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THERMAL INACTIVATION KINETICS OF HORSERADISH PEROXIDASE IN PHOSPHATE BUFFER AND WATER-MISCIBLE ORGANIC SOLVENT MIXTURES

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Introduction

Horseradish peroxidase is one of the most studied peroxidases used as a biocatalyst in the polymerisation of phenol, aniline and their derivatives, and has been shown to be active in a number of organic solvents or aqueous solvent mixtures (1,2). Because of its high thermal resistance and relative simplicity of the assay method, horseradish peroxidase has been used to monitor the adequacy of thermal processing and thus of food stability (3,4,5). Research has also focused on the potential use of peroxidase in waste treatment to remove phenolic compounds from industrial wastewaters (6). Karam and Nicell (6) referred horseradish peroxidase as "undoubtedly one of the most studied enzymes in the relatively new area of enzymatic waste treatment". This enzyme was also found to be a particularly useful tool for immunoassays (7). In literature, the behaviour of enzymes in mixed aqueous-organic media has been extensively studied, particularly in what concerns to their catalytic properties and efficiency (8). There is, however, a lack of information of the interplay of these processes, which afterwards, would address to the understanding of both the thermal inactivation and reactivation kinetics of enzymes and its related mechanisms. This knowledge would ultimately improve the potential and effective use of enzymes in diverse biotechnological processes. To choose the adequate mathematical approach to the kinetic modelling problem is the first step of this procedure. More difficult is to provide a mechanistic understanding of the complex catalytic events, which, would however, give deeper insight into the stability/structure relationships of proteins exposed to different environments. The purpose of this work was to study the thermal stability of horseradish peroxidase in solutions of phosphate buffer and water-miscible solvents (dimethyl sulfoxide and 2-propanol) at three different concentrations (1, 10 and 25% v/v) over a wide range of inactivation temperatures, aiming at a comprehensive mathematical modelling of the thermal inactivation kinetics.

Materials and Methods

Enzyme and organic solvent mixtures

Horseradish peroxidase (R_z 0.98) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aqueous solutions of this enzyme were prepared in distilled water (0.4 mg mL^{-1}) and stored at 4°C. The mixtures containing a water-miscible solvent were prepared in 0.1 mol L^{-1} sodium phosphate buffer (pH 7.0) to the final concentrations of 1, 10 and 25% (v/v). Dimethyl sulfoxide (DMSO; log *P* of – 1.35; Riedel-de Haën, Germany) was stored over 3Å molecular sieves (Riedel-de Haën, Germany) prior to mixing with the buffer solution. The organic solvent 2-propanol (log *P* of 0.13; Riedel-de Haën, Germany) was distilled and dried over 3Å molecular sieves.

Changes in the pH of the buffered solutions upon addition of the organic solvents were measured at room temperature using a pH538 Multical® pH meter (Weilheim, Germany). The pH values varied from 6.90±0.02 to 7.74±0.03 and 6.91±0.03 to 7.26±0.01, respectively, in DMSO and 2-propanol, within the range of concentrations studied. These values were, in general, close to the neutral scale, where optimal thermal stability for horseradish peroxidase was reported (3).

Thermal inactivation

Duplicated aliquots of 1.6 mL of the buffer-solvent solutions were transferred to glass tubes of 15 mm diameter and 50 mm height and immersed in a thermostatic water bath equilibrated at the specified inactivation temperature. Thermal inactivation started when an aliquot of the enzyme solution was added to the solvent mixture in the bath to achieve the concentration of 0.08 mg mL^{-1} of horseradish peroxidase in the final volume of 2 mL. After heating, and for pre-determined exposure times (ranging from 1.5 to 600 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 into an ice bath.

Enzyme reactivation

Horseradish peroxidase thermally inactivated in buffer-solvent mixtures was shown to recover activity after removal from heat and maintenance at low temperature (4°C). To get some insight into the reactivation process and to ensure that experimental data would enable the maximum regeneration of the enzyme, the activity of each thermally inactivated sample at 75°C was assayed after 48 hours from heat treatment and storage at 4°C.

Analysis of the enzyme activity

The enzyme activity was measured according to the Worthington procedure (9). 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, U.K.) UV/VIS kinetics spectrophotometer, every second over one minute. The initial reaction rate (ΔOD min⁻¹) used to define enzyme activity was calculated by linear regression of the measured values of absorbance increase over one minute. 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 effects on the initial activity of the enzyme were found due to the presence of the organic solvents.

Data analysis

In literature, biphasic and multiphasic inactivation kinetics of different enzymes have been reported and adequately analysed by a series-type model (10,11). This model considers the existence of an homogeneous native enzyme population (E_N), which inactivates through a mechanism that includes the formation of an intermediate form of the enzyme, partially inactivated (E_I), that can have different thermal resistance and activity when compared to the native one, to yield an irreversibly inactivated form (E_D):

$$E_N \xrightarrow{k_1} E_I \xrightarrow{k_2} E_D \qquad [1]$$
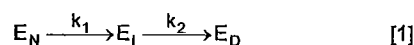
The activity decay is therefore described by (11):

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

where A is the residual activity of the enzyme, A_0 is the initial activity of the enzyme, α is the ratio between the specific activities of the intermediate and native forms (respectively, ξ_I and ξ_N), k_1 and k_2 are, respectively, the reaction rate parameters of the native and intermediate forms, as expressed in equation [1] and t is the heating time.

Linear relationships between the residual activity and heating time were analysed, considering a first-order reaction rate:

$$\frac{A}{A_0} = \exp(-k_2 * t) \qquad [3]$$

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

$$k = k_{ref} * \exp\left[-\frac{E_a}{R} * \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \qquad [4]$$

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 (kJmol⁻¹) and R is the universal gas constant (8.314 Jmol⁻¹K⁻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 (12). In this work, the reference temperature chosen was the mean value of the inactivation temperatures studied, 77.5°C (350.65 K) and 47.5°C (320.65 K), respectively, for DMSO and 2-propanol.

The fitting of the kinetic models to the experimental data is traditionally carried out in a two-step analysis: each model parameter of equations [2] or [3] is determined at each temperature studied and subsequently the estimated rate parameters (k_1 and k_2) are used to obtain the Arrhenius' parameters via linearization of equation [4]. Although this method is essential to assess the adequacy of the model in describing the experimental data, it leads however to higher statistical uncertainty (12,13). Most suitable, the one-step analysis uses a non-linear regression where the temperature dependence (equation [4]) can be directly incorporated into the rate equations [2] or [3], with the latter being directly analysed over all temperatures studied in order to obtain the estimates of α, k_{1ref}, k_{2ref}, E_{a1} and E_{a2}, which, ultimately 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 medium. It was also verified that the estimated values of the α parameter were independent of temperature and therefore, this restriction was also imposed to the model regression procedures.

Non-linear regression for parameter estimation and model building was performed using MATLAB student version Release 12.1 (The MathWorks, Inc., MA, USA).

Results and Discussion

In dimethyl sulfoxide (DMSO) mixtures, and for all the concentrations analysed, the thermal inactivation curves of horseradish peroxidase exhibited a typical biphasic pattern adequately described by the series-type model ($R^2 > 0.99$), as shown in the example of Figure 1. In 2-propanol, a first-order inactivation kinetics was observed (Fig. 2) and the experimental data were analysed using equation [3] ($R^2 > 0.99$). The validity of these kinetic models was further tested by inspection of the resulting residuals, which yielded constancy of variance and tended to follow a normal distribution. The change from first-order to biphasic kinetics, respectively, in 2-propanol and DMSO, may suggest that different mechanisms can play an important role on the thermal inactivation process of horseradish peroxidase in these reaction media. The stability of the horseradish peroxidase was remarkably affected when 2-propanol mixtures were used as reaction media: inactivation temperatures of 45-60°C and 35-50°C were tested, respectively, with 1 and 25 % (v/v) of 2-propanol, whereas higher temperatures (70-85°C) were effectively tolerated by the enzyme in DMSO mixtures, independently of the solvent concentration in the medium. The dependence of the
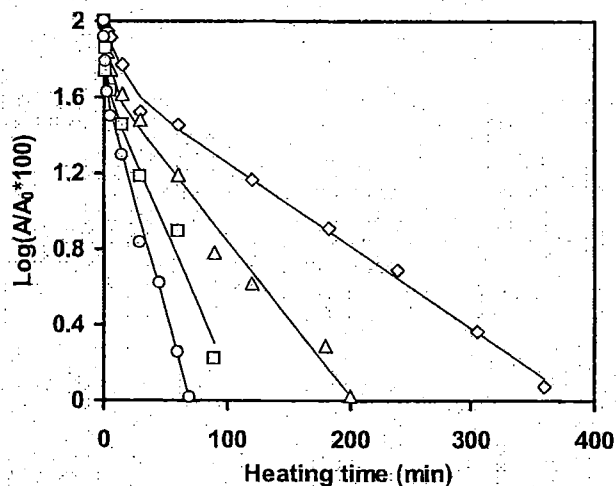

Figure 1 Thermal inactivation kinetics of horseradish peroxidase in buffer with 25% (v/v) dimethyl sulfoxide at 70°C (◊), 75°C (Δ), 80°C (□) and 85°C (O), and fitted curves using the series-type model in a one-step analysis.
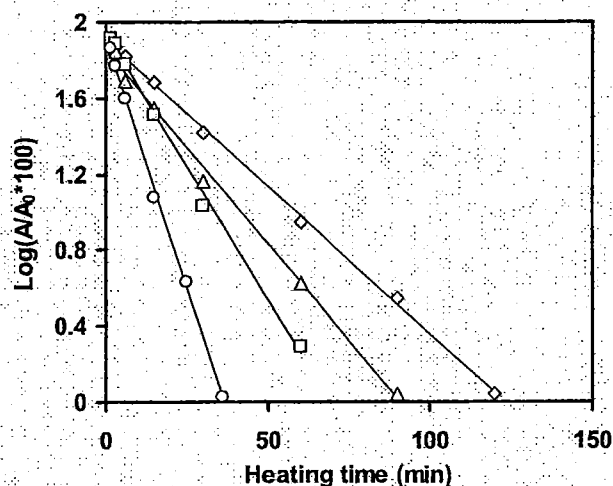
Figure 2 Thermal inactivation kinetics of horseradish peroxidase in buffer with 25% (v/v) 2-propanol at 35°C (◊), 40°C (Δ), 45°C (□) and 50°C (O), and fitted curves using the first-order model in a one-step analysis.

model parameters on both the nature and the concentration of the water-miscible solvent is shown in Table 1. The values of the α parameter increased with increasing DMSO concentration, which indicated that smoother biphasic patterns of the experimental curves were obtained. This finding also suggested that this solvent might afford a protective effect against thermal inactivation in the reaction of formation of the intermediate by yielding intermediate forms of the enzyme with greater relative activity when the concentration of DMSO was increased up to 25% (v/v). The reaction rate for the inactivation of the intermediate was comparatively much slower than that of its formation as the estimates of k_{2ref} were one order of magnitude smaller than k_{1ref} values. In the case of 2-propanol, enzyme stability was particularly affected by its concentration (see Table 1 for k_{2ref} values which considerably decreased when increasing the solvent concentration), thus yielding faster changes in the enzyme conformation and enhanced loss of its catalytic activity. This may be expected as 2-propanol is more hydrophobic than DMSO, and it is reported to be a strong denaturant, disrupting the tertiary structure of the enzyme when its concentration is increased, which ultimately influences enzyme stability (14,15).

Table 1 Kinetic parameters ($\pm$ 95% confidence interval) for thermal inactivation of horseradish peroxidase in mixtures of phosphate buffer with DMSO and 2-propanol

Solvent (% v/v)	α	$k_{1ref} \times 10^{-1}$ (min^{-1})	$k_{2ref} \times 10^{-2}$ (min^{-1})	E_{a1} (KJ mol^{-1})	E_{a2} (KJ mol^{-1})
DMSO [a]					
1	0.21±0.042	2.50±0.68	1.67±0.19	159.7±39.1	121.8±13.6
10	0.33±0.030	5.83±1.42	2.61±0.12	145.6±33.4	135.8±5.42
25	0.42±0.041	3.30±0.88	2.42±0.09	181.0±33.9	114.9±3.71
2-propanol [b]					
1			0.74±0.41		176.8±21.1
10			2.82±0.97		115.2±35.4
25			4.05±1.11		76.2±25.9

[a] estimates of parameters from eqn [2] with T_{ref}=77.5°C; [b] estimates of parameters from eqn [3] with T_{ref}=47.5°C.

Although statistical uncertainty associated to the estimated values of the activation energies do not allow, in general, to define a systematic tendency, there was, however, indication of a decrease of the activation energy (E_{a2}) with increasing concentration of 2-propanol. The magnitude of the

activation energies supports evidence for the occurrence of conformational modification of the enzyme during the inactivation process (16). However, there is the possibility that different events (e.g. covalent changes) might also occur and play a progressively more significant role in the control of the inactivation process when the concentration of 2-propanol was varied from 1 to 25% (v/v). In DMSO mixtures, the activation energies for both reactions (E_{a1} and E_{a2}) were not significantly affected by solvent concentration as their values only showed small fluctuations with no apparent trend. Nevertheless, sensitivity to temperature was found to be greater for the reaction of formation of the intermediate, which probably indicates that conformational changes have greater relative importance in this case.

Some insight into the reactivation kinetics of horseradish peroxidase after heat treatment in buffer-solvent mixtures with subsequent storage of these samples at low temperature was obtained in this study. Maximum regain of enzyme activity was assayed in DMSO thermally inactivated samples after 48 hours at 4°C from heat treatment. The analysis of the kinetic data showed less biphasic profiles of the experimental curves with both α and k_2 model parameters being significantly affected (results not shown). On the other hand, irreversible damages into the structure of the enzyme were caused by the presence of 2-propanol, thus preventing recovery of the enzyme activity after heat removal and dilution of the solvent.

This work clearly demonstrated that the stability of horseradish peroxidase was greatly affected by the concentration of 2-propanol in its environment maybe because of the more deleterious effect of this solvent on the structure of the protein, thus yielding increasingly loss of its catalytic activity. Furthermore, the thermal inactivation kinetic patterns, as well as the enzymatic reactivation process, proved to be dependent on the nature of the water-miscible solvent, which may reflect different mechanisms of inactivation. Future work should be devoted to cast new light on the correlation between the kinetic model parameters and the molecular aspects of interaction between the organic solvents and the enzyme.

Acknowledgements

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