sub>O<sub>2</sub> is generated as a metabolite of enzymic reactions and during photosynthesis (24). Because H<sub>2</sub>O<sub>2</sub> is toxic at low concentrations (10 μM; 24), its accumulation in living tissues should be prevented. For this purpose cells have a variety of defense strategies including antioxidants and detoxifying enzymes.

The antioxidants ascorbate and glutathione participate in both enzymic and nonenzymic H<sub>2</sub>O<sub>2</sub> degradation (28). Enzymes dealing directly with H<sub>2</sub>O<sub>2</sub> are catalases and peroxidases. Peroxidases decompose H<sub>2</sub>O<sub>2</sub> by oxidation of cosub-

strates such as phenolic compounds or antioxidants (11). The 'unspecific' peroxidases in plant extracts are most commonly investigated with the artificial cosubstrate guaiacol and were found to be ubiquitous (11). Peroxidases using glutathione as a cosubstrate have only rarely been identified in plants (7), but peroxidases specific for ascorbate were frequently observed (12, 16). Ascorbate peroxidases are thought to be of particular importance in chloroplasts which are lacking catalase. They catalyze the first step of the ascorbate dependent H<sub>2</sub>O<sub>2</sub> scavenging pathway:



The complete set of enzymes of this pathway has been localized in the cytoplasm (14) and in chloroplasts (10). In the apoplastic space peroxidases have been found that are capable of both production and use of H<sub>2</sub>O<sub>2</sub> (11). The apoplastic peroxide concentration is not only affected by the activities of these enzymes, but also by air pollutants giving rise to H<sub>2</sub>O<sub>2</sub> in aqueous phases such as O<sub>3</sub> (13) or by the deposition of atmospheric H<sub>2</sub>O<sub>2</sub>. Since high concentrations of H<sub>2</sub>O<sub>2</sub> can induce lipid peroxidation and cell injury, it has been suggested that H<sub>2</sub>O<sub>2</sub> consuming processes in the apoplastic space provide an important defense against oxidative damage and, thus, might be essential for determining the H<sub>2</sub>O<sub>2</sub> resistance of plants (3, 23). To date, this hypothesis is supported by only few experimental results: Washing fluids from the extracellular space (EWF<sup>4</sup>) of *Sedum* and pinto bean leaves showed

<sup>4</sup> Abbreviations: EWF, extracellular washing fluid; DTE, dithioerythritol.

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<sup>2</sup> Present address: Department of Biochemistry, University College of Science, 35 Ballygunge Circular Rd., Calcutta 700109, India.

<sup>3</sup> Present address: TU München, Institut für Botanik und Mikrobiologie, Arcisstr. 21, D-8000 München 2, Federal Republic of Germany.

ascorbate peroxidase activity (3, 18). EWFs from spruce and *Sedum* contained ascorbate (4, 5). The levels of ascorbate and ascorbate peroxidase increased in response to ozone (3–5).

Levels of antioxidants and enzymes related to H<sub>2</sub>O<sub>2</sub> scavenging are particularly important in needles of conifers, which persist for many vegetation periods. In spruce levels of antioxidants and unspecific peroxidases, dehydroascorbate reductase, and glutathione reductase have been determined under various experimental, developmental, and environmental conditions (4, 8, 9, 28). However, neither the complete set of H<sub>2</sub>O<sub>2</sub> detoxification enzymes, nor the occurrence of these systems in the apoplastic and symplastic space has been determined in spruce. The objective of this study was to characterize the composition and properties of H<sub>2</sub>O<sub>2</sub> scavenging systems in both fluids obtained from the apoplastic space and total extracts of spruce needles.

## MATERIALS AND METHODS

### Plants

The experiments were performed with needles collected in the morning from approximately 100 to 140-year-old, healthy spruce trees (*Picea abies* L., Karst.). The trees are growing in a mountain region of the Northern Alps (750–850 m above sea level) under clean air conditions with mean concentrations of about 2 nL/L SO<sub>2</sub>, 4 nL/L NO<sub>x</sub>, and 30 nL/L O<sub>3</sub> during the vegetation period (R Sladkovic, K Munzert, personal communication).

### Extraction of Enzymes

Extracts of spruce needles for the determination of enzyme activities were prepared as described previously (20). To determine the activities of dehydroascorbate reductase and monodehydroascorbate reductase, interfering compounds had to be removed. For this purpose, solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was slowly added to crude extracts up to 35% saturation. After centrifugation (10 min, 3200 g), the pigments and Triton X-100, which formed an upper layer, were removed. The supernatant was brought up to 80% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the proteins were pelleted by centrifugation (20 min, 20000 g). The pellet was taken up in 2.5 mL of a buffer containing 50 mM Mes/KOH (pH 6.5). Washing fluids from the apoplastic space were obtained by vacuum infiltration using a modification of the method of Rathmell and Sequeira (22) as elaborated by Pfanz (personal communication). Five grams of freshly cut needles were washed with distilled water and infiltrated for periods of 30 s at -70 kPa in 30 mL of an infiltration solution (50 mM Mes/KOH [pH 6.0]; 40 mM KCl, 2 mM CaCl<sub>2</sub>, 0.1% PVP-10). The needles were thoroughly blotted, filled into a centrifuge tube (90 × 25 mm) with a perforated bottom and placed over an Eppendorf tube. The extracellular washing fluid was collected by centrifugation (10 min, 3200 g).

Before the determination of the enzymic activities, the extracts were desalted over Sephadex G-25 columns (PD-10 or NAP-5, Pharmacia) which had been equilibrated with Mes/KOH (pH 6.5) [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-fractions and EWFs] or with 100

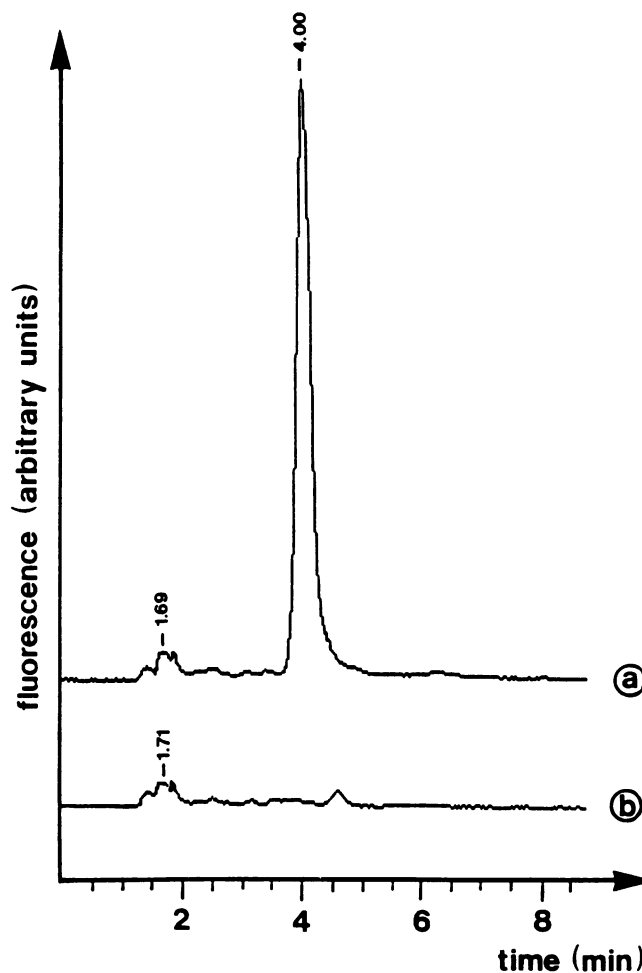
mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.8), 0.5% Triton X-100 (crude extracts).

The media for the extraction of ascorbate peroxidase, dehydroascorbate reductase, and monodehydroascorbate reductase contained additionally 5 mM ascorbate. When ascorbate peroxidase activity was investigated in the EWF, the infiltration media contained 5 mM ascorbate and the EWF was collected in a tube containing 50 μL of a buffered ascorbic acid solution (20 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> [pH 7.0], 5 mM ascorbate).

### Determination of Enzymic Activities

All assays were performed at 25°C and had a final volume of 1 mL. All measurements were performed in triplicates.

Catalase (EC 1.11.1.6) activity was determined as H<sub>2</sub>O<sub>2</sub> consumption measured as the decrease in absorbance at 240 nm according to the method of Aebi (1). The assay contained



**Figure 1.** Chromatographic detection of total ascorbate in extracts of spruce needles. The experimental conditions are described under "Materials and Methods." The chromatograms were recorded (a) without and (b) with a pretreatment with boric acid. The retention time of the fluorescent derivative of dehydroascorbate was  $4.0 \pm 0.1$  min and the peak area corresponded to 56 pmol ascorbate.

**Table I.** Low Mol Wt Components in Extracellular Washing Fluids and Total Extracts from Spruce Needles

Each experiment was performed with current year's needles collected at the end of September from six trees. Samples were individually analyzed in three parallels as described under "Materials and Methods." The standard deviation of the individual experiments was less than 5%. Values in the table are average values of six experiments ( $\pm$  SD). Under the assumption of homogenous distributions, the antioxidant concentrations were calculated using the liquid volumes of needles (541  $\mu$ L/g fresh weight  $\pm$  17  $\mu$ L/g fresh weight) and EWFs (24  $\mu$ L/g fresh weight  $\pm$  13  $\mu$ L/g fresh weight).

	Needle Extract		EWF			
	nmol/g fresh wt		$\mu$ M	pmol/g fresh wt		$\mu$ M
Ascorbate	9220 (1500)		17000	26003 (13402)		1083.4
Glutathione	232	(32)	428	156	(53)	6.5
Cysteine	17	(3)	31	13	(3)	0.5
$\gamma$ -Glutamylcysteine	5	(1)	9	Not detected		

50 mM  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (pH 7.25), 10 mM  $\text{H}_2\text{O}_2$ , and 50  $\mu$ L extract. Controls contained 1 mM aminotriazole (1 mM) to assess nonspecific  $\text{H}_2\text{O}_2$  degradation. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) were determined as described previously (30, 31). Guaiacol peroxidase (EC 1.11.1.7) activity was measured at 436 nm by a modification of the method of Pütter (21). The assay contained 50 mM  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (pH 5.25), 40 mM guaiacol, 10 mM  $\text{H}_2\text{O}_2$ , and 50  $\mu$ L of three different dilutions of the extract. Controls in the presence of boiled extract, in the absence of either extract, or guaiacol, or  $\text{H}_2\text{O}_2$  showed no changes in absorbance. The peroxidase activity was calculated using an extinction coefficient of 25.5  $\text{mm}^{-1} \text{cm}^{-1}$  and a stoichiometry of 4. Glutathione peroxidase (EC 1.11.1.9) activity was measured in a coupled assay at 340 nm after the method of Drotar *et al.* (7). The assay contained 50 mM  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (pH 7.0), 2 mM EDTA, 2.5 units glutathione reductase (Boehringer), 2 mM glutathione, 150  $\mu$ M NADPH, 100  $\mu$ M  $\text{H}_2\text{O}_2$ , 200  $\mu$ L of the extract. Control rates obtained in the absence of extract ( $57 \pm 6 \mu\text{M}/\text{min}$ ), glutathione, NADPH, or  $\text{H}_2\text{O}_2$  were subtracted. Monodehydroascor-

bate reductase (EC 1.1.5.4) was measured by a modification of the method of Borraccino *et al.* (2) at 340 nm. The assay contained 100 mM Tricine/NaOH (pH 8.0), 200  $\mu$ M NADH, 1 mM sodium ascorbate, 100  $\mu$ L enzyme extract ( $(\text{NH}_4)_2\text{SO}_4$ -fraction) and 100  $\mu$ L ascorbate oxidase (1 mg/mL). Control rates in the absence of enzyme, NADH, or ascorbate were found to be negligible. Glutathione reductase (EC 1.6.4.2) was measured by a modification of the method of Foyer and Halliwell (10) at 340 nm. The assay contained 50 mM HEPES (pH 8.0), 0.5 mM EDTA, 250  $\mu$ M NADPH, 500  $\mu$ M oxidized glutathione, and 100  $\mu$ L extract. Control rates obtained in the absence of oxidized glutathione were subtracted. The enzyme activities of glutathione peroxidase, glutathione reductase, and monodehydroascorbate reductase were calculated using an extinction coefficient of 6.22  $\text{mm}^{-1} \text{cm}^{-1}$  for NAD(P)H at 340 nm.

Ascorbate peroxidase (EC 1.11.1.11) activity was measured by a modification of the method of Nakano and Asada (16) at 290 nm. The assay contained 50 mM  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (pH 7.0), 250  $\mu$ M sodium ascorbate, 1 mM  $\text{H}_2\text{O}_2$ , and 100  $\mu$ L of the extract. Control rates obtained in the absence of extract

**Table II.** Activities of  $\text{H}_2\text{O}_2$  Detoxification Enzymes and Subcellular Marker Enzymes in Extracellular Washing Fluids and Total Extracts of Spruce Needles

Experimental conditions were as in Table I ( $\pm$  SD).

	Enzymic Activity	
	Needle extract	Extracellular washing fluid
	<i>nkat/g fresh wt</i>	
Catalase	400 (100)	0.08 (0.01)
Glucose-6-phosphate dehydrogenase	1.08 (0.45)	Not detected <sup>a</sup>
Glyceraldehyde-3-phosphate dehydrogenase	73 (4)	Not detected
Guaiacol peroxidase	2970 (720)	62 (35)
Glutathione peroxidase	Not detected	Not determined
Ascorbate peroxidase	141 (29)	Not detected
Dehydroascorbate reductase	38 (13)	Not detected
Monodehydroascorbate reductase	12 (5)	Not detected
Glutathione reductase	6 (4)	Not detected
Protein (mg/g fresh wt)	22.1 (4.7)	0.007 (0.003)

<sup>a</sup> Detection limit: 0.005 pkat/g fresh weight.

( $4.6 \pm 0.6 \mu\text{M}/\text{min}$ ), ascorbate, or H<sub>2</sub>O<sub>2</sub> were subtracted. Dehydroascorbate reductase (1.8.5.1) was measured as described by Dalton *et al.* (6) at 290 nm. The assay contained 60 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 6.1), 400  $\mu\text{M}$  dehydroascorbate, 5 mM glutathione, and 100 or 200  $\mu\text{L}$  of enzyme extract ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-fraction). Control rates obtained in the absence of extract and in the absence of glutathione were subtracted. Control rates in the absence of dehydroascorbate were negligible. Ascorbate peroxidase and dehydroascorbate reductase activities were calculated using an extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  for ascorbate at 290 nm.

#### Extraction of Antioxidants

Extracts for the determination of the content of glutathione and ascorbate in the needles were prepared as described previously (25). EWF was obtained from 5 g of needles by vacuum infiltration as described by Castillo *et al.* (4). During the centrifugation (10 min, 3200 *g*) the extracellular washing fluid was collected in tubes containing 100  $\mu\text{L}$  0.1 N HCl.

#### Quantification of Thiolcompounds

After reduction of the samples and derivatization of the thiol groups with monobromobimanes, the contents of total glutathione, cysteine and  $\gamma$ -glutamylcysteine were determined in needle extracts and EWFs. The fluorescent derivatives were separated by HPLC (17) as adapted for spruce extracts (25).

#### Quantification of Ascorbate

The total ascorbate contents (oxidized + reduced) in needle extracts and EWFs were determined by a modification of the HPLC method of Speck *et al.* (29). This method is based on the complete oxidation of ascorbate to dehydroascorbate with ascorbate oxidase. Dehydroascorbate is derivatized with *o*-phenyldiamine and the reaction product is detected as a fluorescent compound. If dehydroascorbate is blocked by boric acid before the derivatization, unspecific background fluorescence can be assessed.

Extracts were diluted 10-fold with 0.2 M Na acetate buffer (pH 6.2). Aliquots of 100  $\mu\text{L}$  of this dilution were incubated for 15 min at 37°C with 10  $\mu\text{L}$  ascorbate oxidase (1 mg/mL, Boehringer) and vigorously mixed twice during the incubation period to achieve complete oxidation of the ascorbate. Then 100  $\mu\text{L}$  of a solution containing 3.7 M Na acetate was added to the samples. In control samples the solution contained additionally 3% (w/v) boric acid). The mixture was incubated for 30 min in the dark at room temperature. Then 740  $\mu\text{L}$  of H<sub>2</sub>O and 50  $\mu\text{L}$  of a freshly prepared *o*-phenyldiamine solution (1 mg/mL ethanol, Sigma) was added and the mixture was incubated for 30 min at room temperature. The samples were stable in the dark for at least 24 h. Samples of 50  $\mu\text{L}$  were loaded (Waters, 712 WISP autosampler) on a reversed-phase column (ODS-Hypersil, 125  $\times$  4.6 mm, 5  $\mu\text{m}$ , refill, Bischoff) and separated by HPLC under isocratic conditions with a flow rate of 1 mL/min (Waters pump 510) of an eluent containing 20% (v/v) methanol, 80 mM K<sub>2</sub>HPO<sub>4</sub> (pH 7.8). The fluorescence was detected at an emission wavelength of 450 nm with an excitation wavelength of 350 nm (Fluores-

cence HPLC monitor Shimadzu, RF535). Figure 1 shows a chromatogram of a spruce extract. The recovery of ascorbate standards added to spruce extracts was  $76 \pm 15\%$ . External ascorbate standards which were treated as the samples accompanied every experiment. All determinations were performed in triplicates.

#### Basic Parameters

The protein content was determined in the desalted samples with the BCA reagent kit from Pierce. BSA was used as standard. To obtain the liquid volume of the needles, the dry weight to fresh weight ratio was determined after incubation of fresh needles (0.5 g) for 72 h at 80°C in a drying oven.

## RESULTS

#### Properties of H<sub>2</sub>O<sub>2</sub>-Scavenging Enzymes in Spruce Needles

Some general properties with respect to extraction, assay, and storage conditions of the ascorbate and glutathione related enzymes and guaiacol peroxidases were explored. The activities of ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase were freeze-sensitive. Storage of crude needle extracts at  $-20^\circ\text{C}$  as well as storage of the needles in liquid nitrogen or at  $-80^\circ\text{C}$  led to a complete loss of the ascorbate peroxidase activity and to partial losses in the range of 50 to 80% of the other enzymic activities. By contrast, the activities of the guaiacol peroxidases obtained from needles and the extracellular space were only slightly freeze sensitive. The losses of specific activity were in the range of 10 to 20%, regardless of whether extracts or needles were kept frozen ( $-80^\circ\text{C}$ ).

The losses of enzyme activities during the extraction procedure were assessed for commercially available enzymes. The recovery of guaiacol peroxidase (horseradish peroxidase, Sigma) in spruce extracts was 80%. The recovery of glutathione reductase (yeast glutathione reductase, Boehringer) was 55 to 65%. Inclusion of thiol protecting agents (glutathione, mercaptoethanol) did not improve the recovery of glutathione reductase. Since ascorbate related enzymes were not commercially available, it was not possible to determine recoveries for ascorbate peroxidase, dehydroascorbate reductase, and monodehydroascorbate reductase.

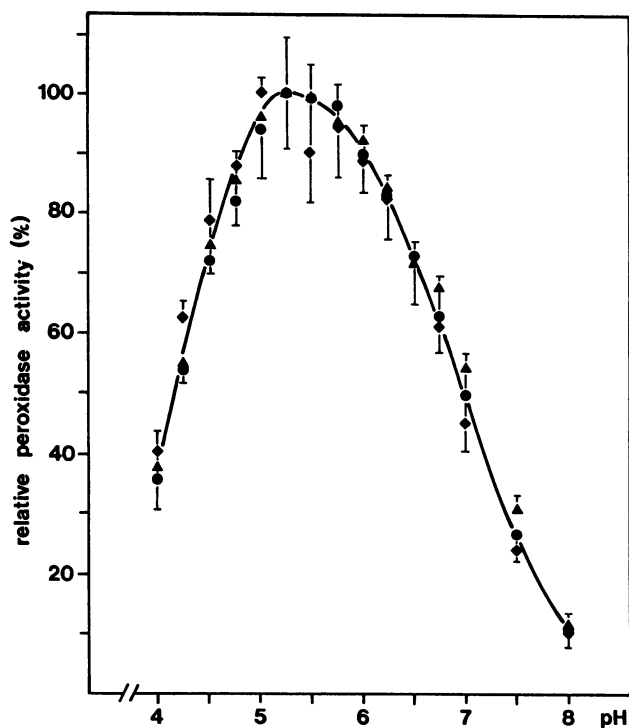
We observed that the activity of ascorbate peroxidase was labile in the absence of ascorbate. Therefore, ascorbate at a concentration of 5 mM was included throughout the preparation and processing of the extracts. Crude extracts contained approximately 30% higher ascorbate peroxidase activities than extracts after gel filtration in the presence of ascorbate. This might indicate either an inactivation despite the presence of ascorbate or the loss of a low mol wt component with ascorbate peroxidase activity similar to that detected in pea extracts (12). The interference of catalase with the ascorbate peroxidase assay was in the range of 10% and was avoided by addition of aminotriazole (500  $\mu\text{M}$ ) to the assay mixture. EDTA which was originally included in ascorbate peroxidase assays, was found to increase the nonenzymic blanks, but did

not affect the enzyme catalyzed rates and, therefore, was omitted.

### Antioxidants in Extracellular Washing Fluids and Total Extracts of Spruce Needles

The amounts of washing fluids (EWF) obtained after vacuum infiltration from the extracellular spaces of spruce needles were generally small. In the present set of experiments  $24 \mu\text{L}$  ( $\pm 13 \mu\text{L}$ ) were obtained per g fresh weight. To determine in these small volumes both the contents of ascorbate and glutathione, highly sensitive detection methods were needed.

For the determination of thiol compounds an HPLC method sensitive up to 1 pmol was available (17, 25). An HPLC method of similar sensitivity relying on the detection of a fluorescent derivative of dehydroascorbate (29) was adapted to determine total ascorbate contents in spruce extracts. Under our experimental conditions the detection limit was 3 pmol. Figure 1a shows a typical chromatogram of a spruce needle extract. The fluorescent derivative of dehydroascorbate was peaking at 4.00 min. If the formation of this



**Figure 2.** Dependency of the guaiacol peroxidase activities on the pH of the assay. The activities of the guaiacol peroxidases from needle extracts ( $\bullet$ ), from EWF ( $\blacktriangle$ ), and from needle extracts obtained after extraction of the EWF ( $\blacklozenge$ ) were determined between pH 4 and pH 8. Under standard conditions, 17, 400, and 17 nkat, respectively, were determined for the three sets of extracts. In the pH range 4 to 6, the pH-values were adjusted using a buffer system of citrate/ $\text{NaH}_2\text{PO}_4$  (0.032–0.052 M). In the pH range 5 to 8, the pH-value was adjusted using a buffer system of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (0.05 M). The experiments were repeated three times. The bars indicate standard deviation.

compound was prevented by blocking dehydroascorbate with boric acid, background fluorescence was detected (Fig. 1b). Unspecific interferences with other compounds did not occur.

Table I shows that ascorbate, glutathione, and cysteine were identified in EWFs.  $\gamma$ -Glutamylcysteine which was present in spruce needles only in small amounts, was not detected in EWFs. Although the concentrations of ascorbate, glutathione, and cysteine in the EWFs were considerable (1.1 mM, 6.5  $\mu\text{M}$ , and 0.5  $\mu\text{M}$ , respectively), the relative portions of these compounds as compared to the amounts present in total needles were fairly low (0.28, 0.07, and 0.07%; cf. Table I). This raised the question as to whether the occurrence of ascorbate, glutathione, or cysteine was caused by unspecific leakage. Leakage of cytoplasmic and chloroplastic marker proteins was not observed (see below), but leakage of low mol wt components cannot unequivocally be ruled out. However, it appears unlikely that membrane injury and unspecific release of symplastic components will lead to a relative enrichment of ascorbate in the EWF (ascorbate:glutathione:cysteine = 2000:12:1) as compared to the relative occurrence of ascorbate, glutathione, and cysteine in total needle extracts (540:14:1). Whether glutathione and cysteine are natural constituents of the apoplastic space or indicate contamination remains unknown.

### Enzymes Related to the $\text{H}_2\text{O}_2$ Metabolism in Extracellular Washing Fluids and in Total Extracts of Spruce Needles

The activities of  $\text{H}_2\text{O}_2$  scavenging enzymes were investigated in EWFs and total extracts of spruce needles. The contamination of the EWF with cellular components was assessed (Table II).

Catalase activities as determined by  $\text{H}_2\text{O}_2$  consumption (1) were observed in needle extracts and at a relative portion of 0.02% of the total activity also in the EWF (Table II). Since the  $\text{H}_2\text{O}_2$  consumption in the EWF was not blocked in the presence of the specific inhibitor of catalase, aminotriazole, it is likely that the observed  $\text{H}_2\text{O}_2$  degradation was due to the catalytic properties of peroxidases rather than to catalase itself (11). This observation indicates that contaminations of the EWF with cellular enzymes might even be lower than 0.02% of the activity found in total needle extracts. This conclusion was supported by the observation that the activities of glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase which are considered as marker enzymes for the cytoplasmic and chloroplastic space, respectively, were not detected in the EWFs (Table II). For instance, a relative contamination of the EWF with 0.02% of the total glucose-6-phosphate dehydrogenase activity would account for 0.22 pkat/g fresh weight and, thus, be 40-fold higher than the detection limit for glucose-6-phosphate dehydrogenase (Table II).

Peroxidase activities with guaiacol as cosubstrate were observed in both extracts of total needles and EWFs (Table II). In the present set of experiments 2.1% of the total activity was extracted with the EWF. The specific peroxidase activity of the EWF was 8.85 kat/kg protein and, thus, by factor of 66 higher than the specific peroxidase activity in extracts from total needles (0.13 kat/kg protein). However, in experiments

conducted with needles from different stands, these figures were found to be extremely variable: The relative portions of the peroxidase activities in the EWFs were ranging from 0.4 to 7% of total peroxidase activities extracted from needles. The specific peroxidase activities in the EWFs were higher by factors in the range between 10 and 400 than in needle extracts.

Glutathione peroxidase activity was not detected in extracts of spruce needles prepared after three different protocols: standard procedure as under "Materials and Methods," standard procedure, but all media contained 5 mM GSH, extraction in phosphate buffer (100 mM, pH 7.0) containing 5 mM EDTA, 10 mM DTE, 1% soluble PVP, 1% Tween 80, 2% casein. Neither crude nor gel filtrated extracts showed glutathione peroxidase activity.

Ascorbate peroxidase activity and the subsequent series of enzymic activities regenerating reduced ascorbate, namely dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase were found in needle extracts (Table II). However, none of these activities was detected in EWFs (Table II). Under the present assay conditions the relative enzymic activities for ascorbate peroxidase: dehydroascorbate reductase: monodehydroascorbate reductase: glutathione reductase were 23:6:2:1.

#### Characteristics of Guaiacol Peroxidase Activities in Extracellular Washing Fluids and Totals Extracts of Spruce Needles

Among the enzymic activities investigated in this paper, only guaiacol peroxidases were present in both total extracts of spruce and EWFs. To determine whether extra- and intracellular peroxidases had similar features, the catalytic properties of guaiacol peroxidases in EWFs, in total needle extracts,

and in extracts obtained from needles after the extraction of EWF were characterized.

#### pH-Dependence

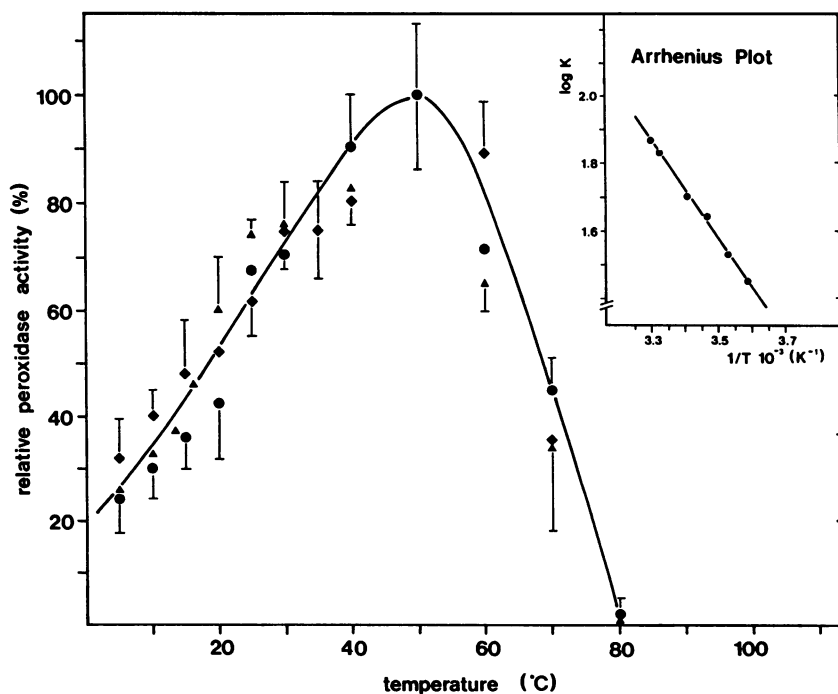
The peroxidase activities in EWFs, total, and residual needle extracts had the same, relatively broad pH-optimum between pH 5 and pH 6 (Fig. 2). At pH 7 the peroxidase activity was approximately 50% and at pH 8 only 10% of its maximum value. The enzyme activity was not affected by different buffers (citrate, phosphate) or by variation of the ionic strength of the assay in the range between 20 and 200 mM buffer concentration.

#### Temperature Dependence

The optimum assay temperature for guaiacol peroxidases in the three sets of extracts, EWF, total, and residual needle extract was 50°C (Fig. 3). At 80°C the peroxidase activities were completely lost. In the physiologically important temperature range between 5°C and 35°C, 25 to 70% of the maximum activity were observed and an activation energy of 27 kJ/M was calculated from the Arrhenius plot (inset, Fig. 3) (26).

#### Kinetic Constants

To investigate the effect of the substrate concentration on the guaiacol peroxidase activity for the three different sets of extracts, *i.e.* EWF, total, and residual needle extract, the concentration of H<sub>2</sub>O<sub>2</sub> was varied between 0.5 and 250 mM and guaiacol was kept at a constant concentration of 40 mM. Vice versa, the concentration of guaiacol was varied between 1 and 160 mM and H<sub>2</sub>O<sub>2</sub> was kept at a constant concentration



**Figure 3.** Dependency of the guaiacol peroxidase activities on the assay temperature. The activities of the guaiacol peroxidases from total (●) and residual needle extracts (◆) and from EWF (▲) were determined at assay temperatures from 5 to 80°C. Under standard conditions the activities were 1, 4, and 147 nkat, respectively. The experiments were repeated three times with extracts obtained from different sets of needles. The bars indicate standard deviation. The inset is an Arrhenius plot of the mean values of the data presented in this figure in the temperature range 5 to 35°C.

**Table III.** Kinetic Parameters of the Guaiacol Peroxidases in Assays Performed with EWF, Total, and Residual Needle Extracts

Under standard conditions the assays contained the following guaiacol peroxidases activities: 0.26, 0.24, and 5.4 nkat (total, residual needle extract, and EWF, respectively). The experiments were repeated three times. The standard deviation was less than 5%. The figures in the table are average values ( $\pm$  SD) calculated from the results obtained with three independent sets of needles. Further explanations are given in the text.

	Needle Extract		EWF
	Total	Residual	
$K_m$ (guaiacol) (mM)	34 (9)	38 (3)	42 (1)
$V_{max}$ (guaiacol) (mM/min)	0.15 (0.01)	0.17 (0.06)	6 (2)
$K_m$ (H <sub>2</sub> O <sub>2</sub> ) (mM)	1.0 (0.5)	1.1 (0.5)	3 (1)
$V_{max}$ (H <sub>2</sub> O <sub>2</sub> ) (mM/min)	0.08 (0.02)	0.08 (0.02)	3.4 (0.8)

of 10 mM. The maximum velocity ( $V_{max}$ ) and apparent  $K_m$  values as calculated from Lineweaver Burke plots are given in Table III. Both, extracellular and total peroxidases had a low affinity for their substrates H<sub>2</sub>O<sub>2</sub> (apparent  $K_m$  values 1–3 mM) and guaiacol (apparent  $K_m$  values approximately 40 mM). The peroxidases in EWF showed  $V_{max}$  values of about a factor of 40 higher than in total extracts. This difference might be caused by the higher purity of the EWF-samples as compared to crude extracts.

## DISCUSSION

### Defense Enzymes

In the present study we investigated the distribution and some properties of enzymes related to the detoxification of hydrogen peroxide in needles from field growing spruce trees. Glutathione peroxidase was not found in spruce needle extracts. Guaiacol peroxidases were found in total needle extracts and with 66-fold higher specific activity in apoplastic washing fluids. In both sets of extracts, the catalytic features of guaiacol peroxidases were similar with regard to substrate affinities, temperature, and pH-optima. Since conifer leaves have apoplastic pH values of about 5.5 (19), the results (Fig. 2) suggest that extracellular peroxidase are likely to operate at their pH optimum, while peroxidases present in compartments with neutral or slightly alkaline pH values might be inhibited up to 50 or 90%.

The major difference between peroxidases in the EWF and total peroxidases was the lack of ascorbate peroxidase activity in the extracellular space. This was surprising, because (a) ascorbate was present in the EWFs of spruce needles (4; and this paper, Table I) and (b) extracellular peroxidases of *Sedum* and pinto bean leaves could use ascorbate as a substrate (3, 18). Since the extracellular ascorbate peroxidases of *Sedum* and pinto bean were obtained by similar extraction procedures (3, 18), it appears unlikely that spruce ascorbate peroxidase remained associated with the cell walls during the extraction.

In spruce needle extracts the activities of the complete set of enzymes necessary to operate the ascorbate-glutathione cycle were found. All enzymes investigated in conifers so far showed high seasonal variations (8, 9). Therefore, the present

results give only a 'snapshot' of the enzymic activities, typical for the period at the end of September. However, similar relative enzyme activities as observed in spruce extracts have also been observed in other plant species (6, 14, 27) and, therefore, might indicate some general feature. Whether or not glutathione reductase which has the lowest rates in the assay, does constitute the rate limiting step remains unknown, because the enzymic activities as determined under optimized substrate and pH-conditions, do not necessarily reflect their *in vivo* activities.

### Antioxidants

The amounts of extracellular ascorbate found in needles of field-growing spruce trees (4.6  $\mu$ g/g fresh weight, Table II) were approximately twofold higher than the amounts found in a previous study with spruce saplings (1.2–2.8  $\mu$ g/fresh weight [4]). To our knowledge, glutathione and also cysteine have not been identified in EWFs before. In EWFs of pinto beans, total sulfhydryl groups have been determined (18) and were approximately, by a factor of 6, higher than the glutathione and cysteine contents found in the EWFs of spruce in this study. Under the assumption that glutathione and cysteine indicated leakage and that the subcellular distribution of glutathione and ascorbate was similar, unspecific leakage of ascorbate accounted for a maximum of 25% of the total amount of ascorbate present in EWFs.

Detoxifying enzymes dealing with ascorbate or glutathione such as ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase have not been detected in the EWF of spruce needles. Therefore, it might be speculated that extracellular ascorbate is involved in nonenzymic defense reactions. Since regenerating systems for ascorbate were apparently lacking in the EWF, the suggested antioxidative function of extracellular ascorbate would require an efficient ascorbate/dehydroascorbate translocator in the plasmalemma membrane.

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