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PROPERTIES OF POLYPHENOL OXIDASE FROM TUBERS OF THE YAM *DIOSCOREA BULBIFERA*

EMMANUEL O. ANOSIKE and AUGUSTINE O. AYAEBENE

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

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Key Word Index—*Dioscorea bulbifera*; Dioscoreaceae; yam tuber; polyphenol oxidase; properties.

Abstract—The subunit MW of *Dioscorea bulbifera* polyphenol oxidase (MW 115 000 ± 2000) determined by SDS–PAGE is *ca.* 31 000 indicating that the enzyme is an oligomeric protein with four subunits. K_i values of various inhibitors and their modes of inhibition have been determined with catechol and pyrogallol as substrates. *p*-Nitrophenol, *p*-cresol, quinoline and resorcinol are competitive inhibitors of catechol binding while only orcinol and *p*-nitrophenol behave in the same way towards pyrogallol as substrate. From the effect of pH on V_{max}, groups with pK values *ca.* 4.7 and 6.8 have been identified to be involved in catalytic activity. The Arrhenius activation energy (E_a) at pH 4.0 is 8.9 kcal/mol between 40–65°. At pH 7.0, the value is 22.1 kcal/mol between 40 and 60°. The enthalpies (ΔH) at pH 4.0 and pH 7.0 are 2.3 kcal/mol and 32.4 kcal/mol respectively. The results are discussed considering the conformational changes of the enzyme during substrate binding.

INTRODUCTION

o-Diphenol: O_2 oxidoreductase (EC 1.10.3.1), polyphenol oxidase, has been isolated and purified from tubers of *Dioscorea bulbifera* [1]. Of all other types of yam tubers tested, *D. bulbifera* showed the highest activity of this enzyme [1]. Macrae and Duggleby [2] described the substrate specificity and effect of inhibitors on the potato enzyme. The thermal denaturation of the enzyme from *Musa cavendishii* has been reported by Padron *et al.* [3]. Various other properties of polyphenol oxidases of plants have recently been reviewed in ref. [4]. In this review, some work on the subunit MW of the enzyme from various sources was reported. Association–dissociation phenomena were observed with the *Neurospora* enzyme [5] while Strothkamp *et al.* [6] showed that the mushroom enzyme contains two types of polypeptide chains. This study, therefore, was partly aimed at providing information on the possible number of subunits per molecule of the yam enzyme as well as on the pK values of some amino acid residues at the active site involved in catalytic activity.

RESULTS AND DISCUSSION

Subunit MW

The subunit MW of the *D. bulbifera* enzyme determined by SDS–PAGE according to the method of ref. [7] was 31 000 ± 2000. The MW of this enzyme determined by gel filtration had previously been reported to be 115 000 ± 2000 [1]. This would indicate that this enzyme is an oligomeric protein with four subunits. Mushroom catechol oxidase had been shown to be polymeric with MW between 116 000 and 128 000 [8–10]. The minimal MW observed by sedimentation was reported to be 26 000–32 000 [11, 12]. The enzyme was initially thought to consist of four identical subunits of MW *ca.* 30 000 each containing one copper atom [9, 11]. However, Strothkamp *et al.* [6] using SDS–PAGE showed that this enzyme contains two types of polypeptide chains: heavy (H, MW 43 000) and light (L, MW 13 400). The *Neurospora* enzyme has been shown to have a MW of 120 000 and by association–dissociation experiments, a monomer MW of 33 000 having one copper atom per molecule has been proposed [5]. This finding has been confirmed in refs. [13, 14]. This indicates the existence of four subunits per polymeric protein molecule of this enzyme. The findings for the *D. bulbifera* enzyme are similar to this and its subunit MW is close to the 29 100 determined for *Streptomyces* tyrosinase by SDS gel electrophoresis and sedimentation equilibrium analysis [15]. Other plant catechol oxidases which have been purified to homogeneity have MWs around 36 000–40 000 [16, 17].

Effects of inhibitors

Table 1 shows the mode of inhibition by various substances of the reaction catalysed by *D. bulbifera* polyphenol oxidase with catechol and pyrogallol as substrates. Compounds which are analogues of the phenolic substrate such as *p*-cresol, *p*-nitrophenol and resorcinol were found to act as competitive inhibitors of catechol binding. Of these analogues, only *p*-nitrophenol inhibited the binding of catechol (Fig. 1a) and pyrogallol competitively. On the other hand, inhibition by orcinol was competitive with respect to pyrogallol and mixed with respect to catechol. This is not surprising since orcinol has three substituents on the benzene ring just as pyrogallol has. On the other

Table 1. Effects of some inhibitors on *D. bulbifera* polyphenol oxidase

Inhibitor	Substrate	Type of inhibition	K_i (mM)
p-Cresol	Catechol	Competitive	0.88
	Pyrogallol	Uncompetitive	0.90
L-phenylalanine	Catechol	Non-competitive	4.33
	Pyrogallol	Uncompetitive	28.60
p-Nitrophenol	Catechol	Competitive	0.17
	Pyrogallol	Competitive	0.25
Diethyldithiocarbamate	Catechol	Non-competitive	0.04
	Pyrogallol	Non-competitive	0.08
Thiourea	Catechol	Mixed	0.07
	Pyrogallol	Non-competitive	0.50
KCN	Catechol	Non-competitive	0.16
	Pyrogallol	Mixed	0.20
NaN_3	Catechol	Non-competitive	1.25
	Pyrogallol	Non-competitive	1.80
Quinoline	Catechol	Competitive	4.25
	Pyrogallol	Non-competitive	4.70
Cinnamic acid	Catechol	Non-competitive	2.0
	Pyrogallol	Non-competitive	1.40
Orcinol	Catechol	Mixed	1.43
	Pyrogallol	Competitive	0.97
Resorcinol	Catechol	Competitive	7.90
	Pyrogallol	Non-competitive	6.40

Reactions were carried out in KPi buffer, pH 7.0. The apparent K_m values for catechol and pyrogallol were 8.9 mM and 6.6 mM respectively.

hand, resorcinol which has two hydroxyl substituents on the benzene ring similar to catechol, competitively inhibited the binding of the latter whereas it non-competitively inhibited the binding of pyrogallol. Surprisingly, *p*-cresol which is a similar analogue to *p*-nitrophenol showed competitive inhibition of catechol binding and non-competitive inhibition towards pyrogallol (Fig. 1b). It has been stated that generally, compounds that affect the site for phenolic substrate

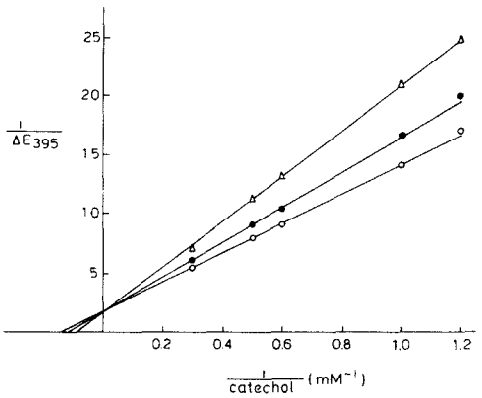

Fig. 1(a). Competitive inhibition of *D. bulbifera* polyphenol oxidase by *p*-nitrophenol with catechol as substrate. Assay conditions were as described in the Experimental. (○), No inhibitor; (●), 0.03 mM *p*-nitrophenol; (△), 0.16 mM *p*-nitrophenol. Enzyme concentration in each assay mixture was 0.042 mg.

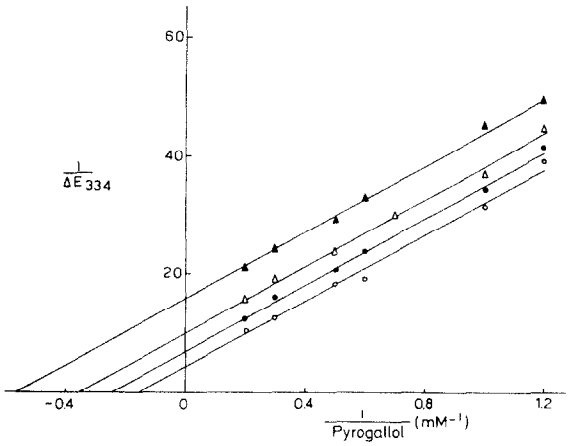

Fig. 1(b). Uncompetitive inhibition of *D. bulbifera* polyphenol oxidase by *p*-cresol with pyrogallol as substrate. (○), No inhibitor; (●), 1.0 mM *p*-cresol; (△), 2.0 mM *p*-cresol; (▲), 3.3 mM *p*-cresol.

show competitive inhibition towards the phenolic substrate [2, 4]. On the other hand, reagents which interact with the copper in the enzyme are said to show non-competitive inhibition towards the phenolic substrate. Cyanide, diethyldithiocarbamate, thiourea and sodium azide are examples of such compounds [18, 19]. All these compounds have been found from this work to show either non-competitive or mixed inhibition towards catechol or pyrogallol as substrates of the *D. bulbifera* polyphenol oxidase

enzyme catalysed reaction. Walker [20] reported that the kinetics of inhibition of catechol oxidase by a series of substituted cinnamic acids indicated that the cinnamic acid derivatives do not act on the site for the phenolic substrate but on a specific 'inhibitor site'. Lerner *et al.* [21] had earlier reached a similar conclusion regarding the inhibition of grape catechol oxidase by phenylhydrazine. The result of this study shows that cinnamic acid non-competitively inhibited the two phenolic substrates tested. Generally, from the K_i values indicated in Table 1 the reagents which normally interact with copper such as diethyldithiocarbamate, thiourea and potassium cyanide seem to bind more tightly to the enzyme than most of the phenolic substrate analogues tested. Unlike tyrosinase of broad beam (*Vicia faba*), which is inhibited by weakly ionizing carboxyl groups [22], *D. bulbifera* polyphenol oxidase is not so affected.

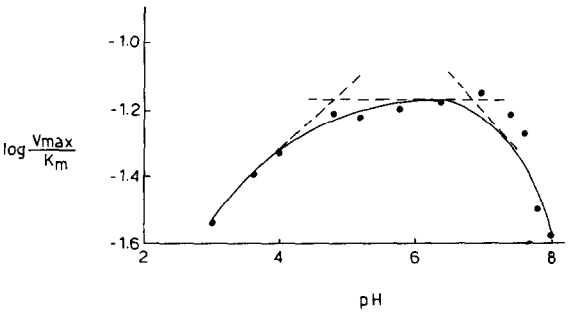

Fig. 3. Effect of pH on log V_{max}/K_m of the reaction catalysed by the *D. bulbifera* enzyme. Tangents to the curves are drawn to have slopes of ± 1 or 0 and they intersect at pH values corresponding to the pK values of acid dissociable groups that are kinetically significant.

Effect of pH

A plot of log V_{max} against pH (Fig. 2) indicates that the catalytic process is dependent on the ionization of two groups on the enzyme–substrate complex. The pK values for these groups were calculated to be *ca.* 4.7 and 6.8. Such values could belong to a carboxylate group and an imidazole group of histidine respectively. This involvement of histidine in catalysis would tend to support the work of Krueger [23] who showed that the change in inhibition of tyrosinase with pH was as a result of a change in the ionization of the imidazole side chains of the enzyme molecule. Figure 3 is a plot of log V_{max}/K_m against pH. From this plot, groups with pK values of *ca.* 4.8 and 6.9 have also been calculated. These would represent two ionizing groups on the free enzyme necessary for catalytic activity. It is likely that these groups are identical to those identified for the enzyme–substrate complex. Lerch and Ettlinger [15] working with tyrosinase from *Streptomyces glaucescens* showed dependence of K_m values on an ionization reaction with a pK of 7.85 ± 0.15 for L-tyrosine methyl ester and 6.47 ± 0.35 for DOPA. Duckworth and Coleman [11] showed that for mushroom tyrosinase, K_m values for all the catechols tested (pyrocatechol, 4-acetylcatechol and 4-formylcatechol) showed dependence on an ionization with a pK_a between 4.9 and 6.0. These pK_a values are similar to those reported here for the *D. bulbifera* enzyme.

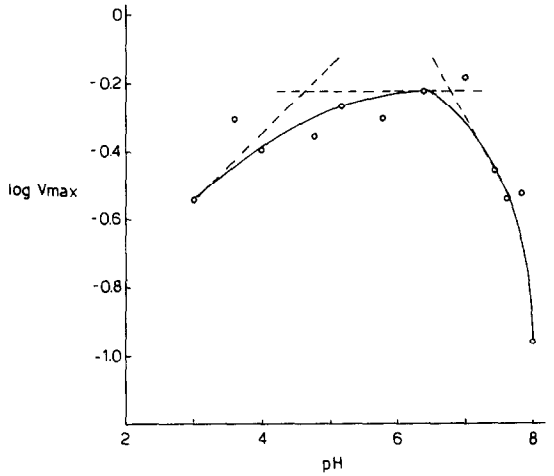

Fig. 2. Effect of pH on log V_{max} of the oxidation of catechol catalyzed by *D. bulbifera* polyphenol oxidase. The various buffer systems used at the various pH ranges are described in the Experimental.

Effect of temperature

The variations of K_m and V_{max} with temperature have been investigated in the range 40–65° and at the two pH values 4.0 and 7.0. The Arrhenius plots of the data as log V_{max} against $1/T$ at pH 4.0 and 7.0 were linear. Table 2 shows the thermodynamic characteristics of thermal activation of the *D. bulbifera* enzyme at the two pH values. At pH 7.0 and 65°, no measurable activity was detected. On the other hand, at pH 4.0 and under the same temperature condition, measurable enzyme activities were detected at various substrate concentrations. It would therefore seem that at the lower pH value, this enzyme resists denaturation, possibly due to conformational change, at the high temperature of 65°. Lerner *et al.* [24] and Lerner and Mayer [25] had postulated that catechol oxidase can undergo conformational changes induced by the substrate oxygen and by pH. It is therefore likely that the enzyme conformation at pH 4.0 is more suited to some degree of binding of the phenolic substrate, catechol, at 65° than at pH 7.0.

Plots of pK_m ($-\log K_m$) against $1/T$ of the data obtained at pH 4.0 and 7.0 were also linear. The enthalpies (ΔH) calculated from these plots were 2.3 kcal/mol and 32.4 kcal/mol respectively. The entropies (ΔS) calculated from these results at various temperatures are shown in Table 2. The values at pH 4.0 are *ca.* 45-times lower than the corresponding ones at pH 7.0 for all the temperatures tested. Since ΔH and ΔS values would represent the changes occurring during the formation of the Michaelis complex, [26], a more considerable conformational change in the enzyme in the course of reaction at pH 7.0 than at pH 4.0 is indicated. These results would support the involvement of a group with a pK value around 6.8 in the catalytic activity of this enzyme. Apart form the work of Himmelwright

Table 2. Thermodynamic characteristics of thermal activation of *D. bulbifera* enzyme at pH 4.0 and 7.0 and at several temperatures

pH	Temperature (°)	Activation energy (E_a) (kcal/mol)	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (cal/mol/°K)
4.0	40	8.9	2.3	1.70	1.9
	50	8.9	2.3	1.82	1.5
	55	8.9	2.3	1.68	1.9
	60	8.9	2.3	1.69	1.8
	65	8.9	2.3	1.69	1.8
7.0	40	22.1	32.4	1.76	97.8
	45	22.1	32.4	2.23	94.8
	50	22.1	32.4	1.93	94.3
	55	22.1	32.4	1.48	94.2
	60	22.1	32.4	0.80	94.8

Reaction mixtures in cuvettes were equilibrated at the appropriate temperatures before the addition of the enzyme which was kept at room temperature (20°) to start the reaction. The activation energies (E_a) were determined from slopes of log V_{max} against $1/T$ as described in Dixon and Webb [29]. The enthalpies (ΔH) were calculated from plots of pK_m against $1/T$. At each temperature, the free energy (ΔG) and entropy (ΔS) changes were obtained from the equations:

$$\Delta G = 2.303RT \log_{10} K_m,$$

$$\Delta S = \frac{\Delta H - \Delta G}{T},$$

where T = °K.

et al. [27] that depicted possible interaction of the oxy site of the effective structure of *Neurospora* tyrosinase with monophenolic and diphenolic substrates, not much further work is reported on the active site of this enzyme. More work is needed in this area that would lead to a better understanding of the mechanism of action the polyphenol oxidases.

EXPERIMENTAL

Chemicals. *p*-Cresol, L-phenylalanine, *p*-nitrophenol, and diethyldithiocarbamate were obtained from Sigma and thiourea, KCN, NaN_3, quinoline, cinnamic acid, orcinol and resorcinol were from BDH Chemicals. All other chemicals used were the best commercial grade available.

Enzyme purification. D. bulbifera polyphenol oxidase was purified as previously described in ref. [1].

Enzyme activity. This was measured as described in ref. [1] using catechol and pyrogallol as substrates.

Determination of subunit MW. The method of Weber and Osborn [7] using SDS–PAGE was employed. The polyacrylamide concn was 10% and the gels were stained for protein after the run with Coumassie Brilliant Blue (1.25 g in 454 ml 50% MeOH plus 46 ml HOAc). The denatured protein samples used as MW markers were BSA, ovalbumin, ribonuclease, myoglobin and cytochrome *c*.

Effects of inhibitors. The modes of inhibition of the various inhibitors were determined from the Lineweaver–Burk [28] plots of the initial velocity data. K_is were determined from Dixon [29] plots.

Effects of pH on K_m and V_{max}. The initial velocity studies were carried out at various pH values with catechol as substrate. 50 mM concn of buffer of each pH value was used in the appropriate assay mixture. The following buffer systems were used over the following pH ranges: 3–5.8, acetate–NaOH; 6.0–7.4, KPi and 7.6–8.0, Tris–HCl.

Effect of temperature. Reactions were carried out at pH 4.0 (50 mM acetate–NaOH buffer) and pH 7.0 (50 mM KPi buffer) with catechol as substrate.

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