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# The effect of pH on the activity of soluble peroxidase in needles of Serbian spruce (*Picea omorika* (Panč.) *Purkinye*): application of a mathematical model

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**Abstract.** We studied the dependence of peroxidase (POD) activity on pH in crude extract of *Picea omorika* (Panč.) *Purkinye* needles and in its acidic and basic fractions, obtained by ion exchange chromatography. Nonlinear regression was applied on the activity data with pH as the explaining variable, using the Levenberg-Marquardt algorithm. Studying crude extract at three different temperatures, the shape of the simulated activity/pH dependences indicated an existence of two components, which was confirmed by mathematical modeling. The kinetic parameters $Act_0$, $K_{EH}$ and $K_{EOH}$ of both components are presented. The curves and pH optima shifted under increasing temperatures towards lower pH values, which was verified after decomposition. Nonlinear regression detected the presence of two components for both fractions, and there is no considerable difference between their pH optima. Our results show for the first time that the sum of components, each described by the mathematical model employed, can be used to explain the complex pH-related POD activity in the extract with two or more enzyme forms simultaneously active.

**Key words:** pH-related POD activity — Mathematical model — *Picea omorika* (Panč.) *Purkinye* — Peroxidase fractions

## Introduction

Endemic species are interesting for fundamental investigations in enzymology. One of the most outstanding endemic species of the European flora that has survived from the Tertiary to the present time is Serbian spruce (*Picea omorika* (Panč.) *Purkinye*), well known for its unusual treetop form. This species is well adapted to severe environmental conditions, such as high irradiation, drought and air pollution, compared with other conifers (Gilman and Watson 1994; Král 2002).

Secretory plant peroxidases (PODs) of class III (E.C. 1.11.1.7.) are localized in vacuoles and the apoplast (Takahama 1992; Ros Barcelo 2000), and can be soluble, ionically and covalently cell wall-bound forms. They are involved in many functions as a consequence of their two cycles: reactive oxygen species (ROS) generation and regulation, $H_2O_2$ level regulation, and oxidation of various substrates (Passardi et al. 2005). They represent an important part of the antioxidative system of plants and catalyze one-electron oxidation of diverse, mainly phenolic, substrates in the presence of $H_2O_2$ (Dunford 1991). Their protective role from free radicals is especially evident during oxidative burst (Lamb and Dixon 1997). Apoplastic PODs participate in the formation of lignins, while both vacuolar and apoplastic PODs participate in suberization during plant infection or wounding (Sato et al. 1993; Bernards et al. 1999). Since Serbian spruce is well-adapted to the type of habitat with pronounced influence of different stressors, the characteristics of its antioxidative protection system

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are of great scientific as well as practical interest. We investigated POD activity in extracts of *P. omorika* needles in the context of its protective system. Since class III PODs are known for their stability under changing temperatures (Aiman and Qayyum 2007), in our previous study we had examined the kinetic parameters of thermal inactivation of PODs from *P. omorika* needles (Laketa et al. 2009) using a mathematical model proposed by Tijskens et al. (Ponne et al. 1996, Tijskens et al. 1997). The effect of pH on enzymatic activity is an important part of enzyme characterization. On the other hand, pH in plants can vary depending on physiological processes (Kurkdjian et al. 1989; Grignon and Sentenac 1991). For these reasons we have extended our research towards mathematical modeling of the pH-related POD activity taking place in *P. omorika* needles. In this study, we investigated dependence of POD activity on pH in crude extract of needles and in its acidic and basic fractions, as obtained by ion exchange chromatography. Generally, enzymes attain maximum activity at a specific pH value as a result of stabilization of the optimal conformation of molecule, which means that there is a highest accessibility of the catalytic site to substrates. In other cases, due to binding of $H^+/OH^-$, conformation changes lead to a decrease in enzyme activity. The cytosolic pH of most plant cells is usually between 7.0 and 7.6, while vacuolar pH falls below 5.5 (Smith and Raven 1979; Felle 1988). pH value in the apoplast of conifer needles is around 5.5 (Pfanz and Dietz 1987; Graber et al. 2003). From the physiological point of view, our interest was to see which pH values are optimal for POD activity in our object of investigation. Our previous studies have shown the presence of multiple POD isoenzymes in the extract of *P. omorika* needles, which can be separated in two pools based on their isoelectric point (pI) values (Bogdanović et al. 2007). Our aim was to see if there is a difference in pH dependence between these two pools of isoenzymes and their contribution to the dependence of POD activity on pH in the extract containing all isoenzymes. Furthermore, we aimed to see if the sum of components, each described by the proposed mathematical model (Tijskens et al. 2001; Seyhan et al. 2002), can be used as a tool to describe a more complex POD activity/pH dependence, where two or more enzyme forms are present. The mathematical model applied to the obtained results can improve our predicting of changes in the enzyme activity over a wide range of pH values and shed more light on the behavior of this particular enzyme.

## Materials and Methods

All reagents and substances used in the experiments were analytical grade and obtained from Sigma (Germany).

*Plant material*

In the experiments we used two-year old needles sampled from 15-year-old *P. omorika* trees grown in a generative seed orchard in Godovik (43° 51' N, 20° 02' E, 400 m), Serbia. Needles were collected in May 2004 and immediately stored in liquid nitrogen until the experiments. The plants used for the experiments were healthy, without any exogenous infection detected. Each of the samples was obtained by mixing the needles of four different trees and immediately stored in liquid nitrogen.

*Enzyme extraction*

The extraction of the samples powdered in liquid nitrogen was done using a buffer containing 0.1 mol/l TRIS-HCl (pH 7.6), with 1 mmol/l DTT, 1 mmol/l EDTA and 2% polyvinylpyrrolidone (PVP), in 1 : 5 (w/v) ratio, during 30 min on ice. The supernatants obtained by centrifugation of homogenates at 12 000 × *g* for 10 min were used for further experiments after a desalting procedure on Sephadex G-25 (NAP-5 column, Amersham Biosciences, Germany).

*Ion exchange chromatography*

The homogenate (crude extract) was brought to 35% saturation with solid ammonium sulfate. After stirring for 30 min, the precipitate was removed by centrifugation at 5 000 × *g* for 25 min. The supernatant was brought to 80% saturation with additional ammonium sulfate and stirred for 1 h. After centrifugation, the precipitate was dissolved in 0.1 mol/l Tris-HCl (pH 7.4) containing 2% PVP, 1 mmol/l EDTA and 1 mmol/l DTT. The sample was dialyzed against 10 mmol/l K-phosphate buffer (pH 7.0). The basic and acidic peroxidase isoenzymes were separated by ion-exchange chromatography on CM-cellulose. The basic POD was eluted from ion-exchange column with 10 mmol/l K-phosphate buffer (pH 7.0) containing 0.5 mol/l NaCl. Under these conditions acidic POD did not bind to the column and was detected in the void volume.

*Determination of enzyme activity*

Measurements of POD activity were done in a thermostated cuvette using a Shimadzu UV-2501 PC spectrophotometer. In the case of crude extract, guaiacol was used as a substrate (Chance and Maehly 1955), as reported in a recent paper by Laketa et al. (2009). For acidic and basic fraction, 92 mmol/l guaiacol and 18 mmol/l $H_2O_2$, or 140 μmol/l coniferyl alcohol and 1 mmol/l $H_2O_2$ were used as substrates. In order to determine the pH-dependent POD activity, 50 mmol/l phosphate/citrate buffer was used with pH values in the

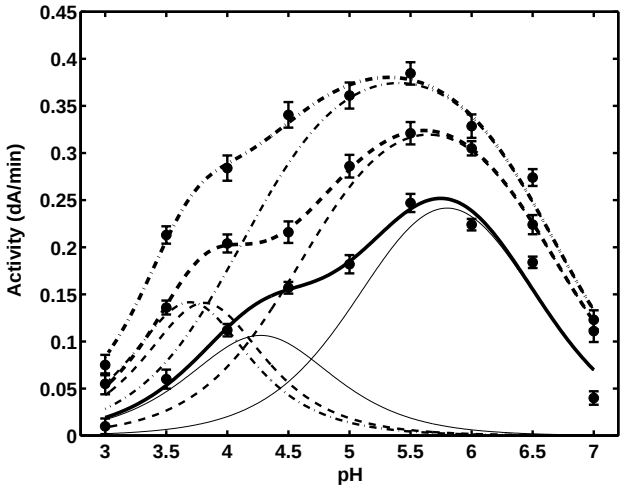


**Figure 1.** The dependence of POD activity on pH in crude extract from *P. omorika* needles, as well as the corresponding regression components, at three different temperatures. Both measured (●) and simulated activities (4°C, solid line; 20°C, dashed line; 40°C, dash-dotted line) are presented. The data points are the average of three measurements with standard error.

range 2.5–8.5. The activity (Act) was expressed as change in absorbance *per* minute.

*Calculations*

The dependence of experimental data on pH was modeled according to Tijskens et al. (2001) and Seyhan et al. (2002) as

$$Act = \frac{Act_0}{1 + \frac{H^+}{K_{EH}} + \frac{K_W}{K_{EOH}}\frac{1}{H^+}} \quad (1)$$

where *Act* stands for the enzyme activity at a particular value of pH. Furthermore, $Act_0 = k_s En_{\text{tot}}$, $k_s$ is the rate constant for the enzyme-substrate reaction, and $En_{\text{tot}} = EnH^+ + EnOH^- + En$ is total enzyme concentration, while $K_W = 10^{-14}$ stands for the water dissociation constant.

When more than one fraction was present in the extract, Eq. (1) was modified to

$$Act = \sum_{i=1}^{n} \frac{Act_{0,i}}{1 + \frac{H^+}{K_{EH,i}} + \frac{K_W}{K_{EOH,i}}\frac{1}{H^+}} \quad (2)$$

where each addend corresponds to one component (fraction). Therefore, in cases where two components were expected, we used Eq. (2); otherwise fitting was performed using Eq. (1).

Nonlinear regression analysis was performed on each series of data separately, with pH as the explaining variable, using Levenberg-Marquardt algorithm supplied with Statistica 6.0 (StatSoft Inc., Tulsa OK, USA). For each curve, parameters $Act_0$, $K_{EH}$ and $K_{EOH}$ were estimated simultaneously. As in Tijskens et al. (2001), in order to reduce the influence of zero and near-zero measured activities, the data were given weights of the values of activities themselves. Quality of fitting was expressed as $R^2_{adj} = 1 - [(\text{SS residual}/(n - m))/(\text{SS total}/(n - 1))]$, where SS residual denotes the error sums of squares, SS total denotes total sum of squares, $n$ is number of experimental points and $m$ is number of fitted parameters.

## Results

Fig. 1 shows the dependence of POD activity on pH in crude extracts of *P. omorika* needles at three different temperatures. The measured as well as simulated activities are presented. Under all three temperatures, the simulated activity/pH dependences, obtained by nonlinear regression analysis, had similar shape indicating the existence of two components. Therefore, using mathematical modeling, the curves were decomposed into two components. On the other side, at increasing temperatures the curves, as well as pH optima, shifted towards lower pH values. After decomposition, this shift was verified for both components and for all three temperatures. The results of nonlinear regression analysis of POD activity *versus* pH are presented in Table 1. The kinetic parameters for both components obtained by curve decomposition are presented. Parameter $Act_0$ of the first component did not depend on temperature. Although mathematical analysis showed two components, the second one had relatively large parameter estimation errors. The values of $K_{EH}$ increased for the first component approximately four times when comparing 4°C and 20°C, and 14 times comparing 20°C to 40°C. For the second component detected, decrease could not be justified again due to a large parameter estimation error. The values of $K_{EOH}$ increased approximately twice (4°C to 20°C) and 15% (20°C to 40°C) in case of the first component, while they decreased for the second component 74 times (4°C to 20°C) and a further 290 times (20°C to 40°C). For each component *i*, pH optimum given in Table 1 was calculated by Eq. (2) as

$$\frac{\partial(Act)}{\partial(pH)} = 0 \Rightarrow (pHopt)_i = 7 + 0.5\log_{10}\frac{K_{EOH,i}}{K_{EH,i}} \quad (3)$$

The values of pH optima for both components decreased with temperature (Fig. 1), i.e. by 0.15 (4°C to 20°C) and 0.25 (20°C to 40°C) for the first component, while the decreases were 0.46 (4°C to 20°C) and 0.12 (20°C to 40°C) for the second component.

**Table 1.** Non-linear regression results of dependence of POD kinetic parameters on pH, using guaiacol as a substrate, in crude extract of *P. omorika* needles, at three different temperatures

| Parameters | Temperature (°C) | | |
|---|---|---|---|
| | 4 | 20 | 40 |
| $Act_{0,1}$ (dA/min )<br>$Act_{0,2}$ (dA/min ) | 0.376 (0.142)<br>0.284 (0.709) | 0.380 (0.007)<br>6.159 (53.096) | 0.371 (0.053)<br>34702 ($2.2 \times 10^{11}$) |
| $K_{EH,1}$ (mol/l )<br>$K_{EH,2}$ (mol/l ) | $5.697 \times 10^{-6}$ (0)<br>$6.407 \times 10^{-5}$ ($2.236 \times 10^{-4}$) | $2.388 \times 10^{-5}$ (0)<br>$7.210 \times 10^{-6}$ ($6.322 \times 10^{-5}$) | $3.434 \times 10^{-4}$ ($1.217 \times 10^{-4}$)<br>$3.706 \times 10^{-12}$ ($2.360 \times 10^{-5}$) |
| $K_{EOH,1}$ (mol/l )<br>$K_{EOH,2}$ (mol/l ) | $2.264 \times 10^{-8}$ (0)<br>$2.246 \times 10^{-10}$ (0) | $4.687 \times 10^{-8}$ (0)<br>$3.042 \times 10^{-12}$ (0) | $5.409 \times 10^{-8}$ (0)<br>$1.053 \times 10^{-14}$ (0) |
| $pH_{opt,1}$<br>$pH_{opt,2}$ | 5.80<br>4.27 | 5.65<br>3.81 | 5.40<br>3.69 |
| $N_{obs}$ | 9 | 9 | 9 |
| $R^2_{adj}$ (%) | 85.0 | 99.7 | 90.1 |

$Act_0$, product of the the rate constant for the enzyme-substrate reaction (*ks*) and total enzyme concentration ($En_{tot}$); $K_{EH}$, the enzyme-$H^+$ equilibrium constant; $K_{EOH}$, the enzyme-$OH^-$ equilibrium constant; subscripts 1 and 2 refer to the two fitting components, defined in Eq. (2). The parameters estimation errors are given in parentheses.

The dependence of POD activity in the acidic and basic fractions on pH is presented in Fig. 2. The data can be examined from two aspects. From the point of view of the substrates used in the experiments, the shape of POD activity *versus* pH, as well as pH optima are similar for guaiacol and coniferyl alcohol in each fraction. On the other side, pH optima differ between the acidic and basic fractions, i.e. the acidic fraction attains maximum activity at pH 5.5, while maximum of the basic fraction is at pH 5.0 for both substrates. However, after nonlinear regression (Fig. 3) it is obvious that there is no considerable difference between pH optima of the acidic (5.44 and 3.67) and basic (5.42 and 3.64) fractions.

Fig. 3 shows the pH-dependent POD activity for crude extract and its two fractions, at the same experimental temperature (20°C), simulated activity *versus* pH curves, as well as the activity components obtained by nonlinear regression analysis of the experimental curves. When subjected to two-component decomposition, the obtained pH optima for crude extract, acidic and basic fractions had similar values. Table 2 shows kinetic parameters of the pH-dependent POD activity of acidic and basic fractions in the extract of *P. omorika* needles. In the case of reaction with both substrates, the parameter $Act_0$ for the first component was higher in the acidic than in basic fraction, 2.4 times for

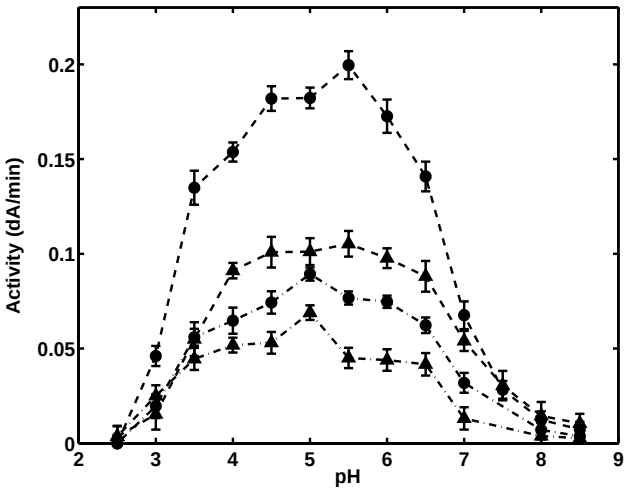


**Figure 2.** Dependence of the POD activity in the acidic (dashed line) and basic (dash-dotted line) fraction on pH, using guaiacol (●) and coniferyl alcohol (▲) as the substrates.

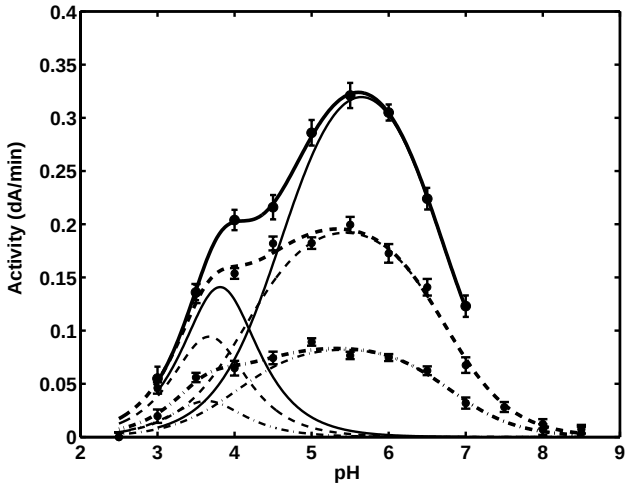


**Figure 3.** Measured (●) and simulated (lines) POD activity as a function of pH, using guaiacol as the substrate, in crude extract (solid line), acidic (dashed line) and basic (dash-dotted line) fraction from *P. omorika* needles, as well as corresponding regression components.

**Table 2.** Non-linear regression results of dependence of POD kinetic parameters on pH, in acidic and basic fraction of the extract from *P. omorika* needles, for two substrates, at 20°C

| Parameters | Guaiacol | | Coniferyl alcohol | |
|---|---|---|---|---|
| | Acidic fraction | Basic fraction | Acidic fraction | Basic fraction |
| $Act_{0,1}$ (dA/min) | 0.211 (0.001) | 0.089 (0.006) | 0.108 (0.004) | 0.059 (0.008) |
| $Act_{0,2}$ (dA/min) | 361 633 ($8.376 \times 10^{11}$) | 62 465 ($1.116 \times 10^{11}$) | 79 777 ($1.41 \times 10^{11}$) | 217 273 ($4.99 \times 10^{12}$) |
| $K_{EH,1}$ (mol/l) | $7.634 \times 10^{-5}$ ($4.953 \times 10^{-5}$) | $9.882 \times 10^{-5}$ ($1.284 \times 10^{-4}$) | $8.025 \times 10^{-5}$ ($1.528 \times 10^{-4}$) | $2.370 \times 10^{-4}$ (0.001) |
| $K_{EH,2}$ (mol/l) | $1.154 \times 10^{-10}$ ($2.67 \times 10^{-4}$) | $2.601 \times 10^{-10}$ ($4.645 \times 10^{-4}$) | $1.391 \times 10^{-10}$ ($2.458 \times 10^{-4}$) | $6.031 \times 10^{-11}$ (0.001) |
| $K_{EOH,1}$ (mol/l) | $5.351 \times 10^{-8}$ (0) | $6.292 \times 10^{-8}$ (0) | $1.178 \times 10^{-7}$ (0) | $4.261 \times 10^{-8}$ (0) |
| $K_{EOH,2}$ (mol/l) | $2.245 \times 10^{-17}$ (0) | $4.240 \times 10^{-17}$ (0) | $9.227 \times 10^{-17}$ (0) | $5.233 \times 10^{-18}$ (0) |
| $pH_{opt,1}$ | 5.44 | 5.42 | 5.58 | 5.13 |
| $pH_{opt,2}$ | 3.67 | 3.64 | 3.91 | 3.47 |
| $N_{obs}$ | 12 | 12 | 13 | 12 |
| $R^2_{adj}$ (%) | 98.2 | 96.5 | 98.7 | 85.2 |

All results are significant. Explanations of parameters are given in Table 1.

guaiacol and 1.8 times for coniferyl alcohol. Unfortunately, large parameter estimation errors prevented us from drawing any conclusions for the second component. In the case of both substrates, no statistically significant differences were detected for the $K_{EH}$ of both components between the acidic and basic fraction. For guaiacol, the value of $K_{EOH}$ for the basic fraction was similar to that of the acidic fraction in case of both components. For coniferyl alcohol, $K_{EOH}$ was 2.8 times lower for the basic fraction than the acidic one in the case of the first component, while it was 17.6 times lower for the second component.

## Discussion

The distribution of experimental points of POD activity measured *versus* pH change in the extract of *P. omorika* needles at different temperatures (Fig. 1) visually indicated the existence of two components. This could further imply an involvement of two amino acids in the catalysis/binding of substrate, presence of at least two isoenzymes or a complex reaction mechanism involving two different forms of oxidized peroxidase (Maehly 1955; Azevedo et al. 2003). Due to the fact that even after purification two peaks continue to exist it is more likely that a complex reaction mechanism is causing the two peaks in the POD activity curve. A two-component decomposition of the activity curves was therefore applied at different temperatures, resulting in high $R^2_{adj}$ values for all three temperatures and showing that the model accounted for 85% variance of POD activity at 4°C, for 99.7% at 20°C and 90.1% at 40°C. The curves obtained by the described mathematical procedure, as well as related components, show a shift towards lower pH values with temperature increase. This may be an effect of thermally-induced fluctuations on intramolecular interactions which are connected with amino acids in the active site. These thermally-induced fluctuations can change the microenvironment of catalytic/binding amino acids and their ionization constants. Temperature had greater effect on the low-pH component, which is visible from the shift of $pH_{opt,2}$ values (Table 1). Similar approach has been applied for analysis of enzyme activity as a function of pH and temperature. Tijskens et al. (2001) reported modeling of temperature and pH dependence of phytase activity using a nonlinear regression model. Seyhan et al. (2002) analyzed temperature and pH dependence of POD and lipase activity in Turkish hazelnut, using the same model.

Large parameter estimation errors for the parameters $Act_0$ and $K_{EH}$ of the second component (Table 1) were probably due to the small number of experimental points and low activity values, or it may be due to a relative applicability of the adopted two component model to the analyzed plant extract used in this experiment. The complexity of our experimental samples might include either the involvement of two amino acids in the catalytic process, different isoenzymes, or enzyme catalytic forms. Consequently, it was difficult to draw conclusions about the effect of temperature on $Act_{0,2}$ and $K_{EH,2}$ values. All equilibrium constants in Eq. (2) – $K_{EH}$, $K_{EOH}$, $K_W$ may depend on temperature under the Arrhenius law. Due to thermal fluctuations of protein structure, ionization constants of the catalytic/binding amino acids can be changed. This can contribute to the values of constants changing with temperature.

Both substrates used for POD reaction are of a phenolic type, coniferyl alcohol being the natural substrate, while guaiacol is the most preferred artificial substrate. This partly explains the similar form of the pH-related activity change for particular POD fractions (Fig. 2), which may reflect

the same mechanism of reaction. Small differences in pH optima between the two fractions may lead to a conclusion that the active enzyme sites are different, which less is likely considering conservation of the catalytic site during evolution (Azevedo et al. 2003). Another explanation may be that small changes in the molecular microenvironment of the catalytic sites, taking place as a result of different titrations of amino acids in the vicinity of active site, are responsible for such behavior.

Considering the fact that there was no pronounced activity maximum in the lower pH region (3 – 4; Fig. 3) for the acidic and basic POD fractions of *P. omorika* needles, it is necessary to use the present model in order to obtain precise data on all pH optima. This approach also prevents possible misinterpretations when relying on the initial dependences of POD activity on pH only by visualization.

Similarity in the values of pH optima for the activity components obtained for crude extract, and acidic and basic fractions (Fig. 3) indicates that the same components take part in the activity *versus* pH dependence in the case of crude, as well as two fractions. These components can originate from two amino acids in one active site of two different catalytic (oxidative) forms of peroxidase in reaction cycle. This can lead us to a conclusion that modeling of pH-related POD activity gives us a superior tool for analyzing a number of components, which can imply either a number of amino acids involved in catalytic mechanism, or a number of catalytic forms of an enzyme. This further may contribute to prediction of changes in enzyme activity. Similarity in pH optima of the activity components obtained for the crude extract, acidic and basic fractions (Fig. 3) may indicate that all POD isoforms in this extract have conserved the catalytic site and retained the same or similar mechanism of catalysis. It means that experimentally observed differences in pI values between the acidic and basic fractions originate from the amino acids located on enzyme surface, rather than from the amino acids involved in catalysis. The small differences in amino acid sequence of the isoenzymes which catalyze the same biochemical reaction, may lead to distinct physical, chemical and kinetic properties (Soltis and Soltis 1990).

The $R^2_{adj}$ values obtained for decomposition of both the acidic and basic fraction activities, in the case of both substrates, were high (Table 2), meaning that the applied model accounted for 98.2% POD activity variance for the acidic fraction and 96.5% for the basic fraction regarding guaiacol. For coniferyl alcohol, the values were 98.7% for the acidic and 85.2 % for the basic fraction. The higher $Act_{0,1}$ value for the acidic than basic fraction, in the case of both substrates, comes from the higher specific/total activity of the acidic fraction than that of the basic fraction. Similar values of $K_{EH}$ between the acidic and basic fractions for both substrates, as well as similar values of $K_{EOH}$ for guaiacol and different for coniferyl alcohol in the two fractions, may be due to different interaction of POD with these two substrates as a consequence of fine differences in their structure.

Our results confirmed, for the first time, that the sum of components, each described by the mathematical model employed Eq. (2), can describe the complex POD activity/pH dependence in the crude extract, acidic and basic fractions of *P. omorika* needles, where two or more enzyme forms are active. The nature of the two components existing in all three fractions is to be characterized and looked into more closely in further studies.

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