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Stability of free and immobilised peroxidase in aqueous–organic solvents mixtures

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Abstract

The activity and stability of horseradish (*Amoracia rusticana*) peroxidase (HRP) free in solution and immobilised onto silica microparticles was studied in the presence of organic co-solvents.

The effect of several hydrophilic organic solvents, namely dimethyl sulfoxide, dimethylformamide, dioxan, acetonitrile and tetrahydrofuran, in the activity and stability of free HRP was studied. From the solvents tested, DMSO led to the highest activities and stabilities. After 2 h of incubation at 35°C, the remaining activity of the enzyme in the presence of 30% of each solvent was less than 30%, with exception of DMSO for which the enzyme remained fully active.

In order to increase stability, HRP was covalently immobilised onto silica microparticles. The half-life of the enzyme in buffer at 50°C increased from 2 to 52 h when the enzyme was immobilised. The stability of both free and immobilised HRP was also studied at 50°C in aqueous mixtures of 3.5, 20, 35 and 50% (v/v) DMSO. Free HRP stability was not affected by the presence of 3.5 and 20% DMSO, but higher contents lead to a more pronounced deactivation. Immobilised HRP stability increased with DMSO content up to 20%, decreasing for higher contents. The enzyme half-life increased more than 300% when changing from buffer to 20% DMSO.

The deactivation of free HRP was modelled using the simple exponential decay, and the deactivation of immobilised HRP was described by a two-step inactivation model.

Keywords: Horseradish peroxidase; Immobilisation; Silica; Dimethyl sulfoxide; Thermostability

1. Introduction

The use of aqueous mixtures of organic co-solvents for conducting enzymatic reactions has become highly promising in biotechnology processes. By total or partial replacement of water as the reaction medium with an organic solvent, hydrophobic substrates can be more efficiently converted, hydrophobic products can be produced with higher yields, and the thermodynamic equilibrium of hydrolytic reactions can be shifted to the synthetic way [1,2]. Moreover, some enzymes are often more stable in water-poor environments than in water [3]. Nevertheless, increasing the concentration of an organic solvent in the reaction medium can lead to the inactivation of enzymes due to reversible changes in the protein structure, while a more prolonged incubation leads to the irreversible inactivation of the enzyme [4,5]. There are several approaches to delay both reversible and irreversible inactivation. One of them is to choose a solvent, which disturbs the catalytically active conformation of the protein to a lesser extent. Another

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possible method is to enhance stability by immobilisation [6].

The covalent immobilisation of an enzyme onto an inorganic support involves the formation of covalent bonds between the enzyme and the support material. These bonds are normally formed between functional groups present on the surface of the support and functional groups from the amino acid residues on the surface of the enzyme. Examples of such groups are the amino groups from lysine and arginine, carboxyl groups from aspartic acid and glutamic acid, hydroxyl groups from serine and threonine, and sulfydryl groups from cysteine [7].

Peroxidases are widely distributed throughout the biological world, being found especially in plants and also in some animal tissues and micro-organisms. The peroxidase from horseradish roots (HRP, E.C.1.11.1.7) is an oxidoreductase enzyme, which has been extensively studied over many years and has been proven to be a helpful tool in biotechnology, namely it has been used in wastewater remediation [8], biosensor construction [9] and certain organic synthesis, e.g. phenolic polymerisation [10]. HRP also functions as an indicator in oxidase-based coupled assays [11], in bioluminescent [12,13] and immunodiagnostic assays [14], in cytochemistry [15] and in DNA probes [16]. HRP occurs as a large family of isoenzymes from which the cationic isoenzyme C is the major one [17].

HRP is a monomeric protein consisting of a single polypeptide chain arranged in two domains and glycosylated at eight specific sites [18,19]. It contains one heme prosthetic group, two calcium ions and four disulphide bridges [20]. The molecular weight is about 44 kDa.

The present work examines enzymatic activity and deactivation in aqueous–organic co-solvent mixtures, using HRP as a model enzyme both free in solution as well as immobilised by covalent attachment to a solid support.

2. Experimental

2.1. Materials

Horseradish (*Amoracia rusticana*) peroxidase isoenzyme C was obtained as a brown freeze-dried powder from Biozyme Laboratories Limited (catalogue name: HRP4). Hydrogen peroxide was purchased from Merck as a 30% (v/v) solution and the substrate used, guaiacol (*o*-methoxyphenol), from Sigma. The organic solvents tetrahydrofuran (THF), 1,4-dioxan, acetonitrile and dimethyl sulfoxide (DMSO) were obtained from Merck; *N*,*N*-dimethylformamide (DMF) and acetone from Riedel-de Haën. Glutaraldehyde was purchased from Merck and 3-aminopropyltriethoxysilane from Sigma. Silica was a gift from a Protuguese chemical company. All other chemical reagents used were of analytical grade.

2.2. Immobilisation of HRP

Enzyme immobilisation by covalent methods usually involves two steps: first, the activation of the support, and second, the coupling of the enzyme to the activated support. Silica microparticles (2.2–2.8 μm) were activated using an organic silanisation technique. A 1 g of silica was suspended in 25 ml of 1% (v/v) 3-aminopropyltriethoxysilane in acetone. The solvent was completely evaporated during 3–4 h in a rotavapor apparatus under low vacuum at room temperature, and the support was then heated at 115°C overnight. This activated support was then suspended in 20 ml of a 2.5% glutaraldehyde and mechanically stirred during 1 h at room temperature. The activated support, now with a yellow/orange colour, was washed a coupled of times with water and one time with 20 mM phosphate buffer at pH 7. Finally, the coupling of HRP was achieved by adding a similar volume of 20 mg ml^{-1} HRP in the same buffer. After 24 h incubation in a cold room, the suspension was centrifuged four times at 14,000 rpm and the immobilised peroxidase suspended in 100 mM phosphate buffer at pH 7 with a support concentration of 25 mg dry silica weight per ml. This suspension was stored at 4°C.

2.3. HRP activity assay

HRP standard activity was measured using hydrogen peroxide and guaiacol (2-methoxyphenol) as substrates. It is known that during the oxidation of guaiacol, the enzymatic reaction is followed by a non-enzymatic polymerisation process that leads to the formation of dimethoxybiphenoquinone, an amber-coloured product, which has maximum absorption around 470 nm [21].

HRP activity was thus determined by monitoring the increase in absorbance at 470 nm using a Hitachi U-2000 spectrophotometer with temperature control at 25°C. This increase is proportional to the rate of H_2O_2 consumption. The activity assay mixture contained 960 μl of 20 mM guaiacol and 20 μl of a solution containing free or immobilised HRP ([free HRP] = 1.30 μg ml^{-1}; [immobilised HRP] = 3.55 μg ml^{-1}). The reaction was initiated by the addition of 20 μl of 100 mM H_2O_2. All solutions were prepared in 100 mM phosphate buffer, pH 7, except the guaiacol solution that was prepared with different contents of DMSO.

In the preliminary studies, the activity of HRP in the presence of different organic co-solvents concentrations was determined using a slightly different methodology. The activity assay mixture contained 200 μl of 100 mM guaiacol, 9 μl of 0.09 mg ml^{-1} free HRP, and 786 μl of an aqueous–organic co-solvent mixture, with different contents of organic solvent. The reaction was initiated by adding 5 μl of 100 mM H_2O_2.

2.4. HRP stability assay

Different enzyme preparations were incubated in a water bath at a fixed temperature. Aliquots were taken at certain time intervals and the residual activity measured according to the following procedure: the activity assay mixture contained 960 μl of 20 mM guaiacol in 100 mM phosphate buffer, pH 7, 20 μl of HRP free or immobilised ([free HRP] = 1.30 μg ml^{-1}; [Immobilised HRP] = 3.55 μg ml^{-1}) and 20 μl of 100 mM H_2O_2. In the preliminary studies, HRP stability was investigated measuring the residual activity in the presence of 30% of the co-solvent in study, according to the procedure described in Section 2.3.

3. Results and discussion

3.1. Co-solvent screening

In a preliminary study, the effect of different water-miscible organic solvents on the activity and stability of free HRP was evaluated. The solvents tested included tetrahydrofuran, acetonitrile, dioxan, dimethylformamide and dimethyl sulfoxide. For all of them, as the co-solvent content increased, HRP activity decreased. Highest activities were observed with DMSO and dioxan. The activity of the enzyme for co-solvent contents higher than 40% (v/v) was lower than 10% of the initial activity, except for DMSO.

Table 1
Residual activity in the presence of 30% co-solvent, respective half-life at 35°C and log P value of the solvent

Co-solvent	$E_{30\%Solvent}/E_{Buffer}$ (%)	$t_{1/2}$ (min)	log P
THF	15	11	0.45
Acetonitrile	10	52	−0.15
Dioxan	21	52	−0.32
DMF	14	73	−0.93
DMSO	27	≫1200	−1.22

The stability of the enzyme also decreased considerably in the presence of these co-solvents, and was greatly influenced by the nature of the co-solvent. Table 1 shows the half-life ($t_{1/2}$) of the enzyme at 35°C, the relative activity ($E_{30\%Solvent}/E_{Buffer}$) in the presence of 30% of co-solvent and the co-solvent log P value. The most striking result is the very high stability obtained with DMSO compared with the other solvents studied.

It is also interesting to notice that the lower the log P value, the higher was the half-life of the enzyme. The log P value corresponds to the logarithm of the partition coefficient of a solvent in an octanol/water system and is often used as a hydrophobicity index — the higher the log P value, the more hydrophobic the solvent is [22]. Gorman and Dordick [23] studied the water-stripping phenomenon of four enzymes including HRP in the presence of a wide variety of solvents and concluded that the amount of water desorbed was roughly independent of the log P values for water-miscible solvents with a log P lower than 1.5 [23]. Thus, the increase in enzyme stability with the log P value is not a universal correlation. Torres et al. [24] have also stated that the log P value cannot be applied for aqueous mixtures containing water-miscible organic solvent.

From these studies, it is clear that in the presence of DMSO, high activities and stabilities were obtained. This co-solvent was then used for further studies.

3.2. Peroxidase activity in aqueous mixtures of DMSO

The activity of the free and immobilised HRP was studied in the presence of different contents of DMSO

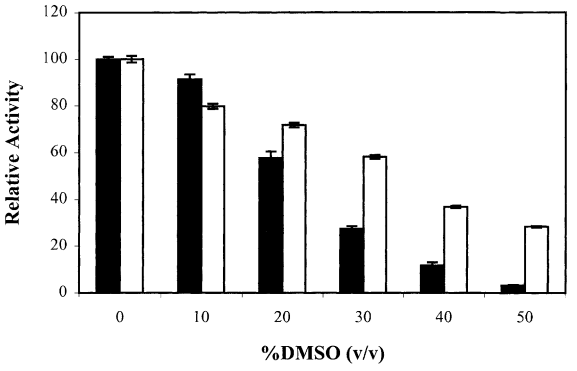

Fig. 1. Relative activity of free (■) and immobilised (□) HRP in the presence of aqueous mixtures of DMSO relative to the activity in pure aqueous system (100% activity is defined as the activity in 100 mM phosphate buffer, pH 7: free HRP exhibited a specific activity of 11.69 ΔAbs ml min^{-1} μg^{-1}, and the immobilised HRP 2.56 ΔAbs ml min^{-1} μg^{-1}).

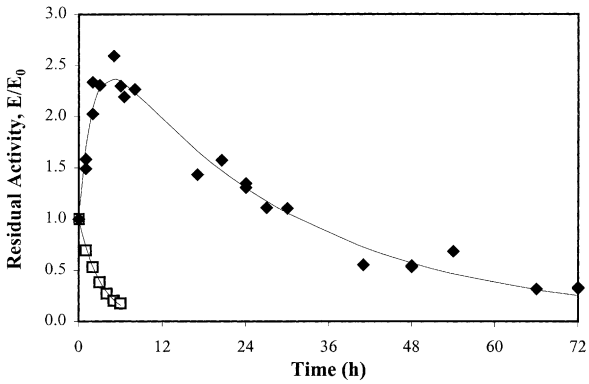

Fig. 2. Deactivation profiles of free (□) and immobilised (◆) HRP at 50°C in phosphate buffer 100 mM at pH 7. [Free HRP] = 1.80 μg ml^{-1} and [immobilised HRP] = 3.55 μg ml^{-1}.

(Fig. 1). On the contrary to what has been observed for other free and immobilised enzymes [25], HRP does not show an enhancement on enzyme activity upon the addition of an organic solvent at low concentration. In fact, the activity of both free and immobilised HRP decreased continuously with an increase in the DMSO content, which is probably due to the toxicity of the organic solvent. Nevertheless, higher activities were obtained with the immobilised HRP than with the free enzyme. This means that the covalent attachment of HRP to the solid support was able to suppress to some extent the protein unfolding due to the presence of an organic solvent.

3.3. Peroxidase stability

Despite the contribution of many authors, the general mechanism of irreversible inactivation of proteins has not yet been clarified. However, it is quite certain that enzyme inactivation involves considerable conformational changes, i.e. the unfolding of the protein molecule. If unfolding is taken as an essential step of enzyme inactivation, then the more rigidly fixed is the protein on the support, the more difficult it is to unfold and, consequently, the harder it will be to destroy the active site and inactivate the enzyme. This is why immobilisation is regarded by many authors as a general and universal method of enzyme stabilisation. In fact, a statistical analysis of available data revealed that only 16% of the enzymes studied showed a decrease in stability after immobilisation [6].

3.3.1. Stability in aqueous media

The application of immobilised enzymes in biotechnology processes has been stimulated due to their well-known advantages. The creation of a covalent bond between the biocatalyst and the support forms a stable conjugate, which is unlikely to dissociate under normal use of the enzyme. The multi-point attachment of the protein molecule to the support can further increase its resistance to thermal inactivation. In fact, as Fig. 2 shows, immobilised HRP is significantly more stable in aqueous solution at 50°C than the free enzyme.

Free HRP deactivates at 50°C according to a simple exponential model: $E_t/E_0 = \exp(-0.0056t)$ with a half-life of 124 min. The exponential decay for free HRP was considered, since it has been reported that native, unmodified peroxidase exhibits a first-order inactivation [26,27]. The value of 0.987 for the correlation coefficient of the adjust to the experimental data confirms the applicability of the model to the HRP inactivation in 100 mM phosphate buffer, pH 7, at 50°C. Nevertheless, modified or immobilised peroxidase does not strictly follow a first-order reaction kinetics [28].

In fact, the shape of the inactivation curve of the immobilised HRP also at 50°C suggests that the inactivation involves an intermediate species, which is more active than the native immobilised enzyme. It

is worth mentioning that the specific activity of this intermediate conformation is, however, lower than the specific activity of the free enzyme (specific activity of immobilised HRP is 22% of the free HRP and the specific activity of immobilised intermediate conformation is 61% of the free HRP). Henley and Sadana [29] have proposed a two-step model deactivation mechanism that involves the irreversible inter-conversion of three-enzyme conformations,

$$E \overset{k_1}{\rightarrow} E_1 \overset{\alpha_1}{\rightarrow} \overset{k_2}{\rightarrow} E_2 \overset{\alpha_2}{} \quad (1)$$

where k_1 and k_2 are first-order deactivation rate constants, E, E_1 and E_2 the native, intermediate and denatured conformations of the enzyme, and α_1 and α_2 the specific activity E_1/E and E_2/E, respectively. It was assumed that both α_1 and α_2 could have non-zero value, since some authors have considered the possible partial retention of activity by the transformed species of hemeproteins, such as peroxidase [28,30]. Assuming that the enzymatic activity, a, is a weighted function of the specific activities of the three different enzyme conformations, then the following equation was adjusted to the inactivation of the immobilised HRP [29]:

$$a = \left(1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right) \exp(-k_1 t) - \left(\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right) \exp(-k_2 t) + \alpha_2 \quad (2)$$

The parameters obtained were the following: $\alpha_1 = 2.78$; $\alpha_2 = 9.20 \times 10^{-3}$; $k_1 = 0.517\,\mathrm{h}^{-1}$; $k_2 = 0.0348\,\mathrm{h}^{-1}$. This means that the intermediate conformation has a relative activity 2.78 times higher than the native enzyme and that the denatured enzyme has a very low activity as it would be expected. The conversion of the native enzyme into the intermediate conformation is quite fast, while the conversion of this form into the denatured form is rate limiting. These results clearly prove that by covalent immobilisation, it is possible to transform HRP in a way that completely changes its deactivation pathway.

3.3.2. Stability in aqueous–organic media

It has been reported that the inactivation of enzymes upon exposure to water-miscible organic solvents takes place immediately [31]. It is believed that these organic solvents strip water from the enzymes, leading to the unfolding of the molecule with exposure of the inner hydrophobic residues and that this denaturation occurs at a much faster rate than in a pure aqueous system.

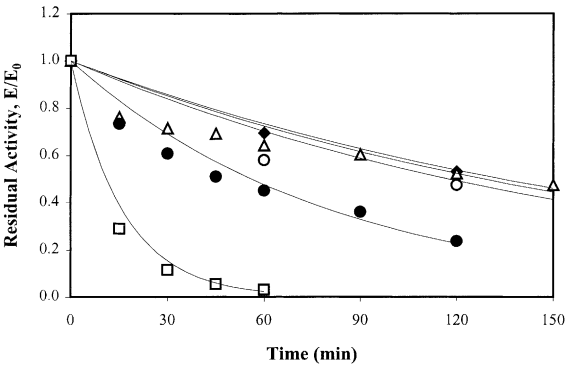

Fig. 3. Deactivation profiles of free HRP at 50°C in the presence of 0 (◆), 3.5 (○), 20 (△), 35 (●) and 50% DMSO (□).

Fig. 3 shows the inactivation of free HRP at 50°C in aqueous mixtures of DMSO, with a co-solvent content ranging from 3.5 to 50% (v/v). The experimental data was fitted using the exponential model. The deactivation equations and respective half-life are summarised in Table 2.

As it can be observed from the deactivation profiles in Fig. 3, the denaturing effect of the organic co-solvent is only observed for DMSO contents higher than 20%. As Table 2 also shows that the enzyme half-life in the presence of up to 20% remains practically constant. However, a severe decrease in stability is observed for higher DMSO contents.

The behaviour of the immobilised HRP in the same aqueous mixtures of DMSO is illustrated in Fig. 4. Deactivation of immobilised HRP in the presence of DMSO for co-solvent contents lower than 50% exhibits a grace period in which activity increases prior to the onset of inactivation. It is interesting to note that

Table 2
First-order inactivation constant (k_d), correlation coefficient (r) and half-life ($t_{1/2}$) of free HRP at 50°C in aqueous mixtures of DMSO

Co-solvent (%)	k_d (min^{-1})	r	$t_{1/2}$ (min)
Buffer	5.6×10^{-3}	0.987	124
3.5	5.9×10^{-3}	0.981	117
20	5.4×10^{-3}	0.954	128
35	12.4×10^{-3}	0.982	56
50	62.4×10^{-3}	0.983	11

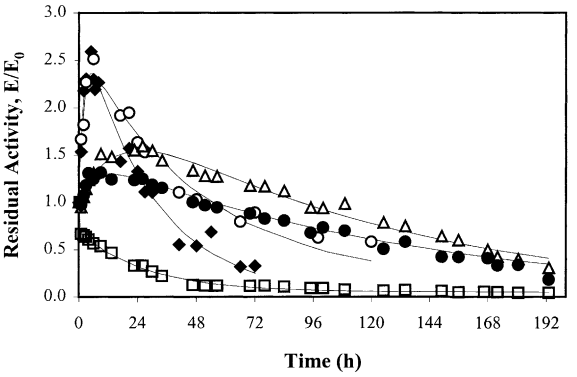

Fig. 4. Deactivation profiles of immobilised HRP at 50°C in the presence of 0 (◆), 3.5 (○), 20 (△), 35 (●) and 50% DMSO (□).

Table 3
Model parameters, correlation coefficient (r) and half-life ($t_{1/2}$) of immobilised HRP at 50°C in aqueous mixtures of DMSO

Co-solvent (%)	α_1	α_2	k_1 (h^{-1})	k_2 (h^{-1})	r	$t_{1/2}$ (h)
Buffer	2.782	0.009	0.516	0.035	0.985	51.8
3.5	2.436	0.016	0.462	0.020	0.969	98.6
20	1.922	0.000	0.067	0.0087	0.976	170.0
35	1.442	0.000	0.152	0.0076	0.985	145.5
50	0.648	0.051	55.744	0.036	0.997	10.0

this increase is higher in pure aqueous media and decreases as DMSO content increases. In the presence of 50% DMSO, no activation is observed, and HRP inactivates immediately when exposed to this solution. Although, maximum activity is reached in pure aqueous media, higher stabilities were obtained in the presence of 20%.

The experimental data was fitted with the two-step model from Henley and Sadana [29] (Eq. (2)), and the values obtained for the model parameters α_1, α_2, k_1 and k_2 are listed in Table 3. As the DMSO content increases, the relative activity of the intermediate conformation (α_1) decreases. The relative activity of the denatured state is very low as expected.

According to this deactivation model, the value of k_1 rate constant represents the rate of formation of the intermediate species, while k_2 represents the deactivation constant. As the co-solvent content increases, the value of the deactivation constant (k_2) decreases until a minimum value is reached for a DMSO content of 35%. This means that in the presence of 35% DMSO, the intermediate enzyme species formed shows the slowest deactivation rate. However, since this intermediate has a specific activity lower than the one of the intermediate formed in 20% DMSO, the highest half-life is observed in a 20% DMSO solution. Regarding the conversion of the native enzyme into the intermediate species, as the co-solvent content increases, k_1 decreases reaching a minimum value at 20% DMSO and increases for higher DMSO contents. In the presence of 50% DMSO, the formation of the intermediate species is very fast ($k_1 = 55.7\,h^{-1}$) and its inactivation occurs shortly after contact with the solvent.

4. Conclusion

This study demonstrated that covalent immobilisation onto silica particles protects HRP from inactivation both in pure aqueous media and in a homogeneous aqueous–organic co-solvent system, with higher stabilities always observed for the immobilised enzyme. DMSO has been revealed to be a very promising solvent to perform biocatalysis with HRP in organic media.

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