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SUBSTRATES AND INHIBITORS OF POTATO TUBER PHENOLASE

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Abstract—The substrate specificity of and effect of inhibitors on potato tuber phenolase are described. For a particular inhibitor, K_I depends upon whether an *o*-diphenol or monophenol is used as substrate, and thus it is proposed that different protein molecules are responsible for the oxidation of *o*-diphenols and the hydroxylation of monophenols. A study of the substrate specificity of the enzyme suggests that the monophenol hydroxylase activity has no biosynthetic significance and that the main function of the enzyme is to provide a defence against wounding and infection by the oxidation of chlorogenic acid.

INTRODUCTION

Two types of phenolase* have been isolated from plant sources. The first type is capable of catalysing both the oxidation of *o*-diphenols to *o*-quinones and the hydroxylation of monophenols. Phenolases of this type include those isolated from potato,[1–3] apple,[4] sugar beet leaf,[5] and broad bean leaf.[6,7] The second type is only capable of oxidizing *o*-diphenols to *o*-quinones and includes those isolated from banana,[8] tea leaf[9] and tobacco leaf.[10]

The relationship between the two activities found in the first type of phenolase is unknown. The oxidation of *o*-diphenols is characterized by a strong product inhibition of the reaction,[11] and the hydroxylation of monophenols is invariably preceded by a lag period. Since the lag period is markedly reduced by the addition of small quantities of an *o*-diphenol it has been suggested that a source of electrons provided by the oxidation of *o*-diphenols is essential for the hydroxylation of monophenols.[12]

Kertesz and Zito[13] have proposed that the hydroxylation of monophenols results from a non-enzymic reaction between *o*-quinone and monophenol. However Dressler and Dawson[14,15] concluded, from a study of copper exchange from mushroom phenolase in the

* The enzyme under study is classified as an *o*-diphenol: O_2 oxidoreductase (E.C.1.10.3.1). Since the monophenol hydroxylase function of the enzyme is of interest we have decided to use the more general term, phenolase, which encompasses both functions of the enzyme.

[1] S. S. PATIL and M. ZUCKER, *J. Biol. Chem.* **240**, 3938 (1965).
[2] D. A. ABUKHARMA and H. W. WOOLHOUSE, *New Phytologist* **65**, 477 (1966).
[3] F. A. M. ALBERGHINA, *Phytochem.* **3**, 65 (1964).
[4] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **7**, 921 (1964).
[5] A. M. MAYER and J. FRIEND, *Nature* **185**, 464 (1960).
[6] T. SWAIN, L. W. MAPSON and D. A. ROBB, *Phytochem.* **5**, 469 (1966).
[7] D. A. ROBB, T. SWAIN and L. W. MAPSON, *Phytochem.* **5**, 665 (1966).
[8] J. K. PALMER, *Plant Physiol.* **38**, 508 (1963).
[9] R. P. F. GREGORY and D. S. BENDALL, *Biochem. J.* **101**, 569 (1966).
[10] R. A. CLAYTON, *Arch. Biochem. Biophys.* **81**, 404 (1959).
[11] D. W. BROOKS and C. R. DAWSON, in *Biochemistry of Copper* (edited by J. PERSACH, P. AISEN and W. E. BLUMBERG), p. 343. Academic Press, New York (1966).
[12] H. S. MASON, *Advan. Enzymol.* **19**, 79 (1957).
[13] D. KERTESZ and R. ZITO, in *Oxygenases* (edited by O. HAYAISHI), p. 307. Academic Press, New York (1962).
[14] H. DRESSLER and C. R. DAWSON, *Biochim. Biophys. Acta* **45**, 508 (1960).
[15] H. DRESSLER and C. R. DAWSON, *Biochim. Biophys. Acta* **45**, 519 (1960).

presence of its substrates, that the enzyme possesses two active sites, one responsible for the hydroxylation of monophenols, the other for the oxidation of *o*-diphenols.

Potato phenolase has been purified from whole tubers,[2] aged discs,[3] and peelings.[1] The preparation from peelings was separated into two components. Both components retained the two activities and it was suggested that the isoenzymes contain the same active site or sites and may be composed of different multiples of the same subunit. Multiple forms of the phenolases from broad bean,[6] green tobacco leaf,[16] tea leaf[9] and mushroom[17] have also been observed. The occurrence of isoenzymes in the latter case has also been explained by association of and dissociation into subunits.[18]

The present study was undertaken to investigate the nature of the two activities of potato tuber phenolase using various substrates and inhibitors.

RESULTS

Substrate Specificity of Potato Phenolase

The enzyme was assayed by measurement of oxygen consumption with a variety of substrates over a range of concentrations. K_M and V were determined for each substrate and the results are shown in Table 1. *p*-Coumaric acid and L-tyrosine were only very slowly oxidized by potato tuber phenolase, even in the presence of small concentrations of *o*-diphenols, and no oxidation of the diphenols 2,3-dihydroxynaphthalene and hydroquinone and the monophenols *p*-nitrophenol, phenol, *o*-cresol and ferulic acid was observed.

TABLE 1. SUBSTRATE SPECIFICITY OF POTATO TUBER PHENOLASE

Substrate	K_M(mM)	V (μl O_2/min/100 μl enzyme)
Chlorogenic acid	1·4	73·1
Caffeic acid	2·1	40·0
L-DOPA	11·8	28·4
Catechol	4·8	52·4
p-Cresol	0·67	2·9
m-Cresol	1·0	0·13
p-Hydroxyphenylpyruvic acid	8·3	0·58
p-Hydroxyphenylpropionic acid	2·2	3·0

Standard assay conditions were used throughout.

The relative rates of oxidation of the various substrates agree with those found for purified potato phenolase,[1–3] and the K_M values are similar to those found for other plant phenolases assayed by measurement of oxygen consumption.[4,7,10] The values are however higher than those obtained for purified potato phenolase assayed by spectrophotometric methods.[1–3] Mayer and his co-workers[19] have pointed out the difficulties in comparing results obtained from different methods of assay.

[16] E. C. SILSER and H. J. EVANS, *Plant Physiol.* **33**, 255 (1958).
[17] J. L. SMITH and R. C. KREUGER, *J. Biol. Chem.* **237**, 1121 (1962).
[18] S. BOUCHILLOUX, P. MCMAHILL and H. S. MASON, *J. Biol. Chem.* **238**, 1699 (1963).
[19] A. M. MAYER, E. HAREL and R. BEN-SHAUL, *Phytochem.* **5**, 783 (1966).

Effect of Inhibitors

A number of monophenols,[7,20–22] diphenols,[22,23] and carboxylic acids[24] have been identified as inhibitors of phenolases, but little information is available about the variation of their inhibitory properties with substrate. The inhibitory effects of the diphenol 2,3-dihydroxynaphthalene, the monophenols *p*-nitrophenol, *p*-coumaric acid and ferulic acid, and the carboxylic acid cinnamic acid on potato tuber phenolase were therefore investigated using several substrates. In each case the type of inhibition shown was deduced from Lineweaver-Burk double reciprocal plots and confirmed by plots of $1/v$ against i.[25] From the points of interception of the latter plots the inhibitor constants, K_i, were deduced. In every

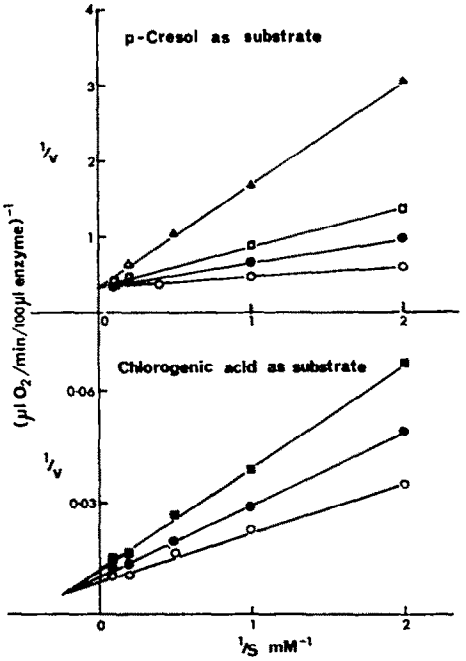

FIG. 1. INHIBITION OF POTATO TUBER PHENOLASE BY *p*-COUMARIC ACID.

Standard assay conditions were used except for the addition of 2·5 µM chlorogenic acid to the *p*-cresol incubation mixtures. This small concentration of an *o*-diphenol reduced the lag period without altering the maximum rate of oxygen uptake. ○, no inhibitor; ●, 10^{-3} M *p*-coumaric acid; □, 2×10^{-3} M *p*-coumaric acid; ■, 4×10^{-3} M *p*-coumaric acid; △, 5×10^{-3} M *p*-coumaric acid.

case good straight lines were obtained and examples of the plots are shown in Figs. 1 and 2. The type of inhibition and K_i for each inhibitor with each substrate are shown in Table 2. 2,3-Dihydroxynaphthalene has previously been identified as a competitive inhibitor of the oxidation of diphenols,[23] and *p*-nitrophenol has been identified as a non-competitive inhibitor of catechol oxidation by broad bean leaf phenolase.[7] L-Tyrosine and L-phenylalanine

[20] H. HEYMANN, Z. ROGACH and R. L. MAYER, *J. Am. Chem. Soc.* **76**, 6330 (1954).
[21] J. BONNER and S. G. WILDMAN, *Arch. Biochem. Biophys.* **10**, 497 (1946).
[22] D. RICHTER, *Biochem. J.* **28**, 901 (1934).
[23] A. M. MAYER, E. HAREL and Y. SHAIN, *Phytochem.* **3**, 447 (1964).
[24] R. C. KRUEGER, *Arch. Biochem. Biophys.* **57**, 52 (1955).
[25] M. DIXON and E. C. WEBB, *Enzymes* (2nd edition), p. 315. Longmans, London (1964).

were shown to have no inhibitory effect on potato phenolase. The latter results should be contrasted with those obtained with mammalian tyrosinase which is competitively inhibited by both DOPA and phenylalanine when tyrosine is used as substrate.[26]

The results in Table 2 show that, for potato tuber phenolase, the properties of an inhibitor depend upon whether an *o*-diphenol or monophenol is used as substrate. Thus for a particular inhibitor the type of inhibition and the inhibitor constant found with *o*-diphenols as substrates are different from those found with monophenols as substrates. L-DOPA appears to be anomalous, sometimes being subject to different type of inhibition and giving a different inhibitor constant from the other *o*-diphenols. However, it is also quite different from the monophenols in its behaviour towards inhibitors.

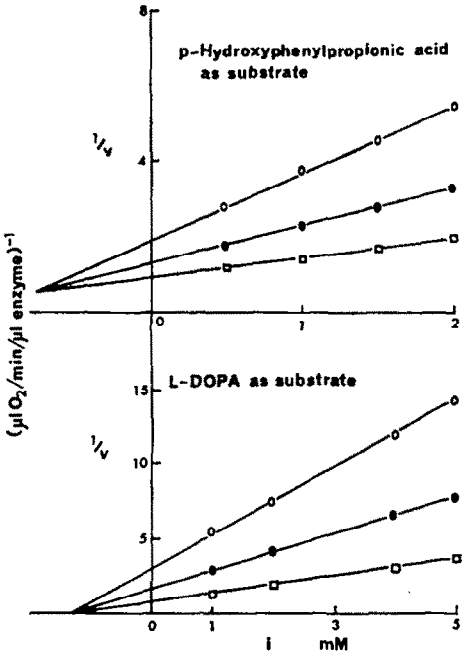

FIG. 2. INHIBITION OF POTATO TUBER PHENOLASE BY *p*-NITROPHENOL.

Standard assay conditions were used except for the addition of 2·5 µM chlorogenic acid to the *p*-hydroxyphenyl propionic acid incubation mixtures. ○, 10^{-3} M substrate; ●, 2×10^{-3} M substrate; □, 5×10^{-3} M substrate.

The different types of inhibition and the different inhibitor constants found with the two types of substrate indicate the existence of two distinct active sites in potato tuber phenolase, one for the oxidation of *o*-diphenols, the other for the hydroxylation of monophenols.

If the sites occur on the same protein molecule one may propose that they are capable of interacting in such a way that monophenols bound at one site would show competitive inhibition of the hydroxylation of other monophenols at the same site and non-competitive or mixed inhibition of the oxidation of *o*-diphenols at the other site. Similarly *o*-diphenols bound at their site of oxidation would show competitive inhibition of the oxidation of other *o*-diphenols and non-competitive or mixed inhibition of the hydroxylation of monophenols.

[26] S. H. POMERANTZ, *Biochem. Biophys. Res. Commun.* **16**, 188 (1964).

In these cases mixed inhibition would mean that the inhibitor affects the affinity of the enzyme for its substrate and yet does not bind at the active site for that substrate.[27] However since the same enzyme species are responsible for the oxidation of *o*-diphenols and hydroxylation of monophenols, K_I, which measures the affinity of the inhibitor for the enzyme, should be independent of the type of substrate. Thus the results in Table 2 do not conform to the simple theory of two interacting active sites on the same protein molecules.

TABLE 2. EFFECT OF INHIBITORS ON POTATO PHENOLASE

Inhibitor	Substrate	Type of inhibition	Inhibitor constant (K_I) mM
p-Nitrophenol	Chlorogenic acid	Non-competitive	2·2
	Caffeic acid	Non-competitive	2·5
	L-DOPA	Non-competitive	1·6
	Catechol	Non-competitive	2·5
	p-Cresol	Mixed	1·1
	p-Hydroxyphenyl propionic acid	Mixed	0·9
Ferulic acid	Chlorogenic acid	Non-competitive	22
	p-Cresol	Competitive	0·45
p-Coumaric acid	Chlorogenic acid	Mixed	5·2
	p-Cresol	Competitive	0·7
2,3-Dihydroxynaphthalene	Chlorogenic acid	Competitive	1·0
	Caffeic acid	Competitive	1·0
	L-DOPA	Competitive	1·2
	Catechol	Competitive	1·4
	p-Cresol	Non-competitive	0·12
	p-Hydroxyphenylpropionic acid	Non-competitive	0·14
Cinnamic acid	Chlorogenic acid	Mixed	6·5
	Caffeic acid	Mixed	7·8
	L-DOPA	Non-competitive	1·7
	Catechol	Mixed	7·8
	p-Cresol	Mixed	3·0
	p-Hydroxyphenylpropionic acid	Mixed	3·2

Standard assay conditions were used except for the addition of 2·5 μM chlorogenic acid to the monophenol incubation mixtures.

A second theory in which the two active sites are on different protein molecules can also be proposed, if each of the species has an additional site which binds the acidic phenols and carboxylic acids. Substances bound at this inhibitor site may interact with the active site. Considering the *o*-diphenol oxidizing form, *o*-diphenol inhibitors will bind at both sites. If the affinity at the active site is much greater than that at the inhibitor site competitive inhibition will be observed, and conversely if the affinity at the inhibitor site is much greater than that at the active site non-competitive inhibition will be observed. If, however, the affinities at the two sites are approximately equal a mixed type of inhibition will be observed. Monophenol inhibitors will only bind at the inhibitor site giving a non-competitive or mixed type of inhibition. Mixed inhibition in the latter case means that the inhibitor affects the affinity of the enzyme for its substrate and yet does not bind at the active site for that substrate.

[27] J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 160. Academic Press, New York (1963).

By a similar reasoning for the monophenol hydroxylating form, *o*-diphenols and carboxylic acids could act as non-competitive or mixed inhibitors and monophenols could act as competitive, non-competitive or mixed inhibitors. Since in the above cases different species are responsible for the hydroxylation of monophenols and the oxidation of *o*-diphenols, K_i will be expected to vary according to which type of substrate is used. Thus the results in Table 2 are in accord with a system involving two different protein molecules, one responsible for the oxidation of *o*-diphenols and the other for the hydroxylation of monophenols.

DISCUSSION

The data obtained from the inhibitor studies indicate that potato tuber phenolase possesses different active sites for the hydroxylation of monophenols and the oxidation of *o*-diphenols. These two sites occur on different protein molecules, and since it has been impossible to separate the two activities by chromatography or electrophoresis[1-3] the two protein molecules must be very closely related, possibly differing only in conformation.

Mallette and Dawson[28] previously suggested that changes in conformation at the active site of phenolases may produce the two activities. They proposed that the enzymes act as hydroxylating systems *in vivo* losing most of their ability to hydroxylate monophenols during isolation owing to a twist or spread of the protein residues to which the copper atoms at the active site are attached. However, a study of the substrate specificity of potato tuber phenolase appears to rule out hydroxylation as a major *in vivo* function of this enzyme. Chlorogenic acid, the major diphenol of potato tubers, is biosynthesized via a hydroxylation of *p*-coumaric or its quinic acid ester,[29] but potato tuber phenolase is almost totally inactive towards *p*-coumaric acid, although the hydroxylation of some other monophenols occurs readily. Indeed it is possible that the major site of chlorogenic acid biosynthesis in potato plants is the leaves, since systems catalysing the hydroxylation of monophenols have been isolated from leaves,[30-32] and the translocation of chlorogenic acid in plants has been demonstrated.[33]

Since no role can readily be ascribed to the monophenol hydroxylating activity of potato tuber phenolase we suggest that this activity has no biosynthetic significance and that the main function of the enzyme is to provide a defence mechanism against wounding and infection by the oxidation of chlorogenic acid.

EXPERIMENTAL

Chemicals

Caffeic acid was recrystallized from water before use. Other chemicals were the best commercial grade available and were used without further purification.

Preparation of Enzyme

An extract was prepared by macerating potato tubers, variety Orion (400 g), in a Waring blender with 600 ml of a 0·5 M solution of sucrose in 50 mM tris buffer, pH 7·0. The homogenate was filtered through cheese-cloth and centrifuged at 75,000 *g*. The supernatant, which contained 90 per cent of the total phenolase activity and was used in all the assays, was collected and stored in a deep freeze. Freezing and thawing had no effect on the activity of the enzyme.

[28] M. F. MALLETTE and C. R. DAWSON, *Arch. Biochem. Biophys.* **23**, 29 (1949).
[29] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[30] S. HATTORI and M. SATO, *Phytochem.* **2**, 385 (1963).
[31] M. SATO, *Phytochem.* **5**, 385 (1966).
[32] P. F. T. VAUGHAN and V. S. BUTT, *Biochem. J.* **104**, 65P (1967).
[33] N. J. MACLEOD and J. B. PRIDHAM, *Phytochem.* **5**, 777 (1966).

Enzyme Assay

In the standard assay enzyme was added to a stirred solution of substrate in 0·1 M K phosphate buffer pH 7·0. This solution had previously been saturated with air. O_2 uptake was measured with a Clark Electrode (Yellowsprings Instrument Co.) and the maximum rate of O_2 uptake was used to determine the velocity of the reaction. With *o*-diphenols as substrate, the maximum rate of oxygen uptake occurred immediately after addition of the enzyme, but with monophenols there was a lag period after the addition of the enzyme before the oxygen uptake became maximal.