Phyton (Horn, Austria)	**Vol. 35**	Fasc. 1	117–129	28. 7. 1995

Properties of Ascorbate-related Enzymes in Foliar Extracts from Beech (*Fagus sylvatica* L.)

By

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With 4 Figures

Received September 9, 1994

Key words: Ascorbate peroxidase, antioxidants, dehydroascorbate reductase, glutathione reductase, monodehydroascorbate radical reductase.

Summary

POLLE A. & MORAWE B. 1995. Properties of ascorbate-related enzymes in foliar extracts from beech (*Fagus sylvatica* L.). – Phyton (Horn, Austria) 35 (1): 117–129, 4 figures. – English with German summary.

The activities of ascorbate peroxidase (APOD), monodehydroascorbate radical reductase (MDAR), dehydroascorbate reductase (DAR), and glutathione reductase (GR) were determined in beech (*Fagus sylvatica* L.) foliage. Foliar buds prior to the emergence of new leaves contained higher activities of APOD and MDAR than mature leaves, but less GR activity and a very small chlorophyll content. DAR activity was below the detection limit in both developmental stages. Under optimum assay conditions for each enzyme, APOD activity (pH 7) was up to 10-times higher than MDAR activity (pH 8). In contrast, when MDAR activity was determined in an assay in which the intrinsic APOD activity was used for the production of monodehydroascorbate radicals (pH 8), half of the maximum MDAR activity was apparently sufficient to cope with the radicals generated. The K_M-values of APOD and MDAR in beech extracts were similar to K_M-values of purified enzymes from other plant sources. The activities of both APOD and MDAR were affected by buffer compounds but not by the detergent used for the extraction. APOD was inactivated by about 70 % in the absence of ascorbate. At 5° C APOD and MDAR retained still 75 % and 50 %, respectively, of their activities observed at 25° C. The results suggest that changes in the physiological and environmental conditions such as pH or temperature may also affect the interaction of the ascorbate-related enzymes in plants.

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Zusammenfassung

POLLE A. & MORAWE B. 1995. Eigenschaften von antioxidativen Enzymen in Blattextrakten der Buche (*Fagus sylvatica* L.). – Phyton (Horn, Austria) 35 (1): 117–129, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung

Die Aktivitäten von Askorbatperoxidase (APOD), Monodehydroaskorbatradikalreduktase (MDAR), Dehydroaskorbatreduktase (DAR) und Glutathionreduktase (GR) wurden in Extrakten aus Blattgeweben der Buche (*Fagus sylvatica* L.) untersucht. In Buchenknospen vor dem Austrieb war die APOD- und MDAR-Aktivität deutlich höher, die GR-Aktivität und der Chlorophyllgehalt jedoch deutlich niedriger als in ausgewachsenen Blättern. Die Aktivität der DAR lag in beiden Entwicklungsstadien unterhalb der Nachweisgrenze. Unter optimalen Testbedingungen für das jeweilige Enzym war die APOD- bis zu 10mal höher als die MDAR-Aktivität. Wenn jedoch die extrakteigene APOD-Aktivität bei pH 8 das Substrat für die MDAR produzierte, war offenbar schon die Hälfte der im selben Extrakt vorhandenen MDAR-Aktivität ausreichend, um die produzierten Askorbat-Radikale zu reduzieren. Die Aktivitäten beider Enzyme wurden durch den verwendeten Puffer beeinflußt, hingen aber nicht von dem zur Extraktion verwendeten Detergenz ab. Die APOD Aktivität wurde ohne Askorbat zu 70 % inaktiviert. APOD und MDAR besaßen bei 5° C noch 75 % bzw. 50 % ihrer Aktivität, die bei 25° C vorlag. Die Ergebnisse weisen daraufhin, daß Veränderungen physiologischer oder umweltbedingter Faktoren, wie pH-Wert oder Temperatur, die Interaktion Askorbat-oxidierender und -reduzierender Enzyme in der Pflanze beeinflussen können.

1. Introduction

H_2O_2 is an inhibitor of photosynthesis at relatively low concentrations (50 % inhibition at 10 µM; KAISER 1979). Since H_2O_2 is produced as by-product of electron transport reactions in chloroplasts and in other subcellular compartments plant cells possess a protective system to prevent unspecific oxidation. In the cytosol and in chloroplasts the reduction of H_2O_2 is catalysed by an ascorbate-specific peroxidase, APOD, which oxidises concurrently ascorbate to monodehydroascorbate radicals (ASADA 1992). Monodehydroascorbate radicals are reduced to ascorbate by MDAR by consumption of NADH or NADPH (ARRIGONI & al. 1981, HOSSAIN & al. 1984) or undergo self-disproportionation yielding ascorbate and dehydroascorbate. Dehydroascorbate is reduced at the expense of glutathione in both enzymatic and non-enzymatic reactions (FOYER & HALLIWELL 1976). Eventually, reduction of glutathione disulfide is achieved by glutathione reductase activity by consumption of NADPH (FOYER & HALLIWELL 1976).

In foliar tissues of deciduous trees only few components of the ascorbate-glutathione related detoxification system have been investigated (POLLE & RENNENBERG 1994). In beech leaves seasonal changes of the ascorbate and glutathione content, and the activity of glutathione reductase have been reported (FRANKE 1965, KUNERT & EDERER 1986, POLLE & al. 1992a). Levels of glutathione reductase and ascorbate were low in spring

during the emergence of the new leaves and high in mature leaves in summer (5 vs 25 nkat / g fresh weight and 2 µmol vs 10 µmol / g fresh weight, respectively; POLLE & al. 1992a, FRANKE 1965). To our knowledge activities of APOD, MDAR, and DAR have not been determined in beech foliage. In the present study the activities of these ascorbate-related enzymes were determined in two stages of leaf development, i.e. in buds prior to the emergence of the new leaves and in mature foliage. The properties of the major enzymatic activities in the assay, APOD and MDAR, and the interaction of these enzymes were characterised in leaf extracts.

2. Materials and Methods

Abbreviations

APOD = ascorbate peroxidase, CHAPSO = 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate, CHES = 2-(N-cylcohexyl amino) ethane sulfonic acid, chl = chlorophyll, DAR = dehydroascorbate reductase, DW = dry weight, FW = fresh weight, MDAR = monodehydroascorbate radical reductase, SD = Standard deviation, tricine = (N-tris(hydroxymethyl) methyl glycine, TRITON X-100 = 4-(1,1,3,3,-tetramethylbutyl)phenol-ethoxylate

Plant materials

Foliage for the charaterisation of enzymatic activities was collected in August 1992 from about 60-year-old beech trees (*Fagus sylvatica* L.) grown in the valley of Garmisch-Partenkirchen (Bavaria, F.R.G.) at about 700 m above sea level. Site characteristics, climatic conditions and air pollutant concentrations were described previously (POLLE & al. 1992b, POLLE & al. 1992c). For the investigation of antioxidative enzymes about 3-months-old leaves and buds were collected in April 1993 from about 2-year-old, potted beech trees grown in a greenhouse. Fresh foliage was extracted and immediately analysed.

Extraction

To extract foliar buds, the brown scales were removed. Bud tissue and leaves were powdered in liquid nitrogen. 400 mg of frozen powder was transferred into 20 ml of ice-cold extraction buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.8, 800 mg insoluble polyvinylpyrrolidone phosphate, 1 % TRITON X-100, 5 mM ascorbic acid) and homogenised (RZR-2000, Heidolph, F.R.G.) (2 times, 30 sec). In some experiments TRITON X-100 was replaced by 2 % CHAPSO. The homogenate was centrifuged at 48000 g (25 min, 4° C). The supernatant was centrifuged again at 12000 g (10 min, 4° C). Aliquotes of 2.5 ml of supernatant were passed through Sephadex G-25 columns (PD-10 columns, Pharmacia, F.R.G.) which had been equilibrated with elution buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.0). For determination of ascorbate peroxidase the elution buffer contained additionally 1 mM ascorbic acid. The purified extracts were used for the determination of enzymatic activities.

Enzyme assays

Standard assays were performed at 25° C in a total volume of 1 ml. APOD activity (ascorbate : hydrogen peroxide oxidoreductase, EC 1.11.1.7) was determined according to NAKANO & ASADA 1987 following the decrease in absorbance at 290 nm.

The assay contained 50 mM KH_2PO_4/K_2HPO_4, pH 7.0, 800 µM ascorbic acid, 2 mM H_2O_2 and 50 µl of purified extract. Controls contained 50 µl elution buffer instead of enzyme extract. Ascorbate POD activity was calculated after subtraction of control rates using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

MDAR activity (NADH : monodehydroascorbate radical oxidoreductase, EC 1.1.5.4) was measured at 340 nm following the decrease in absorbance of NADH and using ascorbate oxidase (Boehringer, F.R.G.) for the production of monodehydroascorbate radicals (Borraccino & al. 1989). The ascorbate oxidase solution (1 mg / 20 ml of 200 mM sodium acetate, pH 6.2) was freshly prepared. Its activity was examined before the measurements of MDAR activity following the oxidation of ascorbate at 290 nm in a reaction mixture containing 100 mM Tricine / NaOH, pH 8.0, 200 µM NADH, 800 µM ascorbic acid. MDAR reductase activity was determined in the same reaction mixture after addition of 50 µl purified extract and 7 nkat ascorbate oxidase. Controls were performed substituting either ascorbate, NADH or enzyme extract by elution buffer. MDAR reductase activity was calculated after subtraction of control rates using an extinction coefficient of 6.12 $mM^{-1}cm^{-1}$.

DAR activity (glutathione : dehydroascorbate oxidoreductase, EC 1.8.5.1) was determined according to Dalton & al. 1986 following the increase in absorbance at 265 nm. The assay contained 60 mM KH_2PO_4/K_2HPO_4, pH 6.1, 800 µM dehydroascorbic acid, 1 mM reduced glutathione, and 100 µl extract. Control rates in the absence of either reduced glutathione or dehydroascorbic acid were negligible. Purified extract was substituted by elution buffer to determine non-enzymatic control rates. DAR activity was calculated after subtraction of non-enzymatic rates using an extinction coefficient of 14 $mM^{-1}cm^{-1}$.

GR activity (glutathione disulfide : NADPH oxidoreductase, EC 1.6.4.2) was determined according to Foyer & Halliwell 1976 following the decrease in absorption at 340 nm. The assay contained 50 mM HEPES, pH 8.0, 0.5 mM EDTA, 250 µM NADPH, 500 µM glutathione disulfide and 100 µl of extract. Unspecific absorption changes were negligible. GR activity was calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

When purified extracts were boiled enzymatic activities were completely lost.

Reference parameters

Pigments were extracted from powdered foliage in 80 % acetone and their concentrations were calculated using the extinction coefficients determined by Lichtenthaler & Wellburn 1983. Dry weight was measured after drying foliar materials for 72 hours at 80° C.

3. Results

Activities of antioxidative enzymes in extracts from foliar buds and leaves.

In order to investigate antioxidative enzymes in two developmental stages of beech foliage, activities of APOD, MDAR, DAR, and GR as well as some reference parameters (dry weight, chlorophyll, carotenoids) were compared in foliar buds and mature leaves. Swollen buds which were collected about two weeks before the emergence of the new leaves showed a lower dry weight content than mature beech leaves (Tab. 1). The living

bud tissues which were still wrapped in brownish scales had only a small pigment content (Tab. 1). Still, APOD and MDAR activities were significantly higher in these tissues than in leaves on the basis of fresh weight (Tab. 2), dry weight or chlorophyll (cf. Tab. 1).

Table 1

General characteristics of foliar buds and leaves of beech (*Fagus sylvatica,* L.). Leaves and buds were obtained from young beech trees grown under greenhouse conditions. Figures indicate means ± SD (n = 3), except for dry matter where pooled samples were analysed.

Parameter	Foliar buds	mature leaves
Dry mass (mg g^{-1} FW)	305	501
Chlorophyll (mg g^{-1} FW)	0.19 ± 0.04	2.57 ± 0.10
Carotenoids (mg g^{-1} FW)	0.06 ± 0.01	0.42 ± 0.01

Miyake & Asada 1992 showed that thylakoid-bound APOD of spinach chloroplasts was rapidly inactivated when extracted with TRITON X-100 but was stable when extracted with CHAPSO. In order to investigate whether the extraction with TRITON X-100 caused a major loss in APOD activity, we investigated the antioxidative enzyme activities also after extraction with CHAPSO. However, regardless of whether TRITON X-100 or CHAPSO were used as detergents, similar enzymatic activities were found (Tab. 2). The variation of APOD activity was less than 10 % and, thus, in the same range as the standard deviation of replicated measurements (Tab. 2). It is, therefore, unlikely that lower APOD activities found in leaf as compared with bud extracts were caused by the specific loss of a thylakoid-bound APOD activity.

Unlike APOD and MDAR, GR activity was lower in buds than in leaves (Tab. 2). DAR was not detected above non-enzymatic rates which amounted to 11 mM s^{-1} (Tab. 2). In mature beech leaves from field-grown trees DAR was also barely detectable (1–6 nkat g^{-1} FW, Morawe, unpublished results), whereas APOD and MDAR activities amounted to about 550 and 200 nkat g^{-1} FW, respectively (see figure legends 1–4). Apparently, in beech foliage MDAR is the key enzyme to keep ascorbate in its reduced, i.e. functional, state.

Characterisation of APOD and MDAR activity in extracts of beech leaves.

Substrates: APOD activity was lost by about 60 to 70 % when extracts were prepared in the absence of ascorbate. Substrate dependence of APOD activity in beech leaf extracts did not show Michaelis-Menten type kinetics because Lineweaver Burke plots were not linear. Half maximum substrate saturation was observed at a concentration of 300 ± 40 µM for ascorbate and 20 ± 4 µM for H_2O_2, respectively (n = 3).

Table 2

Antioxidative enzymes in extracts from foliar buds and mature leaves of beech (*Fagus sylvatica*) using either TRITON X-100 or CHAPSO as detergent for extraction. Leaves and buds were obtained from young beech trees grown under greenhouse conditions. nd = not detected. Figures indicate means ± SD (n = 3).

Parameter	TRITON X-100		CHAPSO	
nkat g^{-1} FW	Buds	leaves	buds	leaves
APOD	1388 ± 79	625 ± 11	1470 ± 57	676 ± 38
MDAR	112 ± 3	82 ± 4	109 ± 2	64 ± 4
DAR	nd	nd	nd	nd
GR	12.5 ± 0.9	28.9 ± 0.6	12.8 ± 1.2	28.2 ± 0.5

Lineweaver Burke transformation of the substrate dependence of MDAR activity yielded linear plots with a K_M-value of 8.4 ± 1.3 µM for NADH (n = 3). When extracts were assayed with NADPH (1 mM) as reductant instead of NADH (1 mM) only 18 % ± 5 % of MDAR activity was observed (n = 3). Because of this low activity, determination of a K_M value for NADPH was not possible with beech extracts.

In spectrophotometric assays the K_M-value of MDAR for monodehydroascorbate radicals can not be determined because of the instability of these radicals. However, substrate requirements of MDAR can be assessed by the determination of the optimum ascorbate oxidase activity in the assay. Maximum MDAR activity was observed at an ascorbate oxidase activity of 6 nkat per ml and more (Fig. 1) which corresponds to a monodehydroascorbate production rate of $v \geq 6$ µM s^{-1}. The steady state concentration of monodehydroascorbate [c] necessary for maximum MDAR activity can be calculated according to $v = k\,[c]\,[c]$. It amounted to 5 µM, if the rate constant $k = 2 \cdot 10^5$ M^{-1} s^{-1} for the spontaneous dismutation of monodehydroascorbate radicals at pH 8 is taken into account (Bielski & al. 1971). According to the data presented in Figure 2, half maximum substrate saturation can be estimated to be about 1.5 µM.

Interaction of APOD and MDAR activity: In order to determine the balance between APOD activity which produces monodehydroascorbate radicals and MDAR which consumes these radicals, MDAR activity was determined by exploiting APOD activity internally present in the extracts for production of monodehydrascorbate radicals instead of externally added ascorbate oxidase. When APOD activity was substrate-limited, i.e. at ascorbate concentrations between 200 and 700 µM, the activity of MDAR increased from about 25 to 50 % of its maximum activity (Fig. 2) and, therefore, was apparently directly dependent on the monodehydroascorbate radicals generated by APOD. However, when APOD was not substrate-limited, i.e. was producing monodehydroascorbate radicals at its maximum rate, MDAR did not increase significantly above

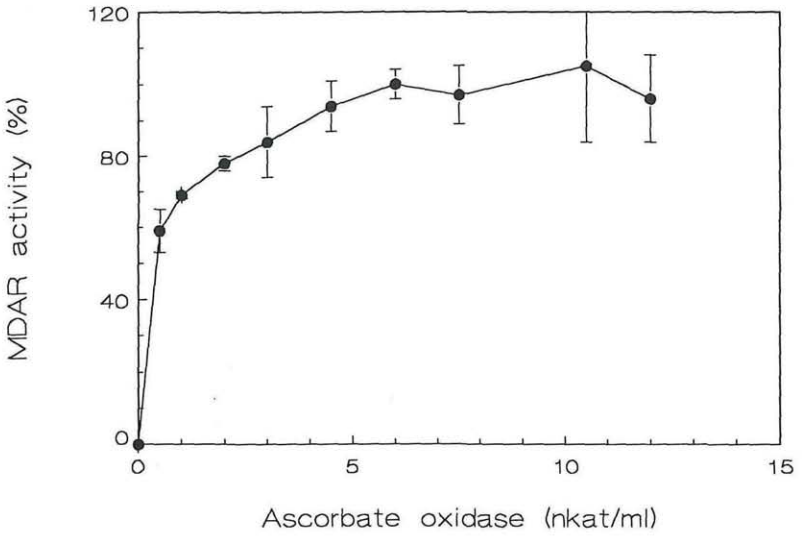

Figure 1: MDAR activity in dependence on monodehydroascorbate radicals generated by increasing ascorbate oxidase activity. (n = 3, ± SD). 100 % corresponded to 211 ± 11 nkat g^{-1} fresh weight.

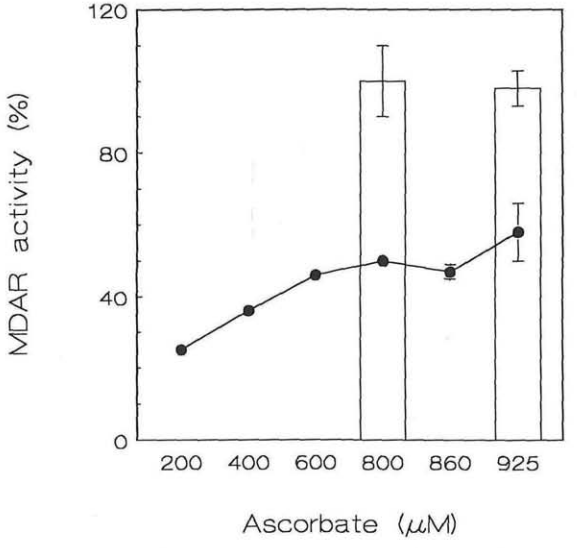

Figure 2: MDAR activity in dependence on monodehydroascorbate radicals generated by APOD at varying amounts of ascorbate (●–●). The assay contained in 1 ml: 100 µl of extract, ascorbate as indicated, 1 mM H_2O_2, 1 mM NADH, 50 mM CHES, pH 8. Bars indicate maximum MDAR activity after addition of 7 nkat ascorbate oxidase. (n = 3, ± SD). 100 % corresponded to 201 ± 11 nkat g^{-1} fresh weight.

50 % of its maximum observed in the presence of ascorbate oxidase (Fig. 2). This result shows that under the present assay conditions the capacity of MDAR for the removal of monodehydroascorbate radicals was greater than the capacity of ascorbate POD for their production, thereby preventing accumulation of these radicals.

Buffer compounds and pH-dependence: The activities of both APOD and MDAR were affected by the buffer compounds used to adjust the pH (Fig. 3 A, B). In the presence of phosphate buffer APOD displayed maximum activities between pH 6 and 7, and was reduced to about 30 % at pH 8 (Fig. 3A). In contrast, in CHES buffer at pH 8 APOD still had 50 to 60 % of its maximum activity (Fig. 3A). Since the non-enzymatic rate of ascorbate oxidation by H_2O_2 increased significantly at acidic pH values (Fig. 3A), standard assays were performed at pH 7.

MDAR activity had a broad optimum between pH 7.5 and 9.5 when CHES or tricine buffer were used, but was inhibited by phosphate buffer (Fig. 3B). For the determination of MDAR activity ascorbate oxidase, which decreased significantly with increasing pH (Fig. 3B), was adjusted to 7 nkat per ml for each of the different pH-values.

Temperature dependence: APOD activity decreased slowly with decreasing temperature and retained at 5° C still 75 % of its maximum activity observed between 20 and 25° C (Fig. 4). At temperatures above 30° C APOD activity decreased significantly. However, non-enzymatic rates increased rapidly with increasing temperature resulting in a net increase in H_2O_2 removal at high temperature (data not shown).

MDAR activity increased in the temperature range from 2° C to 30° C (Fig. 4). At temperatures below 10° C MDAR activity ranged from 40 to 60 % of its activity observed at 30° C. At temperatures above 30° C the assay system did not work linearily. This might have been caused by rapid dismutation of monodehydroascorbate radicals at higher temperatures or unknown effects on ascorbate oxidase.

4. Discussion

In the present study antioxidative enzymes were investigated in beech foliage. Extracts were prepared in the presence of detergents and, therefore, contained a mixture of isozymes from different cellular compartments such as chloroplasts and cytosol. Nevertheless, the kinetic properties of APOD and MDAR activity in beech leaf extracts were similar as compared to enzymes purified from the cytosol or chloroplasts of other plant species. For instance, the K_M-values of MDAR ranged from 4.6 to 8 μM for NADH and from about 0.5 to 1.5 μM for monodehydroascorbate radicals (potato tubers, Borraccino & al. 1986; cucumber, Hossain & Asada 1985; spinach, Hossain & al. 1984; beech, the present study). Ascorbate peroxidase in beech leaves appears to be an allosteric enzyme as also found for ascorbate

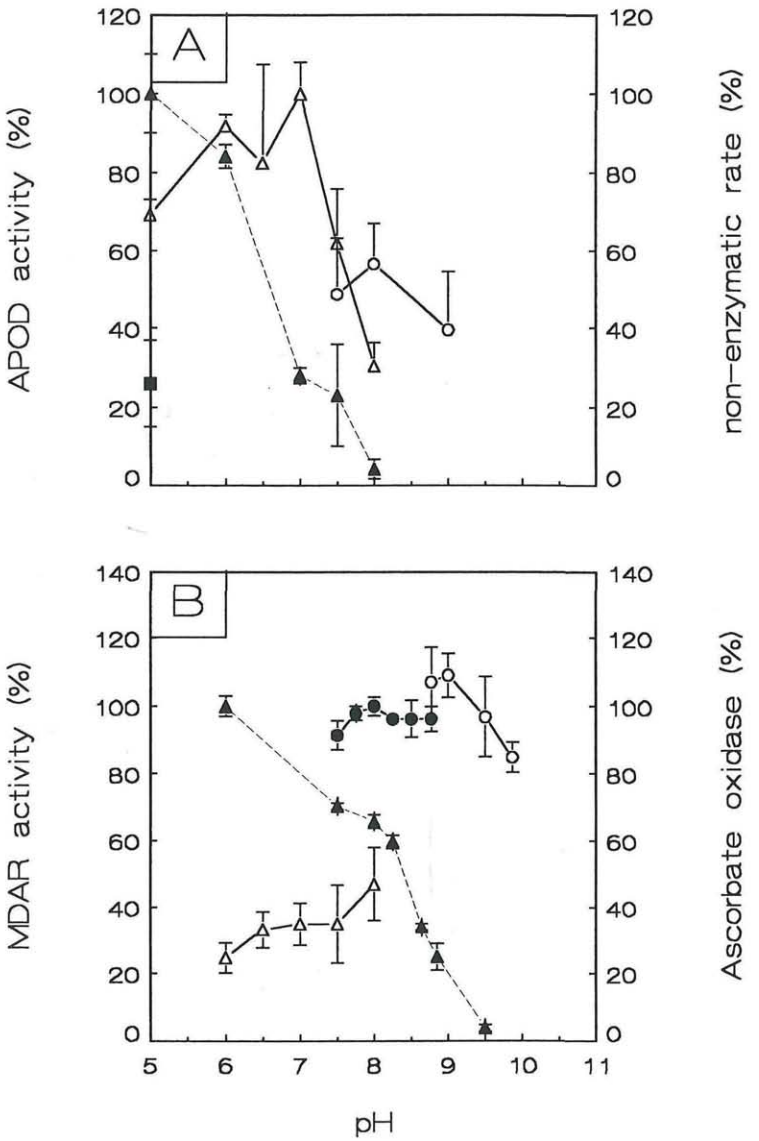

Figure 3: APOD (A) and MDAR (B) activity in dependence on the pH of the assay. The following buffers were used: ■–■ sodium acetate, △–△ K_2HPO_4/KH_2PO_4, ●–● tricine, ○–○ CHES at concentrations of 50 mM and 100 mM in assays for the determination of APOD and MDAR activity, respectively. 100 % corresponded to 521 ± 41 nkat g^{-1} fresh weight for APOD and to 235 ± 24 nkat g^{-1} fresh weight for MDAR activity. (n = 3, ± SD). ▲–▲ indicates the pH dependence of the non-enzymatic rate of ascorbate peroxidation in (A) and the pH dependence of ascorbate oxidase in (B).

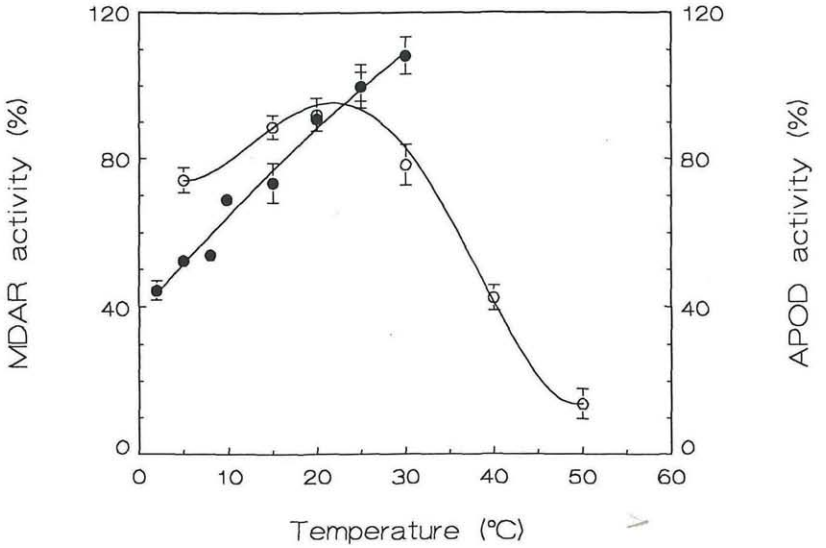

Figure 4: Dependence of APOD (○–○) and MDAR activity (●–●) on the assay temperature. The activity at 25° C was set to 100 % and corresponded to 570 ± 109 nkat g^{-1} fresh weight for APOD and to 190 ± 31 nkat g^{-1} fresh weight for MDAR. (n = 3, ± SD).

peroxidase from root nodules and pea cytosol (DALTON & al. 1987; MITTLER & ZILINSKAS 1991). Most data reported for the half maximum substrate saturation (or K_M-value) of APOD were also in a similar range as found in the present study, i.e. 200 to 400 µM for ascorbate and 20 to 30 µM for H_2O_2 (pea, MITTLER & ZILINSKAS 1991; spinach, NAKANO & ASADA 1987; NAKANO & ASADA 1981; tea, CHEN & ASADA 1989; potato tubers, ELIA & al. 1992; maize cytosol, KOSHIBA 1993).

It has frequently been observed that cytosolic APOD was relatively stable in the absence of ascorbate, whereas chloroplastic APOD lost its activity within a few minutes under these conditions (ASADA 1992). We found that about 30 to 40 % of APOD activity in extracts from beech leaves was resistant towards ascorbate depletion. This portion might have represented cytosolic APOD activity. However, we could not confirm the observation of MIYAKE & ASADA 1992 that an important fraction of APOD activity was inactivated in the presence of TRITON X-100, but was stable in the presence of CHAPSO.

The activities of the ascorbate-related enzymes found in mature beech leaves were in a similar order of magnitude as in other tree species (POLLE & RENNENBERG 1994). A major function of the ascorbate-related system is the removal of photosynthetically produced H_2O_2 (ANDERSON &

al. 1983). Therefore, it was unexpected that the activities of protective enzymes did not increase with increasing chlorophyll (Tab. 1 and 2), but were higher in swollen buds than in mature leaves. It is possible that bud tissues which are highly active prior to the emergence have an increased need for the detoxification of reactive oxygen species. For instance, in germinating wheat seeds and in developing buds of spruce needles significantly enhanced activities of ascorbate peroxidase and superoxide dismutase have also been reported (CAKMAK & al. 1993, KRÖNIGER & al. 1993). In contrast, elevated glutathione reductase activities were neither found in foliar buds of beech (Tab. 2), nor in germination wheat (CAKMAK & al. 1993) or young spruce needles (ESTERBAUER & GRILL 1978).

A balanced interaction between the processes responsible for H_2O_2 removal on one hand and for the regeneration of reduced ascorbate on the other hand is important for the functioning of the antioxidative system. When the activities of APOD and MDAR were determined each in a separate assay under optimum conditions, the capacity of APOD for the reduction of H_2O_2 and, thus, for the production of monodehydroascorbate radicals appeared to exceed the capacity for the removal of these radicals by MDAR up to 10-fold (Tab. 2). As DAR activity (Tab. 2) was relatively small, one might speculate that the turn-over of ascorbate is limited by its regeneration from the oxidised form. However, when oxidation and reduction of ascorbate by APOD and MDAR, respectively, were coupled in an assay system at pH 8 which is relevant for chloroplasts in the light, only 50 % of the maximum attainable MDAR activity was apparently sufficient to remove monodehydroascorbate radicals produced by endogenous APOD activity (Fig. 2). This was probably caused by the high affinity of MDAR for its substrate and by smaller APOD activity at pH 8 than under standard conditions at pH 7 (Fig. 3). In chloroplasts, thylakoids can also directly reduce monodehydroascorbate radicals in the absence of MDAR (MIYAKE & ASADA 1992). In summary, these observations show that it is difficult to conclude from *in vitro* measurements which of the reactions limits the turn-over of ascorbate *in vivo*. They further suggest that changes in the biochemical and physical properties of the subcellular environment such as pH or temperature, which affect the activities of ascorbate-related enzymes, might also modulate the interaction of oxidative and reductive processess.

5. Acknowledgements

We are grateful to C. OSCHINSKI and M. EIBLMEIER for excellent technical assistance and to Prof. Dr. H. RENNENBERG for critical reading of the manuscript.

6. References

ANDERSON J., FOYER C. & WALKER D. 1983. Light-dependent reduction of hydrogen peroxide by intact spinach chloroplasts. – Biochim. Biophys. Acta 724: 69–74.

ARRIGONI O., DIPIERRO S. & BORRACCINO G. 1981. Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. – FEBS Lett. 125: 242–244.

ASADA K. 1992. Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. – Physiol. Plant. 85: 235–241.

BIELSKI B., COMSTOCK D. & BOWEN A. 1971. Ascorbic acid free radicals. I. Pulse radiolysis study of optical absorption and kinetic properties. – J. Am. Chem. Soc. 93: 5624–5629.

BORRACCINO G., DIPIERRO S. & ARRIGONI O. 1986. Purification and properties of ascorbate free-radical reductase from potato tubers. – Planta 167: 521–526.

— DIPIERRO S. & ARRIGONI O. 1989. Interaction of ascorbate free radical reductase with sulphhydryl reagents. Phytochem. 28: 715–717.

CAKMAK I., STRBAC D. & MARSCHNER H. 1993. Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. – J. Exp. Bot. 44: 127–132.

CHEN G.-X. & ASADA K. 1989. Ascorbate peroxidase in tea leaves: occurence of two isozymes and the differences in their enzymatic and molecular properties. – Plant Cell Physiol. 30: 987–998.

DALTON D., RUSSELL S., HANUS F., PASCOE G. & EVANS H. 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soy bean root nodules. – Proc. Natl. Adac. Sci. 83: 3811–3815.

— HANUS F., RUSSELL S. & EVANS H. 1987. Purification, properties, and distribution of ascorbate peroxidase in legume root nodules. – Plant Physiol. 83: 789–794.

ELIA M., BORRACCINO G. & DIPIERRO S. 1992. Soluble ascorbate peroxidase from potato tubers. – Plant Sci. 85: 17–21.

ESTERBAUER H. & GRILL D. 1978. Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. – Plant Physiol. 61: 119–121.

FOYER C. & HALLIWELL B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. – Planta 133: 21–25.

FRANKE W. 1965. Über den Vitamin C Gehalt der Herbstblätter. I. Der Vitamin C Gehalt bis zum Laubabwurf. – Z. Pflanzenphysiol. 53: 289–310.

HOSSAIN M. & ASADA K. 1985. Monodehydroascorbate reductase from cucumber is a flavin adenin dinucleotide enzyme. – J. Biol. Chem. 260: 12920–12926.

— NAKANO Y. & ASADA K. 1984. Monodehydroascorbate reductase in spinach chloroplasts and its participation in the regeneration of ascorbate for scavenging hydrogen peroxide. – Plant Cell Physiol. 25: 385–395.

KAISER W.M. 1979. Reversible inhibition of the Calvin cycle and activation of the pentose phosphate cycle in isolated intact chloroplasts by hydrogen peroxide. – Planta 145: 377–382.

KOSHIBA T. 1993. Cytosolic ascorbate peroxidase in seedlings and leaves of maize (*Zea mays*). – Plant Cell Physiol. 34: 713–721.

KUNERT K. & EDERER M. 1986. Leaf ageing and lipid peroxidation: the role of the antioxidants vitamin C and E. – Plant Physiol. 65: 85–88.

LICHTENTHALER H.K. & WELLBURN A.R. 1983. Determinations of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. – Biochem. Soc. Trans. 603: 591–592.

MIYAKE C. & ASADA K. 1992. Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. – Plant Cell Physiol. 33: 541–553.

MITTLER R. & ZILINSKAS B. 1991. Purification and characterization of pea cytosolic ascorbate peroxidase. – Plant Physiol. 97: 962–968.

NAKANO Y. & ASADA K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in chloroplasts. – Plant Cell Physiol. 22: 867–880.

— & ASADA K. 1987. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. – Plant Cell Physiol. 28: 131–140.

POLLE A. & RENNENBERG H. 1994. Photooxidative stress in tree species. - In: FOYER C. & MULLINEAUX P. (eds.), Photooxidative stress in plants: Causes and amelioration. p. 199–218, CRC Press, Baco Raton.

— MORAWE B. & RENNENBERG H. 1992a. Seasonal variations of glutathione reductase and glutathione in leaves and leaf buds of beech trees (*Fagus sylvatica* L.). – Phyton 32: 99s–102s.

— MÖSSNANG M., SLADKOVIC R., VON SCHÖNBORN A. & RENNENBERG H. 1992b. Field studies on Norway spruce trees at high altitudes. I. Mineral, pigment and soluble protein content of needles as affected by climate and pollution. – New Phytol. 121: 89–99.

— CHAKRABARTI K., CHAKRABARTI S., SEIFERT F., SCHRAMEL P. & RENNENBERG H. 1992c. Antioxidants and manganese deficiency in needles of Norway spruce (*Picea abies* L.) trees. – Plant Physiol. 99: 1084–1089.