ISSN 1330-9862
(FTB-2352)

scientific note

Experimental and Modelling Study of Enzymatic Oxidation of 5-*o*-Caffeoylquinic Acid by Polyphenol Oxidases

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Received: September 26, 2009
Accepted: August 17, 2010

Summary

A set of experiments to investigate the enzymatic oxidation of 5-*o*-caffeoylquinic acid with polyphenol oxidases was carried out in the temperature range from 18 to 39 °C. The kinetic data were obtained in a batch isothermal reactor made of quartz and operated at an optimum pH (approx. 7.0) for enzyme activity, using citrate-phosphate buffer. The decay of 5-*o*-caffeoylquinic acid concentrations was experimentally monitored with a spectrophotometer at 323 nm. A coefficient of molar absorptivity equal to $1.96 \cdot 10^3$ $mM^{-1}m^{-1}$ was found for 5-*o*-caffeoylquinic acid, based on absorbance measurements with standard aqueous solutions. The kinetic experimental results were used to calculate the parameters K_m and v_{max} of the Michaelis-Menten model, which were 0.24 mM and $2.77 \cdot 10^{-4}$ mM/s at 25 °C, respectively. The effects of temperature on the maximum velocity of oxidation of 5-*o*-caffeoylquinic acid and on the rate of enzyme deactivation were well described using the Arrhenius equation.

Key words: chlorogenic acids, 5-CQA, enzymatic oxidation, polyphenol oxidases

Introduction

Chlorogenic acids (CGA) are a family of approximately eighteen identified esters formed between quinic acid and *trans*-cinnamic acids (*1,2*). These simple phenolic species are abundantly found in agricultural and food products (*3–6*), such as coffee beans (*2,7*), shoots of *Camellia sinensis* (*8,9*) and leaves of *Ilex paraguariensis* (*1,10*). Not only is 5-*o*-caffeoylquinic acid (5-CQA) the most abundant member of this particular group of bioactive compounds (CGA) in several plants (*11,12*), but also it is the only one that is available commercially (*1,13*). As a result, it has become a benchmark for evaluating the chemistry of enzymatic oxidation of CGA (*14, 15*).

Reports attributing health benefit in humans to the consumption of CGA have stimulated a recent surge of interest in these compounds (*16–18*). Their pharmacological properties (*13,19*) have been associated with inhibition of cancer (*20–22*) and reduction of coronary diseases (*23–25*). However, CGA are also involved as substrates in several chemical and enzymatic reactions that take place in different stages of food manufacturing, whose occurrence is strictly connected to changes in taste, colour and aroma of these products. At times these reactions are desirable, such as in the fermentation step in the production of black tea (*26,27*). At other times they are not desirable and processing steps are implemented to prevent them, such as the thermal denaturation step undertaken in the processing of mate (*28*).

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In spite of the importance of enzymatic reactions involving CGA in the presence of polyphenol oxidases (PPO) and oxygen, the kinetics of oxidation of these substances is poorly studied (*14*,*15*,*29*,*30*). The effects of pH (*16*,*31*) and temperature (*32*) on the activity of PPO have been experimentally examined, but the optimum pH and the thermostability of PPO depend on the source of the enzyme and on the substrate used for the assay (*30*). Some mechanisms for the enzymatic oxidation of CGA, as well as simplified kinetic models, have been proposed (*14–16*,*30*,*33–37*), but further investigation is necessary.

In this framework, the main aim of this study is to obtain experimental results for the decay of 5-CQA concentration in an isothermal batch reactor operated at different temperatures. The influence of pH on enzyme activity was also examined in order to carry out the kinetic experiments at the optimum value of this variable. A simple theoretical model, incorporating Michaelis-Menten kinetics and enzyme deactivation, was proposed to describe the 5-CQA profiles obtained.

Material and Methods

Experiments

5-*o*-caffeoylquinic acid ($M(C_{16}H_{18}O_9)$=354.3 g/mol, purity 98 %, lot 096K1722) and polyphenol oxidase (5370 U/mg solid, lot 076K7034) used in all the kinetic experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). The same reagents were used in the analytical assays to determine the coefficient of molar absorptivity for 5-CQA and to estimate the enzyme activity over the pH range from 3 to 7. In all these experiments the absorbances of aqueous solutions of 5-CQA were recorded in a spectrophotometer (FENTO 700 PLUS) at controlled temperature and 323 nm, the absorption peak of the substrate. Sample solutions were monitored in cuvettes made of glass with a volume of $10^{-2}\times10^{-2}\times0.025$ m^3.

The calibration curve of absorbance against substrate concentration was obtained with 5-CQA dissolved in ultrapure water. The kinetic assays were done with a mixture of PPO and 5-CQA in a citrate (0.1 M) phosphate (50 mM) buffer, this buffer being recommended for the pH range from 2.6 to 7.0 (*14*), within which the maximum catalytic action of PPO is often found (*16*,*38–43*). Chemical interaction between the buffer and the substrate in the absence of enzymes was checked at the optimum pH for enzyme activity.

The experiments to determine the activity of PPO as a function of pH took place at constant temperature (25 °C), enzyme concentration (0.02 kg/m^3) and substrate concentration (0.057 mM). The absorbances of 5-CQA solutions were monitored at 323 nm and recorded for about 1800 s at 10 s intervals. A polynomial equation was fitted to the resulting profile for decrease in 5-CQA. The derivative of this equation, in μmol/min, at zero time, defines the initial rate of substrate oxidation. The relative enzyme activity is the ratio of substrate consumption rate to the same variable at the optimum pH.

The kinetic experiments were carried out at different temperatures, but with PPO maintained at 0.016 kg/m^3 and the initial concentration of 5-CQA kept at 0.045 mM. Under these conditions the absorbances of 5-CQA solutions were also monitored at 323 nm at different temperatures, for 600 s in time steps varying between 15 and 30 s. A total of 28 measurements of 5-CQA concentrations were performed for each temperature.

Theoretical considerations

The Michaelis-Menten rate equation, with a term taking into account first order enzyme deactivation (*44*), was fitted to the profiles for 5-CQA consumption:

$$-\frac{\mathrm{d}[\text{5-CQA}]}{\mathrm{d}t}=\frac{v_{\max}\mathrm{e}^{-k_\mathrm{d}t}[\text{5-CQA}]}{K_\mathrm{m}+[\text{5-CQA}]} \qquad /1/$$

This model assumes the absence of enzyme inhibition and a constant oxygen concentration.

Eq. 1 is a first order ordinary differential equation, the analytical solution to which is an algebraic expression represented by Eq. 2, which is explicit in the variable t, but implicit in [5-CQA]:

$$t=\frac{-1}{k_\mathrm{d}}\ln\left\{1+\frac{k_\mathrm{d}}{v_{\max}}\left[K_\mathrm{m}\ln(X)+[\text{5-CQA}]_\mathrm{o}(X-1)\right]\right\} \qquad /2/$$

The three kinetic parameters were estimated by fitting Eq. 2 to the experimental profiles obtained at each of the four investigated temperatures. Fitting was done using the simplex method of optimization (*45*) and was based on an objective function that minimized the sum of the absolute squared differences between experimental and calculated reaction times, with the dimensionless concentrations of 5-CQA being considered as the independent variable. The analysis yielded separate values of k_d and $v_{\max}$ for each temperature. The linearized Arrhenius equation was then applied to correlate the values of these two parameters with temperature. In both cases, the least-square method was applied for linear regression.

Results and Discussion

The dependence of the concentration of standard 5-CQA solutions on light absorption was well described by a straight line with an intercept equal to zero, which is in agreement with the Beer-Lambert equation (*15*). A coefficient of molar absorptivity for 5-CQA equal to $1.96\cdot10^3$ mM^{-1}m^{-1} was calculated as the inverse of the slope of this line, divided by the light pathlength through the solution. This value is almost the same as the values reported in the literature for similar investigations involving the same substrate (*46*).

Fig. 1a shows that the coefficient of molar absorptivity of 5-CQA is not affected by pH over the range from 3 to 7. The absorbance of these solutions remained constant over time, indicating that 5-CQA is stable in this buffer. On the contrary, when sodium phosphate buffer was used, a yellow colour formed over time, indicating reaction of the 5-CQA. This buffer had been used by previous workers (*47*), but appeared not to be suitable.

The effect of pH on relative enzyme activity is shown in Fig. 1b. The optimum pH was close to 7, agreeing with previous results involving PPO and 5-CQA (*15*,*30*, *35*). As has been reported previously, the activity drops

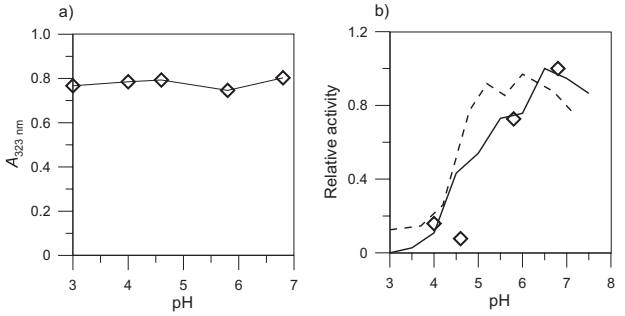

Fig. 1. Effect of pH on the absorbance of 5-CQA in citrate phosphate buffer (a); relative activity of PPO at different pH (b). Symbols=experimental results, solid line=literature (*29*), dashed line=literature (*32*)

off quickly as pH is decreased, reaching very low values around pH=4 (*29*,*32*). Residual activity below pH=3 has been reported (*14*,*32*).

As the linearized plot in Fig. 2 shows, the dependence of the deactivation constants and maximum velocity on temperature was well described by the Arrhenius equation. Eqs. 3 and 4 are the algebraic expressions from which the straight lines shown in Figs. 2a and 2b were obtained, respectively:

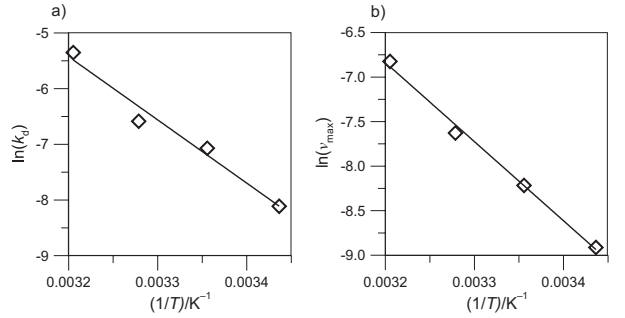

Fig. 2. Influence of temperature on the enzyme deactivation constant (a) and on the maximum velocity of substrate oxidation (b). Symbols=tuned parameters, solid line=Arrhenius equation

$$k_d = 2.628 \times 10^{13} \exp\left(\frac{-11352}{T}\right) \qquad /3/$$

$$v_{max} = 2.403 \times 10^{9} \exp\left(\frac{-8878}{T}\right) \qquad /4/$$

Eq. 3 reveals that the activation energy for the thermal denaturation (*i.e.* E_d) of the PPO that was used is about 94.4 kJ/mol (*i.e.* 11352·8.314 J/mol). Values of E_d are of the order from 170 to 400 kJ/mol for many enzymes (*44*), which means that PPO, with such a low value, is a thermolabile enzyme. The few results that have been reported for PPO confirm low deactivation energies: PPO from yacon roots (*30*), grape juice (*48*) and pineapples (*49*) presented values of approx. 93.7, 83.5 and 80.2 kJ/mol, respectively. The most important practical property of an enzyme with low heat resistance is that enzyme deactivation steps within food manufacturing processes should not consume large amounts of energy, such as contrarily observed in the mate industry (*28*). The implications of the results in terms of appropriate temperature-time treatments for mate processing will be discussed below.

Fig. 3 presents the results for oxidation of 5-CQA over the range from 18 to 39 °C. This figure shows that the kinetic model was always able to represent the experimental results correctly. This is a strong evidence for the reliability of the suggested approach for modelling enzymatic oxidation of 5-CQA with PPO. The absence of experimental runs at different initial substrate concentrations is not typically observed in enzymatic studies because it usually constitutes a primary step for modelling these kinds of catalytic reactions. However, our simplified approach to modelling the oxidation of 5-CQA does not require initial rate data.

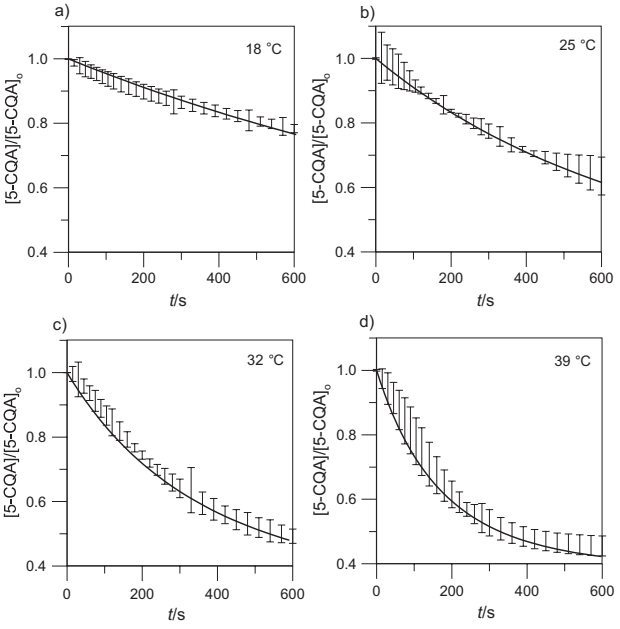

Fig. 3. Experimental (bars) and calculated (solid lines) decay of 5-CQA in the presence of PPO at 18 (a), 25 (b), 32 (c) and 39 °C (d). Error bars define the confidence in the dimensionless concentration of 5-CQA for a 90 % level of probability

The influence of considering enzyme deactivation within the kinetic model is shown in Fig. 4, which compares the best fits of two models: the model that takes enzyme deactivation into account in the manner shown in Eq. 1 and another model in which deactivation is not considered (in which case the exponential term in the numerator of Eq. 1 is set to unity). Only the results at 32 and 39 °C are reported, since at these temperatures the rate of enzyme deactivation is greater, which makes the difference between Figs. 4a and 4b more evident. The region denoted as α in Fig. 4a shows that when a zero rate of PPO is assumed, the model overestimates the disappearance of 5-CQA at longer reaction times (*i.e.* at low values of [5-CQA]/[5-CQA]$_o$). An almost constant slope is also noticed in the kinetic curves under the conditions of simulation that neglect the decrease of enzyme activity. The consequence of it is the absence of the typical transition between the constant-rate and falling-rate pe-

riod that represents the zero and first order mechanism of reaction, respectively. The deviation between the experimental and calculated results in the region β (Fig. 4a) is the concrete effect of this finding.

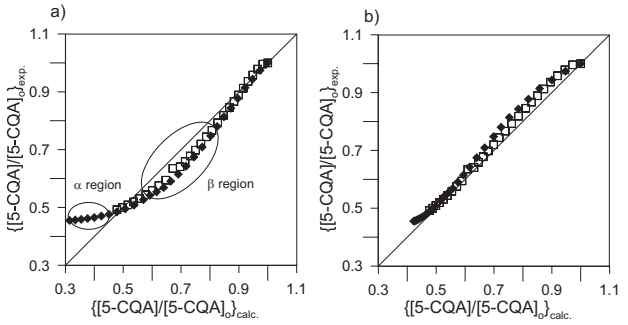

Fig. 4. A comparison between experimental and calculated dimensionless concentration of 5-CQA at 32 (hollow squares) and 39 °C (solid diamonds) by neglecting (a) and considering (b) enzyme deactivation

The influence of time and temperature on the ratio of the concentration of active enzyme to the same variable at time zero is described in Fig. 5. The contours are based on the results obtained with Eq. 5:

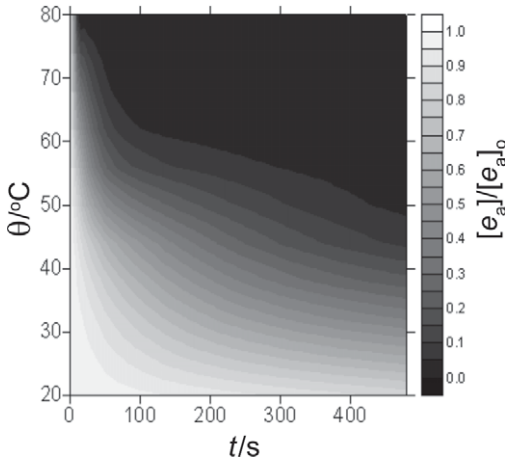

Fig. 5. Influence of reaction time and temperature on the ratio of active enzyme to the same variable at time zero

$$\frac{[e_a]}{[e_a]_o} = \exp\left[2.628\times10^{13} t_{exp}\left(\frac{-11352}{\theta+273}\right)\right] \qquad /5/$$

Fig. 5 indicates that reasonably mild conditions are sufficient for almost full enzyme deactivation. The consequence is that thermal processes applied in the food industry to deactivate polyphenol oxidases should not be overly demanding in terms of energy consumption.

The value of K_m obtained in the fitting procedure was 0.24 mM, which is in good agreement with the results, ranging from 0.04 to 1.5 mM, obtained by other workers under similar operating conditions (*14,15,30,35*). The close agreement between these data is a further evidence of the reliability of the kinetic modelling approach.

Conclusions

Experiments were carried out to investigate the kinetics of enzymatic oxidation of 5-CQA with polyphenol oxidases. The effect of pH on the enzymatic activity of PPO and the influence of reaction time and temperature on the substrate concentration were experimentally examined. A Michaelis-Menten model that accounts for enzyme deactivation correctly reproduced all the experimental results. The Arrhenius equation was able to describe the dependence of both the maximum velocity and the enzyme deactivation constant on temperature. The results also reveal that the polyphenol oxidase used is a thermolabile enzyme with a low energy of activation for thermal denaturation equal to 94.4 kJ/mol. The kinetic parameters K_m and E_d were in close agreement with the data available in the literature and obtained under similar operating conditions.

Nomenclature and Abbreviations

$A_{323\ nm}$	absorbance of aqueous solution of 5-CQA at 323 nm/mAu
5-CQA	5-*o*-caffeoylquinic acid
[5-CQA]	concentration of 5-*o*-caffeoylquinic acid/(mol/m^3)
$[e_a]$	concentration of active enzyme
$[e_a]_o$	initial concentration of active enzyme
E_d	energy of activation for thermal denaturation/(kJ/mol)
k_d	deactivation rate constant/s^{-1}
K_m	Michaelis constant for 5-CQA defined in Eq. 1/mM
M	molar mass of 5-CQA/(g/mol)
PPO	polyphenol oxidases
T	temperature/K
θ	temperature/°C
t	time/s
v_{max}	maximum rate of reaction at infinite 5-CQA concentration/(mM/s)
X	dimensionless 5-CQA concentration

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