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THE SELECTIVE INHIBITION OF *ORTHO*- AND *PARA*-DIPHENOL OXIDASES

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Abstract—*Ortho*- and *para*-diphenol oxidases (DPO's) are often distinguished by substrate specificity tests which are not always unequivocal. This paper suggests that they may be differentiated by their response patterns to certain inhibitors and activators. In general *o*-DPO's ('catecholases') are inhibited by substituted cinnamic acids (cinnamic, *p*-coumaric and ferulic), or polyvinylpyrrolidone (PVP) and may be activated by anionic detergents. By contrast *p*-DPO's ('laccases') are unaffected by cinnamic acids and PVP but are inhibited by cationic detergents such as cetyltrimethylammonium bromide (CTAB). Thus in crude extracts these enzymes may be clearly distinguished by a simple combination of substrate and inhibitor specificity tests.

INTRODUCTION

Enzymes capable of oxidizing dihydroxyphenols are usually classified as one of two types; either *o*-diphenol: O_2-oxidoreductase (EC 1.14.18.1, formerly EC 1.10.3.1), *o*-diphenol oxidase (*o*-DPO), 'catecholase', 'catechol oxidase', 'tyrosinase', etc. or *p*-diphenol: O_2-oxidoreductase (formerly EC 1.10.3.2), *p*-diphenol oxidase (*p*-DPO), 'laccase'. *o*-DPO's are widely distributed amongst the various tissues of many higher and lower plants and they have been intensively studied by many workers (see reviews by Bendall and Gregory [1], Mathew and Parpia [2], Walker [3]). The closely related *p*-DPO's appear to be less common and most investigations have been carried out with enzymes isolated from fungi since, apart from the latex of the lac trees *Rhus vernicifera* and *R. succedanea* [4, 5], there have been few unambiguous reports of *p*-DPO's in higher plants. For example Archer and Palmer [6] claimed that banana produces an *o*-DPO whilst Esterbauer *et al.* [7] reported *p*-DPO activity. Similarly the situation with respect to mushroom DPO is unclear; Lindeberg [8] reported that a *p*-DPO was present in the mycelium and *o*-DPO activity in the fruiting body, however Levine [9] claimed *p*-DPO activity whilst Yamaguchi *et al.* [10] reported an *o*-DPO enzyme in mushrooms.

Experimentally, *o*- and *p*-DPO's are usually differentiated on the basis of differences in substrate specificity. Both enzymes are capable of oxidizing a wide range of *o*-dihydroxyphenols whilst *p*-DPO's also show ability to oxidize *p*-quinol and *p*-phenylene diamine. Käärik [11] used spot tests with *p*-cresol and 1-naphthol to distinguish *o*- and *p*-DPO production by fungi growing on agar plates. More recently Harkin and Obst [12] proposed the use of syringaldiazine as a specific substrate for fungal *p*-DPO's. Furthermore many *o*-DPO preparations, unlike *p*-DPO's show ability to hydroxylate monophenol substrates [1, 9].

Both types of DPO use Cu as a prosthetic group but the reaction mechanisms are quite distinct [1, 9, 13–18] and these differences do not offer a convenient method for the routine differentiation of these enzymes so reliance is usually placed on substrate specificity tests. Unfortunately this specificity is not always unequivocal and Bendall and Gregory [1] showed that not all *o*- and *p*-DPO's exhibit clearly defined patterns of substrate specificity.

An alternate approach is the use of selective inhibitors and Lindeberg [8] and Walker [18] used O_2/CO_2 mixtures to differentiate fungal *p*-DPO's; however this gas mixture is inconvenient and potentially dangerous. Lerner *et al.* [19] reported that phenylhydrazine was a specific inhibitor for *o*-DPO's. Other workers [20–23] have shown that *o*-DPO's from various plants are strongly inhibited by substituted cinnamic acids, however, apart from some preliminary tests by Dr. T. Green (personal communication) there have been no comparable tests with *p*-DPO's. Walker [18] and Lim [24] have reported that several fungal *p*-DPO's were inhibited by cetyltrimethylammonium bromide (CTAB) and other quaternary ammonium compounds, and they suggested that these could be used to selectively inhibit *p*-DPO's. Similarly several workers [25–27] have shown that many *o*-DPO's are inhibited by polyvinylpyrrolidone (PVP). Many plant *o*-DPO's show enhanced activity in the presence of anionic detergents [10, 23, 28–30], but no such activation has been found with fungal *p*-DPO's [18, 24].

In the light of the above reports, we have confirmed and extended these earlier observations in an attempt

to provide a simple procedure to differentiate *o*- and *p*-DPO's by selective inhibition and this procedure is applicable to unpurified enzyme preparations.

RESULTS AND DISCUSSION

The results of the various substrate/inhibitor/activator tests are summarized in Table 1. Some pertinent results from other investigators are also included to lend support to our hypothesis that *o*- and *p*-DPO's may be clearly differentiated by use of selective inhibitors.

Table 1 shows that all *o*-DPO's tested were strongly inhibited by cinnamic and *p*-coumaric acids and, to a lesser extent, by ferulic acid. In most cases the type of inhibition was competitive and Walker and Wilson [20] have shown that competitive inhibition by cinnamic acids became the norm when particulate apple *o*-DPO preparations were rendered soluble. Inhibition by PVP appeared to be a general phenomenon with *o*-DPO preparations from higher plants and the failure to inhibit the mushroom *o*-DPO may be related to the fact that it is of fungal origin. Activation by sodium dodecyl sulphate (SDS) and other anionic detergents was also a property that appeared to be selective for *o*-DPO's.

Table 1. Comparison of substrate/inhibitor/activator responses for *o*- and *p*-DPO's from different sources.

Source of enzyme	Type	Substrate	K_m (mM)	Cinnamic acid Type	K_i (mM)
Apple (fruit) [15, 18, 20, 22]	*o*-DPO	4-Me Catechol	7.50	Comp.	0.22
		Chlorogenic acid	Oxidized	Inhibition	
Banana (pulp)	*o*-DPO	4-Me Catechol	4.50	Comp.	11.25
		Chlorogenic acid	15.00	Non-Comp.	18.06
Mushroom (cap)	*o*-DPO	4-Me Catechol	5.50	Comp.	1.77
		Chlorogenic acid	7.50	Comp.	2.10
*G. cingulata** (strain B)	*o*-DPO	4-Me Catechol	3.0	Comp.	3.3
		p-Phenylene diamine	Not oxidized	Comp.	
Sclerotina sp.*	*o*-DPO	4-Me Catechol	10.00	Non-Comp	2.16
		p-Quinol	Not oxidized		
		p-Phenylene diamine	Not oxidized		
Peach (fruit)	*p*-DPO	4-Me Catechol	6.50	Nil	
		Chlorogenic acid	7.00	Nil	
		p-Quinol	5.00	Nil	
		p-Phenylene diamine	0.90	Nil	
R. vernicifera (latex)	*p*-DPO	4-Me Catechol	16.00	Nil	
		Chlorogenic acid	19.25	Nil	
		p-Quinol	2.00	Nil	
		Toluquinol	4.00	Nil	
		p-Phenylene diamine	20.00	Nil	
Spruce (needles)*	*p*-DPO	4-Me Catechol	18.00	Nil	
		p-Phenylene diamine	10.00	Nil	
*B. cinerea**	*p*-DPO	4-Me Catechol	Oxidized	Nil	
		p-Phenylene diamine	Oxidized	Nil	
C. sanguineus	*p*-DPO	4-Me Catechol	2.50	Nil	
		Chlorogenic acid	5.00	Nil	
		p-Quinol	0.90	Nil	
		p-Phenylene diamine	1.75	Nil	
*C. versicolor**	*p*-DPO	4-Me Catechol	3.8	Nil	
		p-Phenylene diamine	1.6	Nil	
		Chlorogenic acid	Oxidized	Nil	
Trametes sp.	*p*-DPO	4-Me Catechol	8.00	Nil	
		Chlorogenic acid	6.00	Nil	
		p-Quinol	7.00	Nil	
		p-Phenylene diamine	5.00	Nil	
G. cingulata (strain A) [15]	*p*-DPO	Chlorogenic acid	8.5		
	p-DPO	Catechol	3.8		
		p-Phenylene diamine	1.1		

*Limited amount of enzyme restricted number of tests performed.
Comp. = competitive inhibition; Non-Comp. = non-competitive inhibition.

By contrast all the *p*-DPO preparations tested failed to show inhibition by cinnamic acids and PVP, or activation by anionic detergents. However, they were distinguished by their ability to oxidize *p*-quinol and *p*-phenylene diamine and by the inhibition of *p*-DPO activity by CTAB and other quaternary ammonium compounds.

The two contrasting sets of results with the DPO's from *G. cingulata* are of interest and are probably due to strain differences. Sussman and Markert [31] originally claimed *o*-DPO activity in their isolates of *G. cingulata* but reported that this fungus showed wide variations in its DPO activity. Other workers [32–35] have observed that DPO production in many fungi is markedly affected by culture conditions. Nevertheless both enzymes conformed to the predicted patterns of substrate/inhibitor activity.

An explanation of the observed differences in inhibitor response is not immediately apparent but may be related to differences in the nature of the reaction mechanisms and configuration of the active sites of these two enzymes. The failure of cinnamic, *p*-coumaric or ferulic acids to inhibit *p*-DPO's could be advantageous to lignolytic micro-organisms since they would not be affected by lignin decomposition products (T. Green, personal communication). Similarly

Some results (marked with reference) taken from previously published work are included for completeness

Inhibitors						
p-Coumaric acid		PVP	K_i	CTAB		Activator
Type	K_i (mM)	Type	(%w/v)	Type	K_i (mM)	(SDS)
Inhibition		Comp.	0.25	Nil		Activation
Inhibition		Comp.	0.27	Nil		Activation
		Comp.	7.40	Nil		Activation
		Non-Comp.	3.4	Nil		Activation
Non-Comp.	3.48	Nil		Nil		Activation
Comp.	6.79	Nil		Nil		Activation
		Comp.	3.0	Nil		Nil
		Non-Comp.	9.7	Nil		Activation
Nil		Nil		Comp.	12.00	Nil
Nil		Nil		Comp.	3.50	Nil
Nil		Nil		Comp.	2.80	Nil
Nil		Nil		Comp.	1.66	Nil
		Nil		Comp.	2.29	Nil
		Nil		Non-Comp.	3.72	Nil
		Nil		Comp.	0.25	Nil
		Nil		Non-Comp.	5.05	Nil
		Nil		Comp.	5.79	Nil
Nil		Nil		Comp.	10.30	Nil
Nil		Nil		Comp.	4.60	Nil
		Nil		Inhib.		
		Nil		Inhib.		
Nil		Nil		Non-Comp.	6.00	Nil
Nil		Nil		Non-Comp.	23.30	Nil
Nil		Nil		Comp.	3.90	Nil
Nil		Nil		Non-Comp.	16.00	Nil
		Nil		Comp.	2.2	Nil
		Nil		Non-Comp.	4.4	Nil
		Nil		Comp.	2.96	Nil
		Nil		Comp.	5.00	Nil
		Nil		Mixed	5.30	Nil
		Nil		Comp.	4.55	Nil
		Nil		Inhib.		Nil
		Nil		Non-Comp.	1.3	Nil

Table 2. Summary of differential tests for *o*- and *p*-diphenol oxidases

Test	*o*-DPO ('catecholase')	*p*-DPO ('laccase')
Substrate specificity:		
o-dihydroxy phenols	Oxidized	Oxidized
p-dihydroxy phenols and *p*-phenylene diamine	Nil or slow oxidation	Oxidized
*p-cresol	Orange–red colour	—
*1-naphthol	—	Purple colour
syringaldiazine [12]	—	Oxidized
Cinnamic, *p*-coumaric and ferulic acids	Inhibition	Nil
PVP	Inhibition	Nil
CTAB (QAC's, cationic surfactants)	Nil	Inhibition
Sodium dodecyl sulphate (anionic surfactants)	Activation	Nil

*Spot test with fungi [11].

plants which produce *p*-DPO's could have the advantage of a defence mechanism unaffected by DPO inhibitors produced by the invading phytopathogen [21, 36].

In conclusion we suggest that the use of the differences in properties of the enzymes summarized in Table 2 above may provide a simple and convenient procedure for the differentiation of *o*- and *p*-DPO's. Moreover, in some circumstances, they could be used for *in vivo* tests.

EXPERIMENTAL

Enzyme preparations. Crude extracts of diphenol oxidases were prepared from a number of fungal and plant sources using conventional procedures. Enzymic browning, which could cause DPO inactivation, was a problem with some plant prepns and was controlled by addition of ascorbic acid or cysteine [37] to the extraction medium. Fungi potentially capable of producing DPO's were selected by the Bavendamm test: the production of a dark brown zone when the organism was grown on potato dextrose agar containing 1 mM tannic acid. For enzyme production the fungi were cultured at 25° in 250 ml conical flasks containing 50 ml malt extract medium [38]. Both the mycelium and culture filtrate were tested for DPO activity and harvested at time of maximum activity. The reference numbers refer to the Botany Department's culture collection.

Brief details of the various enzyme prepns are as follows. Apple (*Pyrus malus* cv Lobos): A washed suspension of chloroplast debris from young fruit [37]. Banana (*Musa sapientum*): The supernatant from homogenate of banana pulp [6]. Peach (*Prunus persica* cv Paragon): Fruit homogenized in 0.4 M sucrose containing 10 mM ascorbate and 10% (v/v) prewashed Polyclar AT. Centrifuged at 20 000 *g*. Enzyme precipitated from supernatant with cold Me_2CO [39]. Spruce (*Picea omorika*): Needles were stored at −15° until required. To facilitate disintegration they were frozen in liquid N_2 then homogenized in 0.1 M NaPi buffer at pH 7. *p*-DPO activity was found in the supernatant after centrifugation at 20 000 *g* for 30 min. This enzyme was labile at room temp. *Coriolus sanguineus* (No. F44) and *C. versicolor*: The supernatant from a homogenate of mycelium from a 17-day-old culture was used. *Glomerella cingulata*: This organism was isolated from infected apples and its identity kindly confirmed by Dr. A. L. J. Cole. Maximum DPO activity was detected in the mycelial homogenate after 2 days' growth. This *o*-DPO preparation was labile whereas the *p*-DPO isolated by Walker [15] was stable on storage. Mushroom (*Agaricus bisporus*): Prepns from both the cap (pileus) and stalk (stipe) were tested. Tissue was freeze-dried, homogenized in 0.1 M NaPi–citrate buffer (pH 5) containing 10 mM ascorbic acid [10]. Both prepns from the cap and stalk exhibited similar properties so the former was used for all subsequent tests. *Trametes* sp. (No. F64): The filtrate from an 18-day-old culture was used as a source of *p*-DPO. *Botrytis cinerea*: The supernatant from the homogenate of mycelium from a 5-day-old culture. *Sclerotinia* sp.: Supernatant from homogenate of mycelium from 10-day-old culture.

Enzyme assays. DPO activity was assayed by recording the initial rate of O_2-uptake by means of a Clark-type O_2-electrode (Yellow Springs Instruments Co., U.S.A.). The reaction cell contained 0.1 M NaPi–citrate buffer at the optimum pH for each enzyme, and inhibitor or activator in a total vol. of 3 ml at 30°. The reaction was initiated by addition of substrate. K_m, K_i values and the type of inhibition were calculated from these results using Lineweaver–Burk or direct linear plots [40].

The following substrates were routinely used to test for *o*- and *p*-DPO activity: chlorogenic acid, 4-methylcatechol, *p*-phenylene diamine and *p*-quinol. Oxidation of the latter two compounds was taken to indicate *p*-DPO (laccase) activity. Towards the end of this work a sample of toluquinol (1:4-dihydroxytoluene) became available and tests showed it to be superior to *p*-quinol as a substrate for *p*-DPO's.

Chemicals. Most reagents were obtained from B.D.H. Ltd., Sigma Chemica Co. (U.S.A.) or Fluka AG (Switzerland). Soluble PVP (K-25, mean MW 28 000) and Polyclar AT were supplied by General Aniline and Film Corp. (U.S.A.). Me_2CO powder of *R. vernicifera* latex was obtained from Saito & Co., Tokyo.

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