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Enzymatic transformation of sinapine using polyphenol oxidase from *Trametes versicolor*. Effect of pH and temperature and model development

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Abstract

Sinapine, a choline ester of sinapic acid and a main component of the phenolic fraction of rapeseed meals, was enzymatically transformed by an enzyme secreted by a white rot fungus *Trametes versicolor*. A model based on the Theorell–Chance Bi–Bi mechanism that describes the effect of pH, temperature, substrates and enzyme concentrations on the initial reaction rate was developed. The model parameters were estimated from the data regarding the effect of pH and temperature on initial reaction rates using a two-step estimation procedure that was developed in this work. The model predicts experimental data fairly well, and is valid for any pH and temperatures ranges. The optimum pH and temperature of reaction determined experimentally and confirmed by the model are 4.24 and 50 °C, respectively. However, when the effect of temperature on the oxygen solubility is not considered, i.e. oxygen is not the limiting substrate, the model shows that the optimum temperature of reaction is 60 °C. A relation between the temperature and the optimum pH of reaction was proposed.The developed model was used to predict the dynamics of sinapine transformation. The results showed that the investigated enzymatic system includes additional enzymatic reactions between oxygen and the products of sinapine transformation.

Keywords: Sinapine; Polyphenol oxidase; Enzymatic transformation; Model

1. Introduction

Sinapine (SIN), a choline ester of sinapic acid, is the main component of the phenolic fraction of rapeseed meals. It accounts for up to 70% of all sinapic acid esters in canola meal (CM) [1]. It is responsible for the bitter taste of these commodities [2]. Furthermore, the production of tainted eggs is observed when its level in the diet of brown-egg-laying hens exceeds 0.2% [3–5]. This is an important issue for countries such as Great Britain, where almost all of the eggs produced have brown shells [6]. In Canada, where the production of CM reached a level of more than 1 million tons per year [7], the possibilities of replacing soybean products with canola substitutes are being investigated, mainly because of the very good protein composition in CM. One of the requirements for CM to become a sole source of nutrients is to reduce its sinapine level by at least 90% [8]. In this respect, a need for adequate meal quality enhancement processes is still an open issue. The enzymatic reduction of sinapine andother phenolics contents can be an alternative to existing methods that are not fully successful [5,9,10]. We have been investigating such a process using an enzyme preparation secreted by a white rot fungus *Trametes versicolor*. It was reported that this enzyme catalyzed the transformation of sinapic acid derivatives, including sinapine [11]. It was of interest to develop a model that could predict the enzymatic degradation of the sinapine content in CM. Such a model should account for the effects of substrates concentration, pH of the solution and temperature on the enzymatic transformation of sinapine. When the effect of temperature on the reaction rate is investigated, the effect of this variable on the initial oxygen concentration has to be taken into account since oxygen is the second substrate in this reaction.

The purpose of this work was the development of a mathematical model that simultaneously describes the effects of pH, temperature, substrates and enzyme concentrations on the initial rate of the sinapine transformation. The model development was based on the initial rate measurement data

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that were collected in this study. The applicability of this model to predict the dynamics of the sinapine transformation was also considered.

2. Materials and methods

2.1. Enzyme production

A crude enzyme preparation that was used in this study was produced by growing *Trametes versicolor* ATCC 42530 in a semi-defined medium containing (%, w/v): yeast extract (0.5), nutrient broth (0.8), glucose (2.0) and Vogel's minerals (0.5). The culture was incubated at 28 °C in a rotary shaker at 160 rev min^{-1}. The 16-day-old culture broth was centrifuged at 10 000 **g** at 4 °C, and the filter-sterilized supernatant served as the crude enzyme preparation. The enzyme activity was monitored using ferulic acid as a substrate [12].

2.2. Chemicals

Sinapine was purified from commercial canola meal according to Clandinin's method [13]. All other chemicals were of reagent grade.

2.3. Determination of polyphenol oxidase activity

Polyphenol oxidase activity was measured by following changes in the absorbance (328 nm) of the reaction mixture for a given interval of time after enzyme addition. The reacting system consisted of 0.7 ml (0.5 mM) sinapine bisulfate solution, 2.2 ml phosphate–citrate buffer (0.1 M) and 0.1 ml enzyme preparation. The reaction was carried out in 20-ml test tubes placed in a water bath. The buffer and sinapine solutions were heated up to the reaction temperature prior to the addition of enzyme. The reaction was stopped by adding 0.1 ml of 66% trichloroacetic acid (TCA). The solution was transferred to a quartz spectrophotometric cell and the absorbance was recorded at 328 nm. The initial absorbance was measured using the same system to which TCA was added before the enzyme. The enzyme activity was expressed in terms of nanokatals (nmol s^{-1}) [11]. The pH and temperature ranges in these tests were from 2.5 to 7.3 and from 283.2 to 353.2 K, respectively.

The effect of pH was examined at each temperature level. Changes of pH due to the temperature of the system were taken into account using the ATC (automatic temperature compensation) mode on the pH meter. The data were collected under 85 different reaction conditions. Each test was repeated three times. All of the results were used for a parameter estimation procedure using the nonlinear least-squares method based on the Levenberg–Marquardt algorithm [14].

2.4. Dynamics of the sinapine transformation

A 125-ml custom-built glass no-head gas space reactor with a water jacket was filled with a solution of sinapine bisulfate in phosphate–citrate buffer with a desired initial oxygen concentration. The system temperature was controlled by a Haake F3 controller that circulated water through the jacket of the reactor. A magnetic stirrer was used to maintain constant mixing during the reaction. A detailed description of the system is given in [15]. After attaining the required temperature the reaction was started by adding a desired amount of the enzyme stock solution through the sampling port. Changes in the oxygen concentration were measured using a Clark's electrode hooked to a YSI 54 dissolved oxygen meter with a digital readout. Changes in sinapine concentration were measured as follows: 20-μl samples of the reacting solution were withdrawn from the reactor through the sampling port and transferred into 50-μl sampling vials with septum caps containing 5 μl 1 N HCl to stop the reaction. The samples were analyzed for sinapine using high-performance liquid chromatography (HPLC). The extent of oxygen disappearance due to the presence of the Clark's electrode was negligible.

2.5. High-performance liquid chromatography separation

Samples (10 μl) were analyzed using a Waters HPLC system that consisted of a Waters 717 autosampler, a Waters 486 tunable absorbance detector and a Waters 410 solvent delivery pump. The data were acquired at a wavelength of 328 nm and analyzed using the Millennium Waters chromatography system manager. A 120×4.6 mm I.D. Supelcosil LC-8 (5 μm) column was used. The mobile phase consisted of solvent A (0.01 M *n*-dibutylamine and 0.01 M sodium heptanesulfonic acid (pH 2.2)) and solvent B (0.005 M *n*-dibutylamine and 0.005 M sodium heptanesulfonic acid (pH 2.2) modified with 50% acetonitrile). The composition of the mobile phase was changed linearly for 30 min from 2:8 to 7:3 of solvent A to solvent B, then was brought back to the initial composition and the column was equilibrated for 10 min. The flow rate was 1 ml min^{-1}. The temperature of separation was 50 °C.

2.6. Development of the model

It is assumed that the enzymatic transformation of sinapine is an example of the Theorrel–Chance Bi–Bi system [15] where oxygen [O_2] and sinapine [SIN] combine with the enzyme [E] in an obligate order (Fig. 1). Neglecting the presence of products in the reaction mixture, the initial activity equation derived from a steady-state assumption is given by Eq. (1) [16]:

$$v = \frac{V_{max}}{1 + \frac{K_{m,O_2}}{[O_2]} + \frac{K_{m,SIN}}{[SIN]}\left(\frac{K_{i,O_2}}{[O_2]} + 1\right)} \quad (1)$$

Eq. (1) describes the Michaelis–Menten kinetics and is valid for any enzyme concentration for which a linear proportionality of the activity with respect to the enzyme con-

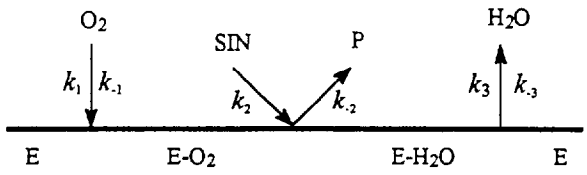

Fig. 1. Theorell–Chance Bi–Bi mechanism for the transformation of sinapine by polyphenol oxidase from *Trametes versicolor*.

centration holds. In order to use Eq. (1) to predict simultaneous effects of the temperature, pH, enzyme and both substrate concentrations on the initial reaction rate, the following assumptions were made.

(i) The enzyme can be considered a dibasic acid which exists in three forms obtained through the protonization and deprotonization of the active site, with only one form (E^-) being active [17]

$$E^{-2} \overset{K_2}{\leftrightarrow} E^- \overset{K_1}{\leftrightarrow} E^0$$

(ii) The thermal deactivation of the active form of enzyme is described by a simple first-order irreversible deactivation mechanism

$$E_a^- \overset{k_d}{\rightarrow} E_d^-$$

(iii) All of the rate constants are defined by the transition state theory

$$k_i = \alpha_i \left(\frac{k_b T}{h}\right) e^{\Delta S_i^{\ddagger}/R} e^{-E_{i,a}/RT} = k_{0,i} T e^{-E_{i,a}/RT}$$

(iv) The protonization and deprotonization dissociation constants are given by the following equation [18]:

$$\ln K_i = -\frac{\Delta G_i^0}{RT}$$

(v) All of the enzyme kinetic parameters are independent of pH [17].

(vi) The concentration of oxygen in the system (mM) at a given temperature is calculated from its solubility, assuming constant (temperature independent) water density and neglecting the effects of salts concentrations (buffer plus sinapine) on oxygen solubility

$$[O_2] = \frac{n_{O_2}}{1 - n_{O_2}} \frac{0.21}{18.0} 10^6$$

where n_{O_2} is the solubility of oxygen at a particular temperature and at an air partial pressure of 101.325 kPa, and is given by the following expression [19]

$$n_{O_2} = e^{-64.2152 + \frac{83.9123}{T/100} + 23.2432 \log \frac{T}{100}}$$

3. Results and discussion

The assumption regarding the mechanism of the reaction that leads to Eq. (1) was based on the fact that the enzymatic transformation of sinapic acid by the same enzyme preparation could be described by the Theorell–Chance Bi–Bi mechanism [15]. Thus, the overall model predicting the initial reaction rates for different conditions was based on Eq. (1). The model was developed in two steps in which the effect of pH and temperature were evaluated separately, and the relationships obtained were combined to get an overall model.

3.1. Effect of pH

It can be shown that using definitions of the dissociation constants, K_1 and K_2, (assumption (i)) and assuming that the overall enzyme concentration does not change, the concentration of the active form of the enzyme is given by Eq. (2) [17]

$$e_0^- = \frac{e_0}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}} \tag{2}$$

Since the initial reaction rate is directly proportional to the enzyme concentration, the combined Eqs. 1 and 2 can be used for the evaluation of initial rates at a given temperature for any hydrogen ion concentration.

3.2. Effect of temperature

To evaluate the effect of temperature on the reaction rate, the relationships between the enzyme kinetic parameters and temperature have to be known. The enzyme thermal deactivation has to be taken into account as well. Here it was assumed that the enzyme deactivates irreversibly. Its deactivation is described by Eq. (3). This type of deactivation mechanism was chosen on the basis of the experimental results [11].

$$e_a^-(t) = e_0^- \, e^{(-k_d t)} \tag{3}$$

Eq. (3) cannot be used in this form in the development of the model based on Eq. (1) because it is impossible to directly incorporate any transient effects into the evaluation of the initial rate measurements. Therefore, a different approach that would account for the enzyme irreversible deactivation process had to be proposed. If the enzyme concentration of the active form can be expressed as a time-average concentration during the reaction, then for any reaction time, t_r, at which the initial rate is measured, such an average enzyme concentration is given by Eq. (4)

$$\langle e_a^- \rangle = \frac{\int_0^{t_r} e_a^- \, dt}{\int_0^{t_r} dt} = e_0^- \frac{1 - e^{-k_d t_r}}{k_d t_r} \tag{4}$$

It should be noted that such a treatment of the deactivation process, i.e. a constant enzyme concentration at a given temperature, is not different from a mathematical point of view from that described by the reversible mechanism that is usually applied for the evaluation of the effect of temperature on enzyme activity [17]. In both cases the enzyme concentration

is assumed to be constant during the reaction. However, the proposed method gives a more realistic picture of the deactivation process.

If Eqs. (4) and (2) are combined and substituted into Eq. (1), the following expression for the initial reaction rates at a constant temperature and at any pH is obtained:

$$v = \frac{\beta k_3 e_0}{1+\frac{h^+}{K_1}+\frac{K_2}{h^+}} \frac{1-e^{-k_d t_r}}{k_d t_r} \frac{1}{1+\frac{K_{m,O_2}}{[O_2]}+\frac{K_{m,SIN}}{[SIN]}\left(\frac{K_{i,O_2}}{[O_2]}+1\right)} \tag{5}$$

In order to use Eq. (5) to predict the initial rate at any temperature, the parameters k_3, k_d, K_1, K_2, K_{m,O_2}, K_{i,O_2} and $K_{m,SIN}$ had to be expressed as functions of this variable. Using assumptions (iii) and (iv), and expressing the enzyme kinetic parameters as the ratios of respective rate constants, the overall model that predicts the initial reaction rates at any temperature, pH, enzyme and sinapine concentrations is given by Eq. (6)

$$v = \frac{\beta e_0}{1+\frac{e^{\Delta G_2^0/RT}}{h^+}+\frac{h^+}{e^{\Delta G_1^0/RT}}} \frac{1-e^{-t_r k_{0,d} T e^{-E_d/RT}}}{t_r k_{0,d} T e^{-E_d/RT}} \times \frac{k_{0,3} T e^{-E_{a,3}/RT}}{1+\frac{\frac{k_{0,3}}{k_{0,1}} e^{-\Delta E_{a,13}/RT}}{[O_2]}+\frac{\frac{k_{0,3}}{k_{0,2}} e^{-\Delta E_{a,23}/RT}}{[SIN]}\left[1+\frac{\frac{k_{0,-1}}{k_{0,1}} e^{-\Delta E_{a,-11}/RT}}{[O_2]}\right]} \tag{6}$$

Eq. (6) has a more complicated form but for the sake of simplicity of representation the oxygen concentration (assumption (vi)) was not expressed as a function of temperature. The more complicated form of Eq. (6) was used for the parameter estimation.

3.3. Parameter estimation

The parameters in the Eq. (6) were estimated using nonlinear regression analysis based on the Levenberg–Marquardt algorithm. However, before any estimation was performed some issues related to the estimation procedure had to be resolved.

First of all, because of the complicated form of the temperature response (many exponential functions), a transformation of the parameters in Eq. (6) was performed. The transformation was done according to Eq. (7), which represents a recommended parameter transformation [20] that helps to overcome difficulties related to the estimation problem often met in the estimation of Arrhenius-like relationships.

$$k_i = k_i^* \, e^{\left[-E_{i,a}\left(\frac{1}{T}-\frac{1}{T^*}\right)\right]} \tag{7}$$

Secondly, the problem related to the lack of any information about the initial values for most of the parameters had to be addressed because very often the convergence of a nonlinear estimation technique depends severely upon the right choice of the initial estimates [21]. Possible ways for generating proper initial guesses are: information from other relevant experiments, a grid search, or a so-called multistage estimation. The last technique relies upon breaking the data set into groups so that auxiliary parameters for each group may be determined [21]. To some extent, all three techniques were employed in this work. It should be pointed out that the parameter transformation used in this work (Eq. (7)) was also useful in overcoming the initial guess problem. For instance, if the value of a rate constant is known at one temperature, then setting T^* equal to this temperature results in the parameter k_i^* being equal to that known rate constant. Then the initial guess for the respective energy of activation can be found by keeping k_i^* constant and performing the estimation with the number of parameters reduced by half.

The estimation of the parameters in the overall model was carried out in two steps. In the first step, the data set was broken into groups of results pertaining to the effect of pH at constant temperature. These groups were used to evaluate the dissociation constants, K_1 and K_2, at a given temperature, according to the new method developed in this work. In the second step, the effect of temperature was analyzed. Instead of performing the estimation either on the entire data set or on the groups of data pertaining to the effect of temperature at constant pH, a new approach based on the results obtained in the first step was proposed.

The principle for the first step was based on the fact that for any given temperature an optimum hydrogen ion concentration (h^+_{opt}) exists for which the initial activity is at its maximum and equal to V_{max}^{opt}. Starting with Eq. (6) it can be shown that the optimum hydrogen ion concentration, h^+_{opt}, is given by

$$h^+_{opt} = \sqrt{K_1 K_2} = 10^{-pH^{opt}} \tag{8}$$

According to assumption (v), the ratio of maximum activity at a given pH to the respective activity at the optimum pH is equal to the ratio of initial rates at respective pH values, regardless of the substrate concentration, and is given by

$$\frac{V_{max}}{V_{max}^{opt}} = \frac{v}{v_{pH}^{opt}} = \frac{1+2\sqrt{K_2/K_1}}{1+\frac{K_2}{h^+}+\frac{h^+}{K_1}} \tag{9}$$

A similar expression was proposed by Segel [16], although the concept behind his formula was to give another method for the evaluation of K_1 and K_2; that method required prior knowledge of pH_{opt} as well as V_{max}. After rearranging, Eq. (9) can be used to estimate the three unknown parameters, v_{pH}^{opt}, K_1 and K_2 from the initial rate measurements at constant temperature and various pHs. These parameters were estimated for each temperature (Table 1) and used in Eq. (9) to predict the effect of pH on the initial activity (Fig. 2). The predicted data agree fairly well with the experimental results and, therefore, the data from Table 1 can be used to obtain a

relationship between the dissociation constants and temperature. As presented in Fig. 3, the anticipated straight line relationship between the natural logarithm of the dissociation constants values and the reciprocal of temperature, usually obtained when the standard enthalpy of change is temperature independent, was not observed. On the other hand, when the dissociation constants are defined using the standard Gibbs energy of reaction, which depends on temperature according to Eq. (10), the data presented in Fig. 3 can be described adequately.

$$\Delta G_i^{(\circ)} = J_i - RT\left(\Delta A_i \ln T + \frac{\Delta B_i}{2}T + \frac{\Delta C_i}{6}T^2 + \frac{\Delta D_i}{2T^2} + I_i\right) \quad (10)$$

However, in order to obtain intuitively acceptable results for the respective dissociation constants outside the range of tested temperatures, the number of parameters in Eq. (10) was decreased by setting ΔA and ΔD equal to zero. This resulted in a negligible decrease in the accuracy of fit, but allowed us to get as general a form of the model as possible. Therefore, the following relationship between dissociation constant and temperature was used in the overall model:

Table 1
Values of the estimated parameters in Eq. (1)

T/K	K_1	Std. error	K_2	Std. error	v_{pH}^{opt}	Std. error
283.2	1.03E−03	1.28E−04	1.01E−05	1.08E−06	1.79E−01	3.27E−03
293.2	4.40E−04	4.63E−05	8.81E−06	8.76E−07	2.45E−01	4.45E−03
303.2	1.95E−03	2.40E−04	3.60E−06	3.54E−07	4.05E−01	6.08E−03
313.2	1.38E−03	9.63E−05	3.07E−06	1.94E−07	0.45171	4.32E−03
323.2	1.45E−03	9.44E−05	1.63E−06	1.10E−07	4.66E−01	3.98E−03
333.2	8.95E−04	9.03E−05	1.41E−06	1.67E−07	4.47E−01	6.73E−03
338.2	4.41E−04	4.73E−05	2.31E−06	2.57E−07	4.15E−01	7.62E−03
343.2	3.01E−04	5.71E−05	2.16E−06	4.84E−07	3.94E−01	1.38E−02
353.2	4.40E−05	7.01E−06	5.28E−06	9.54E−07	2.18E−01	6.09E−03

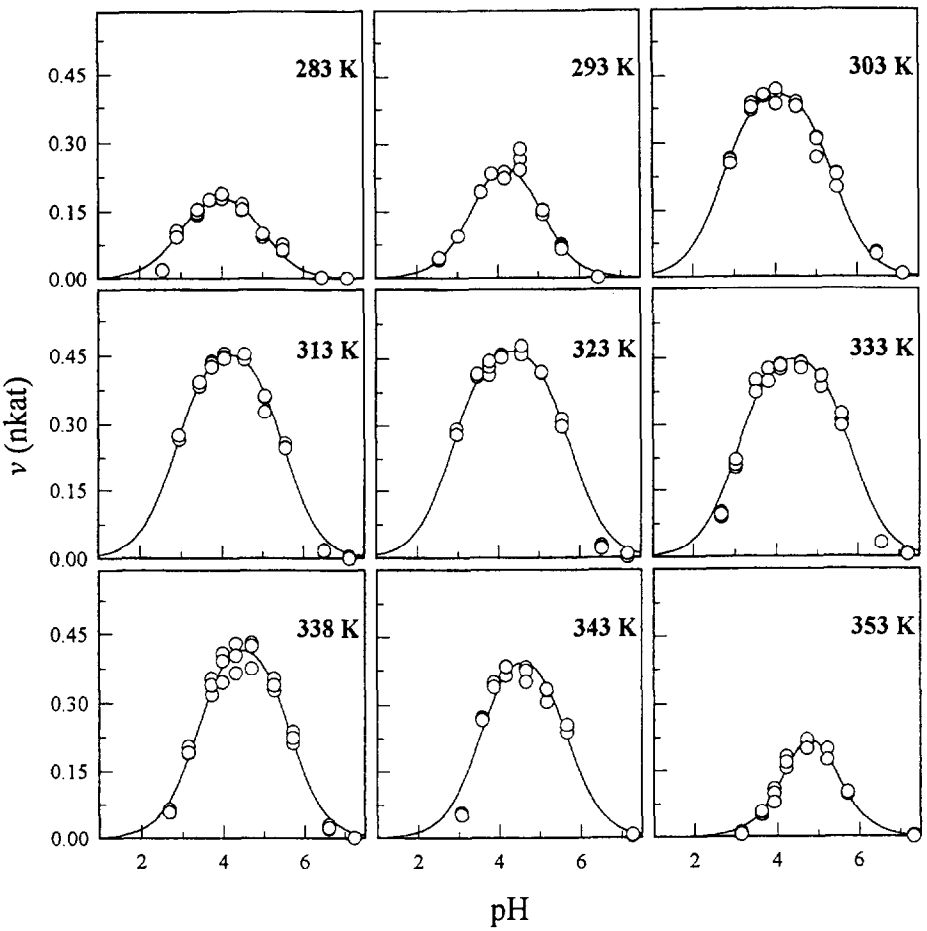

Fig. 2. Effect of pH on the enzyme activity at various temperatures of the system. Symbols represent experimental data, solid lines are predicted values using Eq. (9) with the values of parameters from Table 1.

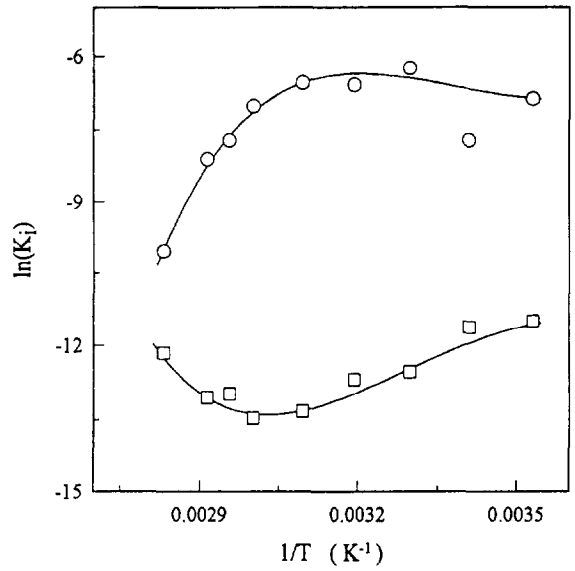

Fig. 3. Relationship between dissociation constants K_1 and K_2 and temperature. Solid lines are predicted data by Eq. (11).

$$K_i = e^{(-J_i/RT + \Delta B_i T/2 + \Delta C_i T^2/6 + I_i)} \quad (11)$$

The estimated values of the parameters in Eq. (11), for each of the dissociation constants, are given in Table 2. These values can be used for the calculation of enthalpy and entropy changes of enzyme protonization and deprotonization reactions. Although from a thermodynamic point of view a deviation from a straight line might be expected [18], the deviation shown in Fig. 3 seems to be rather high. This indicates that the proposed model for pH dependency is an over simplification of the actual situation. Nevertheless, Eq. (11) was used in the final form of the proposed overall model.

Having established K_1 and K_2 as functions of temperature, the next step was to find the initial estimates for the other parameters in Eq. (6). The values for the deactivation energy, E_d, and frequency factor, $k_{0,d}$, were taken from the results obtained in the enzyme deactivation experiments. The respective values for the rate constants k_1 and k_{-1} were taken from relevant experiments regarding enzymatic transformation of sinapic acid. Other initial values of parameters were educated guesses. A rough idea of their values could be obtained using the same technique as used in the first step. That method would require the division of the whole data set into groups of data regarding runs at constant pH, evaluation of the parameters in Eq. (6) for each pH level and then calculation of an average value for each of the parameters. Instead of using this method a new approach was proposed. The estimation of parameters was performed on a special data set created using the estimation results regarding the runs at constant temperature (Table 1). Combined Eqs. (6) and (8) yield Eqs. (12a) and (12b) that allow the introduction of a corrected initial reaction rate, Y_c, that can be calculated using data from Table 1 (Eq. (12a)), and can also be expressed as a function of temperature using all of the parameters of interests (Eq. (12b)).

$$Y_c = v_{pH}^{opt}(1 + 2\sqrt{K_2/K_1}) \quad (12a)$$

$$Y_c = \frac{1 - e^{-t_r k_{0,d} T}\, e^{-E_d/RT}}{t_r k_{0,d} T\, e^{-E_d/RT}} \times \frac{\beta e_0 k_{0,3} T\, e^{-E_{a,3}/RT}}{1 + \frac{k_{0,1}}{k_{0,3}} e^{-\Delta E_{a,13}/RT} \frac{1}{[O_2]} + \frac{k_{0,2}}{k_{0,3}} e^{-\Delta E_{a,23}/RT} \frac{1}{[SIN]} \left[1 + \frac{k_{0,-1}}{k_{0,1}} e^{-\Delta E_{a,-11}/RT} \frac{1}{[O_2]}\right]} \quad (12b)$$

This newly introduced Y_c takes into account the effect of temperature on the optimum pH of the reaction. The data calculated using Eq. (12) are shown in Fig. 4. These data can be used to estimate the parameters in Eq. (12), and its simplicity is considered the greatest advantage of the proposed method (reduced CPU time). Furthermore, the values of the parameters obtained should be more accurate since the effect of temperature on the optimum pH is included in the data. However, after performing the initial estimation runs using this set of data, it appeared that addition of the data regarding the effect of substrate concentration on the initial activity at constant temperature was required (Fig. 4) in order to narrow the parameter space and to obtain more realistic values of the estimates. The estimates obtained after fitting Eq. (12) to this expanded set of data are given in

Table 2
Parameter estimates for the relation between dissociation constants, K_1 and K_2, and temperature

	J	ΔB	$\Delta C \times 10^3$	I
K_1	−1839322.4	15.272	−51.561	−2261.327
K_2	1721477.4	−13.756	45.240	2062.740

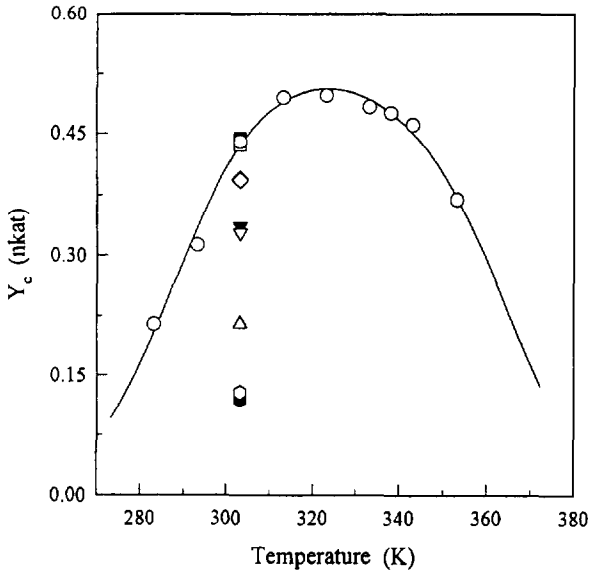

Fig. 4. Relationship between Y_c and temperature. Solid line as predicted by Eq. (12). Sinapine concentration: (○) 0.116 mM, data calculated from Table 1; (□) 0.116 mM; (◇) 0.087 mM; (▽) 0.058 mM; (△) 0.028 mM; (hexagon) 0.014 mM, data obtained at pH 3.7. Closed symbols predicted, open experimental.

Table 3
Estimated energies of activation for the respective rate constants[a]

	k_0	E_a/kJ mol^{-1}
k_1/mM^{-1} s^{-1}	0.1008	4.107
k_2/mM^{-1} s^{-1}	3.7181	10.113
k_{-1}/s^{-1}	8.748×10^{5}	53.379
k_3/s^{-1}	1.241×10^{9}	64.087
k_d/s^{-1}	7.079×10^{9}	105.622

[a] Enzyme concentration given by $e_0=\beta\times v_{enz}/V_{sys}\times dil$; $v_{enz}=0.1$ ml; $\beta=1.25\times10^{-4}$ mmol per ml of enzyme stock solution; $dil=0.05$.

Table 3. They were used to predict the effect of temperature on the Y_c for different sinapine concentrations (Fig. 4). The results obtained indicated that the proposed method resulted in realistic values of the parameters and a fairly good fit.

The values of the respective parameters from Tables 1 and 2 were used for the evaluation of the effects of pH and temperature on the initial activity using Eq. (6). The values obtained are presented in a form of activity-surface (Fig. 5). To check the adequacy of the proposed model, the predicted results were plotted against the experimental ones (Fig. 6). A random distribution of the results indicated that the proposed model predicts the experimental data fairly well, and its accuracy does not depend on the magnitude of the initial rate. Furthermore, a lack of any visible functionality in the residuals with respect to both pH and temperature (Fig. 7) indicates that the model is not restricted to any particular range of these variables.

3.4. Effect of temperature on the optimum pH of reaction

Another interesting result from the study of the effects of pH and temperature on the initial reaction rate was obtained. Since the quantities K_1 and K_2 are functions of temperature,

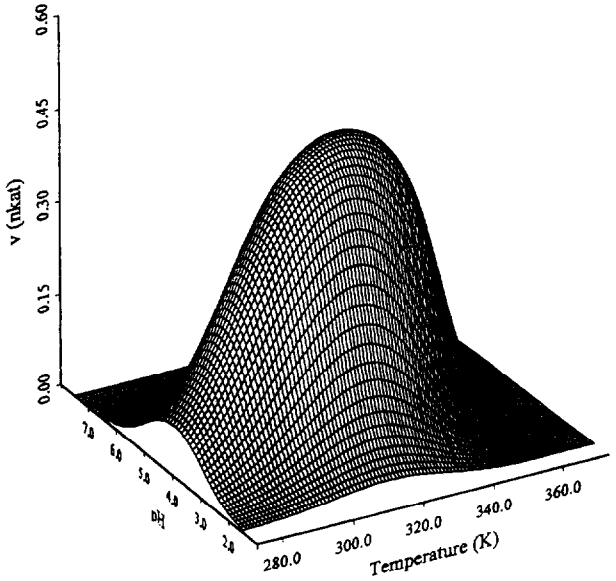

Fig. 5. Effects of temperature and pH on the sinapine oxidase activity in the model system predicted by Eq. (6) for sinapine concentration of 0.116 mM, enzyme dilution 20, and reaction time 10 min.

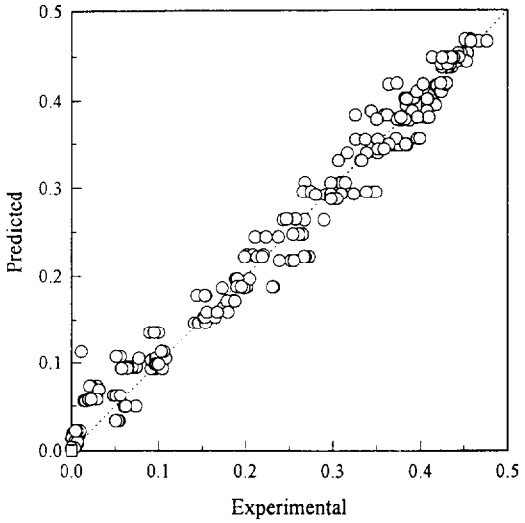

Fig. 6. Comparison between the experimental activities and those predicted by Eq. (6).

according to Eq. (8), the optimum pH for an enzymatic reaction also has to be temperature dependent. Combining Eqs. 8 and 11 yields Eq. (13), which is a general expression for the pH at which the reaction rate at any given temperature will be the highest. This equation is valid for any enzyme that can be considered a dibasic acid (assumption (i)). Fig. 8, a contour plot of activities extracted from Fig. 5, shows how the temperature of the reaction affects the optimum pH. This result implies that the reported optimum conditions for the enzymatic reaction, expressed in terms of optimum pH and temperature, should be closely examined since they are highly correlated. In other words, the optimum condition for the enzymatic reaction should be established from at least two sets of experiments that are focused on the evaluation of the effect of one variable (pH or temperature) at a fixed level of the other.

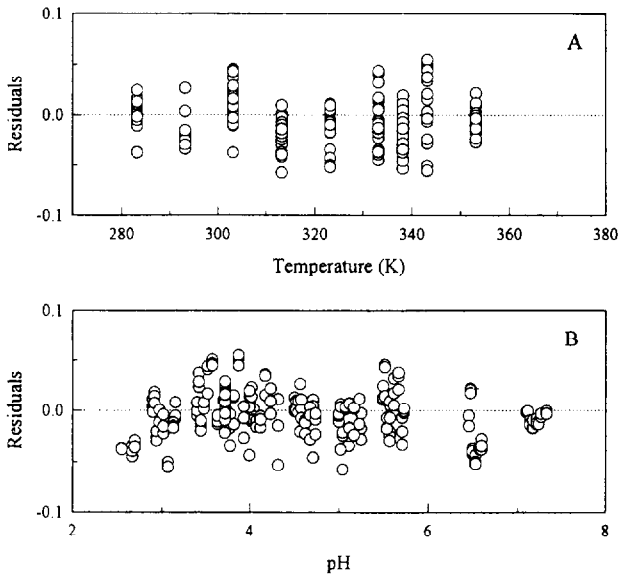

Fig. 7. Plots of residuals for the proposed model, with respect to: (A) temperature; (B) pH.

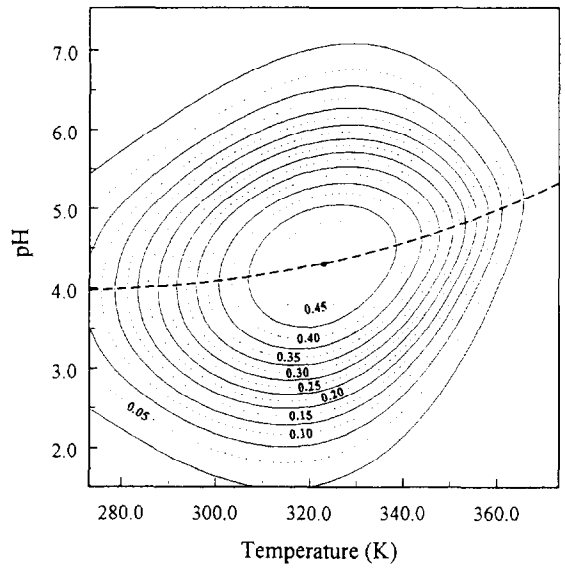

Fig. 8. Effect of temperature on the optimum pH of the enzymatic reaction (dashed line). Constant activity contours as predicted by Eq. (6). Activity expressed in nkat $(ml_{sys})^{-1}$.

$$pH_{opt} = -\log\sqrt{K_2K_1}$$
$$= -0.217\left(-\frac{J_1+J_2}{RT}+(\Delta A_1+\Delta A_2)\ln T + \frac{T}{2}(\Delta B_1+\Delta B_2)+\frac{T^2}{6}(\Delta C_1+\Delta C_2) + \frac{\Delta D_1+\Delta D_2}{2T^2}+I_1+I_2\right) \quad (13)$$

According to Eq. (6), the optimum pH and temperature for this enzymatic reaction are 4.25 and 50 °C, respectively. A detailed experimental study confirmed these results. On the other hand, regardless of the system temperature, if all of the runs were performed at a constant oxygen concentration, the optimum reaction temperature calculated using Eq. (6) would be 60 °C. Consequently, the optimum pH would change the value shown in Fig. 8 to 4.4.

3.5. Dynamics of sinapine transformation

Since, from an engineering point of view, the dynamics of sinapine transformation is of interest, the ability of the proposed model to predict the time changes in sinapine concentration was investigated. When the changes in the oxygen concentration in the system are negligible, the integrated form of Eq. (1) can be used to obtain a progress curve for sinapine. On the other hand, when the oxygen concentration changes during the reaction, the changes in the sinapine and oxygen concentrations have to be described by a system of ordinary differential equations (ODE). Such a system of ODE (Eq. (14)) can be obtained from Eq. (1) expressing the initial activity as a change in the concentration of a given substrate with time. The factor n in Eq. (14) accounts for the stoichiometry of the reaction, and denotes the number of molecules of sinapine that have to be oxidized to reduce a molecule of oxygen.

$$v = -\frac{d[SIN]}{dt} = -\frac{1}{n}\frac{d[O_2]}{dt} \quad (14)$$

To check the model prediction for various conditions of the reaction, the experiments performed were designed to study the effect of substrate and enzyme concentration, pH and temperature on the extent of reaction. The data obtained are shown in Fig. 9(A)–(D). To predict the progress curves for both substrates, the information regarding the stoichiometry of reaction was required. It was expected that the factor n would be the same as for the sinapic acid transformation and equal to 4 [15]. However, it was observed (Fig. 9) that according to the experimental results the ratio of the rates of sinapine and oxygen disappearance varied from 2.73 to 3.9, depending on the reaction conditions. Since all of these results were smaller than the expected value of 4 we assumed that the reason for this could be associated with the presence of additional enzymatic reactions involving the products of sinapine transformation and oxygen. Using the same HPLC separation method that was employed for the sinapine determination, it was possible to detect two such products, X1 and X2. Their concentrations calculated using a sinapine standard curve are presented in Fig. 9. It should be emphasized that these concentration values should be considered only as relative ones, because the actual structures of X1 and X2 are unknown.

The existence of the reactions involving these compounds and oxygen is documented in Fig. 9(B), which shows that the rate of oxygen disappearance remained almost constant even after all of the sinapine was transformed and coincide with the disappearance of X1 and X2. This could suggest that the affinity of the enzyme towards these new substrates, at least at these experimental conditions, is considerable. If this explanation is correct then it can be stated that the rate of oxygen utilization in these tests is higher than that predicted by Eq. (14) because it is a result of multiple reactions. From Fig. 9 it is evident that, depending on the reaction conditions, the effect of the additional reactions on the rate of oxygen consumption can be minimized. For example, the formation of compound X2, which appears after compound X1 is produced, is suppressed by low reaction temperatures (Fig. 9(A)Fig. 9(C)). In both of these cases the ratio of the rates of sinapine and oxygen disappearance, calculated from the experimental data, is much closer to 4 than in the case of higher temperatures (Fig. 9(B)Fig. 9(D)) at which compounds X1 and X2 are noticed much earlier and in larger quantities. The fact that the rate of formation of compound X2 is strongly temperature dependent could indicate that its formation may be governed by thermolysis of compound X1. A similar phenomenon was encountered during the enzymatic transformation of sinapic acid by the same enzyme [15]. Since the compound X2 is also enzymatically transformed, the rate of oxygen utilization can be minimized at low tem-

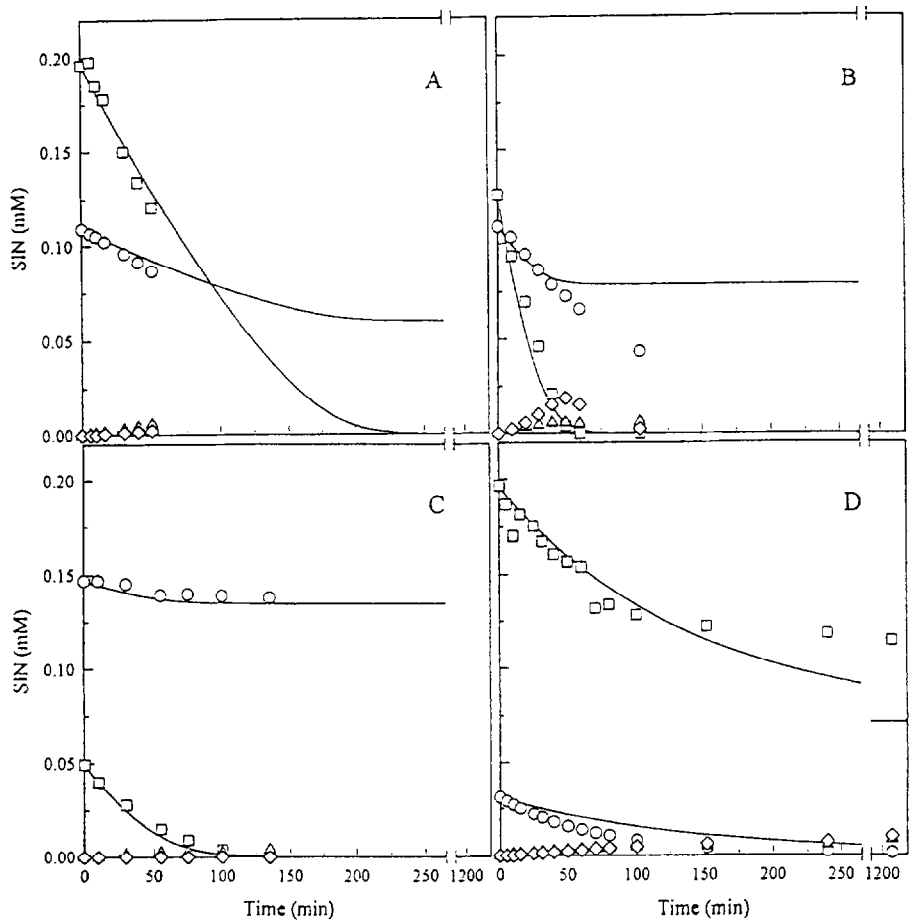

Fig. 9. Effects of temperature, pH, enzyme, sinapine and oxygen concentrations on the concentration–time curves for oxygen (○), sinapine (□), and compounds X1 (△) and X2 (◇). Solid lines represent the predicted data using Eqs. 12 and 12: (A) [SIN] = 0.195 mM, [O_2] = 0.11 mM, v_{enz} = 0.1 ml, pH 4.5, 15 °C; (B) [SIN] = 0.125 mM, [O_2] = 0.11 mM, v_{enz} = 0.15 ml, pH 5.5, 45 °C; (C) [SIN] = 0.05 mM, [O_2] = 0.147 mM, v_{enz} = 0.1 ml, pH 3.1, 15 °C; (D) [SIN] = 0.195 mM, [O_2] = 0.0312 mM, v_{enz} = 0.2 ml, pH 4.5, 30 °C.

peratures of the reaction. In fact, the ratio of sinapine and oxygen disappearance at low temperatures was almost 4. Therefore, this value was used to predict the progress curves for sinapine and oxygen for the four reaction conditions shown in Fig. 9, using Eq. (14) and the values of respective rate constants from Table 3. The results obtained were compared with the experimental ones in Fig. 9(A)–(D). Except for the limitations relating to the presence of more reactions, the model seems to predict the effect of temperature, pH, enzyme and substrate concentration in a reasonable manner. However, a more detailed analysis relating to an investigation of the additional reactions should be performed in order to fully describe this enzymatic system.

4. Conclusions

The enzymatic transformation of sinapine by polyphenol oxidase from *Trametes versicolor* can be described by the Theorell–Chance Bi–Bi mechanism. Based on this mechanism, a model predicting the effect of pH, temperature, enzyme and substrate concentration on the initial reaction rate was developed. The parameters in the model were estimated using the initial rate measurement data collected at 81 initial reaction conditions. The model predicts the experimental results with very good accuracy. It is valid over the whole range of pH and temperature, predicts the effect of substrate concentration (up to 0.15 mM of sinapine and any of the oxygen concentrations) and is valid within the linear response of enzyme concentrations. Using this model, it was found that the optimum temperature for this enzyme can be 60 °C even though 50 °C is determined experimentally. The reason for this discrepancy is the effect of temperature on the oxygen solubility which affects the initial reaction rate. Furthermore, using a derived equation which describes the effect of temperature on the optimum pH for the reaction, it was shown that the optimum pH for this reaction changed from 4.25 at lower temperature to 4.4 at 60 °C. The possibility of using the proposed model to predict changes in the concentrations of sinapine and oxygen with time was also evaluated. It was found that the model gave satisfactory results; a slight discrepancy between experimental and predicted data is probably related to the presence of additional reactions involving the products of the enzymatic transformation of sinapine.

5. Nomenclature

ΔA_i	Constant specific for ith reaction (dimensionless)
ΔB_i	Constant specific for ith reaction (K^{-1})
ΔC_i	Constant specific for ith reaction (K^{-2})
ΔD_i	Constant specific for ith reaction (K^2)
dil	Enzyme dilution factor
$E_{0,i}$	Energy of activation for reaction i (kJ mol^{-1})
E^{2-}, E^-, E^0	Deprotonated, initial and protonated forms of enzyme, respectively
E_a^-	Active form of enzyme
E_d^-	Thermally deactivated form of enzyme
$<e_a^->$	Average concentration of the active form of enzyme during the reaction (mM)
e_0^-	Initial concentration of the active form of enzyme (mM)
e_0	Total initial concentration of enzyme (mM)
ΔG_i^0	Standard Gibbs energy of reaction i (J mol^{-1})
h	Planck constant (J s)
h^+	Concentration of hydrogen ions (M)
h_{opt}^+	Optimum concentration of hydrogen ions (M)
I_i	Constant specific for ith reaction (dimensionless)
J_i	Constant specific for ith reaction (J mol^{-1})
K_1	Deprotonization dissociation constant (M)
K_2	Protonization dissociation constant (M)
K_i	ith dissociation constant (M)
K_{i,O_2}	Inhibition constant for oxygen (mM)
K_{mj}	Michaelis–Menten constant for compound j (mM)
k_{-1}	First-order rate constant (s^{-1})
$k_{0,i}$	Frequency factor for reaction i (mM^{m-1} s^{-1})
k_1	Second-order rate constant (mM^{-1} s^{-1})
k_2	Second-order rate constant (mM^{-1} s^{-1})
k_3	First-order rate constant (s^{-1})
k_b	Boltzman constant ($J K^{-1}$)
k_d	Enzyme decay constant (s^{-1})
k_i	Arbitrary rate constant (mM^{m-1} s^{-1})
k_i^*	Arbitrary rate constant at central or average temperature T^* (mM^{m-1} s^{-1})
m	Reaction order (dimensionless)
n	Stoichiometric coefficient for the enzymatic reaction (dimensionless)
$[O_2]$	Concentration of oxygen (mM)
pH_{opt}	Optimum reaction pH
R	Universal gas constant ($J mol^{-1} K^{-1}$)
ΔS^*	Entropy change ($J mol^{-1}$)
[SIN]	Concentration of sinapine (mM)
T	Temperature (K)
T^*	Central or average temperature (K)
t	Time (s)
t_r	Reaction time (s)
V_{max}	Maximum activity (nkat)
V_{max}^{opt}	Maximum activity at optimum pH (nkat)
v	Initial reaction rate (activity) (nkat)
v_{pH}^{opt}	Initial reaction rate at optimum pH (nkat)
V_{sys}	Volume of the reacting system (l)
v_{enz}	Volume of enzyme added (ml)
Y_c	Corrected initial reaction rate (nkat)
β	Proportionally constant ($mmol_{enz}$ per ml_{enz})

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