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PREPARATION OF EXTRACTS FROM NEEDLES OF OMORIKA (*PICEA OMORIKA* (PANČIĆ) *PURKINYE*) FOR PEROXIDASE ACTIVITY AND ISOENZYME PROFILE ANALYSES

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We studied the effect of nonionic detergent Tween 80 in extraction buffer on the activity and isoenzyme pattern of peroxidase from omorika needles. The activity of soluble peroxidases was studied using UV-VIS spectrophotometry and isoelectrofocusing. Soluble fraction obtained using extraction buffer with detergent had a higher activity and more isoenzymes in IEF pattern in comparison with the corresponding extract not including detergent in extraction medium.

INTRODUCTION

Peroxidases (donor: H_2O_2 oxidoreductase; E.C.1.11.1.7) are a family of heme-containing enzymes that are distributed throughout the plant kingdom. These isoenzymes are differentially expressed in various tissues and organs in plants and

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respond to developmental and environmental cues. Their primary function is to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. Peroxidase activity in cell walls of plants is presumed to be involved with extensin and proline-rich protein cross-linking (COOPER and VARNER 1984), lignification (GASPAR *et al.*, 1982; GASPAR, 1986), suberization (ESPELIE and KOLATTAKUDY, 1985), disease resistance (MEHDY, 1994) and wound-healing (ESPELIE *et al.*, 1986). However, the physiological function of individual isoenzymes is only partially understood and is complicated by the presence of multiple peroxidase isozymes. Induction of peroxidase activity was found for a variety of stress factors: heavy metals (VANGROSVELD and CLIJSTERS, 1994), pathogenic infection, wounding and air pollution (GASPAR *et al.*, 1982). Peroxidase activity and isoenzyme pattern in the spruce needles (*Picea abies* L.) may be used as a reliable criterion of monitoring air and soil pollution (RADOTIĆ *et al.*, 2001).

Because of all mentioned above, it's very important to extract this enzyme appropriately. The preparation of extracts from mature spruce needles is essential for enzymatic analyses (WEIMAR and ROTHE, 1986) In the present paper we report the different isolation conditions of peroxidases from spruce needles.

MATERIALS AND METHODS

Needles were obtained from approximately 100-year-old healthy omorika trees (*Picea omorika* Pančić) in May 2003, at locality Zaovine on mountain Tara (1100 m above see level). The needles were collected from one- and two-year-old branches of omorika. For each analysis, the needles were taken from four trees, which were taken as replicates.

All steps of enzyme extraction were performed at 4°C. The needles were ground to powder; extracts of soluble peroxidase were obtained in the two mediums (0.1 M Tris-HCl buffer pH 7.8, containing 1 mM dithiotreitol, 1 mM EDTA and either 0 or 0.5% (w/v) of the nonionic detergent Tween 80) in 1:5 ratio (w/v). The homogenate was centrifuged at 12000xg for 10 minutes. The supernatant was used for soluble peroxidase activity measurements and isoelectric focusing.

The peroxidase activity was determined spectrophotometrically with guaiacol as a substrate in a total volume of 3 mL. The assay mixture contained 50 mM acetate buffer pH 5.5, 92 mM guaiacol, 18 mM H_2O_2 and 10 µL of the enzyme extracts. The increase in absorbance at 470 nm was monitored with a Shimadzu UV-160 spectrophotometer and the reaction rate was calculated from extinction coefficient for guaiacol of 25.5 $mM^{-1}cm^{-1}$. Control rates were obtained using extraction buffer instead of sample. The activity was referred to the protein content of each enzymic fraction or to the needle fresh weight. The quantity of protein in the enzyme extract was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a reference.

The peroxidase isoenzimes were separated by isoelectric focusing in a pH gradient from 3 to 9 on a 7.5% polyacrylamide gel (using 7% ampholite solution). The isoenzyme patterns were visualized by incubation the gel in 0.1 M

Tris-HCl buffer pH 7.8 whit chloro-1-naphthol and H_2O_2 (LAGRIMINI and ROTHSTEIN, 1987) for 10 min at 25°C.

Statistical analysis of the enzyme activity data was performed using the Mann-Witney ranking test, at the 0.05 level of significance.

RESULTS

When extraction medium with 0.5% Tween 80 was employed, peroxidase activity from omorika needles was significantly higher in comparison with the extraction in the absence of detergent (Tab. 1).

In the absence of detergent in the extraction medium, in peroxidase isoelectrophoretic pattern two groups of isoperoxidases could be observed - cationic isoenzymes with pI 8-9 and anionic isozymes with pI 3-4 (Fig.1). When extraction medium contained Tween 80 the new group of isoperoxidases appeared with pI 7.5-5.(Fig.1).

Table 1. - Activity of peroxidases referred to the protein content of extracts or to the needle fresh weight

Type of extraction buffer	Specific activity (U/FW)	Specific activity (U/mg protein)
0.1 M Tris-HCl buffer pH 7.8, 1 mM dithiotreitol, 1 mM EDTA	40±4	22±3
0.1 M Tris-HCl buffer pH 7.8, 1 mM dithiotreitol, 1 mM EDTA and 0.5% Tween 80	137±4	39±3

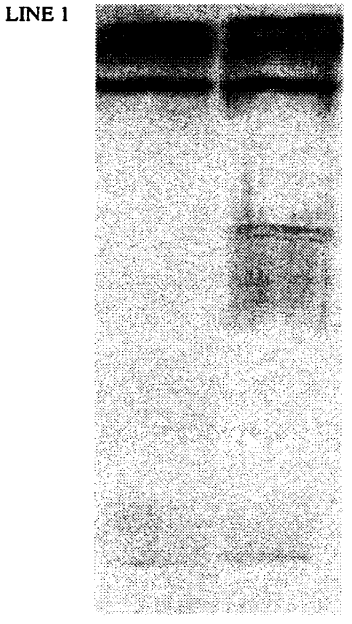

Fig. 1. - Peroxidase isoforms from omorika needles identified in IEF in a pH gradient from 3 to 9; line 1 - Fraction without Tween 80 in extraction buffer; line 2 - Fraction with 0.5% Tween 80 in extraction buffer

DISCUSSION

The obtained results are in accordance with the results of PITEL and CHELIAK (1985) who also obtained an increase in activity of several enzymes from spruce needles when the extraction buffer contained nonionic detergent.

The results of IEF show that the slow-moving anionic isoenzymes attach to the membranes during extraction procedure, and consequently are lost from the extracted supernatant if the extraction medium is without nonionic detergent.

Apoplastic peroxidases and ascorbate are involved in manganese toxicity of *Vigna unguiculata* (FECHT-CHRISTOFFERS *et al.*, 2003.), the activities of water-soluble apoplastic peroxidases were generally significantly higher in Mn treated plants than in control plants (optimum Mn supply); and the new bands became visible, indicating the expression of additional peroxidases isoenzymes.

According to the obtained results, the presence of nonionic detergent in extraction medium is recommendable in case of peroxidase extraction from spruce needles, enabling complete insight into the peroxidase activity and isoenzyme profile. Such an improvement of the isolation procedure is important, since peroxidase, as one of the biochemical parameters, may be involved in the monitoring of environment (KELLER, 1974; RADOTIĆ *et al.*, 2001).

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PRIPREMA EKSTRAKATA OMORIKE ZA ANALIZU AKTIVNOSTI PEROKSIDAZE I IZOENZIMA

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Izvod

Ispitivan je uticaj nejonskog detergenta Tween 80 u ekstrakcionom puferu na aktivnost i izoenzimski sastav peroksidaza u četinama omorike (*Picea omorika* (Pančić) *Purkinye*). Aktivnost unutarćelijske i vanćelijske slobodne peroksidaze ispitivana je korišćenjem UV-VIS spektrofotometrije i izoelektrofokusiranja. Ekstrakti dobijeni korišćenjem ekstrakcionog pufera sa detergentom Tween 80 imaju veću peroksidaznu aktivnost i veći broj izoenzima dobijenih izoelektrofokusiranjem u poređenju sa ekstraktima čiji ekstrakcioni pufer nije sadržao nejonski detergent.

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