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INHIBITION OF THE APPLE PHENOLASE SYSTEM THROUGH INFECTION BY *PENICILLIUM EXPANSUM**

J. R. L. WALKER

Cawthron Institute, Nelson, New Zealand

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Abstract—The light colour of the soft rot of apples infected with *Penicillium expansum* has been shown to be the result of the suppression of the fruit's normal enzymic browning (phenolase system) processes. Rotted apple tissue was found to contain increased amounts of caffeic acid together with an accumulation of *p*-coumaric acid, ferulic acid and the antibiotic patulin. *p*-Coumaric acid and ferulic acid were found to act as competitive inhibitors of catechin oxidation but non-competitive inhibitors for the oxidation of chlorogenic acid.

INTRODUCTION

THE FUNGUS *Penicillium expansum* is the cause of a common storage disorder of apples usually known as "blue mould". The fungus enters the fruit via wounds or lenticels and spreads rapidly throughout the apple flesh giving rise to a light-coloured soft rot. This rotting is brought about by the action of extracellular pectin-degrading enzymes secreted by the fungus and this aspect of the problem has been extensively studied by Cole and Wood.[1,2]

In addition to the ability to secrete pectolytic enzymes, many strains of *P. expansum* secrete the antibiotic patulin and Brian *et al.*[3] have shown that the juice from apples rotted by *P. expansum* may contain up to 1000 mg/l of patulin. More recently, Scott and Somers[4] have shown that the patulin in juice from rotted apples can survive the normal plant processing operations and thus constitute a health hazard. Patulin is also toxic to higher plants and has been implicated in the replant problem in old apple orchards since it may be produced in the soil, by *P. expansum*, from old apple leaves and roots.[5]

The observation that damage by *P. expansum* does not cause much browning around the site of infection (compared with infections caused by *Glomerella* sp. or *Sclerotinia* sp. which give rise to firm, dark-brown lesions) prompted an investigation in this laboratory into the fate of the phenolics and associated enzymes in the rotted tissue. The results of this investigation are reported below.

RESULTS

Comparison of Phenolics in Healthy and Rotted Tissue

The observed reduction in browning of apple fruit rotted by *Penicillium expansum* suggested that the fungus had interfered with the normal enzymic browning process. Cole and

* Part I in the series "Biochemical Aspects of *Penicillium expansum* Infection in Apples".

[1] M. COLE and R. K. S. WOOD, *Ann. Bot. N. S.* **25**, 417 (1961).
[2] M. COLE and R. K. S. WOOD, *Ann. Bot. N. S.* **25**, 435 (1961).
[3] P. W. BRIAN, G. W. ELSON and D. LOWE, *Nature* **178**, 263 (1956).
[4] P. M. SCOTT and E. SOMERS, *J. Agric. Food Chem.* **16**, 483 (1968).
[5] H. BORNER, *Phytopath. Z.* **48**, 370; **49**, 1 (1963).

Wood[2] investigated some aspects of this problem in connexion with their studies of the pectic enzymes secreted by *P. expansum* but the opportunity has now been taken to extend this work in more detail by comparing the levels of the different classes of phenolic compounds in healthy and rotted tissue from several different apple varieties. This was done using the analytical procedures originally developed by Swain and Hillis[6] and the results, given in Table 1, show that there is no marked decrease in the level of phenolics in the rotted tissue.

Variations in the composition of the ethyl acetate-soluble phenolics from healthy and rotted tissue were compared by paper chromatography or TLC on cellulose. Examination of the chromatograms under u.v. light revealed marked differences between the two extracts, notably the appearance in the rotted tissue of free *p*-coumaric and ferulic acid, together with a marked increase in the level of caffeic acid and the appearance of patulin. The identities of the phenolic acids were confirmed by their R_f values in a number of solvent systems, their reactions with chromogenic spray reagents, and their characteristic u.v. absorption spectra. Patulin was identified by co-chromatography with an authentic sample, comparison of u.v. spectra and its characteristic reaction to form a yellow spot on exposure to ammonia followed by spraying with phenylhydrazine and heating at 100°.[7]

TABLE 1. COMPARISON OF LEVELS OF PHENOLICS IN HEALTHY AND ROTTED APPLE TISSUE.
(MEAN OF RESULTS FROM THREE FRUITS)

	Chlorogenic acid (mg/g)		Vanillin-reacting phenolics (as catechin, mg/g)		Leuco-anthocyanidins (as cyanidin, mg/g)	
Apple variety	Healthy	Rotted	Healthy	Rotted	Healthy	Rotted
Jonathan	0·91	0·85	4·2	3·3	2·5	2·3
Granny Smith	0·92	0·76	4·3	3·3	2·3	2·2
Sturmer Pippin	0·85	0·78	3·3	2·3	2·8	1·9
Democrat	1·6	1·5	5·7	5·6	3·6	3·7

Comparison of Phenolase Activity in Healthy and Rotted Apple Tissue

It has been suggested[2] that infection of an apple by *P. expansum* might inhibit the fruit's phenolase system and this hypothesis was tested by comparing the levels of actual and potential browning[8, 9] in homogenates of healthy and rotted tissue. The level of actual browning gave a measure of the availability of the natural phenolase substrates whilst the level of potential browning revealed the total available phenolase activity in the presence of excess substrate. The results of these experiments (Table 2) show clearly that the normal phenolase activity of the apple is almost completely suppressed in the rotted tissue. This observation was confirmed by experiments in which the juice from rotted tissue was added to Warburg flasks containing a preparation of apple phenolase together with a suitable substrate [catechol, chlorogenic acid or (+)-catechin]; the addition of juice from rotted tissue brought about a competitive type of inhibition. A similar type of inhibition effect was also observed when a preparation of chloroplast grana from young tobacco (*Nicotiana tabacum*) leaves was used in place of the apple phenolase.

[6] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agric.* **10**, 63 (1959).
[7] T. YAMAMOTO, *J. Pharm. Soc. Japan* **76**, 1375 (1956).
[8] C. WEURMAN and T. SWAIN, *J. Sci. Food Agric.* **6**, 186 (1955).
[9] J. R. L. WALKER, *N.Z. J. Sci.* **5**, 316 (1962).

TABLE 2. COMPARISON OF LEVELS OF ENZYMIC BROWNING IN HEALTHY AND ROTTED APPLE TISSUE. ACTUAL BROWNING MEASURES BROWNING DUE TO NATURAL SUBSTRATES ONLY WHILST POTENTIAL BROWNING GIVES A MEASURE OF TOTAL AVAILABLE PHENOLASE ACTIVITY IN PRESENCE OF EXCESS SUBSTRATE

	Healthy tissue (degree of browning (units/100 g))		Rotted tissue (degree of browning (units/100 g))	
Variety	Actual	Potential	Actual	Potential
Johathan	6·5	21·0	3·0	4·0
Delicious	11·5	34·0	5·0	8·0
Granny Smith	14·5	31·0	2·5	2·5
Statesman	11·5	33·5	1·0	1·0
Sturmer Pippin	20·0	32·5	1·5	1·5
Democrat	6·5	26·0	1·0	2·0
Dunn's Favourite	12·5	30·0	1·0	1·0

Concurrent with these experiments, it was observed that treatment of the juice from rotted tissue with 10% (w/v) Polyclar AT (an insoluble form of polyvinylpyrrolidone) removed most of its inhibitory effect. Examination of the Polyclar-treated juice revealed a marked reduction in the amounts of phenolic acids present whilst the level of patulin was unaffected.

Inhibition of Apple Phenolase by Phenolic Acids

In the light of the previous observations, and in view of a recent report by Macrae and Duggleby[10] that a number of phenolic acids can act as inhibitors of potato phenolase, the inhibitory effects of phenolic acids upon apple phenolase was investigated in some detail. Values for the inhibitor constant (K_i) and the type of inhibition involved were determined by plotting the reciprocal of the rate of oxidation ($1/v$) against the concentration of inhibitor (i) for two levels of phenolic substrate according to the procedure of Dixon.[11] The results of these experiments are recorded in Table 3 and show that *p*-coumaric acid is a powerful inhibitor of apple phenolase but that the type of inhibition depends upon the phenolic substrate. Similar experiments with a number of antibiotic substances secreted by *Penicillium* sp. showed that patulin, citrinin, penicillic acid and penicillin did not inhibit apple phenolase. Thus it was concluded that the inhibitory action of the juice from rotted apple flesh was due to the presence therein of *p*-coumaric and ferulic acid.

TABLE 3. INHIBITORY EFFECT OF FREE PHENOLIC ACIDS UPON APPLE PHENOLASE

	Substrate			
	Chlorogenic acid		(+)-Catechin	
Inhibitor	Type of inhibition	Inhibitor constant, K_i (mM)	Type of inhibition	Inhibitor constant, K_i (mM)
p-Coumaric acid	Non-competitive	0·13	Competitive	0·03
Ferulic acid	Non-competitive	1·3	Competitive	0·2
o-Coumaric acid	Non-competitive	3·4	Non-competitive	1·8

[10] A. R. MACRAE and R. G. DUGGLEBY, *Phytochem.* 7, 855 (1968).
[11] M. DIXON, *Biochem. J.* 55, 170 (1953).

Formation of Unconjugated Phenolic Acids

A number of workers[12, 13] have used commercial "pectinase" preparations to hydrolyse conjugated phenolic acids and, since *P. expansum* is known to secrete powerful pectolytic enzymes,[1, 2] it seemed reasonable to suppose that these enzymes might also be responsible for the hydrolysis of the chlorogenic and *p*-coumarylquinic acids of the host apple to yield caffeic and *p*-coumaric acids respectively.

A simple experiment was devised to test this hypothesis: 25 ml of juice from rotted apple tissue was treated with 2·5 g of Polyclar AT to remove phenolic compounds and, since this treatment also removed the phenolase inhibition effect, thiourea (0·005 M) was added to block any subsequent phenolase activity. Chlorogenic acid (0·001 M) was then added and the reaction mixture incubated at 30°; if thiourea was omitted, the reaction mixture browned thus confirming the inhibitory action of the unconjugated phenolic acids. Aliquots (5 ml) of the reaction mixture were removed at 4-hr intervals, 0·1 ml conc. HCl was added to stop the reaction, and the sample then extracted with 1·5 ml ethyl acetate. The ethyl acetate extracts were spotted onto a Whatman No. 1 paper and developed in benzene–acetic acid–water[14] (173:72:3). Examination of the chromatogram under long wavelength u.v. light showed that chlorogenic acid disappeared rapidly from the reaction mixture whilst traces of caffeic acid were detected after 8 hr incubation.

DISCUSSION

The results reported above show clearly that the light colour of apple tissue rotted by *Penicillium expansum* is the result of the inhibition of the apple's normal enzymic browning processes which may constitute part of the fruit's defence system. This inhibition of the phenolase system of the apple is brought about by the accumulation of *p*-coumaric and ferulic acids in the rotted tissue. Healthy apples normally contain chloregenic acid and *p*- coumarylquinic acids;[15] the former compound is a substrate for apple phenolase[16, 17] whilst it is most unlikely that the latter is an inhibitor since there is evidence that *p*-coumarylquinic acid is a precursor of chlorogenic acid biosynthesis in potatoes.[12, 18] Difficulty in obtaining *p*-coumarylquinic acid prevented the study of this point. Ferulic acid likewise occurs in plants as depsides with quinic or shikimic acids[18–20] although these compounds have not yet been found in apple fruit.

Infection of an apple with *P. expansum* gives rise to a soft rot and high levels of pectinesterase, polygalacturonase and macerating enzyme[1, 2] are present in the rotted tissue while *p*-coumaric acid, ferulic acid and patulin also accumulate. Preliminary results suggest that the *p*-coumaric acid, and the increased level of caffeic acid, in rotted tissue arise by the hydrolytic action of the fungal pectic enzymes upon chlorogenic acid (caffeylquinic acid) and *p*-coumarylquinic acid. Ferulic acid may be formed by the *O*-methylation of caffeic acid since Finkle and Nelson[21] have shown that treatment of apple juice or slices with a suitable methyl-

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[13] V. C. RUNECKLES, *Can. J. Biochem. Physiol.* **41**, 2259 (1963).
[14] I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, p. 292, Heinemann, London (1960).
[15] A. H. WILLIAMS, *Chem. & Ind.* 1200 (1958).
[16] C. WEURMAN and T. SWAIN, *Nature* **172**, 678 (1953).
[17] J. R. L. WALKER, *Australian J. Biol. Sci.* **17**, 360 (1964).
[18] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).
[19] G. PICTET and H. BRANDENBERGER, *J. Chromatogr.* **4**, 396 (1960).
[20] O. GOLDSCHMID and H. L. HERGERT, *Tappi* **44**, 858 (1961).
[21] R. F. NELSON and B. J. FINKLE, *Phytochem.* **3**, 321 (1964).

ating system (liver *O*-methyltransferase plus *S*-adenosylmethionine) effectively prevented enzymic browning and that ferulic and feruloylquinic acids accumulated. More recently these workers[22] reported the presence of a *meta O*-methyltransferase in the cambial tissue of apple-tree branches. If *P. expansum* secreted an *O*-methyltransferase then ferulic acid could be formed directly from caffeic acid or, less likely, by hydrolysis of feruloylquinic acid derived from chlorogenic acid. *p*-Coumaric most probably originates from *p*-coumarylquinic acid but it could possibly be derived by the action of tyrosine-deaminase upon tyrosine.

Table 3 shows that *p*-coumaric acid is a powerful inhibitor of apple *o*-diphenol oxidase activity although this compound is slowly hydroxylated *in vitro* by phenolases from apple chloroplast grana[17] and from other plants.[23] It will also be seen from Table 3 that the inhibitor constant (K_i) varied widely; from 0·03 mM for the catechin/*p*-coumaric system to 1·3 mM for the chlorogenic acid/ferulic acid system. In addition the type of inhibition depended on the substrate; catechin oxidation was competitively inhibited whereas the oxidation of chlorogenic acid was non-competitively inhibited by *p*-coumaric and ferulic acids. By contrast *o*-coumaric acid, which was included for interest, acted as a non-competitive inhibitor for *both* substrates. The reasons for these divergencies in behaviour are not clear but similar differences in inhibitor/substrate competition were observed by Macrae and Duggleby[10] in their studies on potato tuber phenolase. These authors[10] suggested that the enzyme may possess separate sites for *o*-diphenol oxidation ("active-site") and inhibitor binding ("inhibitor-site") when the inhibitor was an acidic phenol or phenol-carboxylic acid, and that the type of inhibition depended upon the relative affinities at the two sites. The present results lend support to this hypothesis.

EXPERIMENTAL

Apple fruit infected with *Penicillium expansum* were obtained from local orchard packing-sheds and unless otherwise stated the variety "Granny Smith" was used in all experiments. Fruit were selected so that not more than half the apple was rotted, in this way the unrotted portion served as a "healthy" control.

The juice from the infected tissue was obtained by scooping out the soft rotted apple pulp and squeezing it through several layers of fine cloth followed by filtration through Whatman No. 111 paper. Control "healthy" apple juice was prepared by grinding peeled apple flesh in a domestic juice extractor followed by centrifugation at 30,000 × *g*; 1 % (w/v) ascorbic acid was added to control browning. For some experiments, the clarified juices were subsequently freeze-dried.

Estimation of Phenolic Compounds

Healthy or rotted apple flesh (10 g) was homogenized in 70 % (v/v) ethanol, the volume adjusted to 50 ml then the solution clarified by centrifugation followed by filtration. Suitable aliquots were used for the estimation of chlorogenic acid by method of Arnow,[24] vanillin-reacting phenolics by method of Swain and Hillis[6] and leuco-anthocyanidins by method of Swain and Hillis.[6] Analyses were made for at least three fruits.

Estimation of Enzyme-Catalysed Browning[8,9]

This was done by homogenizing 20 g of healthy or rotted tissue in 20 ml of pH 7·0 0·1 M phosphate buffer then adding 10 ml of the homogenate to test-tubes containing (*a*) 10 ml 0·1 % $Na_2S_2O_5$ (control) or (*b*) 10 ml water (actual browning; no added substrate) or (*c*) 10 ml 0·001 M-Catechol (potential browning). The tubes were incubated at 30° for 20 min, filtered and the optical density of the filtrate recorded in a colorimeter fitted with an Ilford No. 621 (450 nm peak) filter. The degree of browning was recorded as units/100 g fruit, where 1 unit corresponds to a difference in optical density of 0·1.[9]

[22] B. J. FINKLE and R. F. NELSON, *Biochem. Biophys. Acta* **78**, 747 (1963).
[23] M. SATÔ, *Phytochem.* **5**, 385 (1966).
[24] L. E. ARNOW, *J. Biol. Chem.* **118**, 531 (1937).

Chromatography and Identification of Phenolic Acids

Whole or freeze-dried extracts of healthy and rotted apple tissue were extracted with ethyl acetate and the extracts concentrated before examination by chromatography on paper (Whatman No. 1 or 3MM) or thin layers of cellulose (Whatman CC41). The solvent systems benzene/acetic acid/water (136:72:3) (BzAc)[14] or 2% (v/v) acetic acid (HAc) were found to give the best separations. Chromatograms were visualized under long and short wave u.v. light, before and after exposure to NH_3. Patulin showed up as a dark absorbing spot under short wavelength (254 nm) u.v. light. Phenolic acids were also located and identified by their colour reactions when chromatograms were sprayed with diazotized *p*-nitraniline or sulphanilic acid. U.v. absorption spectra of the individual phenolic acids, isolated from a chromatogram on Whatman No. 3MM paper, were also measured.

Measurement of Inhibitor Activity

This was done by means of conventional Warburg techniques or by means of an O_2-electrode (Rank Bros., Bottisham, Cambridge) coupled to a Heathkit EUW-20AE recorder; the latter technique was more convenient for determination of K_i values. Warburg flasks or the O_2-electrode cell contained 0·1 M phosphate-citrate buffer, pH 5·0, enzyme, inhibitor and 4 or 10 μmoles substrate in 3 ml of solution. All experiments were carried out at 30° in an atmosphere of air. The apple phenolase was prepared as described previously.[17]

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