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# Apoplasmic CuZn-Superoxide Dismutase in *Pinus sylvestris*

By

S. STRELLER[1], H. SCHINKEL[1] & G. WINGSLE[1]

Key words: Apoplast, isoenzymes, *Pinus*, superoxide dismutase.


## Summary

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An apoplasmic CuZn-superoxide dismutase (SOD; EC 1.15.1.1) was found in needles of Scots pine. The enzyme was isolated from extracellular washing fluid (EWF), and is a novel isoenzyme since its $NH_2$-terminal amino acid sequence differs from that of a previously described apoplasmic isoform. The inclusion of 0.3 M KBr in the extraction buffer of EWF doubled the amount of active SOD extracted from needles, which suggests an ionic binding of this apoplasmic SOD in Scots pine. This apoplasmic SOD was also detected in EWF obtained from extraxylematic tissue (phloem and cambium) while it was absent in xylematic tissue.


## Introduction

The apoplastic compartment of cells is often disregarded in physiological processes though it has an important function in growth, differentiation, wound repair and resistance to pathogen invasion (ADAIR & MECHAM 1990). Only little enzyme activity is detectable in this compartment, mainly comprising of hydrolases, peroxidases (CASSAB & VARNER 1988) and other oxidoreductases (DEAN & ERIKSSON 1992). It is believed that active oxygen species (AOS) like superoxide and hydrogen peroxide are principally generated by three enzymes in the apoplast: cell wall localised peroxidases, diamine oxidases or plasma membrane-bound NAD(P)H-oxidases. These AOS are involved in the

[1] Department of Forest Genetics and Plant Physiology, Faculty of Forestry, Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden.

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physiological processes mentioned above and take part in a complex regulation and defense system; the mechanisms of which are yet to be clarified.

The disproportionation of superoxide anion radicals to molecular oxygen and hydrogen peroxide by SOD was for a long time regarded as an exclusive symplasmic reaction. Recently, though, extracellular SOD was identified in human tissues (HJALMARSSON & al. 1987) and the trematode *Schistosoma mansoni* (HONG & al. 1993) in the bacterium *Nocardia asteroides* (BEAMAN & al. 1983), and in *Pinus sylvestris* (STRELLER & WINGSLE 1994). These findings were confirmed by immunolocalisation studies (OGAWA & al. 1996).

This study investigates the existence of true isoenzymes in the apoplast of various tissues of Scots pine. Moreover, the possibility of ionic binding of apoplasmic SODs to the plasma membrane was examined by adding the chaotropic salt KBr to the extraction buffer.

Abbreviations: AOS, active oxygen species. SOD, superoxide dismutase. GR, glutathione reductase. PEPCase, phosphoenolpyruvate carboxylase. EWF, extracellular washing fluid. PAGGE, polyacrylamide gradient gelelectrophoresis. PVP, polyvinylpyrrolidone.

## Materials and Methods

To analyse the presence of apoplasmic SODs in different tissues, the stems of 8-year old field-grown pine (*Pinus sylvestris* L.) were washed and then separated into xylem and phloem/cambium by peeling off the bark (SUNDBERG & al. 1990). The two fractions (xylem and phloem/cambium) were then washed and vacuum-infiltrated in 50 mM K-phosphate buffer (pH 7), 0.5 mM EDTA, 0.3 M KBr and 0.5 % (w/v) polyvinylpyrrolidone (PVP) for 10 minutes. After that, the fractions were centrifuged at 2000g for 10 minutes to collect the extracellular washing fluid (EWF) (SÖDING 1939). PVP to a final concentration of 4% (w/v) was added and the samples were then precipitated with $(NH_4)_2SO_4$ (30%; w/w). The supernatant was desalted over a 3x40 cm Sephadex G-50 column (Pharmacia LKB, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 8.5), 0.5 mM EDTA. The samples were then concentrated in a Microsep microconcentrator (Filtron Technology Corporation, Northborough, MA, USA). The apoplasmic SODs were detected by native PAGGE (acrylamide gradient 8-25%).

Needles were treated in the same way except that the supernatant after the 30% (w/v) $(NH4)_2SO4$ precipitation was desalted over PD-10 columns (Pharmacia LKB, Uppsala, Sweden).

Crude extracts of the various tissues were prepared by grinding in a kitchen mixer (Moulinex, France). The extraction buffer used was the same one of the infiltration experiments mentioned above, except that it contained 4% (w/v) PVP and no KBr.

All measurements of enzyme activity were done with the desalted and concentrated supernatants from the 30% (w/v) $(NH_4)_2SO_4$ precipitation.

To estimate the leakage from cells into the apoplast during preparation and infiltration of the samples, assays of two marker enzymes were performed, phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31) and glutathione reductase (GR; EC 1.6.4.2) according to GARDESTRÖM & EDWARDS 1983 and WINGSLE 1989. The activity of SOD was determined using the direct assay of MARKLUND 1985.

For the purification of apoplasmic SOD, the EWF from 12.4 kg (FW) needles was collected, precipitated with $(NH_4)_2SO_4$ and desalted over PD-10 columns. Further purification steps are indicated in Fig. 1.

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The $NH_2$-terminal sequence of about 40 pmol of protein was determined using the Edman-sequencing method (Model 477 A protein/peptide sequencer coupled with the on-line model 120 A PTH analyser; applied Biosystems, Foster City, CA, USA).

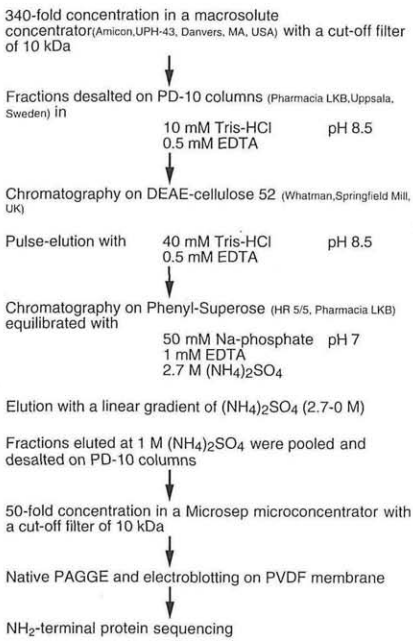


Fig. 1. Purification of apoplasmic SOD from the EWF of 12.4 kg (FW) needles. The chart contains only the steps after vacuum-infiltration and 30% $(NH_4)_2SO_4$ precipitation (see Materials and Methods).

## Results and Discussion

The two-step purification of apoplasmic SODs from EWF of needles provided a recovery of 3.9% of the initial total SOD activity detected and a yield of 26 µg protein (data not shown). The SOD activity of the partially purified enzyme was 32,000 units (mg protein)$^{-1}$, corresponding to 830 units (mg protein)$^{-1}$ in the McCord & Fridovich 1969 xanthine oxidase/ cytochrome c assay (Marklund 1985).

The partially purified proteins were applied to native PAGGE and the apoplasmic SOD isoenzyme (apoSOD 2) was isolated by means of western transfer. Its first 19 $NH_2$-terminal amino acids (except position 12) were determined (Fig. 2). Comparison with the $NH_2$-terminal sequence of apoSOD 3 (Streller & Wingsle 1994) revealed an analogy of 71% (12/17 amino acids), while the similarities towards chloroplastic and cytosolic SODs were lower (56% and 50%, respectively).

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Cytosolic CuZn-SOD
SOD 4 T L L K A V V V L D G A A D V K G V V T F

Chloroplastic CuZn-SOD
SOD 3a E A K K A V A V L K G D S Q V E G V V T L

Apoplasmic CuZn-SOD
SOD 2 V K A V A V L R G D K X I T G T V K F
SOD 3 X K A V A V L R L D K A I L V K V V F

Fig. 2. $NH_2$-terminal sequences of apoplasmic SODs in alignment to cytosolic and chloroplastic SODs.

The chaotropic salt KBr had no significant influence on the amount of total extractable SOD activity from needles, but increased the extractable SOD activity in EWF (Fig. 3). This suggests that apoplasmic SODs may be ionically bound, similarly to apoplasmic acidic peroxidases (SCHLOSS & al. 1987).

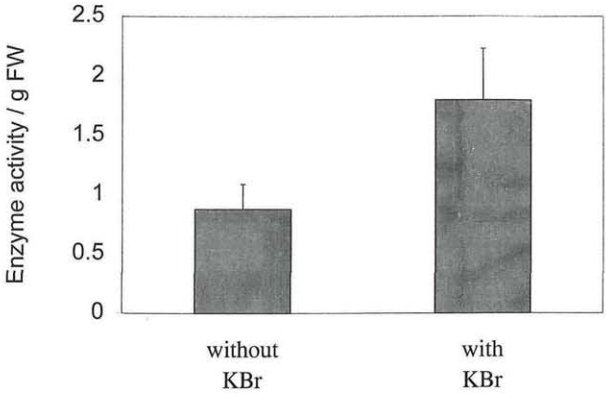


Fig. 3. Effect of 0.3 M KBr on extractable SOD activity in EWF. Means ± SD are given (n=3).

When apoplasmic SODs were extracted from stems, these enzymes were only detected in the EWF from phloem/cambium (Fig. 4). As the peeling of the wood causes damage to cells, the amount of leakage of intracellular compounds into the apoplast is considerable. By assaying two intracellular enzymes, this leakage was calculated to amount to 1% on freshweight basis (data not shown); this is approximately thirty times more than in needles (STRELLER & WINGSLE 1994). Though not significantly, does the study of symplastic marker enzymes nevertheless indicate that SODs are existing in the apoplast (Table 1).

The function of apoplasmic SODs is as yet unclear and more investigation is needed in this field. Pathogene defense and lignification are two possible processes where these SODs might be involved (STRELLER 1996).

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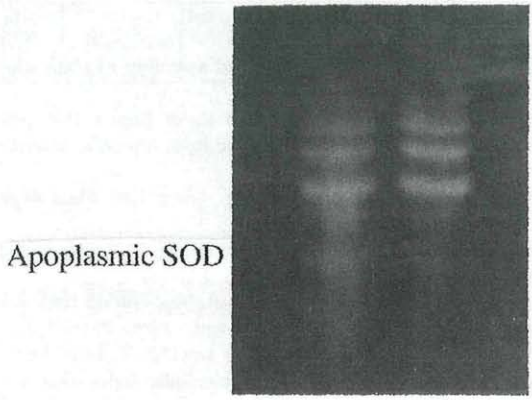


Fig. 4. Native PAGGE (acrylamide gradient 8-25%) of concentrated EWF (left) and concentrated crude extract (right) both from phloem/cambium fraction. Six units SOD activity, as measured in the direct assay of MARKLUND 1985, were applied in both lanes. In the EWF one additional band below the bands of the cytoplasmic SODs was detectable.

Table 1. Specific enzyme activity in EWF and crude extracts. Results are given in units/mg protein for SOD and in nkat/mg protein for PEPCase and GR. Values are means±SD from three isolations.

| | SOD | PEPCase | GR |
|---|---|---|---|
| ***Phloem*** | | | |
| EWF | 1488 ± 144.5 | 1 ± 0.2 | 3 ± 1.1 |
| crude | 2752 ± 934.3 | 7 ± 2.5 | 13 ± 4.0 |
| EWF/ crude | 0.54 | 0.14 | 0.23 |
| ***Xylem*** | | | |
| EWF | 1440 ± 646 | 2 ± 1.8 | 17 ± 3.3 |
| crude | 1545 ± 128.9 | 4 ± 0.7 | 13 ± 0.6 |
| EWF/ crude | 0.93 | 0.5 | 1.3 |

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