Characterization of glutathione S-transferases in needles of Norway spruce trees from a forest decline stand

PETER SCHRÖDER and ANDREAS E. WOLF

Summary Glutathione S-transferases (GST) detoxify many electrophilic xenobiotics, including several volatile organic compounds and pesticides. The GST activity for the conjugation of several xenobiotic substances was isolated from needles of Norway spruce (Picea abies L. Karst.) trees from a forest decline stand in the northern Alps. Trees that exhibited different degrees of damage were selected from several stands in an altitude profile. The GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in crude protein extracts of needles showed a seasonal pattern with highest activity during summer. The GST activity exhibited a strong dependence on the altitude of the stand showing highest activities in trees growing in the valley and lowest activities in trees growing in the summit regions of the mountain. When cytosolic GST from needles of healthy and damaged trees was purified, trees of healthy appearance exhibited three distinct GST isozymes with activities for the conjugation of CDNB and 1,2-dichloro-4-nitrobenzene (DCNB), whereas severely defoliated trees exhibited four GSTs with additional activity for the conjugation of ethacrynic acid. The main GST isozymes catalyzing the conjugation of CDNB differed in molecular weight, isoelectric point and catalytic properties between damaged and healthy trees.

Keywords: detoxification, isozymes, Picea abies, xenobiotics.

Introduction Glutathione S-transferases (GST, E.C. 2.5.1.18) catalyze the nucleophilic attack of reduced glutathione (GSH) on electrophilic alkylating substrates and are known to act as detoxification enzymes for numerous organic xenobiotics (Frear and Swanson 1973, Lamoureux and Rusness 1989), including chlorinated nitrobenzenes, diphenyl ethers, thiocarbamates, chlorotriazines. These enzyme activities have been found in more than 30 plant species (see Lamoureux and Rusness 1993, Schröder 1993). Schröder and Berkau (1993) purified and characterized cytosolic glutathione S-transferases from the needles of healthy Norway spruce trees (Picea abies L. Karst.). They found at least two GST isozymes that differed in physicochemical and catalytic properties. The isozymes were homodimers consisting of two identical subunits with molecular weights of 24 and 26 kD and isoelectric points of 4.3 and 6.0, respectively. Both isozymes conjugated 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB), whereas only one isozyme was able to conjugate the pesticides fluoroafen and alachlor at appreciable rates.

The occurrence of this enzyme class in gymnosperms implies that GST may be ubiquitous in higher plants, and that it may play an important role in plant secondary metabolism (Lamoureux and Rusness 1993). Although cinnamic acid is one of the few natural substrates that have been described (Diesperger and Sandermann 1979), the role of GST in the detoxification of organic pollutants is well documented and is of particular interest in the context of man-made pollutants and the forest decline phenomenon.

Studies of forest decline in the alpine regions of Europe indicate that a coincidence of numerous natural and anthropogenic stress factors is responsible for the decrease in tree vitality. Airborne pollutants seem to be of special importance. In addition to the inorganic pollutants SO\(_2\), NO\(_x\), and related compounds, volatile organic compounds, such as halogenated hydrocarbons and pesticides, are emitted in large amounts in industrialized countries (over 2 million Mg year\(^{-1}\) in Germany, UBA 1992). After long-range transport to remote mountain areas (Elling et al. 1987), these volatile organic compounds may be deposited on tree leaves (Herterich und Herrmann 1991). Model experiments (Frank and Frank 1986) indicate that these volatile organic compounds have phytotoxic effects, such as pigment bleaching. Thus, detoxification of these xenobiotics is essential for plant survival. Because detoxification can take place by GST-catalyzed conjugation of the xenobiotic molecule to the tripeptide glutathione (GSH), we tested the hypothesis that assaying GST activity with halogenated aromatic model compounds such as CDNB as substrates provides a measure of the detoxification capacity of trees toward organic pollutants such as halogenated olefins and substituted phenols. Additional objectives of the study were to: obtain information about GST activity in needle extracts of damaged and healthy spruce trees from different stands; correlate GST activity with degree of damage (i.e., extent of needle loss),

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1 This paper is dedicated to Prof. Dr. Wolfgang Große on the occasion of his 60th birthday.
Materials and methods

Sample sites

We studied several 80- to 140-year-old alpine Norway spruce stands on the southwestern slope of Wank Mountain close to Garmisch-Partenkirchen, Germany. Three sites at 750 -- 800 m above sea level (valley), 1150 -- 1270 m (middle altitude) and 1650 -- 1700 m (summit region) were chosen, and six to eight Norway spruce trees on each site were selected and characterized by percentage needle loss (Table 1).

Plant material

Experiments were performed on current-year, two- and three-year-old needles. Twigs were harvested from the sunlit parts of the crown. The trees were divided into two groups: (a) healthy trees = needle loss up to 25% and (b) damaged trees = needle loss above 30% according to the protocol of the PBWU (Projektgruppe Bayerns zur Erforschung der Wirkung von Umweltschadstoffen). Needles were frozen and stored in liquid nitrogen immediately after harvest.

Chemicals

Both CDNB (1-chloro-2,4-dinitrobenzene) and DCNB (1,2-dichloro-4-nitrobenzene) were obtained from Kodak (Rochester, NY). Ethacrynic acid ([2,3-dichloro-4-((2-methylene butyryl)-phenoxy)acetic acid], GSH (reduced glutathione), PVP K 30 (polyvinylpyrrolidone), EDTA and affinity chromatography material were from Sigma Chemical Co. (St. Louis, MO); 2-mercaptoethanol and Nonidet P 40 were from Fluka Chem. (Neu-Ulm, FRG); PD 10 columns, FPLC (Fast protein liquid chromatography) equipment and chemicals were from Pharmacia (Freiburg, FRG). All other chemicals used were research grade commercial materials.

Preparation of crude enzyme extracts

Crude enzyme extracts were prepared by a modification of the procedure described by Schröder and Berkau (1993). Briefly, up to 4 g of frozen needles was pulverized in liquid nitrogen in precooled mortars and extracted at 4 °C with 10 vol (w/v) of 0.1 M Tris-HCl buffer (pH 7.8) containing 20 mM 2-mercaptoethanol, 5% PVP K 30, 2 mM EDTA, 0.5% Nonidet P 40, 5 mM GSH and 3 µg ml⁻¹ Pepstatin A. After homogenization for 30 s with an Ultra Turrax, the crude extract was centrifuged at 20,000 g for 30 min. Samples were desalted by gel filtration (PD-10, Pharmacia, Freiburg, FRG) before measurement of GST activity. A 0.1 M potassium phosphate buffer (pH 7.8) containing 1% PVP K30, 5 mM EDTA, and 0.25% Nonidet K 40 (Schröder et al. 1990a, 1990b, Schröder and Berkau 1993) yielded 20 ± 2% lower GST activities for the conjugation of CDNB than the Tris buffer system.

Enzyme purification

Proteins in the crude extract were precipitated by the addition of ammonium sulfate. The 40–80% ammonium sulfate fraction was centrifuged, the pellet was resuspended in 25 mM potassium phosphate buffer and desalted on a PD-10 column. The samples were then subjected to affinity chromatography on a sulfobromophthalein-linked agarose column. The column was rinsed with 25 mM potassium phosphate buffer and GST was subsequently eluted with a 1 to 20 mM glutathione gradient. The fractions containing GST activity were pooled and loaded on an FPLC Mono-Q HR 5/5 anion exchange column and eluted with an NaCl gradient (Schröder and Berkau 1993). Protein fractions were analyzed by gel electrophoresis with commercially available gels (8–25% polyacrylamide gel gradient in the presence and absence of SDS) in a PhastSystem. Isoelectric focusing (IEF) was carried out between pH 3 and 9. Gels were stained with silver (Blum et al. 1987) for visualization of proteins.

Enzyme assay and protein determination

Each step of the purification procedure was followed by the determination of GST activity as previously described by Habig et al. (1974) and Schröder et al. (1990a). Briefly, aliquots of the enzyme extracts were added to 100 mM potassium phosphate buffer at pH 6.4 containing 1 mM CDNB (ε₃₄₀nm= 9.6 mM⁻¹ cm⁻¹) dissolved in EtOH, and 1 mM GSH.

Table 1. Characterization of trees at Sites W1, W4 and W7 on Wank Mountain close to Garmisch-Partenkirchen according to the protocol of the PBWU (Projektgruppe Bayerns zur Erforschung der Wirkung von Umweltschadstoffen). Sites W1, W4 and W7 are at altitudes of 1650–1700 (summit region), 1150–1270 (middle altitude) and 750–800 (valley) m a.s.l., respectively.

<table>
<thead>
<tr>
<th>Summit region</th>
<th>Middle altitude</th>
<th>Valley</th>
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<tbody>
<tr>
<td>No.</td>
<td>Needle-loss (%)</td>
<td>Degree of damage</td>
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<tr>
<td>W1/90</td>
<td>20</td>
<td>1</td>
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<tr>
<td>W1/86</td>
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<td>W1/85</td>
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<td>W1/84</td>
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The formation of a glutathione conjugate was followed spectrophotometrically at 340 nm in a Perkin Elmer dual beam spectrophotometer. The conjugations of DCNB ($\varepsilon_{345\text{nm}} = 8.5$ mM$^{-1}$ cm$^{-1}$) and ethacrynic acid ($\varepsilon_{270\text{nm}} = 5.0$ mM$^{-1}$ cm$^{-1}$) were assayed by the same procedure except that, in the case of DCNB, potassium phosphate buffer at pH 7.5 was used. The reactions were started by the addition of enzyme. Control reactions were measured without enzyme or substrate. Protein content of the samples was measured by the method of Bradford (1976) with bovine serum albumin as a standard protein.

The GST-catalyzed conversion of CDNB to a glutathione conjugate was detected in crude protein extracts of the needles of each of the investigated trees. The GST activity was highly variable and ranged from 0.5 to 15 nkat g$^{-1}$ (Figure 1). The high variability of the GST activity was associated with (a) an annual pattern of enzyme activity, (b) the altitude at which the trees were growing, (c) the degree of defoliation of the trees, and (d) the age of the needles.

**Results**

The GST-catalyzed conversion of CDNB to a glutathione conjugate was detected in crude protein extracts of the needles of each of the investigated trees. The GST activity was highly variable and ranged from 0.5 to 15 nkat g$^{-1}$ (Figure 1). The high variability of the GST activity was associated with (a) an annual pattern of enzyme activity, (b) the altitude at which the trees were growing, (c) the degree of defoliation of the trees, and (d) the age of the needles.

Figure 1 summarizes the time course of GST activity in needles of Norway spruce trees during six harvesting periods from November 1989 to November 1991. Generally, GST activity for the conjugation of CDNB trees was low during winter and spring (Figures 1A, B and F) and elevated in summer and autumn (Figures 1D and E). The seasonal pattern was most evident in trees growing in the valley at 750 m above sea level.

In trees with little needle loss, an altitude-dependent pattern of GST activity was observed. Needles of healthy trees in the

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Figure 1. Activity of GST in needles of six healthy and six damaged Norway spruce trees in an altitude profile during the course of a year. Each bar represents the median of triplicate GST assays with CDNB as substrate. Sampling sites were at 750 (valley), 1150 (middle) and 1650 (summit) m above sea level (see Table 1). Statistical treatment of the data is presented in Table 2. Pattern denomination: dense cross hatch = 3-year-old needles of healthy trees; black = 3-year-old needles of damaged trees; cross hatch = 2-year-old needles of healthy trees; dense hatch = 2-year-old needles of damaged trees; empty = current-year needles of healthy trees; and wide hatch = current-year needles of damaged trees.

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**Statistical analysis**

Data were subjected to statistical testing using STATGRAF software. Data were tested for normal distribution, variance and statistical difference between samples from damaged and healthy trees. Means and medians are only given when normal distribution was observed. Significance levels are given in the figure captions.
valley generally had higher GST activity than needles of healthy trees in the summit region of the mountain. During some of the sampling periods (Figures 1B–E), a linear relationship between GST activity and altitude was observed in needles of healthy trees. In severely defoliated trees, there was no significant altitude dependence of GST activity, except for the samples taken in early spring, when foliar GST was low in the trees in the valley and high in the trees in the summit region (Figure 1C). Needle age contributed to the variability in GST activity especially during winter and spring. As shown in Figure 1, GST activity appeared to decrease with increasing needle age.

There was a large difference in GST activity between healthy and damaged trees. Throughout the year, damaged trees from the stand at an elevation of 750 m exhibited generally lower GST activity (53%) than healthy trees. In the middle-elevation stands, this relationship was somewhat concealed (93%); however, in the summit region, the GST activity of damaged trees was significantly higher (177%) than in healthy trees (Table 2).

In addition to differences in total GST activity between healthy and damaged trees, there were differences in the catalytic properties of the enzymes from 2-year-old needles of healthy and damaged trees growing at the site with an elevation of 1200 m. The $K_m$ values for GSH in the GSH/CDNB system were similar for healthy and damaged trees (0.36 ± 0.03 and 0.38 ± 0.04 mM, respectively), but the $V_{max}$ values differed significantly between healthy and damaged trees (0.074 ± 0.006 and 0.055 ± 0.005 ± 0.005 µkat m g$^{-1}$, respectively). The affinity of the enzyme for CDNB was significantly higher in healthy trees (0.2 ± 0.03 mM) than in damaged trees (0.35 ± 0.03 mM), and the velocity of the reaction was 25% higher in needle extracts from healthy trees compared with damaged trees (0.059 ± 0.005 versus 0.047 ± 0.005 µkat m g$^{-1}$).

To obtain more detailed information about the kinetic properties of GST in healthy and damaged trees, the enzyme was purified from needles collected from the Norway spruce stand in the valley (750 m a.s.l.) where the largest differences in GST activity between damaged and healthy trees were observed (Figure 1). The GST activity for the conjugation of CDNB in crude needle protein extracts of the healthy and damaged trees chosen for the study of GST kinetics was 32 and 16 nkat g$^{-1}$, respectively. However, the activities were almost equal when calculated on a protein basis (Table 3).

The enzyme in needle extracts from healthy trees was purified 307-fold and recovery was about 3% of the total GST activity (Table 3). The corresponding values for the enzyme in needle extracts from damaged trees were 255-fold and 3%. Anion exchange chromatography on a MONO-Q bed was the most effective step of the purification procedure. Native gel electrophoresis on gradient gels indicated that the purified proteins containing GST activity were homogeneous (insets in Figure 2).

The elution profile of GST activity from the FPLC anion exchange column differed for needle extracts from healthy (Figure 2A) and damaged trees (Figure 2B). The GST from needle extracts of healthy trees eluted in three peaks. Each peak had GST activity for the conjugation of DCNB, but only two of the peaks contained GST activity for the conjugation of CDNB. Peak 1 of GST activity, which eluted from the MONO-Q column together with the bulk protein of the sample (Figure 1C), contained activities for the conjugation of CDNB and DCNB in the ratio of 1 to 10. Peak 2 of GST contained activities for the conjugation of CDNB and DCNB in the ratio of 2 to 1. No GST activity was detectable for the conjugation of DCNB.
of ethacrynic acid. In contrast, GST from needle extracts of damaged trees eluted from the Mono-Q column in four peaks (Figure 2B). Each peak contained activity for the conjugation of ethacrynic acid, and three of the peaks also contained activity for the conjugation of DCNB. Two peaks had significant but low CDNB activity and one peak contained high CDNB activity. Activities for the conjugation of CDNB and DCNB in Peak 1 were in the same ratio (1 to 10) as for healthy trees. Activity for the conjugation of DCNB was absent in Peak 2, whereas activities for the conjugation of CDNB and DCNB were present in a one to one ratio in Peak 3. Thus, in damaged trees, DCNB conjugation is lower but still comparable to that in healthy trees, whereas GST activity for the conjugation of CDNB is quite different.

Kinetic analysis of the purified GST from damaged and healthy trees revealed that purified GST from damaged trees had a lower affinity for CDNB substrate ($K_M = 0.116 \text{ mM}$) than purified GST from healthy trees ($K_M = 0.074 \text{ mM}$). The maximum reaction rate was also lower in the purified enzyme preparation from damaged trees than from healthy trees ($V_{max} = 267$ versus $296 \text{ nkat mg}^{-1}$).

The differences in catalytic behavior of the two GSTs were associated with differences in physicochemical properties. The GST isolated from needles of healthy trees consisted of two identical subunits with a molecular weight of 23 kD, whereas the GST from damaged trees was a homodimer with a subunit size of 24.5 kD. Isoelectric focusing on Phast gels revealed that the GST from healthy trees had a pI of 4.3–4.7, whereas the GST isolated from damaged trees had a pI of 5.8–6.2.

Discussion

In all of the investigated trees, GST was present, but activity varied between 0.5 and 15 nkat g$_{fw}$^{-1}. This result is in the range of GST activity given by Schröder and Berkau (1993) for healthy Norway spruce trees (6.5 nkat g$_{fw}$^{-1}). The occurrence of annual patterns of GST activity and an uneven distribution in an altitude profile may be correlated with the general behavior of other enzymes and metabolites, such as glutathione or ascorbate (Schupp and Rennenberg 1992, Bermadinger-Stabentheiner et al. 1991, Bermadinger-Stabentheiner 1994). We found a large difference in GST activities between trees of healthy appearance and severely defoliated trees.

Although we used synthetic substrates for the determination of enzymatic activity, the results strongly support a role for GST in the catalysis of detoxification reactions of organic
pollutants associated with forest decline in the alpine regions of Germany. The finding that GST decreases with altitude in needles of healthy trees but not in damaged trees indicates differences in the metabolism of these trees that could be associated with certain isoforms of the enzyme. We found that Norway spruce needles contained different GST isozymes depending on the severity of damage sustained by the tree. The occurrence of GST isozymes with different kinetic properties and a differing spectrum of substrates was also found by Anderson and Gronwald (1991), who isolated GSTs from different strains of *Abutilon theophrasti* and found that the atrazine-resistant strain exhibited a higher GST activity against atrazine than the susceptible strain. Timmermann (1989) reported on the occurrence of three GST isozymes in corn; two of the isoforms had an inducible activity against CDNB. In an earlier study of GST in Norway spruce, Schröder and Berkau (1993) separated two distinct GST isozymes with activity for CDNB and DCNB, but only one isozyme was able to conjugate herbicides like alachlor or fluorozinid. In the present study, we were able to separate three GST isoforms by using GSH-bromosulphophthalein agarose agarose affinity chromatography followed by anion exchange chromatography on a Mono-Q column.

The main GST activity peak in healthy trees was assumed to be identical to the enzyme designated GST II by Schröder and Berkau (1993). This GST is a homodimer consisting of 23 kD subunits and exhibits a pI of 4.4. In damaged Norway spruce trees, the GST of the main peak consists of subunits that are significantly larger (24.5 kD) than the subunits of GST II and have a pI around 6.0. We conclude that either an isoform is overexpressed as a result of the stress caused by the decline phenomenon or the Norway spruce population is composed of two provenances, one having the 24.5 kD GST as the main GST isozyme and one having the 23 kD GST as the main GST isozyme. It is not known whether the occurrence of different GST isoforms in Norway spruce trees at different stages of damage is associated with a differing potential for detoxifying organic xenobiotics by way of GSH conjugation. We speculate that trees lacking the “correct” isozyme pattern might not be able to decompose the toxic compounds present at a given site, even if their overall GST activity is high. This would explain needle losses and decreasing vitality of selective trees in the stand.

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**References**


