ABSTRACT

The kinetics of catechol oxidation catalyzed by polyphenol oxidase in two-phase systems with mixtures of turpentine and heptane as the solvent phase is investigated, with particular reference to the effect of partitioning of catechol on the enzyme kinetics. Theory is developed to derive a relationship between the apparent $K_m$ and the intrinsic or "true" $K_i$. The theory predicts that substrate partitioning should not change $V_{max}$, but that the relationship between the apparent and intrinsic $K_i$ values should depend on the phase volume ratio and the partition coefficient. The theory shows good agreement with the results and gives a consistent $K_i$ value.

Keywords: polyphenol oxidase, enzyme kinetics, two-phase system, partition effect.

INTRODUCTION

Mushroom polyphenol oxidase is amongst the many enzymes that have been shown to be catalytically active in a variety of organic solvents (Dodacka, 1988; Kazazian and Kilbanov, 1985; Zakas and Kilbanov, 1980). Polyphenol oxidases oxidize catechol to quinone by converting oxygenases that can catalyse the oxidation of monophenol to orthophenol and subsequently to ortho-quinone. In particular, polyphenol oxidase catalyses the oxidation of catechol to quinone. Although the polyphenol oxidases exhibit suicidal behaviour during the oxidation of catechol to o-quinone, various potential applications have been suggested, such as thermal abuse sensor (Becrider, et al., 1986), phenol removal from industrial wastewaters (Atlow, et al., 1984 and Sun., et al., 1992), and the auto-selective oxidation of phenols and N-acetyl L-proline ethyl ester in organic solvents (Kazazian and Kilbanov, 1985). The work reported here is part of a study of polyphenol oxidase activity in selected biphasic aqueous-solvent systems.

It is well known that the nature of the organic solvents can markedly affect the reaction kinetics and stability of an enzyme, which is usually reflected in changes in $K_m$ and $V_{max}$. The solvent inhibition to β-hydroxyxesteryl dehydrogenase was found to be competitive when using ethyl ether, ethyl acetate and butyl acetate (Carrea, et al., 1979). For 5α-hydroxysteroid dehydrogenase, the inhibition depended on the nature of the substrate. The inhibition of ethyl acetate on this enzyme was mainly noncompetitive with androstenediol, meanwhile when using cholic acid as a substrate, the inhibition was found to be mixed (Carrea, et al., 1988). There seems to be no general rule concerning the apparent inhibitory effect of various solvents on a particular enzyme, and solvents must be tested individually. However, there is some ambiguity in interpreting the significance of changes in these apparent parameters in two-phase system. In particular, it is important to be clear which substrate concentration is to be used in calculating the kinetic parameters, since the concentration can be referred either to the total volume of the two phase system, or the aqueous phase, where the reaction takes place or the organic phase.

Indonesian Food and Nutrition Progress, 1998 Vol.3 no. 1
There are three ways in which the solvent can influence the enzyme kinetics in two-phase systems: a) mass transfer effect b) solvent-enzyme interaction, and c) partitioning effect. The most obvious difference between aqueous and non-aqueous systems is that enzymes are generally more soluble in organic solvents and therefore are subject to different diffusional limitations than when they are in aqueous media (Kamat, et al., 1992). However, the reaction usually takes place in well stirred media, and it is usually assumed that mass transfer is very fast so that the rate of reaction is a limiting factor. In a typical system, a plot of initial velocity against rotating speed shows that external mass transfer limitation were not significant in the rotating speed greater than 200 rpm (Chalalaksamamukul, et al., 1992).

Direct solvent-enzyme interactions may also lead to changes in the kinetic parameters. For example, the organic solvent may affect the affinity of the substrate for the enzyme, particularly when the substrate is bound by non-polar interactions. This is because of the effects of different solvents on the enzyme.

Another important factor is that partition of substrate between the organic phase and the aqueous phase can affect the kinetic parameters. For example, the organic solvent may affect the affinity of the enzyme for the substrate. In order to establish the role of direct interactions on enzyme activity, the partition coefficient formula can be used to determine the apparent values of the constants K and V, which may be used with some success for predicting the consequences of changing solvents with different properties.

The partitioning between the organic phase and the aqueous phase implies that even in the absence of mass transfer limitations the equilibrium concentration of substrate near the enzyme is different from its value on the solvent or in the whole mixture. The partition coefficient formula can be used to establish the real reason for changes in their apparent values in a two-phase system.

**THEORY**

We consider a typical biphasic system in which the enzyme is in the aqueous solution and the relation therefore proceeds essentially in the aqueous phase. Here we also consider how the apparent or measured enzyme kinetics are affected by the biphasic nature of the system. If we assume that the system is not mass transfer limited, that is that the transfer of substrate from the organic phase to the reaction site is fast in comparison with the reaction, then it is reasonable to assume that at any time during the reaction the substrate concentrations in the two phases are equal (equilibrium) and thus related by the partition coefficient.

The apparent K values will normally be determined using the overall substrate concentration, whereas in fact the appropriate substrate concentration to use is that in the aqueous phase since it is there that the reaction occurs. Thus we need to derive a relationship between the apparent measured K and the true K. The true K will correspond to the conditions in the aqueous phase if it has the concentration defined per unit volume of the aqueous phase. In what follows we use the symbols K and V to denote the intrinsic or aqueous phase parameters and K and V to denote the apparent values.

At equilibrium, the volume ratio, R, is defined by

\[ V = \frac{R}{V} \]

V and V are the volume of organic and aqueous phases, respectively. The partition coefficient, P, can be defined by

\[ C_p = \frac{C_p}{C} \]

C and C are the concentrations of substrate in the organic and aqueous phases. Assuming that solvent and water are immiscible and neglecting their mutual solubilities, a mass balance on the substrate gives:

\[ V C_p + V C = V C \]

C is the overall substrate concentration. Thus from equations 2 and 3:

\[ C_p = \frac{(V + V) C}{(V + V) R} \]

Then using \( V = V \) from equation (1):

\[ C_p = \frac{(1 + R) C}{(1 + R)} \]

Assuming Michaelis-Menten kinetics in the aqueous phase, the reaction rate is given by:

\[ V = \frac{V C}{K + C} \]
In terms of the overall substrate concentration we can define the apparent rate, \( V' \):

\[
V = \frac{V_{\text{max}} C}{K_{\text{m}}' + C}
\]  

(6)

Substituting (4) into (5)

\[
V = \frac{V_{\text{max}} C}{K_{\text{m}}' + \alpha C} = \frac{V_{\text{max}} C}{(K_{\text{m}}' + \alpha C) + C}
\]  

(7)

Comparing (6) and (7):

\[
V_{\text{max}}' = V_{\text{max}}
\]

(8)

\[
K_{\text{m}}' = K_{\text{m}}/\alpha
\]

(9)

From equations (1) and (4),

\[
P = \frac{V}{V'N} \text{ and } \alpha = (1 + R)/(1 + RP)
\]

(10)

If \( V' >> V \), the \( R >> 1 \). Moreover, if \( P \) is also sufficiently large that \( RP >> 1 \), from equation (10):

\[
\alpha = 1/P
\]

(11)

and from equation (9):

\[
K_{\text{a}} = K_{\text{m}}/\alpha = K_{\text{m}}P
\]

(12)

Thus, the aqueous phase or intrinsic \( K_{\text{a}} \) can be calculated from values of the apparent Michaelis constant \( K_{\text{m}}' \) and the partition coefficient \( P \).

**MATERIALS AND METHODS**

**Materials**

Pycnocatechol, L-proline-t-butyl ester, l-aryl alcohol, NaHPO₄, and Na₂HPO₄ were purchased from Sigma. Hexane was purchased from FSA Laboratory. Polyphenol oxidase was extracted from freeze-dried mushroom powder (Agaricus bisporus) (Utami, 1992).

Catechol oxidation by polyphenol oxidase in mixtures of l-aryl alcohol and hexane

Throughout this work, catechol and L-proline-t-butyl ester were dissolved in the solvent mixture and enzyme was added to the solvent as an aqueous solution of polyphenol oxidase in phosphate buffer, pH 7.0.

Polyphenol oxidase activity was measured by determining the amount of coloured product formed with the L-proline-t-butyl ester. The solvent mixtures used were mixtures of l-aryl alcohol and hexane in the volumetric proportions 25:75, 50:50, and 75:25 respectively and labelled as 25LA-75H, 50LA-50H and 75LA-25H. Solvents were pre-saturated with phosphate buffer, pH 7.0. The enzyme was added as aqueous polyphenol oxidase in phosphate buffer, pH 7.0, and in all experiments accounted for 0.3% v/v of the total reaction mixture. Catechol and L-proline-t-butyl ester were dissolved in given solvent mixture with the same concentration ratio. Then the buffered enzyme was added and mixed at 300 rpm at 25°C using a submerged magnetic stirrer. Catechol concentration in the reaction mixture was in the ranges of 0.1 mM-2.0 mM. Samples were withdrawn at defined intervals and their absorbance was measured at 509 nm. Initial rate of reaction was determined using the first three linear points in this region. Enzyme specific activity is expressed as micromoles of coloured product per minute per mg protein using an extinction coefficient for reaction product at 509 nm of 3320 M⁻¹cm⁻¹. Protein concentration in the concentrated mushroom polyphenol oxidase was determined by the method of Lowry et al (1951).

**Assay for polyphenol oxidase in aqueous solution**

Determination of polyphenol oxidase activity is modification from the method of Rizpeki and Wase (1999). The enzyme activity was measured by monitoring the accumulation of the colour produced from the reaction between o-quinone and L-proline. 0.01 ml enzyme was added and mixed quickly with 299 ml of substrate solution, and the increase in absorbance at 525 nm was measured with time. The substrate solution contained 3.33 mM catechol and 16.7 mM L-proline in 0.1 M sodium phosphate buffer, pH 6.5. The enzyme specific activity is expressed as micromoles coloured product per minute per mg of protein, using the measured molar extinction coefficient for the reaction product of 4700 M⁻¹cm⁻¹.

**Assay for polyphenol oxidase in hexane**

In a separate experiment the catalytic oxidation of catechol in hexane was carried out using 1 g of non
porous glass beads coated with polyphenol oxidase which was added into hexane containing 0.1 mM catechol and 3 mM α-naphthylamine as a nucleophile. It should be noted that α-naphthylamine was not chosen as a nucleophile for other biphasic experiments because of its poor solubility and its incompatibility. Catechol was added to the hexane by introducing 0.5 ml of 20 mM catechol in 0.1 M phosphate buffer, pH 7.0. Thus the main water source came from the substrate solution. The reaction volume was 100 ml with 0.5% v/v water. The suspension was shaken at 250 rpm using a rotary shaker at 25°C. Periodically, aliquots of liquid were withdrawn and their absorbance was measured at 515 nm. The enzyme specific activity is calculated using the measured molar extinction of 3100 M⁻¹cm⁻¹.

Partition coefficient

A defined amount of catechol was dissolved in a mixture of lauryl alcohol and hexane. The solvent mixtures were always presaturated with phosphate buffer, pH 7.0. Then water was added into the solvent mixture to achieve a 1:1 volume ratio of organic solvent and water. This was mixed at room temperature (± 25°C) until equilibrium had been established. The mixture was then left to stand for some time to allow the organic solvent and water to separate. The catechol concentrations in organic solvent before and after equilibrium were determined spectrophotometrically at 278 nm. The concentration of catechol in the water phase was calculated from a mass balance on catechol in the organic solvent before and after equilibrium. Experiments were carried out using a range of initial concentrations of catechol (0.1 mM - 2.0 mM). The partition coefficient of catechol is expressed as the molar ratio between the catechol concentration in the organic solvent (C_o) and in water (C_w).

RESULTS AND DISCUSSION

Partition coefficient

The equilibrium concentrations of catechol in the lauryl alcohol-hexane and water phases are shown in Table 1. The equilibrium concentrations of catechol in 75LA-25H and 50LA-50H were higher than in water. Conversely, the equilibrium concentration of catechol in 25LA-75H was smaller than in water. This is because catechol is more soluble in lauryl alcohol than in water and much more soluble in water than in hexane.

Table 1. Equilibrium concentrations of catechol in 75LA-25H and 50LA-50H were higher than in water. Conversely, the equilibrium concentration of catechol in 25LA-75H was smaller than in water. This is because catechol is more soluble in lauryl alcohol than in water and much more soluble in water than in hexane.

<table>
<thead>
<tr>
<th>Equilibrium concentration of catechol (mM) in</th>
<th>75LA-25H</th>
<th>50LA-50H</th>
<th>Water</th>
<th>25LA-75H</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.013</td>
<td>0.023</td>
<td>0.050</td>
<td>0.090</td>
<td>0.043</td>
<td>0.062</td>
</tr>
<tr>
<td>0.161</td>
<td>0.052</td>
<td>0.152</td>
<td>0.065</td>
<td>0.085</td>
<td>0.123</td>
</tr>
<tr>
<td>0.320</td>
<td>0.100</td>
<td>0.287</td>
<td>0.136</td>
<td>0.179</td>
<td>0.255</td>
</tr>
<tr>
<td>0.657</td>
<td>0.227</td>
<td>0.381</td>
<td>0.254</td>
<td>0.355</td>
<td>0.463</td>
</tr>
<tr>
<td>0.801</td>
<td>0.245</td>
<td>0.624</td>
<td>0.290</td>
<td>0.288</td>
<td>0.616</td>
</tr>
<tr>
<td>1.592</td>
<td>0.358</td>
<td>1.259</td>
<td>0.564</td>
<td>0.516</td>
<td>1.219</td>
</tr>
</tbody>
</table>

In this case, the partition coefficient is equal to the ratio of the equilibrium catechol concentrations in a mixture of lauryl alcohol-hexane and water. Table 2 summarises the experimental values of partition coefficients, which were all found to be constant over the range of concentrations investigated. The partition coefficients increase with increasing concentrations of the more polar lauryl alcohol. The partition coefficients were greater than unity for alcohol-rich mixtures. It means that the catechol concentration in the lauryl alcohol was higher than in the aqueous phase. Therefore, the higher the concentration of lauryl alcohol in the solvent mixture, the higher the solubility of catechol, increasing the equilibrium concentration of catechol and thus the partition coefficient.

Table 2. Partition coefficient of catechol between solvent mixture and water

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Partition Coefficient (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75LA-25H</td>
<td>3.087</td>
</tr>
<tr>
<td>50LA-50H</td>
<td>2.175</td>
</tr>
<tr>
<td>25LA-75H</td>
<td>0.726</td>
</tr>
<tr>
<td>Hexane</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Kinetic analysis

The effect of catechol concentration on the rate of reaction in various mixtures of lauryl alcohol-hexane
was investigated. Under the reaction conditions used here the maximum apparent loss in enzyme activity after two minutes was about 2.1% of the original activity. The decline in product colour has an insignificant effect on the estimated initial rates, and previous experiments also showed that enzyme was not inactivated by L-proline-t-butyl ester (Utami, 1992). The effect of catalase concentration on the initial rate of enzyme reaction in solvent mixtures with composition of 7SLA-2SII, 50LA-50H and 2SLA-7H is shown in figure 1.

![Figure 1](image)

**Figure 1.** Effect of catalase concentration on the rate of coloured product formation in various alcohol-hexane mixtures (--- 7SLA-2SII; --- 50LA-50H; --- 2SLA-7H). The initial rate of reaction was expressed as μmole coloured product per minute per mg of protein.

Apparent Kₐ values (i.e., based on the overall concentration measures) calculated from the initial rate data are shown in Table 3. The higher the concentration of hexane in the solvent mixture the smaller Kₐ. When hexane was the only organic solvent used, Kₐ was very small (0.02 mM). For comparison the Kₐ value for catalase in aqueous solution is 0.33 mM.

**Table 3.** Michaelis constant of polypehophenol oxidase for catalase in organic solvent and water

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Apparent Kₐ (mM)</th>
<th>Vₐmax (μmole product/minute/mg protein)</th>
<th>Apparent phase Kₐ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7SLA-2SII</td>
<td>2.44</td>
<td>5.76</td>
<td>0.79</td>
</tr>
<tr>
<td>50LA-50H</td>
<td>1.08</td>
<td>5.75</td>
<td>0.86</td>
</tr>
<tr>
<td>2SLA-7H</td>
<td>0.60</td>
<td>4.73</td>
<td>0.83</td>
</tr>
<tr>
<td>Water</td>
<td>0.02</td>
<td>4.00</td>
<td>0.71</td>
</tr>
<tr>
<td>Water</td>
<td>0.33</td>
<td>11.20</td>
<td>0.33</td>
</tr>
</tbody>
</table>

The enzyme assay was carried out under standard conditions using various catalase concentrations. Apparent Kₐ and Vₐmax values were calculated from Hanes plot.

In the biphasic system experiments, the substrate (catalase) was distributed between the solvent (lauryle alcohol and hexane) and aqueous phases, with the enzyme in the aqueous phase. The mass transfer limitations were not important since the experiments were carried out with intense stirring. Thus, assuming equilibrium distribution of catalase between the dispersed phase, and using the fact that the volume ratio and partition coefficients allow the simplified version of the theory to be used, the intrinsic aqueous phase Kₑ can be calculated from equation (12).

When the concentration of hexane in the solvent mixture increase, the solvent becomes more hydrophobic, and then P = C₈/C₆ decreases. Thus higher hexane concentrations correspond to higher values of α. Since Kₐ = Kₑ/α, the higher the concentration of hexane in the mixture of lauryle alcohol-hexane the smaller should be the apparent Michaelis constant, Kₐ.

Values of the intrinsic aqueous phase Kₑ calculated from the experimental data and equation (12) are shown in Table 3. The values are remarkably constant, supporting the hypothesis that differences in the apparent Kₐ values in the different concentration solvent mixtures in an aqueous-organic solvent system are due to the partition effect of the substrate between the two phases. However, it will be seen that the average intrinsic Kₑ value is greater than the corresponding value for catalase in aqueous solution (0.33 mM). This suggests that in addition to the partitioning effect, the affinity of catalase for the enzyme decreases in hexane and in mixtures of lauryle alcohol-hexane, presumably due to direct interactions between the solvent and the enzyme's active site.

First, consider the enzyme in nearly anhydrous hexane. In this case it seems likely that organic solvent will be present very close to the active site. The substrate specificity of enzymes stems from their ability to utilize the free energy of bonding with substrate to facilitate the reaction. Since the net binding energy is the difference between the binding energies of the substrate with the enzyme and with the surrounding solvent (Fersht, 1975), replacing water with organic solvent could change the substrate specificity, which would be reflected in a change in the kinetic constants. This is consistent with results found in the case of chymotrypsin where the main driving force for enzyme-substrate binding derives from hydrophobic interactions between the side chain of the amino acid substrate and the binding pocket of the enzyme. In aqueous solutions, the
CONCLUSIONS

This study shows that the values of the apparent Michaelis constant (Km) for catechol increase with increasing lauryl alcohol concentration on the solvent phase of biphasic mixtures. This is consistent with the changes in the partition coefficient of catechol between water and the corresponding solvent, which increased with increasing lauryl alcohol concentration. The simple theory developed here to relate the apparent Km and intrinsic phase Kc works well, including the results from the same reaction in nearly anhydrous hexane, giving a consistent value for Km. We believe that most likely explanation for the consistently high value of the Michaelis constant in comparison to its measured value in an azeotropic system is due to the ability of water to solubilize some solvent from the organic phase, and the subsequent effect of the organic solvent at the active site of the enzyme, thus decreasing the affinity of catechol for the enzyme.

This study also shows that the apparent catalytic rate constant in solvent mixtures decreased with increasing hexane concentration. Partition theory does not explain this effect. It appears that other forms of enzyme/solvent/solvent interaction affect the apparent catalytic rate constant.

ACKNOWLEDGEMENT

This work was supported by Indonesian Development Project. The author wishes to thank Dr. I. A. Kozlov for his helpful advice during this work.

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