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Inactivation and reactivation kinetics of horseradish peroxidase in phosphate buffer and buffer–dimethylformamide solutions

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Abstract

The influence of the regeneration of horseradish peroxidase activity at 4 °C on both the thermal inactivation and the reactivation kinetics of the enzyme was studied in phosphate buffer and a mixture of this buffer with 10% (v/v) *N*,*N*-dimethylformamide, at temperatures ranging from 70 to 85 °C. A series-type model was fitted to the experimental data that exhibited typical biphasic patterns, which became less pronounced as enzyme activity was regenerated. The kinetic parameters were found to be, in general, significantly different in both inactivation media. The magnitude of the activation energies for both the reactions of formation and inactivation of the intermediate enzyme indicated that conformational changes might play a major role in both processes when the solvent is added. A logarithmic model, although lacking a theoretical background, was able to predict the enzyme activity regeneration upon storage of the inactivated samples at 4 °C. There was evidence that the rate of reactivation may be dependent on the amount of intermediate formed in the first reaction or remaining after inactivation, as suggested by both the inactivation temperatures and extent of heating time relationships verified in this work.

Keywords: Horseradish peroxidase; Thermal inactivation; Dimethylformamide; Reactivation; Kinetic parameters

1. Introduction

Peroxidases (EC 1.11.1.7) are enzymes ubiquitously found in bacteria, fungi, plants and animals that act as oxidoreductases and efficiently catalyse the oxidation of a variety of compounds [1]. Horseradish peroxidase is the most studied peroxidase used as a biocatalyst in the polymerisation of phenol, aniline and their derivatives, and being shown to be active in a number of organic solvents or aqueous solvent mixtures [2,3]. Because of its high thermal resistance and relative simplicity of the assay method, horseradish peroxidase is used to monitor the adequacy of thermal processing and thus of food stability [4–6]. Research has also focussed on the potential use of peroxidases in waste treatment to remove aromatic compounds from wastewaters of industries [7]. Karam and Nicell [7] referred horseradish peroxidase as "undoubtedly one of the most studied enzymes in the relatively new area of enzymatic waste treatment".

The knowledge and understanding of horseradish peroxidase thermal inactivation and reactivation kinetics and its related mechanisms would offer new possibilities in improving the potential and effective use of this enzyme in such diverse and broad areas. In literature, thermal inactivation of horseradish peroxidase has been reported emphasising the analysis of the kinetics of the process and covering a large range of experimental conditions [8–10]. The potential regeneration of peroxidase activity in thermally processed

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fruits and vegetables may be affected by several factors, among them the operational conditions of thermal processing [11], the species of vegetables and also the type of isoenzymes [12]. There was some evidence that regain of peroxidase activity after thermal inactivation could be inherently attributed to the fraction of ionically bound isoperoxidases as reported for mango [13] or Japanese radish [14]. Earlier studies had experimentally confirmed that both the inactivation and reactivation processes could be, in part, related to the dissociation/reassociation of heme from/to the protein [15]. Dissociation of the prosthetic (heme) group from the active centre of the enzyme was, however, found to be pH dependent and occurring more rapidly at pH values lower than 5 [16], with complete detachment reported at pH 2.4 and 25 °C [17]. Teipel and Koshland Jr. [18] followed the enzyme reactivation measuring both optical rotation and fluorescence changes which occurred within 1 min, although the maximum extent of activity regain was not attained before 40–750 min. Spectral analysis were also performed during the reactivation process and enabled to found multiple enzyme forms, such as several intermediates as well as the inactivated and native forms. In the case of horseradish peroxidase thermal inactivation in the presence of organic solvents, no study was found correlating their presence to the kinetics of the spontaneous reactivation process of this enzyme.

The main objective of this work was to study the kinetics of the inactivation and reactivation of horseradish peroxidase stored at 4 °C, previously thermally inactivated in phosphate buffer and the same buffer with 10% (v/v) dimethylformamide, over a wide range of inactivation temperatures (70–85 °C), aiming at a comprehensive mathematical modelling of the processes. In order to achieve this, a systematic and comparative study of the effect of activity regeneration on both the inactivation and reactivation kinetics of this enzyme in both reaction media was carried out.

2. Materials and methods

2.1. Enzyme solutions

Peroxidase from horseradish (RZ 0.98) was purchased from Sigma. Aqueous solutions of 0.08 mg ml^{-1} of horseradish peroxidase were prepared in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.0, or in a mixture of this buffer with *N*,*N*-dimethylformamide (DMF; Reagente Puro Erba, Italy) to the final concentration of 10% (v/v). The water-miscible solvent was stored over 3 Å molecular sieves (Riedel de Haën, Germany) prior to mixing with the buffer solution.

Changes in the pH of the buffered solution upon addition of the organic solvent were measured at room temperature using a pH538 Multical® pH meter (Weilheim, Germany). The pH value obtained was within the neutral scale (7.03 ± 0.01), which has been reported to be of most importance to achieve the highest thermal stability of horseradish peroxidase [4]. In order to minimise the temperature dependence of pH, the range of inactivation temperatures used was kept sufficiently narrow (within 15 °C).

2.2. Thermal inactivation of HRP

The thermal inactivation was performed by placing eppendorf tubes of 15 mm diameter and 50 mm height, in duplicated, filled with 1.5 ml of enzyme solutions in a thermostatic water bath, previously equilibrated at the desired temperatures of inactivation: 70, 75, 80 and 85 °C. After heating, for pre-determined time intervals (ranging from 1.5 to 245 min, with the maximum time removal depending upon both the temperature and the reaction medium), 100 μl aliquots were rapidly removed from tubes and immediately diluted and cooled in 150 μl sodium phosphate buffer 0.1 mol l^{-1}, pH 7.0, in eppendorf tubes immersed in ice water.

2.3. Enzyme reactivation

Horseradish peroxidase thermal inactivated in both the buffer and the mixture buffer–DMF was shown to recover activity after removal from heat and storage at low temperature (4 °C). Therefore, to ensure that experimental data would cover the overall reactivation process until achieve maximum regeneration of the enzyme, the activity of each thermal inactivated sample, kept at 4 °C, was always assayed after 5, 15 and 30-min and 1, 2, 8, 24 and 48-h heat treatment.

2.4. Analysis of the enzyme activity

The activity of both the inactivated and reactivated horseradish peroxidase samples was measured

according to the Worthington procedure [19]. The increase in optical density upon addition of an aliquot of 50 μl of the enzyme solution to 1.45 ml of substrate solution was measured at 510 nm and 25 °C using a Jenway 6405 (Essex, UK) UV-Vis kinetics spectrophotometer, every second over 1 min. The initial reaction rate (ΔOD min^{-1}) was used to express enzyme activity and was calculated by linear regression of the measured values of absorbance increase over 1 min. The substrate solution had a concentration of hydrogen peroxide of 9.75 mmol l^{-1}, a phenol concentration of 0.16 mol l^{-1} and 4-aminoantipyrine concentration of 2.3 mmol l^{-1}, in a 0.1 mol l^{-1} phosphate buffer, pH 7.0. In preliminary experiments, no significant differences were found for the initial activity of the enzyme in buffer only and in the mixture buffer–DMF.

2.5. Data analysis

Biphasic and multiphasic patterns of the inactivation experimental data of different enzymes have been reported and adequately described by a series-type model [8,20], which was found to be preferable to the most often used two-fraction model [21].

The series-type model considers an homogeneous native enzyme population (E_N), which inactivates by a mechanism that includes the formation of an intermediate form, partially inactivated (E_I), that can have different thermal resistance and specific activity compared to the native form, to yield an irreversibly inactivated form (E_D):

$$E_N \xrightarrow{k_1} E_I \xrightarrow{k_2} E_D \quad (1)$$

The activity decay is therefore described by [20]:

$$\frac{A}{A_0} = \left(1 - \frac{\alpha k_1}{k_1 - k_2}\right) \exp(-k_1 t) + \frac{\alpha k_1}{k_1 - k_2} \exp(-k_2 t) \quad (2)$$

where α is the ratio between the specific activities of the intermediate and native forms (respectively, ξ_I and ξ_N) and k_1 and k_2 are, respectively, the reaction rate parameters of the native and intermediate forms, as expressed in Eq. (1).

Temperature dependence of the rate parameters, in general, follows an Arrhenius-type relationship according to:

$$k = k_{ref} \exp\left[-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (3)$$

where k is the rate parameter at temperature T, k_{ref} is the rate parameter at a reference temperature T_{ref} (K), E_a is the activation energy (kJ mol^{-1}) and R is the universal gas constant (8.314 J mol^{-1} K^{-1}). The use of a finite reference temperature is most important, as it decreases the correlation between the pre-exponential factor, k_{ref}, and the activation energy, E_a, thus improving parameter estimation [22]. In this work, the reference temperature chosen was the mean value of the inactivation temperatures studied, 77.5 °C (350.65 K).

Usually, the estimation of the kinetic parameters is carried out by the so-called two-step procedure: firstly, Eq. (2) is used to obtain the values of the parameters α, k_1 and k_2 using non-linear regression and then the activation energies (E_{a1} and E_{a2}) are calculated using a linearised form of Eq. (3). While this approach is essential to assess the adequacy of the model in describing the experimental data, it leads to higher statistical uncertainty [22,23]. Alternatively, Eqs. (2) and (3) can be combined to obtain directly the values of α, k_{1ref}, k_{2ref}, E_{a1} and E_{a2} using non-linear regression, in a one-step analysis, which provides higher precision estimates and avoids the estimation of intermediate parameters. Thus, to increase precision, a model imposing the above referred relation between the rate parameters of the series-type model and temperature was fitted to the complete set of experimental data, for all the temperatures tested for each inactivation media. It was verified that the estimated values of the α parameter were independent of temperature [24] and therefore, this restriction was also imposed to the model regression procedures.

Parameter estimation and model building were performed by non-linear regression using Stata 5.0 (Computing Research Centre, Santa Monica, USA).

3. Results and discussion

3.1. Inactivation kinetics as a function of the reactivation time

A typical biphasic profile was found for all the experimental data as illustrated in Fig. 1, which was

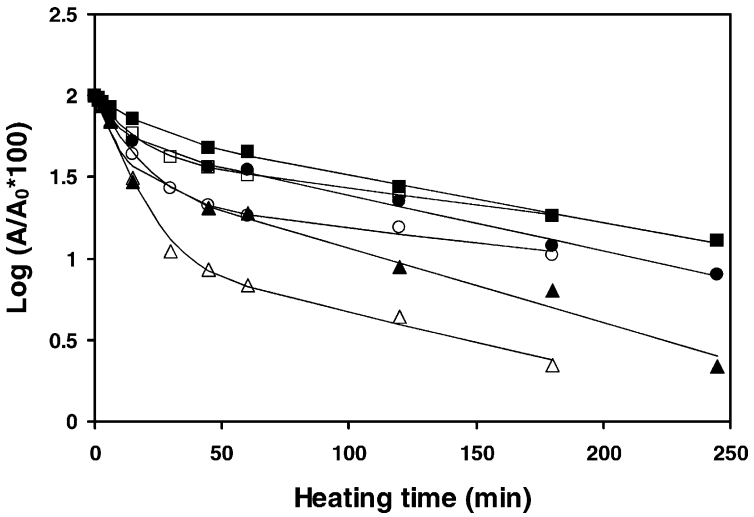

Fig. 1. Inactivation kinetics of peroxidase at 70 °C in buffer (open symbols) and buffer with 10% (v/v) DMF (closed symbols) after 5 min (△/▲), 2 h (○/●) and 24 h (□/■) of reactivation at 4 °C and best fitted curves using the series-type model.

adequately described by the series-type model. Resulting residuals proved to yield constancy of variance and tended to follow a normal distribution, thus showing the validity of the model. The coefficients of determination (R^2) were above 0.99.

The results clearly show that reactivation affected the estimated parameters α, k_{1ref} and k_{2ref} (Fig. 2). The values of the α parameter significantly increased with the time of reactivation, for both inactivation media (Fig. 2a), which thus reflect the change of the experimental curves profile into a less pronounced biphasic pattern, as shown in Fig. 1. In the presence of dimethylformamide, greater values for the α parameter were found, indicating that the solvent may afford a protective effect against thermal inactivation in the first reaction of the inactivation process thus yielding an intermediate form of the enzyme with higher catalytic activity.

Both rate parameters, k_{1ref} and k_{2ref}, appeared to decrease with increasing time of reactivation (Fig. 2b and c, respectively), although, in general, relatively large errors were associated to these parameters. On the other hand, the reaction for the inactivation of the intermediate was found to be slower than that of its formation (k_{2ref} values were one order of magnitude smaller than k_{1ref} values) and greater values for both rate parameters were obtained in the presence of dimethylformamide. The overall effects on thermal stability of the enzyme, in terms of residual activity, due to the different values of α, k_{1ref} and k_{2ref} for the two inactivation media, resulted in a higher residual activity with the solvent (see Fig. 1). This observation leads to the conclusion that the organic solvent affords a protective effect to the enzyme in this case. Different patterns were verified for some of the other temperatures, clearly indicating that to compare stability in the two media, one must take into account the combined effect of the three kinetic parameters. The same holds true, in general, when comparing stability of enzymes that show a biphasic profile as in this work.

Although statistical uncertainty associated to the estimated values of the activation energies do not allow defining a systematic tendency, there is some indication for a decrease of E_{a1} and increase of E_{a2} with time of reactivation in both media (results not shown). Nevertheless, sensitivity to temperature was found to be significantly greater in the presence of the solvent, which may indicate that conformational changes have a greater relative importance in both the formation and inactivation of the intermediate in this medium [25].

3.2. Regeneration kinetics as a function of the heating time

The relationship between the regeneration of enzyme activity after thermal treatment and the time of

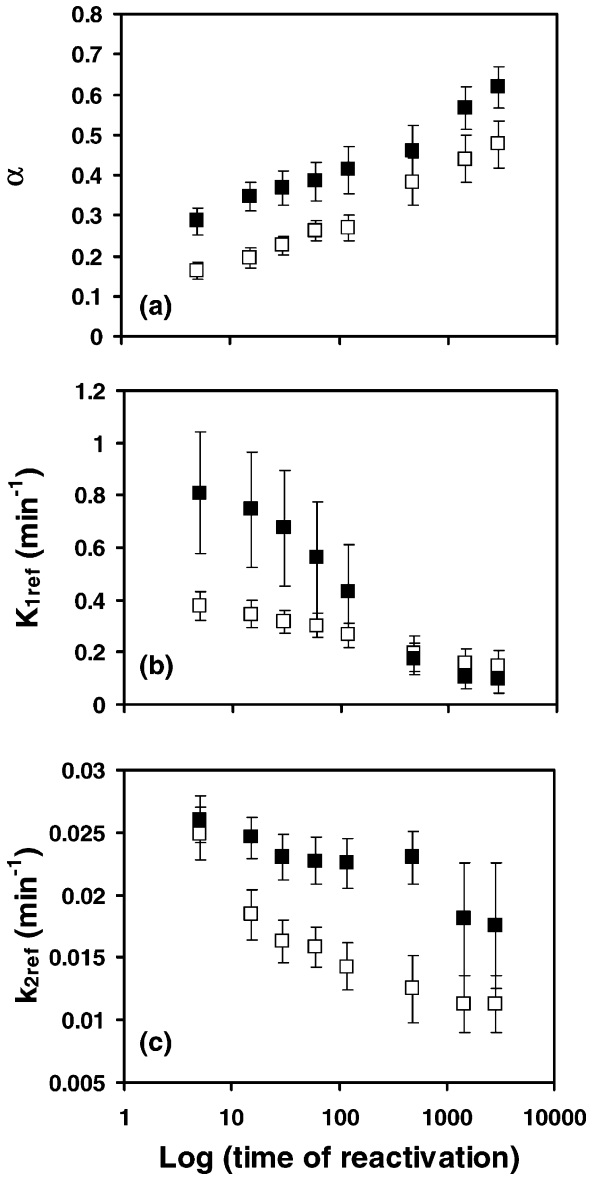

Fig. 2. Dependence of the series-type model parameters on the time of reactivation, and their 95% confidence intervals, at 4 °C of peroxidase thermal inactivated in buffer (□) and with DMF (■): (a) α; (b) k_{1ref}; and (c) k_{2ref} (the logarithmic time scale is used to enhance the visualization of parameters' variation at smaller times).

storage has been reported to follow other kinetics than first- or second-order: after a steep increase of the activity, an equilibrium stage is attained where the values of the enzyme activity are not significantly increased with increasing time of storage [26]. These overall results were similar to those obtained in this work, although, in the present case, a systematic analysis of the regeneration process was carried out. Attempts were made to find an empirical mathematical model that could adequately describe the activity regeneration with the reactivation time, which was found using the following logarithmic equation:

$$\frac{A}{A_0} = a \ln t_r + b \quad (4)$$

where a is the rate parameter and b the intercept (activity at $t_r = 0$).

This analysis was performed up to 2 h of reactivation after which there was no further activity

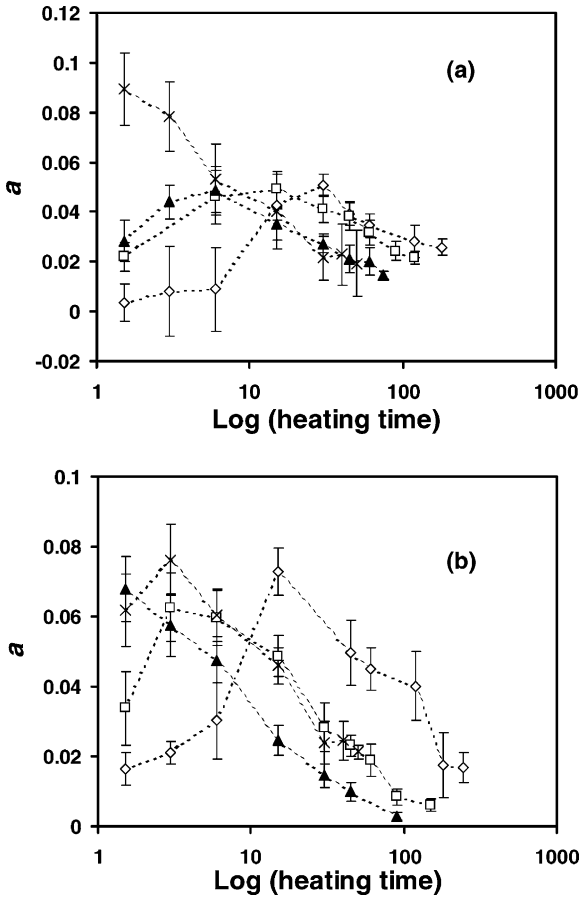

Fig. 3. Variation of the rate of reactivation (parameter a) with both the inactivation temperature (70 °C (◇), 75 °C (□), 80 °C (▲) and 85 °C (×)) and the heating time for peroxidase in (a) buffer and (b) with DMF.

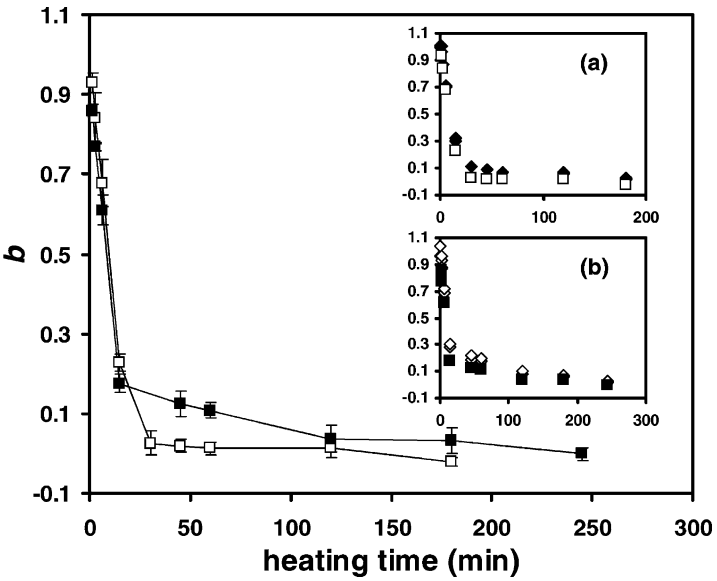

Fig. 4. Predicted residual activity for peroxidase without reactivation (parameter *b*) in buffer (□) and with DMF (■) at 70 °C. The smaller plots represent the relationship between the predicted values of parameter *b* (□,■) and the experimental data obtained for residual enzyme activity after 5 min of reactivation (◇,◆) in (a) buffer and (b) with DMF.

increase. The parameter *a*, which measures the rate of the regeneration process showed no significant differences for both media. A systematic trend was, however, found for most of the experiments with the *a* values reaching a maximum value (peak) (Fig. 3). An interesting occurrence is that the experimental heating times to reach both, the maximum value of the parameter *a* in Eq. (4) and the inflection point of the curves predicted by Eq. (2) discussed in the preceding section, by each temperature and reaction media, were about the same. This inactivation time corresponds to the moment when formation of the intermediate has been completed and only inactivation occurs. Therefore, the rate of reactivation increases throughout the time needed to "accomplish" the reaction of formation of the intermediate form of the enzyme (before reaching the peak), showing that the reactivation process may be determined, at this stage, by the amount of enzyme which has been transformed into the intermediate form. Moreover, the values of the parameter *a* were found to be greater at higher temperatures, in spite of the relatively larger errors in the estimation of this parameter, indicating that increasing the temperature increases the rate of the reaction for the formation of the modified enzyme and thereafter the rate of reactivation. Interestingly, after complete formation of the intermediate (after the maximum value of *a*), opposite trends are observed for the dependence of the rate parameters (*a* values) on both the temperature of inactivation and the extent of heating time. All these observations support the reasoning described above for the dependence of the rate of reactivation (*a*) on the amount of intermediate present in the inactivation media.

The parameter *b* was also estimated for each temperature and heating time, yielding the predicted values of enzyme activity when no reactivation occurs ($t_r = 0$), as can be seen in Fig. 4 for buffer and buffer–DMF at 70 °C. It is interesting to find that the *b* values were, as expected, always close and smaller than the experimental data obtained for 5 min of reactivation, as illustrated in the smaller plots of Fig. 4, supporting the validity of the model used.

4. Conclusions

Inactivation of horseradish peroxidase in phosphate buffer and a mixture of this buffer containing 10% (v/v) dimethylformamide showed biphasic profiles,

which were adequately described by the series-type model. In the mixture, the enzyme intermediate was formed and inactivated faster than in buffer alone, although, it retained higher activity, which resulted, in some cases, in a protective effect on the enzyme against inactivation in the presence of the solvent. Enzyme reactivation affected the inactivation profiles and their related estimated parameters, yielding smoother biphasic patterns. A simple logarithmic equation proved to fit well the enzyme activity regeneration as a function of the reactivation time, with a maximum value for the rate of reactivation found at the time at which the greatest amount of the intermediate is expected to be present. The methodology used in this study also allowed estimating the residual activity of the enzyme after heating, when reactivation has not yet occurred.

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