Phytochemistry, 1972, Vol 11, pp 89 to 94 Pergamon Press Printed in England

SPECIFICITY OF AN *o*-DIPHENOL OXIDASE FROM *PRUNUS AVIUM* FRUITS

GAETANO LANZARINI, PIER GIORGIO PIFFERI and ARTURO ZAMORANI

Istituto di Tecnologie Chimiche Speciali, Facoltà di Chimica Industriale, Università di Bologna, Bologna, Italy

(*Received* 10 *March* 1971)

Abstract—An *o*-diphenol oxidase extracted from sweet cherry fruits (*Prunus avium*) was purified 17-fold, and the recovery of enzyme activity was 5 6% The optimum pH with catechol as substrate was 4·2 The enzyme exhibited only catecholase activity and was inactive with monophenols The specificity of the enzyme toward 19 substituted catechols was examined Substituents in position 3 caused a decrease in the affinity for the enzyme, probably due to steric hindrance. Electron-donating substituents in position 4 enhanced enzymic activity, whereas electron-attracting substituents in the same position reduced or eliminated the oxidation

INTRODUCTION

o-DIPHENOL oxidases are of widespread occurrence in the plant kingdom and exert their activity on a large number of substrates.[1,2] They are commonly referred to as catecholases, tyrosinases, cresolases, polyphenol oxidases or phenolases All these enzyme entities oxidize *o*-diphenols to *o*-quinones and are inactive on ascorbic acid; only some of them can also catalyse hydroxylation of monophenols in the *ortho*-position.[3] Generally, the ratio of monophenolase to *o*-diphenolase activities varies according to the source of the enzyme and the method used for extraction and purification.

o-Diphenol oxidase contains copper as its active prosthetic group, and according to Kubowitz[4–6] and Keilin[7] the copper is present in the enzyme as the bivalent ion. More recently other authors[8–11] using ESR have demonstrated that the Cu^{2+} content of the oxidase is very small and that the active enzymic form is associated with Cu^{+} ions. This is in accordance with the fact that phenolases are easily inactivated by CO, a typical complexing agent for monovalent copper The part played by copper in the enzymic reaction has been indicated by the formation of an enzyme-oxygen bond a Cu^{+}—O_2 interaction would activate molecular oxygen, and the reaction would occur according to the scheme

[1] W. D BONNER, *Ann Rev Plant Physiol* **8**, 427 (1957)
[2] D W BROOKS and C R DAWSON, in *The Biochemistry of Copper* (edited by PEISACH, P. AISEN and W E BLUMBERG), p 343, Academic Press, New York (1966)
[3] D A. ROBB, L W MAPSON and T SWAIN, *Phytochem* **4**, 731 (1965)
[4] F KUBOWITZ, *Biochem Z* **292**, 221 (1937)
[5] F KUBOWITZ, *Biochem Z* **296**, 443 (1938)
[6] F KUBOWITZ, *Biochem Z* **299**, 32 (1938)
[7] D KEILIN and T MANN, *Proc Roy Soc B* **125**, 187 (1938)
[8] T NAKAMURA, *Biochem Biophys Res Commun* **2**, 11 (1960)
[9] H S MASON, E SPENCER and T YAMAZAKI, *Biochem Biophys Res Commun.* **4**, 236 (1961).
[10] I YAMAZAKI, H S MASON and L H PIETTE, *J Biol Chem* **235**, 2444 (1960)
[11] I YAMAZAKI and L H PIETTE, *Biochem Biophys Acta* **50**, 62 (1961)

proposed by Mason [12] Other authors[2,13–15] have studied the effects of inhibitors and of substitution in the ring of phenolic substrates on the velocity of the enzymic reaction, on the basis of these results it can be assumed that oxidation occurs via an electrophilic attack

In the present paper the results of investigations on an *o*-diphenol oxidase from sweet cherry fruits (*Prunus avium*) are reported After purification the enzyme showed neither cresolase nor laccase activity, whereas it acted very effectively on catechol (catecholase, E C 1 10 3.1, *o*-diphenol oxygen–oxidoreductase) The purpose of this research was to determine the affinity of the purified *o*-diphenol oxidase for some 3- or 4-substituted *o*-diphenols

RESULTS

Extraction and Purification of o-*Diphenol Oxidase*

Unless otherwise stated, all operations were conducted at temperatures between −2° and +4° in sodium phosphate buffer at pH 7·0 Pitted cherries, pre-frozen at −20°, were homogenized and extracted in acetone The acetone powder (20 g) was ground with 100 ml of 20 mM buffer and after repeating this three times, the extract was centrifuged and the sediment was discarded The supernatant was then dialysed against 2 mM buffer (step 1) Ammonium sulphate was added to 30% saturation, the solution centrifuged at 23,000 *g* for 20 min, and the supernatant redialysed against 2 mM buffer (step 2) Ammonium sulphate was then added to 85% saturation and the ppt obtained on centrifuging (23,000 *g*, 20 min) was taken up in 0 1 M buffer and dialysed (2 mM buffer) (step 3) This solution was chromatographed on DEAE-cellulose (Whatman DE 23), eluting with 25 mM buffer, (step 4) The eluate fractions containing the enzyme were combined and chromatographed on DEAE-Sephadex A-50, eluting with 50 mM buffer (step 5) To follow the course of purification of the enzyme, catecholase activity was measured at each step by determining the initial velocity of reaction V_o, with catechol at pH 4 2 as the substrate, since preliminary experiments had shown that this was the optimal pH of the *o*-diphenol oxidase The increase in extinction at 420 nm due to the products of oxidation of catechol was used as a measure of the oxidation of this substance

TABLE 1 ACTIVITIES OF *o*-DIPHENOL OXIDASES FROM SWEET CHERRIES (20 g ACETONE POWDER) AS MEASURED WITH CATECHOL AT pH 4 2 AS SUBSTRATE, FOR THE VARIOUS PURIFICATION STEPS

Step	Protein		Enzymic activity		Specific activity,	Recovery	Purification
	Total, mg	mg/ml	Total units	Units/ml	units/mg protein	(%)	
1 Crude extract (650 ml)	1632	2 454	15,700	23 60	9 61	100	1
2 After dialysis following 30% saturation (720 ml)	1037	1 440	9360	13 00	9 40	60	1
3 After dialysis following 85% saturation (120 ml)	193	1 607	5670	45 25	29 40	36	3
4 After DEAE-cellulose column chromatography (145 ml)	23 6	0 163	1907	13 10	87 30	12	9
5 After DEAE-Sephadex column chromatography (70 ml)	5 3	0 076	882	12·60	166 00	5 6	17

The amount of enzyme which gives, as initial velocity of reaction (V_o), a $\Delta E_{420/1'}$ value of 0 050 is here defined as the unit of enzyme activity

[12] H S MASON, *Advances in Enzymology* **19**, 131 (1957)
[13] A M MAYER, *Phytochem* **1**, 237 (1962)
[14] A M MAYER, E HAREL and Y SHAIN, *Phytochem* **3**, 447 (1964)
[15] L L INGRAHAM, *J Am Chem Soc* **76**, 3777 (1954)

The results of the analyses are shown in Table 1 Except for the crude extract (step 1) which showed a low cresolase activity, all the extracts were found to possess catecholase activity only The course of purification in the last steps was also followed by means of acrylamide-gel electrophoresis, demonstrating the efficiency of the DEAE-Sephadex column chromatography. While the enzyme preparation from step 4 separated into 3 protein bands on electrophoresis, only one of which was active on catechol at pH 4·2, only the band with phenol oxidase activity appeared on the electropherograms after DEAE-Sephadex column chromatography. Moreover, when the enzyme from purification step 4, i e after DEAE–cellulose column chromatography, was assayed for catecholase activity at various pH values, the velocity of oxidation of catechol was found to exhibit two maxima at pH 4·2 and 6 2, respectively, after further purification by DEAE–Sephadex chromatography (step 5), only the maximum at pH 4 2 was still present (Fig. 1)

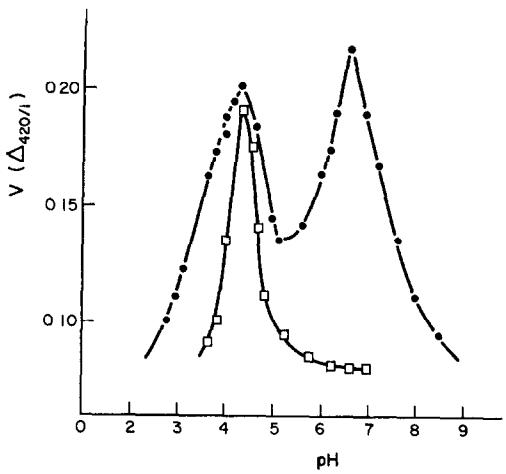

FIG 1 CHANGES IN OXIDATION VELOCITY OF CATECHOL (V_0) AS A FUNCTION OF THE pH OF REACTION SYSTEM, FOR THE ENZYME OBTAINED IN PURIFICATION STEPS 4 ● AND 5 □

Enzyme Specificity

The specificity of the purified enzyme was tested using catechol,[16] 3- or 4-substituted catechols and 2,3-dihydroxynaphthalene as substrates; the substituent groups were chosen among the simplest and most significant in order to evaluate their effect on the catecholase activity of the enzyme For each of the substrates the wavelength at which the corresponding oxidation products exhibited maximum absorption was previously determined For the substances with no maxima in their absorption spectra, the 420 nm wavelength was chosen for spectrophotometric determinations (Table 2) Moreover, for each of the substrates the pH value was determined at which the enzyme exhibited maximum activity as evaluated by V_0 measurements. In all cases, the optimum pH values differed only slightly from that for catechol and were in the range 4–4 6 Subsequently, the specificity of the enzyme toward the various substrates was evaluated at the optimum pH's Readings were taken at the wavelengths of maximum absorption, and the following two parameters were measured· (1) the

[16] H. U. BERGMEYER, *Methods of Enzymatic Analysis*, p 34, Academic Press, New York (1965).

TABLE 2 ENZYME SPECIFICITY TOWARD DIFFERENT SUBSTRATES, EXPRESSED AS AMOUNT OF OXIDIZED ASCORBIC ACID AND AS VELOCITY OF FORMATION OF OXIDATION PRODUCTS (VALUES GIVEN WITH RESPECT TO THOSE FOR CATECHOL, ASSUMED AS EQUAL TO 100)

Substrate	λ_{max} absorption of oxidation products (nm)	Optimum pH	Oxidized ascorbic acid	V_o of formation of oxidation products
Catechol	420	4 2	100	100
4-Methylcatechol	496	4 0	136	133
3-Methylcatechol	420*	4 2	31	31
3,4-Dihydroxyphenylacetic acid	420*	4 6	115	105
3,4-Dihydroxybenzoic acid	420*	4 0	10	9
2,3-Dihydroxybenzoic acid	—	—	0	0
Ethyl-3,4-dihydroxybenzoate	—	4 0	0	0
3,4-Dihydroxypropiophenone	436	4 1	106	105
ω-Chloro-3,4-dihydroxypropiophenone	—	—	0	0
4-Chlorocatechol	410	4 1	130	128
2,3-Dihydroxy-4-methoxybenzoic acid	—	—	0	0
3,4-Dihydroxybenzaldehyde	420*	4 3	22	19
3,4-Dihydroxybenzonitrile	436	4 1	14	12
3,4-Dihydroxycinnamic acid	420*	4 2	113	119
3,4-Dihydroxyphenylalanine	482	4 1	23	24
4-Nitrocatechol	—	—	0	0
Chlorogenic acid	420*	4 5	141	142
2,3-Dihydroxynaphthalene	—	—	0	0
2,3-Dihydroxyphenylsulfonic acid	—	—	0	0

* Value arbitrarily chosen, since it was difficult to determine the wavelength at which the oxidation product exhibited maximum absorption

amount of ascorbic acid oxidized in a given time,* (ii) the rate at which the substrate was oxidized after all the ascorbic acid had been converted to dehydroascorbic The values obtained (Table 2) are given with respect to those for catechol

DISCUSSION

An examination of Table 2 shows good agreement between measurements based on the oxidation time of ascorbic acid and the measurements of the velocity of reaction V_o. This confirms once more that ascorbic acid, in the concentrations used for the analytical determinations, does not interfere with the enzyme-substrate reaction [17–19] As regards the effect of the substituent groups on enzyme specificity, the following considerations can be made

Steric Effect

The decrease of reactivity in the 3-substituted substrates (3-methylcatechol, 2,3-dihydroxybenzoic acid, 2,3-dihydroxy-4-methoxybenzoic acid, 2,3-dihydroxyphenylsulfonic acid) can be attributed to steric hindrance, since it occurred independently of the electron-

* The velocity of the reaction between ascorbic acid (AH_2) and quinone (see below) is greater than that at which the quinone itself is formed, thus preventing measurable amounts of oxidized substrate from remaining in the reaction medium as long as some ascorbic acid is present AH_2 + *o*-quinone → A + *o*-diphenol

[17] B LUDWIG and J M NELSON, *J Am Chem Soc* **61**, 2601 (1939)
[18] L L INGRAHAM, *J Am Chem Soc* **78**, 5095 (1956)
[19] W SCHARF and C R DAWSON, *J Am Chem Soc* **80**, 4627 (1958)

donating or electron-attracting character of the substituent (for instance, compare 3-methylcatechol and 2,3-dihydroxybenzoic acid with the 4-methyl and 3,4-dihydroxy isomers)

Inductive Effect

The presence of an electron-donating group in the 4 position results in a greater reactivity of the substrate This is particularly evident as regards chlorogenic acid, 4-methylcatechol, 4-chlorocatechol and 3,4-dihydroxycinnamic acid. In contrast, electron-attracting groups cause a decrease of affinity or a total inactivation; this is the case with 4-nitrocatechol, 3,4-dihydroxybenzonitrile, 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde The only exception is 3,4-dihydroxypropiophenone, which exhibits a greater affinity than catechol The lack of enzymic activity toward 2,3-dihydroxynaphthalene should be attributed to the remarkable chemical stability of this molecule

Chemical oxidation of *o*-diphenols has been supposed to occur *via* the formation of the phenate radical-ion [20–22] This mechanism of reaction cannot be assumed for the enzyme studied, for the following two reasons (i) the very acid pH of the reaction system (4·2) does not favour the formation of the above ion; (ii) electron-attracting substituents, favouring the formation of the same ion, should increase substrate reactivity, but no such increase was observed here Our results can rather be explained by the hypothesis[12,23,24] which regards an electrophilic stage as a factor governing oxidation rate This probably results from the formation of an enzyme-substrate complex in which the copper of the enzyme participates directly Being aromatic substances with π orbitals available, phenolic substrates can form complexes with transition metals, even in their lower-valency state [25] Substrates with electron-donating substituents allow the enzyme-substrate complex to be formed more easily, and *o*-diphenol oxidase should therefore have a greater activity with them, which is in accordance with our results

EXPERIMENTAL

Plant material and phenolic substrates Sweet cherries picked at maturity were kept at −20°, since freezing was found to increase the amount of extractable enzyme All phenolic substrates were supplied by FLUKA (their degree of purity was in no case lower than 99%), except for 2,3-dihydroxyphenylsulfonic acid, which was prepared by the method of Gentsch [26]

Measurement of enzymic activity Aq catechol (1 ml of 175 mM), citrate–phosphate buffer (2 ml of 0 5 M, pH 4 2) and the enzyme preparation (0 5 ml) were placed in a spectrophotometer cell, at 25°, in the order given Extinction was measured at 420 nm 30 sec after the addition of the enzyme, and the measurement was repeated every 15 sec up to 3 min from the beginning of the reaction The graphic method described by Bergmeyer[16] was used for calculating V_o The amount of enzyme under the above conditions, giving a V_o corresponding to a $\Delta E_{420/min}$ of 0 050 is defined as enzyme unit

Protein determination The protein content of the enzyme extracts from purification steps 1, 2 and 3 was determined by the Kjeldahl method [27] For the other purification steps, the protein content was determined by measuring extinction at 280 nm and comparing the values obtained with those for albumin solutions of known concentrations

[20] P W Davies, *Physical Techniques in Biological Research* (edited by W L Nastuk), Vol 4, p 137 Academic Press, New York (1962)
[21] F R Hewgill, T J Stone and W A Waters, *J Chem Soc* 408 (1964)
[22] T J Stone and W A Waters, *J Chem Soc* 1488 (1965)
[23] H S Mason and W L Fowlks, *J Am Chem Soc* **77**, 2914 (1955)
[24] B J B Wood and L L Ingraham, *Nature, Lond* **205**, 291 (1965)
[25] L E Orgel, *An Introduction to Transition Metal Chemistry—Ligand Field Theory* Wiley, New York (1960)
[26] C Gentsch, *Ber Chem* **43**, 2108 (1910)
[27] J. L Bailey, *Techniques in Protein Chemistry*, p 299, Elsevier, New York (1962)

Column chromatography on DEAE-cellulose Whatman DE 23 DEAE-cellulose (25 g, exchange capacity 1 meq/g) was treated according to the procedure described by Neukom [28] The column (1·8 × 90 cm) supplied with a cooler, was equilibrated with 5×10^{-3} M NaH_2PO_4 buffer (pH 7) Elution was carried out with $2\,5 \times 10^{-2}$ M phosphate buffer (pH 7) collecting 4-ml fractions

Column chromatography on DEAE-Sephadex DEAE-Sephadex A 50 (2 g, exchange capacity, 3 5 meq/g) was dispersed in $2\,5 \times 10^{-2}$ M NaH_2PO_4, placed in a column (1 4 × 10 cm), and equilibrated with the same buffer The enzyme was eluted with 5×10^{-2} M NaH_2PO_4 buffer (pH 7)

Acrylamide-gel electrophoresis The gel was prepared by polymerization of a mixture containing 2 5 g of cyanogum (American Cyanamid Co), 24 ml of 0 2 M tris HCl buffer (pH 6 5), 0 1 ml of 7% ammonium persulphate, and 70 μl of *N,N,N',N'*-tetramethyldiamine Protein bands were stained with amido black, other electropherograms were dipped into 0 17 M catechol solution in citrate-phosphate buffer (pH 4 2), *o*-diphenol oxidase caused a deep yellow band

Measurement of the wavelength at which oxidation products exhibited maximum absorption Enzymic oxidation was carried out as described for the determination of enzymic activity After 3 min the reaction was stopped by adding sufficient NaF to a final concentration of 10^{-1} M Absorption spectra were plotted over the range 200–400 nm

Determination of optimum pH's for the various substrates The phenolic substrates (0 2 M, 1 ml normally as aq solns, but for insoluble substrates, dissolved in a 9 1 mixture of H_2O and EtOH) were placed in a spectrophotometer cell with 2 5 ml of 0 1 M citrate–phosphate buffer,[29] pH range 2 5–6, and 0 3 ml of solution of purified enzyme Spectrophotometric determinations were carried out at the wavelength of maximum absorption of the oxidation products

Measurement of specificity The phenolic substrates (1 ml of 30 mM), 0 2 ml of 10^{-5} M EDTA, 0 5 ml of 6×10^{-4} M ascorbic acid, 1 ml of 0 1 M citrate–phosphate buffer at the optimum for pH the substrate and 0 3 ml of solution of purified enzyme were placed successively in a spectrophotometer cell, at 25° and absorbances were read at the wavelength of maximum absorption The time elapsing from the addition of the enzyme to the reaction system and the appearance of a spectrophotometrically detectable absorption was measured This time corresponded to that necessary for the oxidation of all the ascorbic acid present Spectrophotometric readings were then repeated at 15-sec intervals From the time necessary for the oxidation of the ascorbic acid, the amount of ascorbic acid oxidized in the same time in the presence of the various substrates was calculated with respect to catechol The results of the spectrophotometric determinations were used for calculating the V_o for each substrate with respect to that for catechol Ascorbic acid blanks, in which the enzyme was thermally inactivated were incubated in the range pH 4 2–4 6 and no loss of ascorbic acid was observed

Acknowledgement—The authors thank Dr Osvaldo Jannuzzi for his collaboration during this research The work was supported by a grant from the National Research Council of Italy

[28] H NEUKOM and H DEUEL, *Helv Chim Acta* **43**, 64 (1960)
[29] T G MCILVAINE, *J Biol Chem* **49**, 183 (1921)

Key Word Index—Prunus avium, Rosaceae, cherry, *o*-diphenol oxidase, substrate specificity