ENZYME BIOCATALYSIS IN AOT/ISOOCTANE REVERSED MICELLES

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Enzyme biocatalysis in reversed micelles

Traditionally, enzymes have been used in an aqueous medium, but reversed micelles became attractive, especially where lipophilic substrates and/or products are employed and a low water content is desirable. Several additional advantages of reversed micellar systems on enzyme biocatalysis such as shift of thermodynamic equilibrium on the synthesis processes, minimising side reactions (reverse hydrolytic reactions, polymerisation, others), high interfacial areas of contact (10–100 m^2/ml), rigorous control of the amount of water present in the reversed micellar system, and many others could be pointed out. The examples of enzymes studied in reversed micelles and catalysed reactions are numerous inclusively in our research group (Table 1). Among these processes some have potential industrial applications in areas such as: food, pharmaceuticals, chemicals and bioremediation.

Table 1. Some examples of application of reversed micellar systems on enzyme biocatalysis by our research group.

Enzyme	Biocatalysis reaction	Reversed micellar system
Cutinase	Ester synthesis	Phosphatidylcholine
C. viscosum lipase B	Olive oil hydrolysis	AOT/isooctane
α-chymotrypsin	Dipeptide synthesis	TTAB/octanol/heptane
phospholipase A2	Lecithin hydrolysis	AOT/lecithin
V. mirtyllus peroxidase	Guaiacol oxidation	CTAB/hexane/isooctane
Cutinase	Alcoholysis reactions	CTAB/hexane/hexanol
P. citrinum lipase	Triolein hydrolysis	AOT/isooctane
Horseradish peroxidase	Guaiacol oxidation	AOT/isooctane

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The main disadvantages are associated with product recovery and enzyme re–use that continue still difficult to achieve. There are only very few examples and all of them involve the use of membranes such as ultrafiltration. However, due the nature of the dynamics of reversed micelles the surfactant molecules cross the membrane in the form of small aggregates or more likely as monomers.

MATERIALS AND METHODS

Chemicals: Horseradish peroxidase (HRP) was obtained from Biozyme, AOT and guaiacol from Sigma while hydrogen peroxide 30% (v/v) was obtained from Merck.

HRP activity assay: HRP activity was measured using guaiacol as substrate and the resulting enzymatic polymerisation product, dimethoxybiphenoquinone, measured by the absorption at 470 nm. The activity values measured in this work are presented in DAbs/min units.

Reversed micelles of AOT containing HRP were prepared by adding appropriate amounts of a HRP solution in 20 mM phosphate buffer pH 7 into 5 ml of a 100 mM AOT solution in an organic solvent. After vortex mixing for a few seconds, clear micellar solutions were obtained. 200 μl of 500 mM guaiacol in isooctane were then added to the reversed micelles. After waiting for 5 min, 1 ml of this solution was added to a 1.5 ml stirred cuvette and the reaction was initiated with the addition of 5 μl of 100 mM H_2O_2 solution. The reaction was then followed for 1 min and activity was calculated from the increase in absorbance at 470 nm.The substrate concentrations are expressed in overall system (solvent plus water). Thus, all experiments were performed keeping the overall concentration of enzyme and substrates constant, unless otherwise stated.

HRP stability: HRP preparations solubilized in reversed micelles having different water content values, AOT and enzyme concentrations were incubated in capped glass vials in a thermostated bath at 20 and 35°C. Samples were taken at intervals during the incubation and the residual enzyme activity was determined as described above.

RESULTS AND DISCUSSION

HRP enzymatic reaction in reversed micelles

The HRP catalysed conversion of guaiacol in reversed micelles is a complex process. This involves the collision of HRP and H_2O_2 containing micelles with exchange of contents and the subsequent oxidation of the enzyme by peroxide. In the next step the substrate guaiacol is transported through the micellar interface where it forms a complex with the enzyme, which is further reduced to its native form. The phenoxy radicals formed are then transported back to the organic phase where they undergo a polymerization reaction. This polymerization can also occur inside the micelles, with the final product being transported to the organic phase. Since the substrate diffusion rate is much higher that the enzymatic reaction rate, we assume that the process is not limited by mass transfer.

Example of some determinant factors for enzyme biocatalysis in reversed micelles

Organic solvents

Reversed micellar solutions containing HRP were prepared in different organic solvents with a Wo of 15. HRP was active in solvents such as hexane, heptane, octane, isooctane, decane, undecane and decalin. No activity was however detected in cyclohexane, dodecane, tetradecane and tetralin, since it was not possible to encapsulate the enzyme in AOT reversed micelles with these solvents at a Wo of 15. The HRP in the reversed micelles shows to be stable after being incubated at 20°C for 5 and 21 h (Fig. 1). The highest levels of microencapsulated HRP activity were obtained with heptane, isooctane and decane. Reversed micelles prepared with shorter and longer hydrocarbon chains gave rise to lower activities. The highest levels of activity retention, after 21 h incubation, were obtained with the solvents isooctane and decalin. It is possible to conclude that these results follow the general trend in

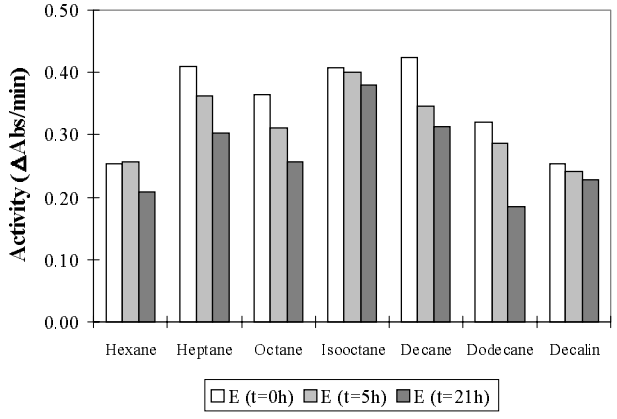

Figure 1. Effect of the organic solvent on the activity of HRP encapsulated in 100 mM AOT reversed micelles and in the residual activity after 5 and 21 h of incubation at 20°C.

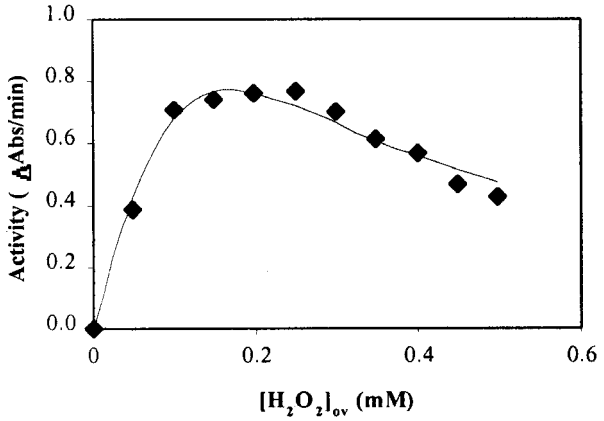

Figure 2. Effect of the hydrogen peroxide on the activity of HRP encapsulated in 100 mM AOT reversed micelles in isooctane at pH 7.

which solvents with high log P values were less detrimental for the enzyme stability.

Kinetics of reactions in reversed micelles

The simple model to explain the kinetic behaviour of biocatalysts in reversed micelles is based on a two consecutive diffusion–steps: the intermicellar diffusion (collision between micelles containing enzyme and micelles with substrate) and intramicellar diffusion. The kinetics of reactions catalysed by microencapsulated enzymes in reversed micelles usually obey to the classical Michaelis–Menten model but different from the ones observed in aqueous solution and the turnover numbers (Kcat) are, in general, of the same order of magnitude. For K_M, which has the dimensions of concentration it is associated with substrate affinity. Changes in this value as compared to aqueous solution are due to some dependency with the partition of the substrate in the reversed micelles. The effect of hydrogen peroxide in the activity of HRP encapsulated in AOT reversed micelles, at a constant Wo value of 10, was studied in the range 0.05 to 0.5 mM (Fig. 2). As this figure shows an increase in HRP activity when hydrogen peroxide concentration is raised up to 0.25 mM but in the contrary there is an inhibitory effect for concentrations higher than this. The experimental data was fitted with a Michaelis–Menten kinetic equation with substrate inhibition.

Wo value

The catalytic activities are dependent on the size of the micelles, i.e., on the Wo parameter. A common feature is that normally the optimum Wo is correspondent to a micellar size comparable to that of the protein to be encapsulated and concomitantly with necessary to attain maximal activity as the different physico–chemical properties of the water inside the reversed micelle compared to bulk water can affect enzyme activity. Another decisive factor in choosing the Wo values is the type of catalytic reaction. Hydrolytic or

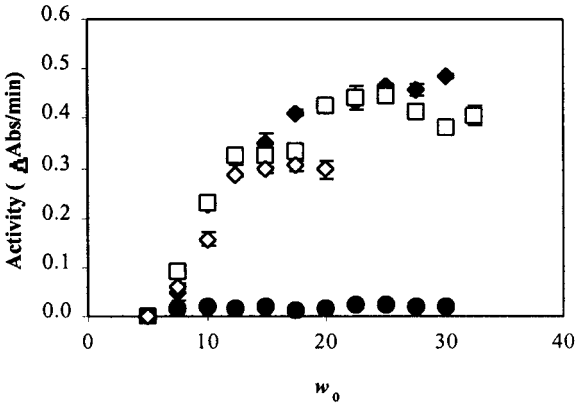

Figure 3. Effect of the water content (Wo) on the activity of HRP encapsulated in 100 mM AOT reversed micelles in isooctane at pH 7 (♦), 8 (□) and 9 (●) and in decalin at pH 7 (◊).

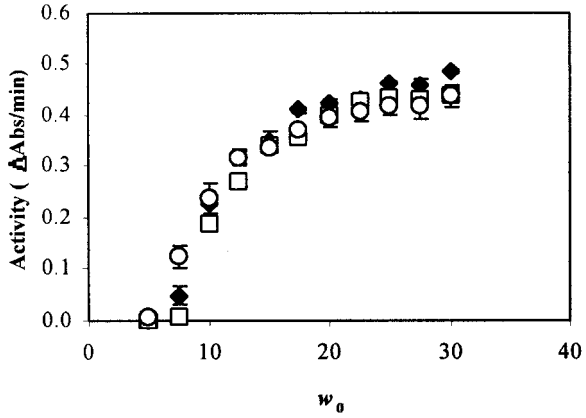

Figure 4. Effect of the water content (Wo) on the activity of HRP encapsulated in AOT reversed micelles in isooctane at pH 7 for different concentrations 25 (o), 100 (♦) and 250 mM (□).

synthetic reactions have different needs for water and this also accounts for the overall reversed micellar catalytic system performance. By medium engineering it is possible to design systems with optimal enzyme activity, without adverse effects on stability.

The activity profile of HRP was determined as a function of the Wo value (Fig. 3). The enzyme activity increases with increasing water content (Wo) over the range 5–17.5 and remains fairly constant for higher water contents. This hyperbolic profile may due to the fact that increasing the water content in a micellar system, concomitantly, increases the size of the reversed micelles. This means that the water pool becomes larger and more water is thus available to hydrate the enzyme, which remains fully active for Wo values greater than 17.5. The explanation for these values can be done through the Stokesradius of a globular protein (r_p) determined using an empirical expression based on the protein molecular weight (MW).

$$r_p\ (Å) = 0.38 + (MW)\ 0.4$$

Since the molecular weight of HRP is 44 kDa, its radius is estimated to be 27 Å. Using the expression of the average radius of the inner water cavity,

$$r^{RM}\ (Å) = 1.64*Wo$$

it is possible to calculate, for the Wo in these experiments, for which the inner water cavity equals the size of the protein that is 16.7. This explains the fact that for water content values higher than 17.5 the activity remains constant (Fig. 3), i.e., the size of the reversed micelles is greater than the protein and hence there is enough free water to hydrate the enzyme.

Surfactant concentration

The effect of the AOT concentration in the activity of HRP microencapsulated in AOT reversed micelles in isooctane has been investigated at pH 7. Fig. 4 shows the activity profile of HRP as a function of the water content for three different AOT concentrations, namely 25, 100 and 250 mM. Changing the surfactant concentration in a reversed micellar system at a constant water content results in an alteration in the micelle concentration without affecting their size and other physical properties. Hence, the activity of solubilized HRP should not depend on the surfactant concentration at these experimental conditions (Fig. 4).

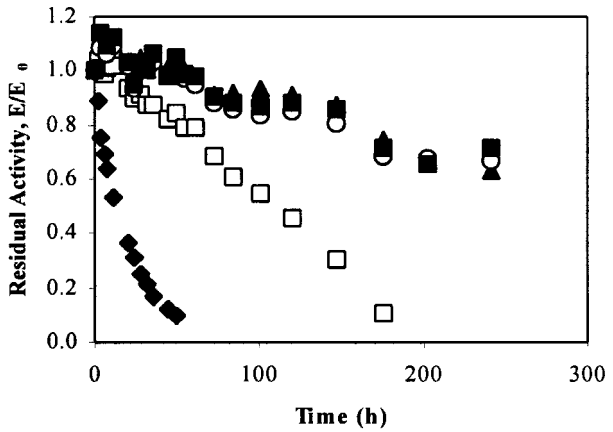

Figure 5. Stability profile of HRP encapsulated in 100 mM AOT reversed micelles in isooctane at AOT 20°C at different water contents: 10 (♦), 15 (□), 20 (▲), 25 (○) and 30 (■).

HRP stability in reversed micelles

The stability of enzymes solubilized in organic solvents is highly dependent on the water content of the system and surfactant concentration. The effect of water content on the stability of HRP solubilized in AOT reversed micelles at 20°C in isooctane can be seen in Fig. 5. As the water content increases, the inner water cavity of the reversed micelles and the water layer that hydrates the enzyme, both increase. This effect can explain the increase in the retention of enzyme activity with changes in the water content.

REFERENCES

[1] A.M. Azevedo, L.P. Fonseca, D.L. Graham, J.M.S. Cabral and D.M.F. Prazeres (2001) Behaviour of horseradish peroxidase in AOT reversed micelles. Biocat. Biotrans., 19, 213–233.