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EXTENSION OF ISOCONVERSIONAL APPROACH TO ENZYME INACTIVATION FOR SOME UNCOMMON SYSTEMS

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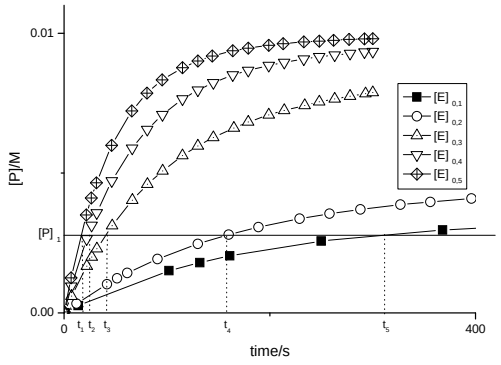
This work reports simple and costly-efficient methods to detect and evaluate the degree of operational inactivation of a (bio)catalyst in homogenous media. The isoconversional method, toghether with the qualitative Selwyn test for enzyme inactivation were applied to peroxidase-catalyzed oxidation of guaiacol and capsaicin by H_2O_2. This approach requires only the progress kinetic curves of the reaction products, even when dealing with a single reaction or with a reaction network. The catalyzed oxidation of a substrate (capsaicin) derived from an extract with unknown concentration as well as the oxidation of a substrate (guaiacol) with multiple product formation were kinetically monitored using spectrophotometric methods. The estimated first order inactivation rate constants k_{in} were of the same order of magnitude (10^{-3} s^{-1}) but the comparison to other similar inactivation constants of phenolic compounds oxidation suggests a strong dependence of these constants on the specificity of the reducing substrates.



INTRODUCTION

Peroxidases are probably the most widely used enzymes in green industry mainly due to their low substrate specificity, catalyzing the oxidation with hydrogen peroxide of a large number of reducing substrates, particularly phenols and aromatic amines. [1] Their potential applications in the cellulose industry, [2] degradation of xenobiotics in water, [3] as antioxidants, [4] as indicators in food processing, in biosensors[4] or for the synthesis of many chemicals and pharmaceuticals [5] are partly limited by their thermal and operational stabilities. [6]

Therefore, many recent works were focused on their stability and inactivation, especially in the case of fruit and vegetable processing, where the peroxidases activity and stability must be decreased in order to limit unwanted oxidation processes. Horseradish peroxidase (HRP) uses hydrogen peroxide or other organic peroxides as electron acceptors to oxidize a wide range of reducing substrates. [1] Mechanistically, the (HRP) catalyzed oxidation with H_2O_2 proceeds by two-electron oxidation and forming of an oxidized intermediate (Compound I) which further reacts with the reducing phenolic substrate to form an intermediate with an oxoferryl group (Compound II).[7] In the reaction with the phenolic substrate, Compound II can be converted into the native enzyme or can react with excess H_2O_2 being transformed into a peroxy protoporphyrin radical (Compound III) which is inactive. Inactivation of peroxidases from different sources was extensively studied in literature and several models for inactivation were proposed. [6-10] Apparently, the

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inactivation constants depend on the particularities of reducing substrates as well as of the complexity of organic radicals and oxygenated reactive species generated during the overall oxidation process. For example, the apparent first-order rate constants were found 2.5 10^{-3} s^{-1} for H_2O_2 decomposition, [11] 3.3 10^{-3} s^{-1} for reaction with m-chloroperoxybenzoic acid, [12] 1.44 10^{-2} s^{-1} for 2-aminophenol oxidation[13] and 2 10^{-4} s^{-1} for L-3,4-dihydroxyphenylalanine (L-DOPA) oxidation. [14]

In this paper we present a kinetic investigation of HRP inactivation during the oxidation by H_2O_2 of two relevant phenolic compounds both containing a 4-hydroxy-3-methoxybenzyl group: guaiacol and capsaicin. The guaiacol was chosen as a "reference" since it is the most used substrate in assaying for activity of peroxidase. [15] Capsaicin, an alkaloid responsible for chillies hot taste, having an analgesic and anticarcinogenic potential, [16] is a well known antioxidant. The inactivation of HRP was investigated through the analysis of the extended progress curves using both a qualitative test proposed by Selwyn,[17,18] which uses plots of product (or substrate) concentration *vs*. initial enzyme concentration multiplied by time, and the isoconversional method, [19] which requires plots of reaction rate *vs.* time at the same conversion (or an amount directly correlated to conversion) recorded for different initial concentrations of enzyme. The advantage of the isoconversional method results from the possibility to estimate the overall kinetic inactivation constant without assuming an inactivation mechanism; it was previously used to detect the suicide inactivation of catalase at high substrate concentration, thermo-inactivation of urease, [19] and for estimating the kinetic inactivation constant of HRP in the oxidation of 2-aminophenol [13] and L-DOPA. [14]

DISCUSSION

Isoconversional method

According to the isoconversional method, [19] the isoconversional times (t_1, t_2, t_3...t_j.) corresponding to a chosen conversion of substrate (designated here with the lower index 1), measured as the product or substrate concentration, $(C_j)_1$, were estimated from the extended kinetic curves C_j = f(time) obtained for the same initial substrate concentration and different initial enzyme concentrations $[E]_{0,j}$, as illustrated in Fig. 1.

The method requires also the knowledge of the first derivative $\left(dC_j/dt\right)_1$ at each point. For a reliable estimation of both isoconversional time and first derivative, a suitable function must be fitted on each extended curve.

For a first order inactivation, the first derivative $\left(dC_j/dt\right)_1$ calculated for the chosen value of $[P]_1$, depends on the isoconversional time t_j according to the following equation. [19]

$$(dC_j/dt)_1 = f(C_j)_1 \cdot [E]_{0,j} \cdot e^{-k_{in} \cdot t_j} \qquad (1)$$

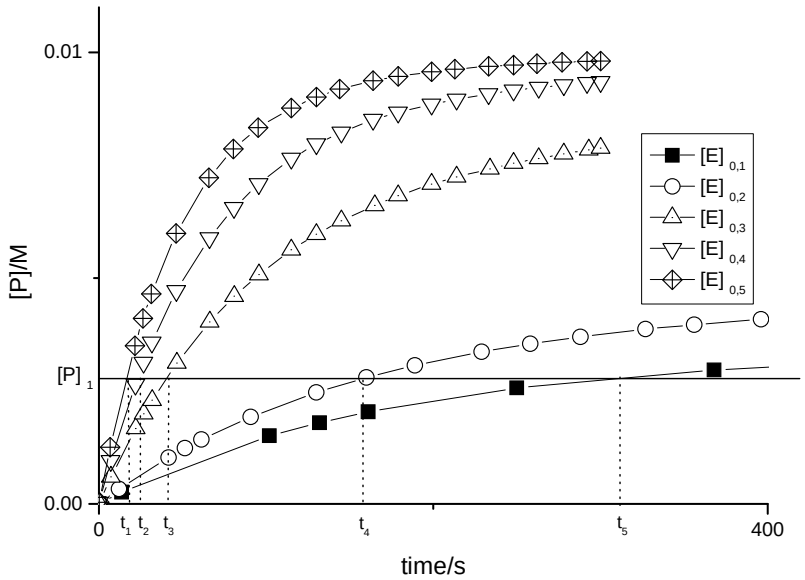


Fig. 1 – Illustration of the isoconversional method: the progress curves [P] *vs.* time for different initial enzyme concentrations $[E]_{0,j}$ and the same initial substrate concentration intersect the isoconversional line $[P]_1$ at different isoconversional times.

The overall inactivation rate constant k_{in} can be estimated either directly through nonlinear regression analysis or through linear regression analysis using the equation:

$$\ln[(dC_j/dt)_1 \cdot 1/[E]_{0,j}] = \ln f(C_j)_1 - k_{in} \cdot t_j \quad (2)$$

where the slope is equal to the overall inactivation rate constant. It should be noted that the inactivation rate constant resulted from the present analysis is a composite property including both the thermal and operational inactivation and will be designated as the overall inactivation rate constant.

An inspection of equation (1) shows that the concentration C_j can be replaced by a proportional property like absorbance, without changing the value of the inactivation constant, the procedure becoming simpler. The same is true for enzyme extracts when the enzyme concentration is not known but is proportional to other measurable property.

When there is no proof for a direct proportionality between the concentration C_j and the absorbance A_j, a polynomial of second or third order can be used for more complex systems:

$$C_j = a_0 + a_1 \cdot A_j + a_2 \cdot A_j^2 + a_3 \cdot A_j^3 \quad (3)$$

where a_0, a_1, a_2, a_3 are fitting parameters. It can be seen that, for a chosen absorbance, the derivative (dC_j/dt) is proportional with (dA_j/dt):

$$(dC_j/dt) = (a_1 + 2a_2 \cdot A_j + 3a_3 \cdot A_j^2) \cdot$$
$$\cdot (dA_j/dt) = \alpha_j \cdot (dA_j/dt) \quad (4)$$

In isconversional conditions (the same conversion, the same product concentration and consequently the same absorbance) $\alpha_j = \alpha$ (a constant value for the entire family of kinetic curves obtained with different initial concentrations of enzyme), equation (2) takes the form:

$$\ln[(dA_j/dt)_1 \cdot 1/[E]_{0,j}] =$$
$$= \ln[f(C_j)_1/\alpha] - k_{in} \cdot t \quad (5)$$

The best results (which give the best fit and randomly distributed residuals) for the calculation of derivatives dA_j/dt were obtained for a three parameter function available from ORIGIN 8.0 software, of the form:

$$A_j = y_0 + \gamma \cdot \exp(-t/\beta) \quad (6)$$

where y_0, γ, and β are fitting parameters. At each point t_j, the derivative is given by:

$$(dA_j/dt)_1 = -(\gamma/\beta) \cdot \exp(-t_j/\beta) \quad (7)$$

Guaiacol oxidation

Guaiacol is widely used in the activity assays for almost all the classes of peroxidases, even if the reaction leads to the formation of secondary products [20,21] absorbing at the same wavelength as the main product (tetraguaiacol). The oxidation of 0.2 mM guaiacol and 0.026 mM H_2O_2 in the presence of different initial HRP concentrations ranging between 4 10^{-8} – 2 10^{-7} M, at pH = 7.0 was followed spectrophotometrically at λ = 470 nm for 15 min. The variation of products absorbance in time is given in Fig. 2.

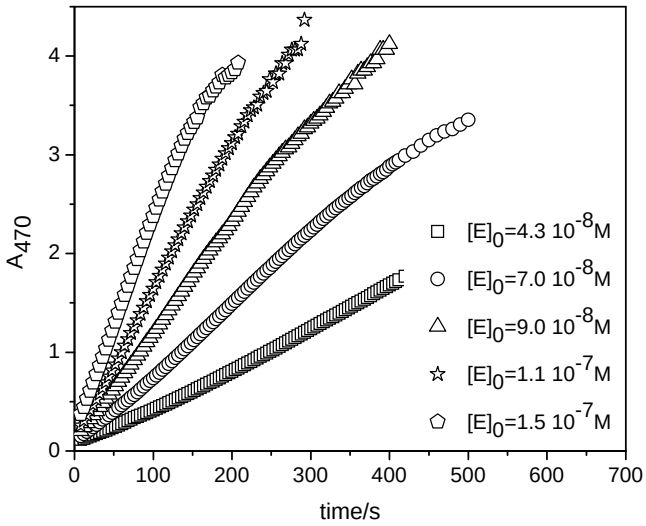


Fig. 2 – Variation of products absorbance in time for the oxidation of guaiacol at different initial enzyme concentrations (0.2 mM guaiacol and 0.026 mM H_2O_2, pH = 7).

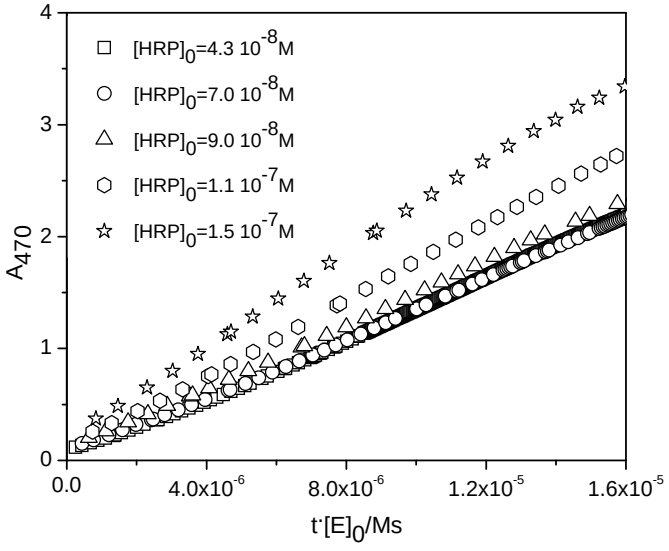
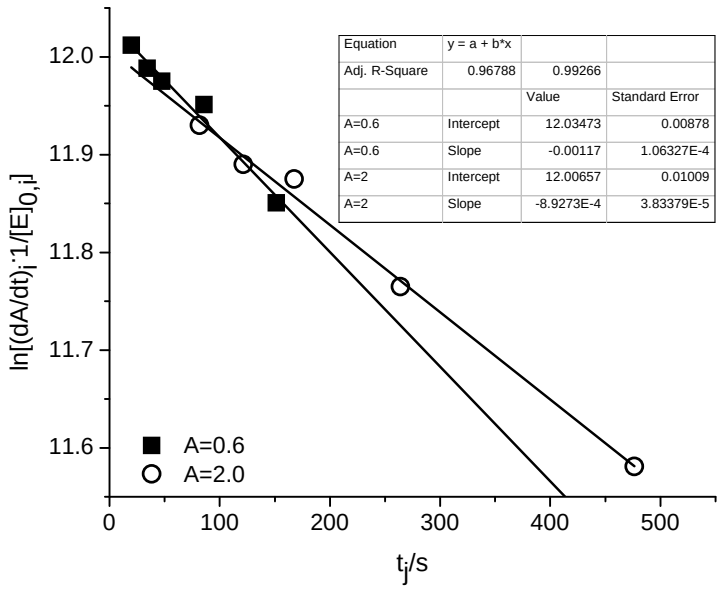


Fig. 3 – Selwyn test for the inactivation of HRP during the oxidation of guaiacol (0.2mM guaiacol, 0.026 mM H_2O_2, pH = 7).

In order to verify if peroxidase is inactivated during the reaction, the qualitative Selwyn test[17] was performed. From Fig. 3 it can be observed that inactivation of enzyme does occur, the plots A=f(t $[E]_0$) being non-overlapped.

Once the enzyme's inactivation being detected, the estimation of the overall inactivation rate constant was carried out with the isoconversional method. The isoconversional times t_j and the corresponding derivatives $\left(dA_j/dt\right)$ were estimated for two different products absorbances: A = 0.6 and A = 2. Even though the differences between the two conversions are quite large, the correlation parameters do not appear to differ significantly.

The plot $\ln\left[\left(dA_j/dt\right)\cdot 1/[E]_{0,j}\right]$ *vs.* isoconversional time t_j is linear (Fig. 4), indicating that the inactivation can be described appropriately by a first order process. The overall inactivation rate constants k_{in}, in the presence of both substrates, were estimated from the linear regression on equation (5), leading to an average value of (1.032±.021) 10^{-3} s^{-1}. This value is in good agreement with literature data obtained at low hydrogen peroxide concentrations. [20,21]



Equation	y = a + b*x		
Adj. R-Square	0.96788	0.99266	
		Value	Standard Error
A=0.6	Intercept	12.03473	0.00878
A=0.6	Slope	-0.00117	1.06327E-4
A=2	Intercept	12.00657	0.01009
A=2	Slope	-8.9273E-4	3.83379E-5

Fig. 4 – Estimation of the overall inactivation rate constant using the isoconversional method. The data represent the results obtained for guaiacol oxidation, using different initial enzyme concentrations.

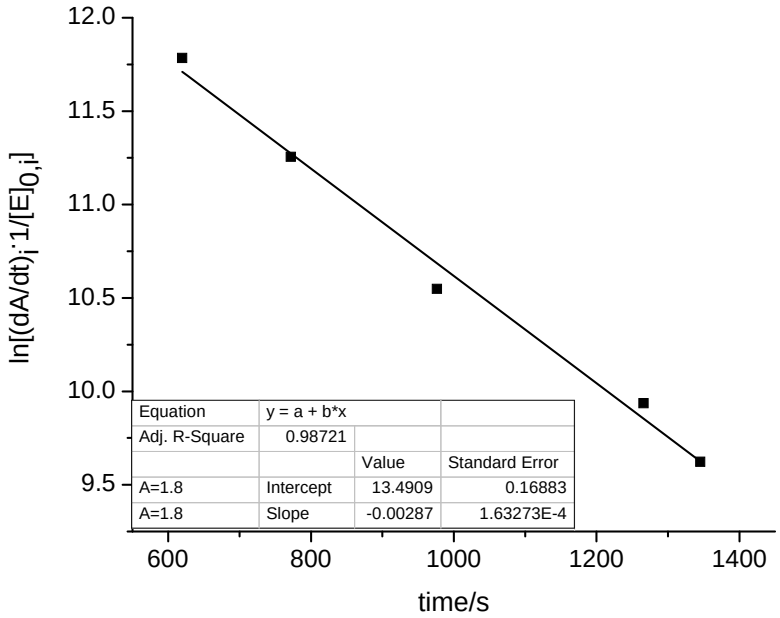


Fig. 5 – Linear regression estimation of inactivation rate constant of HRP during capsaicin oxidation with H_2O_2.

Capsaicin oxidation

The oxidation of capsaicin extract from *Capsicum annuum*, having an initial absorbance A_{262}=1.5, with 0.026 mM H_2O_2 in the presence of different initial concentrations of HRP ranging from 4 10^{-8} to 2 10^{-7} M , at pH = 7.0 was carried out spectrophotometrically at λ=262 nm for 1 hour. This wavelength corresponds to the formation of oxidizing products, identified in literature as dimers of capsaicin, 5,5'-dicapsaicin and 4'-O-5-dicapsaicin ether, a polymeric product with a mean polymerization degree of 15, and capsaicin-protein copolymers.[22] Following the same procedure described above, the isoconversional times t_j were estimated for A = 1.8 and the overall inactivation rate constant was estimated by linear regression on $\ln\left[(dA_j/dt)\cdot 1/[E]_{0,j}\right]$ *vs.* t_j dependence (Fig. 5), as (2.87 ± 0.16) 10^{-3} s^{-1}. This value, exceeding the one obtained for guaiacol oxidation suggests a higher efficiency of HRP inactivation. It is worth mentioning that in this case the value of substrate concentration is not needed.

EXPERIMENTAL

Guaiacol was obtained from Sigma. Capsaicin was extracted from hot peppers (*Capsicum annuum*). The alcoholic capsaicin extract was obtained macerating 10 grams of dried red hot peppers in ethylic alcohol for 48 hours in the absence of light. For the analysis the filtered solution was used. H_2O_2 obtained as a 30% solution from Merck was diluted and the concentration was measured spectrphotometrically using the molar absorptivity coefficient ε_{240nm} = 39.4 $M^{-1}cm^{-1}$. [23] All other chemicals were of analytical grade and were purchased from Sigma. HRP obtained from Sigma with a specific activity of 1280 U/mg solid matter (ABTS method) was prepared in a 0.02 M sodium phosphate buffer with pH 7.0. The final enzyme concentration in the reaction mixture ranged between 1 10^{-8} and 5 10^{-7} M and was determined spectrophotometrically using $\varepsilon_{403\ nm}$ = 102000 $M^{-1}cm^{-1}$. [24]

HRP activity was determined spectrophotometrically with a JASCO V-350 using H_2O_2 as oxidizing substrate and guaiacol or capsaicin as reducing substrates. The reaction was carried out at room temperature for 1 hour with 3mL of the reaction mixture containing HRP, reducing substrate and H_2O_2.The characteristic absorbances of reaction products were measured in time to obtain extended kinetic curves.

CONCLUSIONS

The operational inactivation of HRP during the oxidation of two mono-phenols (guaiacol and capsaicin) with H_2O_2 was detected through Selwyn test and the corresponding overall inactivation rate constants were evaluated using the isoconversional procedure: k_{in} = (1.032 ±.021) 10^{-3} s^{-1} for guaicol and k_{in} = (2.87 ±0.16) 10^{-3} s^{-1} for capsaicin oxidation. This work proves that the isoconversional method is applicable even for systems where the measured reaction progress is non-linearly related to substrate or product concentration. Its relative simplicity makes it easy to use in different steps of technological processes, especially in food processing or in experimental design when enzyme inactivation is required.

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