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Application of Factorial Design to the Optimization of Peroxidase Activity in Reverse Micelles of *bis*(2-ethylhexyl)Sodium Sulfosuccinate/ Isooctane

Ana Morna-Gomes,[1] Maria Luisa Serralheiro,[2] Pedro Salema Fevereiro,[3] and Maria Raquel Aires-Barros*,[1]

[1]Centro de Engenharia Biológica e Química, Laboratório de Engenharia Bioquímica, Instituto Superior Técnico, Av. Rovisco Pais, 1000 Lisboa, Portugal, E-mail: pcbarros@alfa.ist.utl.pt; [2]Centro de Ciência e Tecnologia de Materiais, Faculdade de Ciências de Lisboa da Universidade de Lisboa, Departamento de Química e Bioquímica, 1700 Lisboa, Portugal; and [3]Laboratório de Biotecnologia de Plantas, ITQB, Quinta do Marquês, 2780 Oeiras, Portugal

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Abstract

Two cationic peroxidases isolated from *Vaccinium myrtillus* were encapsulated in reverse micelles of *bis*(2-ethylhexyl)sodium sulfosuccinate/isooctane. By using a central composite design, some relevant parameters for the enzymatic activity, such as surfactant and water concentration, pH, and buffer molarity, were analyzed. With the results obtained from this experimental planning, the response surface curves were established. The maximum specific activity obtained (0.19 m*M*/min · m*M* of enzyme) was approximately the same for both peroxidases, but the experimental conditions under which this value was attained differed considerably.

Index Entries: Peroxidase; reverse micelles; activity; factorial design; response surface.

Introduction

Peroxidases (EC 1.11.1.7) are heme-containing enzymes that are widely distributed in plants *(1)* and catalyze the oxidation of a wide range

*Author to whom all correspondence and reprint requests should be addressed.

of molecules using hydrogen peroxide as an electron acceptor. These enzymes are implicated in several physiological processes including lignin biosynthesis *(1)*, indole-3-acetic acid metabolism *(2)*, pathogen resistance *(3,4)*, and response to stress *(5)*. However, the understanding of the physiological roles of plant peroxidases is still incomplete owing to the existence of many different isoenzymes and the high diversity of in vitro substrates.

The plant peroxidases have been used as an important component of reagents for clinical diagnosis and for laboratory experiments either as a part of enzymatic kits for analytical chemistry or for detection of immunological reactions *(6)*. These enzymes are able to catalyze different types of reactions, such as the hydroxylation, dimethylation, sulfoxidation, and oxidation of different organic compounds *(7)*. It has been suggested that they can be used in the treatment of wastewater containing phenolic compounds, in the synthesis of organic compounds *(8)*, and in the removal of peroxides from foodstuffs and industrial residues *(9)*.

One limitation in the use of peroxidases is the hydrophobicity of many potential organic substrates *(10)*. Recently chemical modification of the enzyme, by ligation to polymers such as polyethylene glycol *(7)*, hexadecylamine, and octylamine *(10)*, or by conjugation with cortisol *(11)*, or even by modification of lysine residues by thioacylation *(12)*, has enabled a significant increase in the stability of peroxidases in organic solvents, without major losses in activity, increasing its potential uses.

The reversed micellar system allows the utilization of organic solvents as reaction media, since the enzyme is in the aqueous pool of the reversed micelles and is protected against the solvent toxicity. Furthermore, encapsulation in reversed micelles provides a very high interfacial area (the water pool has a diameter of 10–100 nm) to minimize mass-transfer limitations, allow the solubilization of hydrophilic and hydrophobic substrates, and often enhance catalytic enzyme activity.

Encapsulation of peroxidases in reversed micelles can increase the activity toward hydrophobic substrates *(13,14)* and improve enzyme stability and reaction yields. Synthesis of polyethylphenol by horseradish peroxidase is greatly improved using reversed micelles, owing to solubilization of the hydrophobic substrate in the organic micellar phase *(15)*. Reversed micellar systems in which the activity and stability of *Vaccinium myrtillus* peroxidases are higher than in aqueous solution have also been described *(14)*.

In the present study a cell suspension culture of *V. myrtillus*, showing high levels of peroxidase activity in the culture medium *(16)*, is used. Seven extracellular isoenzymes were identified; of these the two predominant cationic enzymes *V. myrtillus* C1 peroxidase (VMPxC1) and *V. myrtillus* C2 peroxidase (VMPxC2) were purified *(16)* and characterized *(17)*. The two cationic peroxidases microencapsulated in reversed micelles of *bis*-(2-ethylhexyl)sodium sulfosuccinate (AOT) in isooctane are used to catalyze the oxidation of guaiacol in the presence of hydrogen peroxide to obtain tetraguaiacol. Isooctane was chosen as the organic solvent because of its log

P value (logarithm of the partition coefficient in an octanol/water two-phase system) of 4.5 (higher than 4), which makes this solvent nontoxic for the enzyme.

The effects of experimental parameters such as the amount of water, buffer molarity, pH, and surfactant concentration on the activity of the two microencapsulated cationic peroxidases were evaluated using a factorial design of 2^4 variables expanded further to a central composite design (CCD) according to Barker *(18)* and Box et al. *(19)*. The application of the factorial planning allows the distinguishing of interactions among factors that, with the classical experimental methods, are not detectable. This methodology also requires fewer experiments.

Materials and Methods

Enzymes

The peroxidases used were obtained from the culture medium of *V. mirtyllus* suspension cell cultures. The two cationic peroxidases obtained, VMPxC1 and VMPxC2, were then purified *(16)* and characterized *(17)*. Both enzymes are glycosylated, with p*I*s close to 9.0 and mol wts of 34,000 Da for VMPxC1 and 38,000 Da for VMPxC2.

Chemicals

Surfactant AOT (99% pure) was purchased from Sigma (St. Louis, MO). Isooctane (99.5% pure) was from Riedel-de-Haen (Seelze, Germany), and guaiacol (>99% pure) and hydrogen peroxide (30.0% w/w) were from BDH (Poole, England). Salts (analytical reagent grade) were from Merck (Darmstadt, West Germany).

Preparation of Reverse Micelles

Reverse micelles of AOT containing the VMPxC1 peroxidase (83×10^{-5} μ*M*) and the VMPxC2 peroxidase (166×10^{-5} μ*M*) were obtained by injecting appropriate amounts of a stock peroxidase solution in aqueous buffer (25 m*M* phosphate buffer, pH 6.5) into 5 mL of AOT (100 m*M*) in isooctane containing the substrate guaiacol (5 m*M*). After vortex mixing for a few seconds, clear micellar solutions were obtained.

Peroxidase Activity Assays

Three milliliters of the micellar solution containing the guaiacol (5 m*M*) was added to a 3-mL cuvet, and the reaction was started by the addition of 5 μL of hydrogen peroxide (33 m*M*). The reactions were followed for 1 min by reading the increase of absorbance at 470 nm. The extinction molar coefficient of the oxidation product, $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$, was used for calculation of the activities. The water content of the micellar solutions was quantified using a Mettler DL18 Karl Fisher titrator (Merck, Darmstadt, Germany). w_0 was defined as the molar ratio (water)/(surfactant).

Table 1
Parameter Values of Central Composite Design
Experimental Plan Used to Study Activity of Peroxidases Encapsulated in Reverse Micelles of AOT/Isooctane

	Level				
Factor	–2	–1	0	+1	+2
AOT (m*M*)	50	75	100	125	150
Water (m*M*)	500	750	1000	1250	1500
Buffer molarity (m*M*)	10	25	40	55	70
pH	4.6	5.4	6.1	6.9	7.6

Factorial Design for Optimization of the System

The influence of the surfactant and the water concentration as well as the pH buffer molarity on peroxidase activity were studied by applying a factorial design, known as central composite design *(19)*. The factors were varied according to the values shown in Table 1.

A second-order model relating all the factors and taking into account the interactions among the factors was applied to the experimental data (Eq. 1):

$$y = b_0 + \sum_{i=1} b_i x_i + \sum_{i<j} b_{ij} x_i x_j + \sum_{i=1} b_{ii} x_i^2 + e \qquad (1)$$

in which y refers to the system response under each condition tested, and x to the factor studied. Since the number of factors studied is four, these summations will have four terms. The parameter b in the equation was calculated by multiple linear regression analysis *(20)*. The statistical analysis of the empirical models obtained *(20)* indicated a coefficient of multiple determination of 0.978 and 0.945 for VMPC1 and VMPC2 enzymes, respectively. The response surface curves were drawn for both enzyme forms.

Results and Discussion

Effect of pH and Buffer Molarity on C1 Peroxidase–Specific Activity

The influence of buffer molarity and pH on the activity of cationic peroxidase, C1, was studied at different values of AOT and water concentration. There is no unique response surface curve for all the combinations studied. The response surface depends on the AOT and water concentration values of the system. In Fig. 1A the influence of ionic strength on the C1 peroxidase–specific activity is shown for 50 m*M* AOT and 1000 m*M* water. These values were chosen from a previous analysis of the enzymatic activity at different conditions, owing to the higher activities obtained. From the analysis of Fig. 1A, it can be seen that the buffer molarity does not play a central role on the C1-specific activity and that the pH value is crucial for the peroxidase activity. An optimum value of about 5.4 is obtained for all buffer molarity values studied.

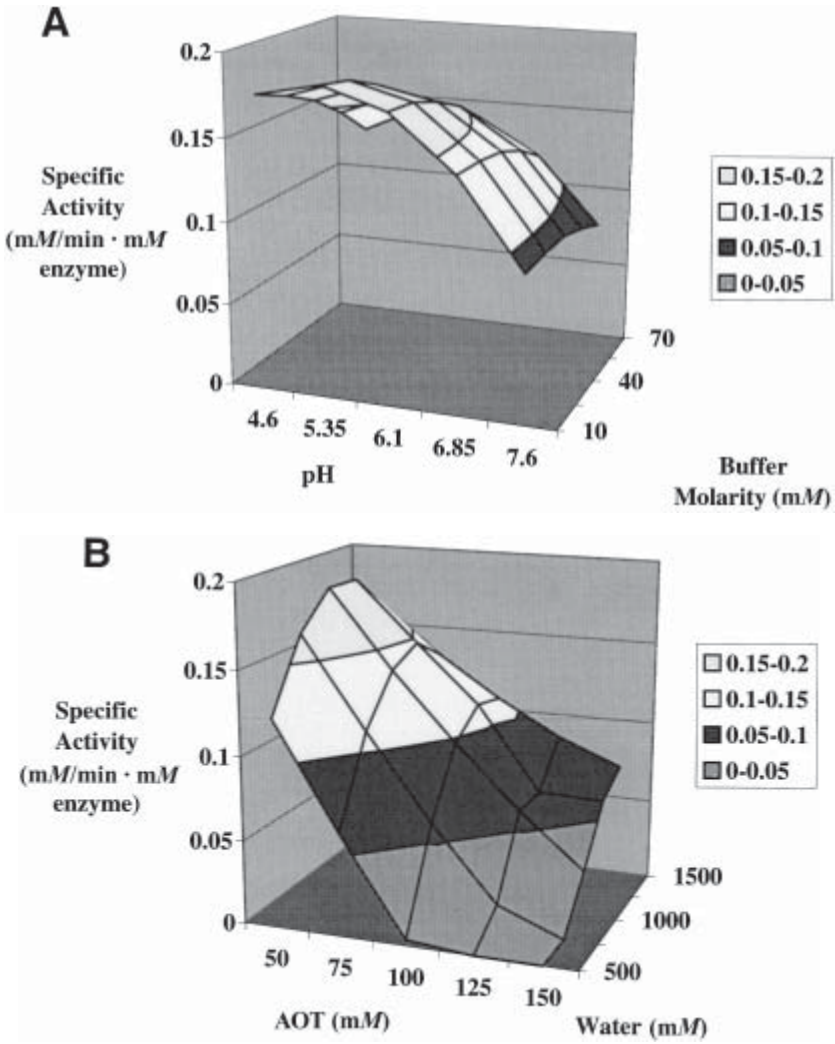

Fig. 1. Response surface curves for VMPxC1 specific activity in reverse micelles of AOT/isooctane. **(A)** Effect of pH and ionic strength at 50 m*M* AOT and 1000 m*M* water; **(B)** effect of AOT and water concentration at pH 5.4 and 10 m*M* buffer concentration.

Effect of AOT and Water Concentration on C1 Peroxidase–Specific Activity

Figure 1B shows the influence of AOT and water concentration on the specific activity of peroxidase. This response curve is set up for buffer molarity of 10 m*M* and pH of 5.4. At these conditions, higher specific activities were obtained (Fig. 1A). The surfactant and the water concentration have a significant effect on the enzyme behavior. For all the water concentration values studied, the increase in AOT concentration brings about a decrease in the C1 peroxidase–specific activity. According to Martinek et al. *(21)*, the increase in surfactant concentration at a fixed value of water

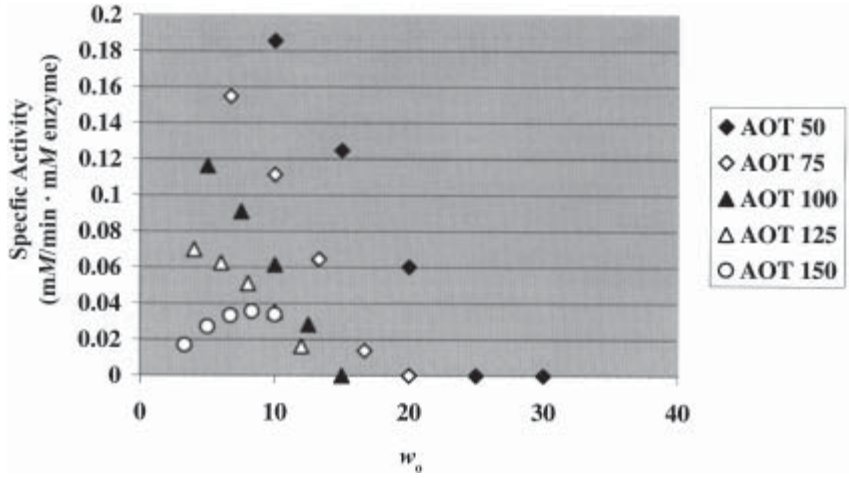

Fig. 2. Effect of the w_0 on the VMPxC1 specific activity at different AOT concentrations for pH 5.4 and 10 m*M* buffer concentration.

concentration will lead to an increase in the number of reverse micelles with smaller dimensions. By increasing the water concentration in the system, this effect can be reversed. The highest specific activity obtained for C1 in AOT/isooctane reverse micelles was 0.19 m*M*/min · m*M* of enzyme, at 50 m*M* AOT, 1000 m*M* water, corresponding to a w_0 of 20, and 10 m*M* buffer molarity at pH 5.4.

For the same w_0 values, but with different AOT and water concentrations, quite different specific activity for the peroxidase was obtained (Fig. 2). For instance, at a w_0 of 20 an activity of 0.19 m*M*/min · m*M* of enzyme was obtained when the AOT was 50 m*M* against a value 32% lower when the AOT was raised by 25 m*M*. These findings seem difficult to explain based solely on the increase in the number of reverse micelles given by the increase in the surfactant concentration together with an increase in the water concentration at the same ratio (maintaining the w_0 constant). It appears that the simultaneous increase in the surfactant and the water concentration will form reverse micelles with a bigger radius, in which the enzyme will lose the stabilizing contact between its ionic surface-charged groups and the surfactant polar-head groups. This could explain the lower specific activity found under these experimental conditions. The same argument was used to explain the higher stability found for α-chymotrypsin in reverse micelles of TTAB/heptane/octanol when both the surfactant and the water concentration were raised at the same w_0 *(22)*.

Effect of pH, Buffer Molarity, AOT, and Water Concentration on C2 Peroxidase–Specific Activity

The effect of the same parameters on C2 peroxidase–specific activity was evaluated. Figure 3A shows the response surface curve for the influence of buffer molarity and pH at 50 m*M* AOT and 500 m*M* water ($w_0 = 10$).

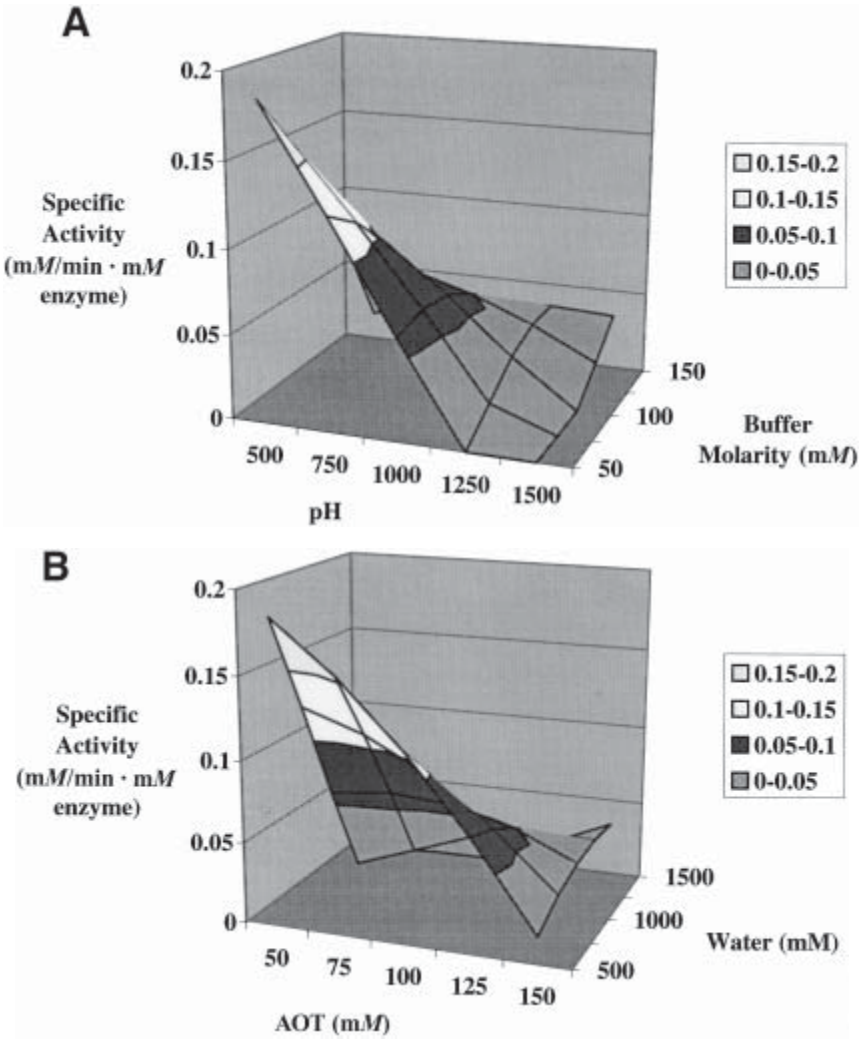

Fig. 3. Response surface curves for VMPxC2 specific activity in reverse micelles of AOT/isooctane. **(A)** Effect of pH and ionic strength at 50 m*M* AOT and 500 m*M* water; **(B)** effect of AOT and water concentration at pH 7.6 and 70 m*M* buffer concentration.

A previous study of the same factors at different values of AOT and water was carried out. The response surface curves varied according to the parameters of the reverse micelles, but the higher specific activity values were obtained for 50 m*M* AOT and 500 m*M* water concentration, precisely the shown values. These results are corroborated by the response surface curves shown in Fig. 3B. Figure 3B shows the effect of AOT and water concentration for a buffer molarity of 70 m*M* and at pH 7.6, which were the best values found for the C2-specific activity in this study.

For C2 peroxidase, an increase in the buffer molarity led to an increase in the specific activity (Fig. 3A). Laame et al. *(23)* showed that the increase

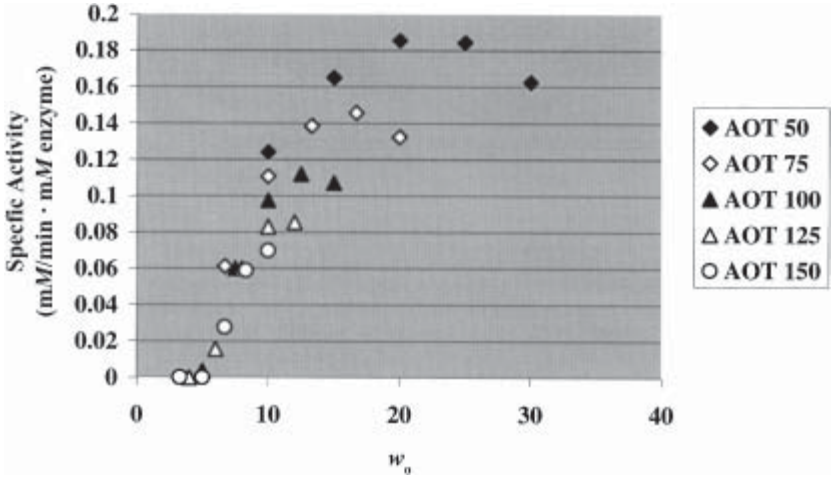

Fig. 4. Effect of the w_o on the VMPxC2 specific activity at different AOT concentrations for pH 7.6 and 70 m*M* buffer concentration.

in the number of charged ions inside reverse micelles allowed the formation of micelles with smaller diameters, because these ions gave a protective shield against the repulsions found between the surfactant polar heads.

Analysis of Fig. 3B also shows that C2 peroxidase prefers reverse micelles with smaller diameters. The highest specific activity, 0.19 m*M*/min · m*M* enzyme, was found for 50 m*M* AOT and 500 m*M* water concentration, corresponding to a w_0 of 10. The effect of increasing the reverse micellar radius is notorious for the smaller AOT concentration in which the C2-specific activity diminishes drastically with an increase in water concentration. For C2 peroxidase, the AOT surfactant had a negative effect on the enzymatic specific activity (Fig. 4). For the same w_0, the lowest surfactant concentration led to a higher enzymatic specific activity.

The different behavior of C1 and C2 peroxidases encapsulated in reverse micelles is probably correlated with their different physical, kinetic, and structural properties. Both are heme-containing glycoproteins with similar p*I*s and only small differences in molecular weight, which could be owing to the different glycosylation patterns. From glucosamine analysis, the number of glycans and the percentage of carbohydrate present in the two peroxidases was estimated *(17)*. Apparently VMPxC2 is more glycosylated than VMPxC2, in accord with the higher molecular weight observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. From analysis of the magnetic circular dichroism spectra of the two enzymes *(17)*, differences were detected that may be related to differences in the coordination of the heme iron. The VMPxC1, owing to similarities to horseradish peroxidase, must have a penta-coordinated heme iron, whereas VMPxC2 can have a mixture of penta- and hexa-coordinated iron.

Conclusion

The optimization process of peroxidases from *V. myrtillus*, VMPxC1 and VMPxC2, in reverse micelles of AOT/isooctane leads to the same specific activity (0.19 m*M*/min · m*M* of enzyme) at different optimal conditions. The highest specific activity for VMPxC1 was obtained with 50 m*M* AOT and 1000 m*M* water ($w_0 = 20$) at a buffer molarity of 10 m*M* at pH 5.4. For VMPxC2, the optimal conditions found correspond to 50 m*M* AOT and 500 m*M* water ($w_0 = 10$), at pH 7.6 with a buffer molarity of 70 m*M*. Differences in the composition of the glycosylated residues and in the coordination of the heme iron are probably responsible for the differences observed.

The global analysis of the optimum process by the application of the factorial design allows investigators to obtain the required information at lower costs and takes into account the interactions of factors that with the classical methods are not possible.

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