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Influence of Storage Temperature on Total Peroxidase Activity in Crude Extracts from *Picea abies* L. Karst. Needles*

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Peroxidases are enzymes with numerous functions in plant cells and are, therefore, interesting objects of current biochemical research. The purpose of this work was to investigate the effect of 30 days storage at different temperatures on the peroxidase activity in crude extracts from one-year old needles of *Picea abies* L. Karst. Total peroxidase activity was detected in the extract reaction with guaiacol and H_2O_2. Colour product was quantified by absorbance measurement at 470 nm. Measurements were performed daily in the first 10 days, and on the 12th, 14th, 16th, 18th and 30th day of the experiment. Extracts stored for 30 days were subjected to polyacrylamide gel electrophoresis, incubated with peroxidase substrates, and the resulting bands were quantified. Three different dependences on storage temperature were noticed. Extracts kept at –196 °C and –20 °C displayed no change in activity, samples kept at +24 °C and +37 °C decreased the activity, while those kept at +4 °C manifested a considerable rise in activity. Electropherogram analysis revealed two isoenzymes, with total activities resembling those obtained spectrophotometrically. There was no difference in isoenzyme appearance between samples. Recommendations regarding enzyme extract storage are given.

INTRODUCTION

Peroxidases are enzymes with numerous biochemical and physiological roles in higher plants.[1] They participate in plant growth, differentiation and development processes, including auxin catabolism, ethylene biosynthesis, plasma membrane redox systems and the generation of H_2O_2, cell wall edification, lignification and suberization, as well as response to pathogens.[2] Of particular interest nowadays is the role of peroxidases in H_2O_2-mediated signalling processes as the response to abiotic and biotic stresses in plants.[3,4] In general, peroxidases are enzymes that oxidize a variety of hydrogen donors at the expense of peroxide or molecular oxygen. Consequently, there are several areas where peroxidases, owing to their oxidative nature, can replace current chemical oxidation techniques, meeting the environmental demands of future technologies.[5]

At the moment, our interest is focused on peroxidases in the needles of *Picea abies* L. Karst., the evergreen plant that has been the object of investigation in our laboratory for almost twenty years. In this period, different parameters were explored during the proliferation of vegetative buds into young shoots bearing the current-year

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needles and their maturation. The main characteristics of proliferating buds were a significant increase in mitotic activity,[6] different size and distribution of nuclei in leaf primordia[7] and the enlargement of embryonic tissue,[8] as well as bursting of the lignified tracheary elements into the embryonic shoot, disappearance of callose from sieve areas in the wall of the cup-like structure and diffusion of pectic substances from the middle lamellae in the wall of crown cells.[9] Dynamic of changes in the amount of photosynthetic pigments in buds and, after bud break, in young needles, as well as plastid ultrastructure,[10,11] functional characteristics of the photosynthetic apparatus[12] and the appearance of vacuolar polyphenols[13] were also investigated. Total soluble protein amount was determined during the bursting time[14] in order to support the conclusions about the events during bud break and needle elongation.

A considerable peroxidase activity can be detected in the crude preparation of the soluble protein extract from spruce needles. For this reason, our future research will focus on peroxidases isolation and further enzyme and isoenzyme characterization (*i.e.*, kinetic measurements, agonist and antagonist effects, *etc.*), which demands a stabile and reliable enzyme preparation for a longer time period. Therefore, the aim of our present study was to examine the influence of storage temperature on total peroxidase activity in crude extracts of *Picea abies* needles in a period of 30 days, and to define the optimal storage temperature for these particular enzymes.

EXPERIMENTAL

Crude Enzyme Extract Preparation

One-year old needles were sampled from the same tree of *Picea abies* on three occasions, at time intervals of two months, starting at the end of August. Sampling was performed in the morning, and was followed by subsequent extract preparation. Fresh needles were minced into small pieces and powdered in liquid nitrogen. Soluble proteins were extracted with 1 M Tris-HCl buffer, pH = 8.0, with the addition of polyvinylpyrrolidone (PVP) and centrifuged 20 min at 18000 rpm and +4 °C. The green supernatant was collected, while the sediment was extracted two more times using the same buffer. United extracts were distributed into small, capped bottles and stored at different temperatures (–196, –20, +4, +24, and +37 °C).

Total Peroxidase Activity Measurement

Total peroxidase activity was detected in the extract aliquots of fresh needles. A modified version of the method of Siegel and Galston[15] was used for enzyme activity measurement. The reaction mixture contained 5×10^{-3} M reduced, colourless guaiacol and 5×10^{-3} M H_2O_2 in 0.2 M phosphate buffer, pH = 5.8. The reaction was started by adding 200 μL of enzyme extract to 800 μL of reaction mixture. The absorbance increase of the oxidized, brown coloured guaiacol at 470 nm was recorded spectrophotometrically using an Analytic Jena SPECORD 40. Measurements were performed daily for the first ten days, and on the 12th, 14th, 16th, 18th and 30th day of the experiment. For each day of each experiment, samples were prepared and analyzed in triplicate.

Electrophoretic Analysis

Enzyme extracts stored for 30 days at different temperatures were subjected to native electrophoresis on 10 % polyacrylamide gels in 1.5 M Tris-glycine buffer, pH = 8.3. After electrophoretic separation, the gels were incubated with the guaiacol and H_2O_2 like in the total peroxidase activity measurements in solution. The brown colour bands obtained, resulting from enzyme activity, were (after gel scanning) analyzed and quantified densitometrically, using the computer program ImageJ 1.32e.

Statistical Evaluation

For each experimental sample, the relative peroxidase activity was calculated:

$$a\,/\,\% = \frac{\text{total enzyme activity at day } X \text{ of storage}}{\text{total enzyme activity at the day of isolation}} \times 100$$

The mean relative activities were further calculated for the same day of all the three separate experiments. The analysis of variance was applied to test the variability of enzyme activity between the different storage temperatures on each measurement day.[16] Calculated F-values were plotted to better understand the trend of activity changes during the storage period.

Graphical presentations were performed using Microsoft Excel.

RESULTS AND DISCUSSION

The »basal« peroxidase activities at the day of extraction were 0.226, 0.640 and 0.823 ΔA_{470} min^{-1} in the first, second and third experiment, respectively. The original measurements were further expressed as relative activities, *i.e.*, as percent of the activity at the day of extract preparation (which was defined as 100 %). The mean values and standard deviation of nine relative activities obtained for each experimental day of each experiment were plotted against the different storage temperatures applied and are presented in Figure 1.

Three different temperature dependences could be observed:

– Samples kept at –20 °C and –196 °C displayed similar activity throughout the whole experimental period, with negligible variations.

– Activity of samples kept at +4 °C increased already on the second day by about 50 % compared to the day of extract preparation, and this trend continued, reaching

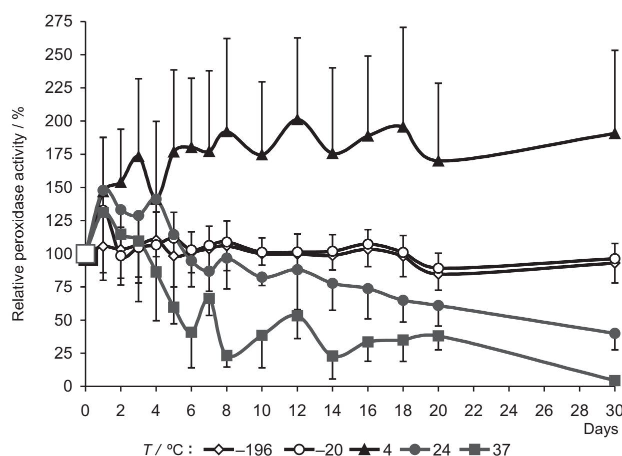

Figure 1. Effect of storage temperature on the relative peroxidases activity during the period of 30 days. Each point represents the mean value of nine measurements.

its maximum on the 12th experimental day, when the activity was 100 % higher compared to the day of extract preparation. This highly increased activity remained constant throughout the remaining experimental period.

– Samples kept at +24 °C and +37 °C generally displayed a decrease in activity. After 30 days, the activities of samples kept at +37 °C were almost negligible, while those kept at +24 °C retained about 40 % of the activity at the day of extraction. Interestingly, the first five days for +24 °C, and the first three days for +37 °C, the activities were increased, by almost 50 % at the former, and about 20 % at the latter storage temperature.

Generally, the variability in enzyme activity was more pronounced in the first several days of experiment, and the greatest variations were noticed for samples kept at +4 °C. Even the extracts stored at –20 °C increased the activity by 25 % 24 hours after preparation, but returned to the original value after 24 hours.

The change of variability during the experiment was tested by the analysis of variance, and the calculated *F*-values were plotted for each experimental day (Figure 2).

A permanent increase of *F*-values could be observed, which was additionally confirmed by the trend calculation (positive trend line equation with $R^2 = 0.8228$). Also, from the *F*-values obtained, it was evident that the observed differences between the activities of samples kept at different temperatures became significant already on the second day after extract preparation ($P<0.05$), the third day the significance rose to 0.001, and remained as high or even higher until the end of the experiment. In other words, differentiation of the peroxidase activities in extracts started as soon as 24 hours after preparation, increased later on, and became more and more pronounced until the end of the experiment.

Electrophoretic patterns of peroxidase activities on the 30th experimental day are presented in Figure 3a.

Under native conditions, at pH = 8.3, only two isoenzyme bands could be detected in all samples. The intensity of bands, depending on enzyme activity, resembled the results obtained by activity measurement in solution. This was confirmed by the densitometric analysis, which is given in Figure 3b and Table I.

Differences in peroxidase activities in the extract kept 30 days at various storage temperatures determined electrophoretically were not so pronounced as those determined spectrophotometrically, but displayed the same trends. The highest activity belonged to +4 °C, followed by –196 °C and –20 °C, while the lowest activity was detected in samples kept for 30 days at +37 °C. Isoenzyme profile was not influenced by different storage temperatures, either qualitatively (number of isoenzymes) or quantitatively (relative participation of isoenzymes in total peroxidase activity).

Literature data on the stability of plant peroxidase preparations at various storage temperatures vary, mostly depending on the enzyme source. The best explored is

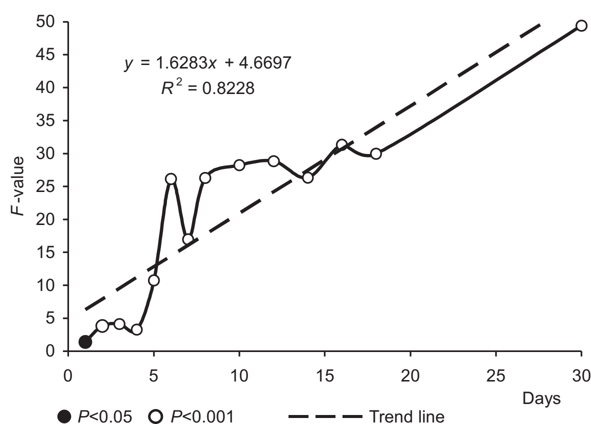

Figure 2. Changes of the variability (expressed as *F*-values) and trend (represented by the trend line and the appertaining equation with R^2-value) during the 30 day experimental period.

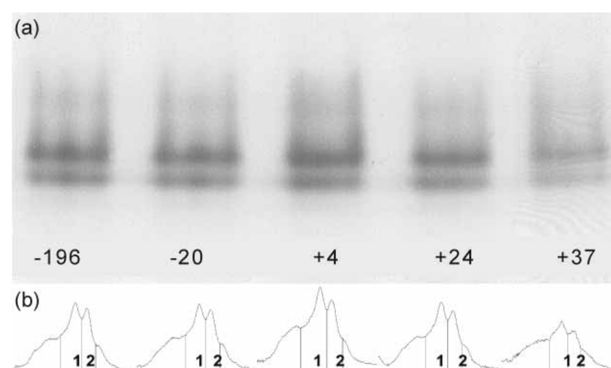

Figure 3. (a) Electropherogram of crude protein extracts kept for 30 days at different storage temperatures, after incubation in reaction mixture containing peroxidase substrates. Electrophoretic conditions: 10% polyacrilamide separation gel, pH = 8.3 (1.5 M Tris-glycine buffer). (b) Densitograms of protein bands in each lane of the electropherogram presented in Figure 3a.

Table I. Densitometric analysis of electropherograms presented in Figure 3b[a]

Temperature	Band 1	Band 2
°C	%	%
–196	63,3	36,7
–20	65,6	34,4
+4	65,3	34,7
+24	64,9	35,1
+37	64,8	35,2

[a] The area under the two identified peaks was calculated for each electrophoretic lane. The relative participation of each band in the total bands area is expressed in percents.

horseradish peroxidase, which according to the latest report,[18] displays a loss of activity of almost 30 % when stored at +4 °C and +10 °C. The Worthington Catalog declares a high purified horseradish peroxidase stability (9–12 months at 8 °C), and recommends storage temperature of 2–8 °C.[19] Based on this relatively good stability, they recommend peroxidase-labelled immunoglobulins as immuno-histological probes for demonstration of tissue antigens, and for the quantitative determination of soluble and insoluble antigens in enzyme amplified immunoassay systems. Also, the use of the highly specific, sensitive and very stabile horseradish peroxidase with a chromogenic donor has proven very useful for assay systems producing hydrogen peroxide – *i.e.,* in the determination of glucose or galactose by the respective oxidase or in the determination of certain L-amino acids in conjunction with L-amino acid oxidase.

The activity of peroxidases can be influenced by different agents, which can also change their thermal stability. Karaseva *et al.*[20] reported on horseradish peroxidase-catalyzed oxidation of *o*-phenylenediamine, activated with melamine, and proposed a rapid, highly accurate, and simple analytical test system for quantitative melamine determination. Transition metals (Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}) also affect the horseradish peroxidase activity either as activators, or as inhibitors, depending on concentrations applied.[21] Such metal ions are potent inductors of conformational and functional stability of horseradish peroxidase. At the same time, other substances, like arylhydrazides, proved to be potent inhibitors of peroxidases containing haem, like horseradish peroxidase isoenzyme C.[22] Activity stimulation by ions (Mg^{2+}) has been also observed for tobacco peroxidase, at low pH-values.[23]

Peroxidases from other sources display different stability at various storage temperatures. For example, tea peroxidase is, unlike the horseradish one, more stable at 10 °C than at +4 °C.[18] Peroxidase from grape inactivated after heating at 60 °C for 1–10 minutes,[24] while an extremely temperature-stabile peroxidase has been reported in leaves of African oil palm tree.[25] This peroxidase retained its full activity at the optimal pH-value even after heating to 70 °C for 60 minutes. In a report dealing with spruce peroxidases, complete loss of ascorbate peroxidase activity was observed in crude extracts from needles stored at –20° C and –80 °C, while identically treated guaiacol peroxidases in the same preparations were only slightly freeze sensitive.[26] Losses of activity were in the range of 10–20 %, regardless of whether extracts or needles were kept frozen. The effect of higher temperatures on spruce peroxidases has not been tested to date.

Our results confirmed the presumption that the optimal storage temperature for crude peroxidase preparation from spruce needles is –20 °C or lower, as it is recommended for most commercially available enzymes. At this temperature, the enzyme is stable for a period of at least one month, without the need to add any stabilizing substance. Also, the enzyme inactivation at +25 °C and +37 °C could be predicted, and attributed to microbial or/and proteolytic degradation.[17] Increases in enzyme activities obtained at +4 °C were not anticipated. The constant rise in the first 5 days of storage might be the result of the changes in their native environment, after enzymes extraction from their biological environment, and/or possible liberation of potential activator(s). At higher temperatures, the inactivating effects may be predominant, while at lower temperatures (+4 °C) these inactivating effects may not have a chance.

CONCLUSIONS

The results obtained in this study suggest the following conclusions:

– Optimal storage temperatures for the crude protein extract from spruce needles containing peroxidase activity are –20 °C and lower.

– It is not recommended to perform experiments with the enzyme extract at room temperature or higher.

– Experiments using the same sample for a longer period of time may be performed at +4 °C, but enzyme preparation must be kept at +4 °C for at least 5 days before the experiment starts, to insure a constant enzyme activity for the next 25 days.

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SAŽETAK

Utjecaj temperature pohranjivanja na aktivnost ukupnih peroksidaza u nepročišćenom ekstraktu iglica smreke (*Picea abies L. Karst.*)

Elizabeta Has-Schön, Hrvoje Lepeduš, Ljiljana Jerabek i Vera Cesar

Peroksidaze su enzimi s brojnim funkcijama u biljnim stanicama. Stoga su ovi enzimi zanimljiv predmet suvremenih biokemijskih istraživanja. Svrha ovoga rada bila je ispitati utjecaj pohranjivanja kroz 30 dana pri različitim temperaturama na aktivnost ukupnih peroksidaza u nepročišćenom ekstraktu iz jednogodišnjih iglica smreke. Aktivnost ukupnih peroksidaza određivana je reakcijom proteinskoga ekstrakta s gvajakolom i H_2O_2. Količina obojenoga produkta proporcionalna je apsorbanciji na 470 nm. Mjerenja su vršena svaki dan prvih 10 dana, a nakon toga u slijedeće dane pokusa: 12., 14., 16., 18. i 30. Ekstrakti pohranjeni 30 dana bili su podvrgnuti nativnoj elektroforezi na poliakrilamidnome gelu te inkubirani sa supstratima peroksidaza, a intenzitet obojenja vrpci kvantitativno je određen. Uočene su tri različite ovisnosti o temperaturi pohranjivanja. Ekstrakti čuvani na –196 °C i –20 °C nisu promijenili aktivnost, uzorcima čuvanim na +24 °C i +37 °C aktivnost se smanjila, dok se onima čuvanim na +4 °C povećala. Analiza elektroferograma pokazala je postojanje dva izoenzima, pri čemu je zbroj njihovih pojedinačnih aktivnosti za svaku temperaturu pohranjivanja sličan onoj izmjerenoj spektrofotometrijski. Nije bilo razlike u broju i udjelu izoenzima između uzoraka. Preporuča se određeni način pohranjivanja enzimskoga ekstrakta iz iglica smreke.