Kinetics and Substrate Partitioning in the Polyphenol Oxidase-Catalysed Oxidation of Catechol in a Two-Phase System

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ABSTRACT

The kinetics of catechol oxidation catalysed by polyphenol oxidase in two-phase systems with mixtures of lauryl alcohol and hexane 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_m*. The theory predicts that substrate partitioning should not change* V_{max}*, but that the relationship between the apparent and intrinsic* K_m *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_m *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 (Doddema, 1988; Kazandjian and Klibanov, 1985; Zaks and Klibanov, 1986). The polyphenol oxidases are copper-containing 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 (Beoriu, *et al,* 1986), phenol removal from industrial wastewaters (Atlow., *et al.,* 1984 and Sun., *et al.,* 1992), and the regio-selective oxidation of phenols and N-acetyl L-tyrosine ethyl ester in organic solvents (Kazandjian and Klibanov, 1985). The work reported here is part of a study of polyphenol oxidase activity in selected biphasic aquoeus-solvent system.

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-hydroxysteroid dehydrogenase was found to be competitive when using ethyl ether, ethyl acetate and butyl acetate (Carrea, *et al.,* 1979). For 3α-hydroxysteroid dehydrogenase, the inhibition depended on the nature of the substrate. The inhibition of ethyl acetate on this enzyme was mainly noncompetitive with androstanediol, 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 ambiquity 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.

There are three ways in which the solvent can influence the kinetics in two-phase system: a) mass transfer effect b) solvent-enzyme interaction, and c) partitioning effect. The most obvious difference between aqueous and nonaqueous system is that enzymes which catalyse reaction in organic solvents are insoluble 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 showed that external mass transfer limitation were not significant in the rotating speed greater than 200 rpm (Chulalaksananukul, *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 binding to the active site of the enzyme, particularly when the substrate is bound by non-polar interactions. This is concern of much recent work aimed at elucidating the effects of different solvents on enzyme behaviour.

Another important factor is that partition of substrate between the organic phase and the aqueous phase change the concentration of substrate near the enzyme. We argue here that it is important to first understand the consequences of partitioning in order to establish the role of direct interactions on enzyme activity, since interpretation based on equating the apparent K_m with enzyme/substrate specificity may confuse the consequences of changed solubility with other forms of enzyme/substrate interactions.

Substrate 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. Theory to predict the effect of partitioning on V_{max} and K_m is therefore needed, in order 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 at equilibrium and thus related by the partition coefficient.

The apparent K_m 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_m and the 'true' K_m. The 'true' K_m would correspond to the conditions in the aqueous phase ie with the concentration defined per unit volume of the aquoeus phase. In what folows we use the symbols K_m and V_{max} to denote the intrinsic or aqueous phase parameters and K'_m and V'_{max} to denote the apparent values.

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

$$V_o = R\,V_w \qquad (1)$$

V_o and V_w are the volume of organic and aqueous phases respectivelly. The partition coefficient, P. can be defined by

$$C_o = P\,C_w \qquad (2)$$

C_o and C_w 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:

$$V_w C_w = (V_w + V_o)\,C - V_o C_o \qquad (3)$$

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

$$C_w = \frac{(V_o + V_w)C}{(V_w + V_o)P}$$

, Then using $V_o = R\,V_w$ from equation (1):

$$C_w = \frac{(1+R)\,C}{(1+RP)} = \alpha\,C \qquad (4)$$

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

$$V = \frac{V_{max}\,C_w}{K_m + C_w} \qquad (5)$$

In term of the overall substrate concentration we can define the apparent rate, V':

$$V = \frac{V'_{max}\, C}{K'_m + C} \qquad (6)$$

Substituting (4) into (5)

$$V = \frac{V_{max}\, \alpha\, C}{K_m + \alpha\, C} = \frac{V_{max}\, C}{(K_m/\alpha) + C} \qquad (7)$$

Comparing (6) and (7):

$$V_{max} = V'_{max} \qquad (8)$$

$$K'_m = K_m/\alpha \qquad (9)$$

From equations (1) and (4),

$$R = V_o/V \quad \text{and} \quad \alpha = (1 + R)/(1 + RP) \qquad (10)$$

If $V_o >>> V_w$, the $R >>> 1$. Moreover, if P is also sufficiently large that $RP >> 1$, from equation (10):

$$\alpha = 1/P$$

and from equation (9): (11)

$$\text{Apparent } K_m = K'_m = K_m P \qquad (12)$$

Thus, the aqueous phase or intrinsic K_m can be calculated from values of the apparent Michaelis constant K'_m and the partition coefficient P.

MATERIALS AND METHODS

Materials

Pyrocatechol, L-proline-t-butyl ester, lauryl alcohol, Na_2HPO_4 and NaH_2PO_4 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 lauryl 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 lauryl 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 presaturated 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 µmoles of coloured product per minute per mg protein using an extinction coefficient for reaction product at 509 nm of 3320 $M^{-1}cm^{-1}$. 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 Rzepecki and Waite (1989). 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 2.99 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 µmole coloured product per minute per mg of protein, using the measured molar extinction coefficient for the reaction product of 4700 $M^{-1}cm^{-1}$.

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-napthylamine as a nucleophile. It should be noted that α-napthylamine 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 spesific activity is calculated using the measured molar extinction of 3100 $M^{-1}cm^{-1}$.

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 concentration of catechol is lauryl alcohol-hexane and in water phases**

Equilibrium concentration of catechol (mM) in					
75LA-25H	**Water**	**50LA-50H**	**Water**	**25LA-75H**	**Water**
0.073	0.023	0.060	0.030	0.043	0.062
0.161	0.052	0.152	0.065	0.085	0.123
0.320	0.106	0.287	0.136	0.179	0.235
0.657	0.227	0.583	0.254	0.355	0.463
0.801	0.245	0.624	0.300	0.428	0.616
1.592	0.518	1.259	0.564	0.916	1.219

In this case, the partition coefficient is equal to the ratio of the equilibrium catechol concentrations in a mixture of lauryl alcohol-hexane and in 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 alchohol-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**

Solvent	Partition Coefficient (P)
75LA-25H	3.087
50LA-50H	2.175
25LA-75H	0.726
Hexane	0.028

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 catechol concentration on the initial rate of enzyme reaction in solvent mixtures with composition of 75LA-25H, 50LA-50H and 25LA-7H is shown in figure 1.

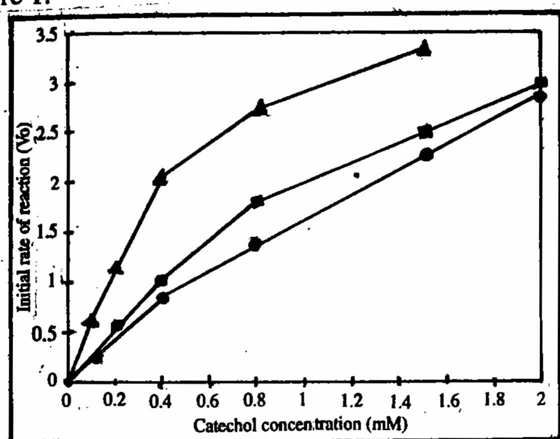

Figure 1. **Effect of catechol concentration on the rate of coloured product formation in various alcohol-hexane mixtures (–•–: 75LA-25H; –■: 50LA-50H; –▲–: 25LA-75H). The initial rate of reaction was expressed as µmole coloured product per minute per mg of protein.**

Apparent K_m values (ie 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 K'_m. When hexane was the only organic solvent used, K'_m was very small (0.02 mM). For comparison the K'_m value for catechol in aqueous solution is 0.33 mM.

Table 3. **Michaelis constant of polyphenol oxidase for catechol in organic solvent and water**

Solvent	Apparent K_m (mM)	V_{max} µmole product/min/mg protein	Aqueous phase K_m (mM)
75LA-25H	2.44	5.76	0.79
50LA-50H	1.88	5.75	0.86
25LA-75H	0.69	4.73	0.83
Hexane	0.02	4.10	0.71
Water	0.33	11.26	0.33

The enzyme assay was carried out under standard conditions using various catechol concentration. Apparent K_m and V_{max} values were calculated from Hanes plot.

In the biphasic system experiments, the substrate (catechol) was distributed between the solvent (lauryl 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 catechol 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 K_m can be calculated from equation (12).

When the concentration of hexane in the solvent mixture increase, the solvent becomes more hydrophobic, and then $P = C_0/C_w$ decreases. Thus higher hexane concentrations correspond to higher values of α. Since $K'_m = K_m/\alpha$, the higher the concentration of hexane in the mixture of lauryl alcohol-hexane the smaller should be the apparent Michaelis constant, K'_m.

Values of the intrinsic aqueous phase K_m 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 K_m 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 K_m value is greater than the corresponding value for catechol in aqueous solution (0.33 mM). This suggests that in addition to the partitioning effect, the affinity of catechol for the enzyme decreases in hexane and in mixtures of lauryl 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 diference between the binding energies of the substrate with the enzyme and with the surrounding solvent (Fersht, 1985), 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

nonpolar amino acid substrates partition into the hydrophobic active site. In nonpolar organic media, this partitioning is reduced and then the apparent K_m increases (Zaks and Klibanov, 1986).

The active site of polyphenol oxidase has a hydrophobic character, all of its substrates have an aromatic nucleus (Robb, 1984). Thus the main driving force in polyphenol oxidase-substrate binding is probably hydrophobic interactions between catechol and the enzyme. Then the presence of non-polar hexane would alter the hydrophobic interactions, leading to an increase in the intrinsic K_m.

In the two-phase experiments the solvent was presaturated with phosphate buffer prior to use. Thus there was no question of further partitioning of essential water from the enzyme to the organic solvent. On the other hand, a possible explanation of the increased intrinsic K_m values could stem from the ability of lauryl alcohol-hexane mixtures to dissolve in the water phase. The presence of either solvent in the water phase, even at low concentration, could in principle cause a deterioration in the catalytic properties of the enzyme, since as argued, because the solvent plays an important role in all types of enzyme-substrate interactions, any substitution of water by non polar organic solvent would be expected to have an impact on the substrate specificity of the enzyme.

Experiments show that the measured V_m' values in the solvent mixtures are consistently lower than V_m in aqueous solution (11.26 μmole product per minute per mg protein). There is also some evidence that V_m' decreases with increasing hexane concentration. The partitioning theory predicts that partition of substrate between two phases it self should not affect V_m. Thus, it appears that factors other than substrate partition between two phases, probably direct hexane enzyme interactions also affect the catalytic constant.

Whilst it is clear that the many variables that control enzymatic activity in organic media are not yet fully understood, we believe that the fact that the simple partitioning theory gives consistent K_m values for the two phase systems substantiates our argument that reducing the observed values to their intrinsic counterparts by using equations (10) or (12) provides a proper basis on which to assess the importance of other influences of the solvent system on enzyme behaviour. Clearly, more kinetic experiments with various enzyme solvent systems should be carried out to test this theory over a range of conditions.

CONCLUSIONS

This study shows that the values of the apparent Michaelis constant (K'_m) for catechol increase with increasing lauryl alcohol concentration 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 relates the apparent K'_m and intrinsic phase K_m works well, including the results from the same reaction in nearly anhydrous hexane, giving a consistent value for K_m. We believe that most likely explanation for the consistently high value of the Michaelis constant in comparison to its measured value in an aqueous system is due to the ability of water to solubilise 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/ substrate/solvent interaction affect the apparent catalytic rate constant.

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