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ORIGINAL ARTICLE

The influence of composition and structure of water-in-oil microemulsions on activities of Iraqi Turnip peroxidase

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Abstract The activity of the enzyme Iraqi Turnip peroxidase (ITP) is studied in a reverse microemulsion composed of chloroform, aqueous buffer, sodium dodecylsulfate (SDS) and alcohols of the homologous series 1-propanol to 1-hexanol through the measurements of absorbancy of the product of oxidation at the wavelength of 470 nm in the course of reactions. The ITP catalyzed reaction is the oxidation of guaiacol by hydrogen peroxide. Maximum enzyme activity was obtained at ω_0 (molar ratio of water to surfactant) = 8. It was found that the oxidation reaction obeyed Michaelis–Menten kinetics in the investigated concentration rang (0.08–0.8 mM) of the substrate, and the Michaelis constant K_m and maximal reaction rate V_m were determined. The enzyme inhibition caused by the alcohols in microemulsions is a consequence of both the solubility of the alcohols in the buffer and the flexibility of the interfacial film.

1. Introduction

In recent years, enzymes have extensive application as catalysts for organic reactions in water-in-oil (o/w) microemulsion (Stamatis et al., 1999; Jing et al., 2003; Bauduin et al., 2005). They work at ambient temperature with large selectivity, specificity and minimal side-product formation (Dordick, 1991; Schmid et al., 2001). Water-in-oil microemulsion is thermodynamically stable, isotropic, optically transparent solution. Dispersion of water in oil stabilized by surfactant molecules. Often, as in the present case, the formation of microemulsion requires the presence of a cosurfactant. Depending on the composition and temperature, such systems can have very different microstructures. The enzyme is solubilized in the water droplets of the microemulsion, while the hydrophobic substrates are dissolved in the continuous, organic phase. The reactions take place at the oil/water interface and the products are distributed to the oil phase or the water phase. The enzyme molecules can be entrapped in the water pools, avoiding direct contact with organic solvent that potentially denatures to

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enzyme. The w/o microemulsion environment represents a medium where the aqueous/organic interface is very large (approximately 100 m^2/ml) (Verhaert and Hihorst, 1991; Carvalho and Cabral, 2000; Klyachko and Levashov, 2003). In this system, the activity of enzyme is related to the microstructure of the w/o microemulsion (Jing et al., 2003).

Peroxidase (EC 1.11.1.7) is an enzyme widely distributed in nature. It catalyzes the oxidative coupling of phenolic compounds using H_2O_2 as oxidizing agent (Kay et al., 1967). Peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, rigidification, and lignification of cell walls and protection of plant of tissues from damage and infection by pathogenic microorganism. In vitro, this enzyme is widely employed in microanalysis. Currently, peroxidases are used also in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals. There have been a few studies of the peroxidase reactions in bulk water in w/o microemulsions (Chauhan and Sahoo, 1999; Gebicka and Pawlak, 1997; Setti et al., 1995; Parida et al., 1991; Eremin and Metelitsa, 1985; Klyachko et al., 1984; Martinek et al., 1982; Bauduin et al., 2005). Therefore, in the present studies, we report the characterization of oxidation of guaiacol catalyzed by Iraqi Turnip peroxidase (ITP) in w/o microemulsions formed in chloroform/aqueous buffer/sodium dodecylsulfate (SDS):alcohols of the homologous series 1-propanol to 1-hexanol (2:1) system. The influences of the characteristic parameters of this system, such as molar ratio (ω_0) of water to SDS, the concentrations of substrate and enzyme, 1-alcohol carbon number on activity are examined.

2. Experimental

2.1. Materials

Peroxidase (specific activity 5333 units/mg protein) was produced from germinated seeds of Iraqi Turnip by Sakran (2008). Purified water was taken from a Millipore Milli-Q system (electrical conductivity < 10^{-6} S m^{-1}). The buffer contained phosphate solution (concentration c = 0.05 mol/l), pH 7.4. Chloroform and series alcohols used from 1-propanol to 1-hexanol (purity > 99%) were purchased from Merck. SDS (purity > 99%) was obtained from El-nasr pharmaceutical chemicals Co. The substrates chosen were hydrogen peroxide (was purchased from Merck, 30% (w/w) medical. Extra pure) and guaiacol (was supplied from sigma chemical company, USA).

2.2. Preparation of microemulsion

Microemulsions were prepared by the addition the desired amount of surfactant mixture in which SDS and alcohols of the homologous series 1-propanol to 1-hexanol were mixed at a weight ratio of 2:1 to chloroform (10 ml). In this system, the phosphate buffer solutions (0.05 mol/l, pH 7.4) were then added to, and the final ω_0 value was adjusted by the addition of the amount of the buffer. The mixture was briefly shaken until an optically clear single-phase solution was formed.

2.3. Preparation of the reaction mixtures

The reaction mixtures were prepared by the addition 10 μl of a solution containing desired concentration of guaiacol to 1 ml of the microemulsion media. Then 0.5 ml of a solution containing 9 mM H_2O_2 and finally 50 μl of a solution containing ITP (330 μg/ml) were added. The final addition of ITP initiates the oxidation of the guaiacol. The concentrations of ITP and guaiacol in the microemulsion reaction system were 16.5 μg/ml and ranging from 0.08 to 0.86 mM, respectively.

2.4. Determination of enzyme activity

The kinetics of guaiacol oxidation was studied spectroscopically at a constant temperature of 25 °C using a varian cary 3E spectrometer. The temperature (T) of cell compartment was maintained with Haake circulator KT 33. The progress of the reaction was estimated from the absorption at 470 nm of oxidized form of guaiacol, which was detected during the first 2 min after the addition of ITP. One enzyme unit is the change in molarity (M) per minute under assay conditions. Molar extinction coefficient, 26,600 M^{-1} cm^{-1} was used to convert absorbance into molarity (Keyhani et al., 2000).

3. Results and discussion

3.1. Effect of "water pool" size in w/o microemulsion and enzyme concentration on enzyme activity

The size of water pools in w/o microemulsion occurs to be the most important factor that determines the reaction rate and it has been studied recently (Jing et al., 2003). The size of water pools in w/o microemulsion strongly dependent on ω_0. Altering ω_0 influences on stability of the microemulsion. At a constant overall enzyme concentration ($[E]_{ov}$) of 16.5 μg/ml and constant total SDS concentration of 0.014 mol/l, the effect of ω_0 on the ITP activity in the w/o microemulsion was determined under the conditions of pH 7.4 and 25 °C. The experimental results showed bell-shaped curve with a maximum value of V at ω_0 = 8 as presented in Fig. 1. In the system under study, the peroxidase contacts with the water pool inter-

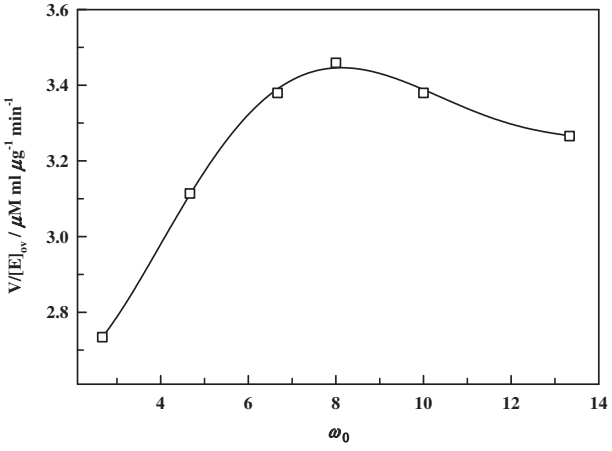

Figure 1 Dependence of $V/[E]_{ov}$ on ω_0 at 25 °C, pH = 7.4, [guaiacol] = 0.03 mol/l, [SDS] = 0.014 mol/l, and $[E]_{ov}$ = 16.5 μg/ml in SDS/1-hexanol/$CHCl_3$/buffer system.

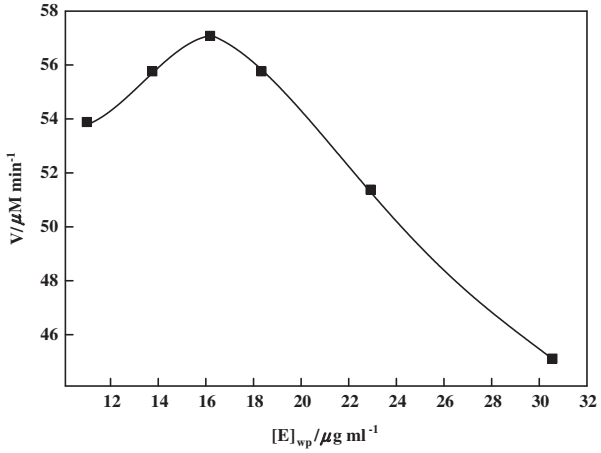

Figure 2 Dependence of initial reaction rate on enzyme concentration in the water pool at 25 °C, pH = 7.4, [guaiacol] = 0.03 mol/l, [SDS] = 0.014 mol/l, and $[E]_{ov}$ = 16.5 μg/ml in SDS/1-hexanol/$CHCl_3$/buffer system.

face and guaiacol is partitioned between the organic phase and interface. Therefore, the reaction most likely carried out at interface or close to the interface and the peroxidase concentration in water pool may affect the initial reaction rate. With $[E]_{ov}$, SDS concentration, substrate concentration, pH, and temperature being fixed at 16.5 μg/ml, 0.014 mol/l, 0.03 mol/l, 7.4 and 25 °C respectively; the initial reaction rates were measured for various concentrations of ITP in water pool ($[E]_{wp}$) and are presented in Fig. 2. The experimental results illustrated that the activity of ITP in the SDS microemulsion system had a typical bell-shaped dependence on $[E]_{wp}$ with a maximum value of initial reaction rate at $[E]_{wp}$ = 16.2 μg/ml. The existence of optimal ω_o for ITP activity was probably related to conformational changes of the protein, to the changes in the water structure in the hydration shell, and to the water activity in the microemulsion (Jing et al., 2003).

3.2. Effect of the number of carbon atoms in the alcohols on enzyme activity

The influence of the alkyl chain length of 1-alcohols on the enzymatic activity was tested in w/o microemulsion. This

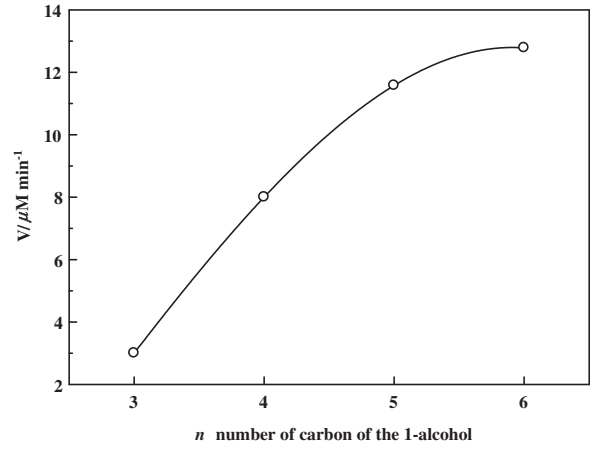

Figure 3 Dependence of initial reaction rate on *n*, the number of carbon atom of the 1-alcohol as co-surfactant.

was done to evaluate the maximal enzymatic inhibitions caused by each alcohol. As shown in Fig. 3, the results exhibited the maximal enzymatic activity in presence of 1-hexanol. Thereby, an increase of inhibition was observed for shorter alcohols (1-propanol and 1-butanol). The denaturation of horse radish peroxidase by 1-alcohols from 1-butanol to 1-octanol has been extensively studied (Bauduin et al., 2005). It was concluded that the inhibition influence of the alcohols depends both on the solubility of the alcohols in the buffer and the flexibility of the interfacial film which is usually expressed in terms of an elastic constant or an elastic modulus (De Gennes and Taupin, 1982). The very low enzymatic activity values in the SDS/1-propanol/$CHCl_3$/buffer system can be explained by the relatively high solubility of 1-propanol in the aqueous buffer system (solubility of 1-propanol in water at 25 °C (w/w) = 0.0740). Due to 1-propanol is essentially dissolved in the aqueous droplets leading to a strong inhibition of the water soluble enzyme. Moreover, 1-propanol acts as cosurfactant with the particular feature that the SDS/1-propanol film is a highly flexible. It is also possible that ITP has a certain affinity to the interface. As consequence, the affinity of the enzyme to the highly flexible film also exposes the enzyme to the denaturating chloroform phase, a fact which further decreases the activity.

In SDS/1-hexanol/$CHCl_3$/buffer system, the initial enzymatic activity increases up to a maximum. The high V value of 12.78 μM min^{-1} in the 1-hexanol system is due to the low solubility of 1-hexanol in water (solubility of 1-hexanol in water at 25 °C (w/w) = 0.0058). It is known that 1-hexanol yields a more rigid film than shorter alcohols do (Bauduin et al., 2005) and obviously the 1-hexanol molecules are so strongly incorporated in the film that their inhibition effect is negligible.

3.3. Effects of substrate concentration on enzyme activity

The enzyme-catalyzed kinetics has been investigated in SDS-based w/o microemulsions (SDS/1-hexanol/$CHCl_3$/buffer). It was well known that enzyme-catalyzed reactions usually obey Michaelis–Menten kinetics represented by the following equation:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m} \cdot \frac{1}{[S]} \quad (1)$$

where

$$V_m = k_{cat}[E] \quad (2)$$

K_m, V_m, $[S]$, and $[E]$ represent the Michaelis constant, maximum activity, the concentration of substrate, and the concentration of enzyme, respectively. When $[E]_{ov}$ was kept constant (16.5 μg/ml), a series of initial reaction rates for various guaiacol concentrations ([guaiacol]) were measured under the constant assay conditions: [SDS] = 0.03 mol/l, ω_0 = 8, pH = 7.4 and T = 25 °C. A profile of V against guaiacol concentration is shown in Fig. 4. Good linear relationship between $1/V$ and 1/[guaiacol] indicates the validity of Eq. (1) to oxidation of guaiacol catalyzed by ITP. The values of K_m and V_m were determined from the slope and the intercept of the straight line of Lineweaver–Burk plot (Fig. 5), which were 0.244 mM and 39.53 μM/min, respectively. The K_m value for guaiacol in w/o microemulsion was lower than the value of 13.6 mM reported for guaiacol in buffer solution (Sakran, 2008). The

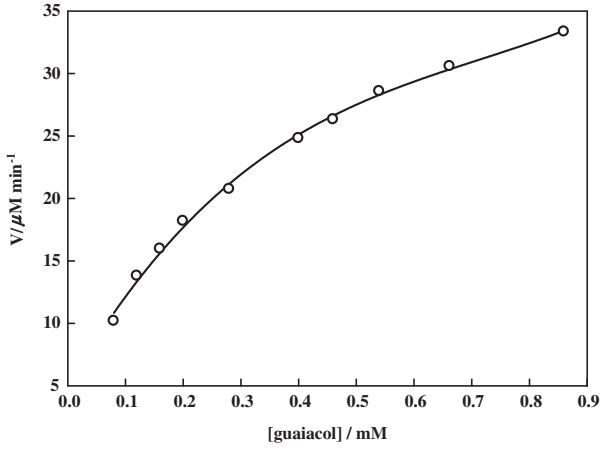

Figure 4 Dependence of initial reaction rate on substrate concentration at 25 °C, pH = 7.4, $\omega_0 = 8$, and $[E]_{ov} = 16.5$ μg/ml in SDS/1-hexanol/$CHCl_3$/buffer system.

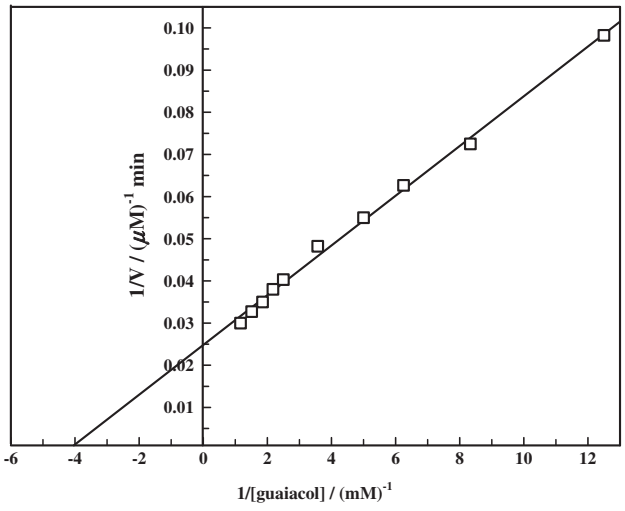

Figure 5 Lineweaver–Burk plot of Iraqi Turnip peroxidase action on guaiacol in the presence of hydrogen peroxide at 25 °C, pH = 7.4, $\omega_0 = 8$, and $[E]_{ov} = 16.5$ μg/ml in SDS/1-hexanol/$CHCl_3$/buffer system.

lowest K_m with w/o microemulsion and the highest V_m/K_m exhibited that ITP has greater tendency toward guaiacol in the presence of w/o microemulsion. The tendency due to increasing the interfacial area that leads to an increase in the number of substrate molecules available to react (Verhaert and Hihorst, 1991; Carvalho and Cabral, 2000; Klyachko and Levashov, 2003).

4. Conclusions

We have studied the activity of Iraqi Turnip peroxidase in w/o microemulsions with various composition ranges through the catalysis of guaiacol oxidation using H_2O_2 as oxidizing agent. An important point to mention is that in reverse SDS microemulsion system, the optimum values for this bioconversion to be 8 and 16.2 μg/ml for the molar ratio of water to SDS and enzyme concentration in water pool, respectively. When the overall concentration was constant, the enzyme activity was found to be dependent on the size of the water pool. This phenomenon was shown as a bell-shaped curve. The inhibition effect of the alcohols depends both on the solubility of the alcohols in the buffer and the incorporation velocity of the alcohols in the interfacial film. Thus an increase in the length of the 1-alcohol leads to an increase in enzymatic activity. The lowest enzymatic activity observed for 1-propanol is explained both by the relatively high solubility of 1-propanol in the aqueous buffer and the highly flexible SDS/1-propanol film. The Lineweaver–Burk plot has presented as a straight line with the values of K_m and V_m being 0.244 mM and 39.53 μM/min, respectively. This behaviour indicated the validity of Michalis–Menten kinetics. The lowest K_m with w/o microemulsion compared with reported in buffer solution exhibited that peroxidase has greater tendency toward guaiacol in the presence of w/o microemulsion.

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