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MULTIPLE FORMS OF SUPEROXIDE DISMUTASE IN THE APOPLAST AND WHOLE-NEEDLE EXTRACT OF SERBIAN SPRUCE [*PICEA OMORIKA* (PANČ.) PURKYNĚ]

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Abstract – Activity and isoenzyme composition of superoxide dismutase in the apoplast and whole-needle extract of *Picea omorika* (Panč.) Purkyně, was studied. Total SOD activity of the soluble fraction of the needle extract exceeded markedly that of the apoplastic SOD. Several acidic and two slow-migrating basic isoforms were found in the whole extract. Extracellular SOD had an extremely acidic isoform. Using specific inhibitors, we identified Cu/Zn- and MnSOD forms in the total extract, but only MnSOD in the apoplast. The Fe- isoform was not present in a detectable amount.

Key words: Antioxidative response, enzyme activity, free radicals, isoelectrofocusing

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INTRODUCTION

After the Ice Age, the habitat of *Picea omorika* [(Panč.) Purkyně] became rather narrow (approx. 10000 km^2), occupying exclusively the region of the middle and upper courses of the Drina River – on the present territory of Serbia and Bosnia (J o v a n o v i ć, 1970). Serbian spruce is more tolerant to air pollution and drought compared with other conifers (G i l m a n and W a t s o n, 1994; K r á l, 2002). In cultivated form, it is used throughout Europe as an ornamental due to its elegant shape and pollution resistance.

One of the basic functions of plant cells is their ability to respond to fluctuations in their environment. Environmental and xenobiotic stresses, as well as the developmental process induce production of reactive oxygen species (ROS) in plants, which are generally linked with free radical processes (E l s t n e r, 1982; H e n d r y and C r a w f o r d, 1994). The enzyme superoxide dismutase (SOD) has an important role in the antioxidative response of plant cells to damaging conditions, as an effective quencher of reactive intermediary forms of oxygen and peroxide radicals, and consequently in the related H_2O_2 increase (S c a n d a l i o s, 1993). This enzyme has also beenemployed as a biomarker for the early recognition of environmental pollution (S c h u l z and H ä r t l i n g, 2001). In the present work, we studied activity and the isoenzyme profile of superoxide dismutase in the apoplast and whole-needle extract of *Picea omorika*. This is the first study of SOD in the given species. The obtained results were compared with data known for the other coniferous species. The aim was to observe the difference in activity and isoenzyme forms between extracellular and intracellular SOD in *P. omorika*, as well as to see whether this species differs in SOD characteristics from other conifers. As a coniferous species, Serbian spruce can be used to monitor the environment and its SOD as a biochemical parameter. More generally, such studies may be of assistance in expanding the range of Serbian spruce.

MATERIALS AND METHODS

Needles were collected from healthy Serbian spruce trees about 50-year old growing in the Botanical Garden in Belgrade. All needle samples were taken from the trees between 9:00 and 11:00 h and transported on ice within 15 min to the laboratory.

Frozen needles were powdered in liquid nitrogen in a mortar. The powder was resuspended in the extraction buffer (0.1 M TRIS-HCl, pH 7.6, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5% Tween 80, and 2% PVP) in a 1:5 (w/v) ratio. The homogenates were stirred on ice for

1 hour and centrifuged at 12000g for 10 min. The supernatant was desalted on Sephadex G-25 and used for whole-needle SOD activity measurements and zymogram detection. The apoplastic SOD fraction was obtained by vacuum infiltration of the fresh needles. The needles were deeply cut at the base and the tip and washed with distilled water. They were then infiltrated at -70 kPa for 5 min, 50 mM K-phosphate buffer (pH 6) being used as the infiltration buffer. After infiltration, the needles were centrifuged for 20 min at 1200g. Contamination of the apoplast-washing fluid by symplastic compounds was estimated from the presence of malate dehydrogenase (MDH) in the given fluid. Activity of MDH was assayed spectrophotometrically by measuring NADH oxidation at 340 nm (B a r a s z a h and B r a d s h a v, 1975) in the 0.1 M K-phosphate buffer (pH 7.4) containing 0.2 mM NADH and 0.2 mM oxaloacetic acid at 25^0C. One unit was defined as the amount of enzyme which produces 1 μmol of NAD^+ per min. The apoplastic fluid was desalted on Sephadex G-25.

Superoxide dismutase activity was determined by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals in 100 mM K-phosphate buffer (pH 7.8) (M c C o r d and F r i d o v i c h, 1969) in the presence of 100 μM EDTA and 20 μM sodium azide, an inhibitor of peroxidases (F l o h é and O t t i n g, 1984). The reaction was monitored at 550 nm. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of cytochrome c reduction. The presence of Cu/Zn-, Fe-, and MnSODs was investigated using specific inhibitors (A s a d a *et al*., 1975, B r i t t o n and M a l i n o w s k i, 1978): KCN inhibits Cu/ZnSOD, but does not affect MnSOD and FeSOD, while H_2O_2 inactivates Cu/ZnSOD and FeSOD without affecting MnSOD. Potassium cyanide in a final concentration 5 mM was added to the reaction mixture before the addition of H_2O_2 in a final concentration of 5 mM. Activity of MnSOD was determined in the presence of both 5 mM KCN and 5 mM H_2O_2; FeSOD activity was obtained by subtracting MnSOD activity from that yielded in the presence of 5 mM KCN, while Cu/ZnSOD activity was calculated as the difference between total activity and that of MnSOD and FeSOD.

Enzyme activities were referred to fresh weight of the samples. Protein concentration in the needle extracts was determined by the method of L o w r y *et al*. (1951) with bovine serum albumin as the standard.

Soluble SOD isoenzymes were separated in a pH gradient of from 3 to 9 (using 5% ampholite solution) on 7.5% polyacrylamide gel. Iisoenzymes were stained using the procedure of B e a u c h a m p and F r i d o w i c h (1971) involving 30-min incubation of gels with $2.5 \cdot 10^{-5}$ M NBT and $8.44 \cdot 10^{-6}$ M riboflavin in the dark and subsequent irradiation with UV light. In order to distinguish between Mn-, Cu/Zn- and FeSOD isoforms, activity staining was performed in gels previously incubated at 25°C for 40 min in 100 mM K-phosphate buffer (pH 7.8) containing 5 mM CN^- or 5 mM H_2O_2.

RESULTS AND DISCUSSION

Total protein content, as well as SOD activity of the soluble fraction of the needle extract, markedly exceeded that of apoplastic SOD (Table 1). Activity of MDH was not detected in the extracellular washing fluid (Table 1), indicating that there was no apoplastic contamination by intracellular compounds. The extracellular SOD consisted only of an extremely acidic (pI 3) isoform (Fig. 1). Using specific inhibitors, Cu/ZnSOD (pI 3.5-4) and MnSOD (pI around 3, 4.5-5, 7-7.5, and 9) forms were identified in the total extract. In the apoplast only MnSOD was observed, representing 0.07% of total Mn SOD in needles. The Fe- isoform was not present in a detectable level.

The data on SOD activity and isoenzyme profile are not abundant for conifers. The first report on SOD activity in these species was presented by H u t t u n e n and H e i s k a (1988) for *Pinus silvestris* needles from different localities in Finland. In later studies, several SOD isoforms with pI of about 10 were extracted from Scots pine (*Pinus silvestris*) needles by K a r p i n s k a *et al*. (2001). S c h i n k e l *et al*. (1998) found SOD in extraxylematic bark tissue of needles, as well as in seedlings of *P. silvestris*. They showed that the isoelectric point of these extra-

Table 1. Protein content and superoxide dismutase activity in whole needle extract and apoplast of *Picea omorika* (Panč) *Purkyné*. Data are presented as means (± SE, n=4). [a] control of apoplastic contamination by intracellular compounds

	Protein (mg · g FW^{-1})
Whole needle extract	3.03 ± 0.08
Apoplast	0.00091 ± 0.00004
	Enzyme activity (U · g FW^{-1})
Whole needle extract	
Total SOD	270 ± 6
Cu/ZnSOD	105 ± 14
MnSOD	165 ± 13
Apoplast	
Total SOD (MnSOD)	0.12 ± 0.01
Malate dehydrogenase	not detected [a]

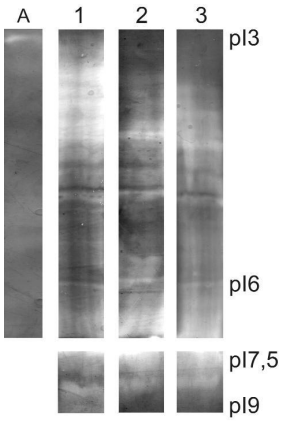

Fig. 1. Isoelectrophoretic pattern of superoxide dismutase from *P. omorika* needles. Lane A: apoplastic SOD; the pattern is the same before and after inhibition with CN^- and H_2O_2. Lanes 1-3: whole needle extract SOD without inhibition and after CN^- and H_2O_2 inhibition, respectively. Band staining procedure is described in Materials and Methods.

cellular forms was distinctly higher than that of cellular SOD. The major isoform was purified and showed to be a Cu/Zn form (being 0.1 % of total Cu/ZnSOD in needles) and to have a high pI value. There were no indications of its role in the lignification process. Several acidic (pI 3-6) and two slow-migrating basic (pI 7.5-8) isoforms were found in the whole extract. K a r l s s o n (2003) also found extracellular Cu/ZnSOD with a high pI value in *P. sylvestris*. The author proposed that the enzyme is important in lignification, as a source of H_2O_2. In germination of *P. silvestris*, five Cu/ZnSOD isoforms were also found. T a n d y *et al*. (1989) extracted SOD from the needles of red spruce (*Picea rubens* Sarg.) and two *Pinus* species, loblolly pine (*Pinus taeda* L.) and Scotch pine (*Pinus silvetris* L.). They found different isoforms in the two families; the response of these isoforms to various external stress conditions was also studied. Our results show that SOD isoforms with both high and low pI values are present in *P. omorika*. In the apoplast of Serbian spruce only the extremely acidic isoform is present, contrary to the case of pine, where extremely high-pI forms were detected. In *P. omorika,* extracellular SOD completely consisted of the Mn-isoform, which is different from the case of pine, where extracellular SOD is the Cu/Zn isoform. Typically, Cu/ZnSOD is found in the cytosol or extracellular space in all eukariotes and various prokaryotes. The MnSOD isoform is present in the mitochondrial matrix of eukaryotic cells and in the cytoplasm of prokaryotic cells, while FeSOD is found in many bacteria and chloroplasts of a few higher plants. Comparison of deduced amino acid sequences from these three different types of SOD suggest that Mn- and FeSOD are more ancient types of SODs, and that the Cu/Zn type probably evolved separately in eukaryotes (S m i t h and D o o l i t t l e, 1992). H e r n á n d e z *et al*. (2001) found an MnSOD isoform in the *Pisum sativum* apoplast. The results obtained in the present work are the first evidence of an extracellular MnSOD in conifers. Contrary to the case of pine, where Cu/Zn isoforms were found to be with high pI values, in *P. omorika* these isoforms are acidic. These results show that SOD in *P. omorika* has specific characteristics in comparison with data available for other coniferous species. Such characteristics may have been developed in *P. omorika* under influence of its habitat as a basic built-in feature of the antioxidative system in this species.

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РАЗЛИЧИТЕ ИЗОФОРМЕ СУПЕРОКСИД ДИСМУТАЗЕ У АПОПЛАСТУ И ЕКСТРАКТУ ЧЕТИНА ОМОРИКЕ [*PICEA OMORIKA* (PANČ.) PURKYNĚ]

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Проучавана је активност и изоензимски састав супероксид дисмутазе у апопласту и екстракту целих четина оморике [*Picea omorika* (Panč.) Purkyně]. Укупна активност супероксид дисмутазе у солубилној фракцији екстракта четина је била значајно већа од активности супероксид дисмутазе у апопласту. У екстракту целих четина нађене су неколико киселих и две спо-ро-мигрирајуће базне изоформе. Ванћелијска супероксид дисмутаза садржи само једну екстремно киселу изоформу. Користећи специфичне инхибиторе, Cu/Zn- и Mn- супероксид дисмутазне форме су идентификоване у екстракту целих четина, док је у апопласту нађена само Mn- супероксид дисмутаза. Fe-изоформа није присутна у мерљивој концентрацији.