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FUNGAL DEGRADATION OF PHLORIDZIN

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Abstract—A variety of fungi were shown to degrade phloridzin to phloretin, phloroglucinol and phloretic acid. Phloretic acid in turn was metabolized to *p*-hydroxybenzoic acid. *Aspergillus niger* yielded cell-free preparations which catalyzed the hydrolysis of phloretin to phloroglucinol and phloretic acid.

INTRODUCTION

IN ADDITION to improving our understanding of the turnover of naturally occurring organic compounds, the study of the microbial degradation of flavonoids also promises to yield information on some interesting and perhaps novel enzymes. For example, in studies on the fungal degradation of the flavonol glucoside, rutin,[1,2] it was found that extracellular enzymes yielded phloroglucinol carboxylic acid, protocatechuic acid, rutinose and carbon monoxide from its breakdown. The production of carbon monoxide in these reactions warrants further detailed investigation at the enzymological level. Microbial enzymes undoubtedly exist for the breakdown of stilbenes, aurones and all the other flavonoids typically produced by higher plants.

Because of its implication in a "soil sickness" associated with old orchard sites, the dihydrochalcone, phloridzin, a constituent of *Malus* species, has attracted attention. Non-sterile soil,[3] races of the fungus *Venturia inaequalis*[4] and species of *Aspergillus* and *Penicillium*[5] have been reported to degrade phloridzin to phloroglucinol, phloretic and *p*-hydroxybenzoic acids. More recently, Grochowska[6] was able to demonstrate, with crude extracts from the sap of apple spurs, the hydrolysis of phloridzin and phloretin to phloroglucinol and phloretic acid. We have been screening a number of fungi for their ability to degrade phloridzin in order to discover a good source of this activity for enzyme studies. This report is concerned with the results of a survey in which we have found that the ability to degrade phloridzin is quite a common feature of fungi. Moreover, we have obtained evidence for the presence of an enzyme which hydrolyzes phloretin to phloroglucinol and phloretic acid.

RESULTS AND DISCUSSION

The ability of various fungi to degrade phloridzin was assessed mainly by the appearance of compounds such as phloretin, phloroglucinol, phloretic acid and *p*-hydroxybenzoic acid in the culture medium. The R_f values of these compounds in four solvent systems, together

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[5] G. H. N. TOWERS, unpublished work cited on p. 280 in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), Academic Press, New York (1964).
[6] M. J. GROCHOWSKA, *Bull. Acad. Polon. Sci.* **15**, 455 (1967).

TABLE 1. CHROMATOGRAPHIC CHARACTERISTICS OF PHENOLIC COMPOUNDS FORMED IN CULTURE FLUIDS OF FUNGI ADMINISTERED PHLORIDZIN

Compound	R_f values in solvents*				Color reaction with diazotized *p*-nitroaniline + NaOH
	A	B	C	D	
Phloridzin	0·23	0·10	0·54	0·81	Yellow–orange
Phloretin	0·05	0·25	0·86	0·87	Yellow
Phloroglucinol	0·54	0·10	0·63	0·75	Brown–orange
Phloretic acid	0·65	0·95	0·68	0·97	Violet
p-Hydroxybenzoic acid	0·50	0·80	0·45	0·93	Pink

* TLC, cellulose as stationary phase. Solvents: A, formic acid–water (2% v/v); B, benzene–methanol–acetic acid (45:8:4 by volume); C, propanol–butanol–2 N NH_4OH (6:1:3 by volume); D, butanol–acetic acid–water (4:1:2·2 by volume).

TABLE 2. CHROMATOGRAPHIC ANALYSES OF CULTURE FLUIDS OF FUNGI CULTIVATED IN PHLORIDZIN*

Organisms possessing phloretin-hydrolase activity		Organisms possessing only β-glucosidase activity		Organisms causing other changes on phloridzin	
Aspergillus chevalieri	(803)	*Candida albicans*	(297)	*Aspergillus giganteus*	(808)
A. citrosporus	(820)	*C. albicans*	(298)	*Candida guilliermondi*	(274)
A. flavus	(807)	*C. guilliermondi*	(273)	*Cryptococcus laurentii*	(899)
A. flavus-oryzae	(811)	*Cryptococcus albidus*	(900)	*Penicillium stipitatum*	(856)
A. fumigatus	(815)	*Monascus purpureus*	(227)		
A. japonicus	(817)	*Penicillium cyclopium*	(890)		
A. melleus	(821)	*P. funiculosum*	(844)		
A. nidulans	(810)	*P. intricatum*	(852)		
A. niger Schiemanni (mutant)	(812)	*P. italicum*	(849)		
A. niger	(814)	*P. janthinellum*	(864)		
A. niger	(872)	*P. notatum*	(851)		
A. niger	(878)	*P. patulum*	(871)		
A. sydowi	(816)	*P. variable*	(863)		
A. terreus	(818)	*Pseudoeurotium sp.*	(241)		
A. terreus	(819)	*Rhodotorula mucilaginosa*	(873)		
Botrytis cinerea	(921)	*Shanorella spirotricha*	(240)		
Carpenteles javenicum	(203)	*Torula cremoris*	(910)		
Emericella variecolor	(296)	*Torulopsis sphaerica*	(876)		
Penicillium adametzi	(845)	*Trichoderma viride*	(920)		
P. camemberti	(846)				
P. canescens	(866)				
P. charlesii	(857)				
P. chrysogenum	(847)				
P. claviforme	(848)				
P. clavigerum	(922)				
P. digitatum	(843)				
P. fellutanum	(861)				
P. griseofulvum	(869)				
P. herquei	(894)				
P. lilacinum	(850)				
P. lilacinum	(923)				
P. oxalicum	(860)				
P. patulum	(870)				
P. patulum	(902)				
P. thomi	(853)				
Sporobolomyces roseus	(901)				
Talaromyces vermiculatis	(251)				

* Number in parentheses indicate UBC strain numbers.

with their color reactions with diazotized *p*-nitroaniline, are given in Table 1. Solvent A was used in routine studies. Analyses were carried out after 6, 15 and 30 days of growth respectively. As can be seen from Table 2, thirty-seven out of the sixty organisms screened degraded phloridzin to produce phloretin, phloroglucinol and/or phloretic acid. Earlier observations suggested a difference between *Aspergillus* and *Penicillium* in that members of the former genus utilized the phloretic acid moiety of phloridzin in preference to the phloro-

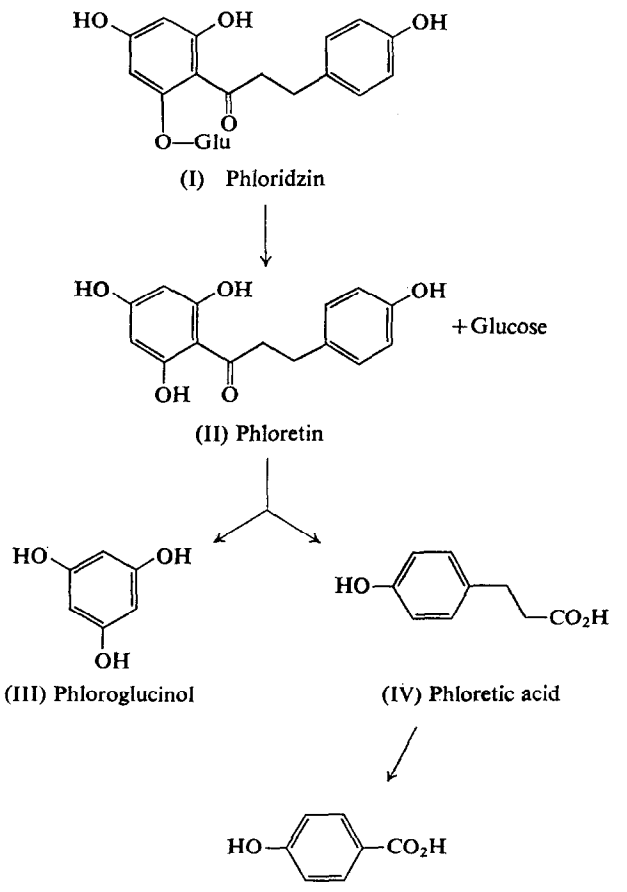

FIG. 1. SCHEME FOR THE PARTIAL DEGRADATION OF PHLORIDZIN BY FUNGI.

glucinol nucleus. Subsequent studies, however, indicated that this was not always so although is was found that all the *A. niger* strains included in the survey showed the presence of only phloroglucinol in growing culture fluids. This was particularly so with strain 814 which was subjected to a more detailed study. For ease of operation phloretin was used instead of phloridzin as the substrate in replacement studies as well as in investigations with cell-free enzyme preparations. Replacement studies with mycelial mats of this strain clearly indicated the disappearance of phloretin from the media and the production of phloroglucinol and phloretic acid. Crystalline phloroglucinol was isolated from the medium of a 6-day-old growing culture

of this strain and the u.v. and i.r. spectra of the isolate were found to be identical with that of an authentic sample. Likewise, with cell-free enzyme preparations, phloretin was hydrolyzed to phloroglucinol and phloretic acid which were identified by chromatography and spectrophotometry. To the best of our knowledge, this is the first report on the solubilization of this enzyme, which we have called phloretin hydrolase, from a micro-organism. Further studies relating to the purification and properties of this enzyme are under way and will form the subject matter of a later communication. In replacement studies with mycelial mats of the same strain of *A. niger*, grown on phloridzin, it was possible to demonstrate the conversion of phloretic acid to *p*-hydroxybenzoic acid. Eventually *p*-hydroxybenzoic acid disappears from the replacement medium (24 hr) indicating its further metabolism. It can be seen from Table 2 that nineteen organisms possessed only β-glucosidase activity indicated by the accumulation of phloretin alone in the medium. *A. giganteus*, *P. stipitatum*, *Candida guilliermondi* (274) and *Cryptococcus laurentii* did not even produce phloretin. There was evidence, however, of the metabolism of phloridzin although the products have not been identified. The overall sequence of the fungal degradation of phloridzin as suggested by these experiments is indicated in Fig. 1. The further metabolism of phloroglucinol is under investigation.

EXPERIMENTAL

Chemicals

Phloretin was prepared by acid hydrolysis of phloridzin. Phloretic acid was isolated from an ether extract of an alkaline hydrolyzate of phloridzin with $BaCO_3$.

Organisms

All the strains employed in the investigation were from the U.B.C. culture collection (numbers given in parentheses) and were cultivated as still cultures at room temperature in a medium containing KH_2PO_4, 1·0 g; $MgSO_4$, 0·5 g; KNO_3, 2·0 g; yeast extract (Difco), 0·5 g; Fe, 0·2 mg; Zn, 0·2 mg; Mn, 0·1 mg; Cu, 0·2 mg; and distilled water to 1 l. To this medium was added phloridzin at a final concentration of 0·05 per cent. The basal mineral solution was sterilized at 126° for 20 min and the phenolic substrate was aseptically incorporated. Uninoculated controls were always maintained and were subject to the same chemical analyses.

Chromatography

Two per cent formic acid–water (v/v) was employed for the routine TLC chromatographic separation of the various intermediates of phloridzin and phloroglucinol degradation on cellulose (20 × 20 cm plates). The spots were located on chromatograms by spraying with diazotized *p*-nitroaniline.

Analyses of Culture Fluids of Fungi

The various fungal strains screened in the survey were inoculated into the media as spore or cell suspensions. At the end of 6, 15 and 30 days of growth 5 ml of culture filtrate was aseptically withdrawn and acidified to pH 2·0 with 1 N HCl and extracted with 10 ml of ethyl acetate by repeated shaking. The ethyl acetate extracts were dried, filtered and evaporated to dryness. The residues were dissolved in 1 ml of ethanol for chromatography.

Replacement Techniques

Six-day-old mycelial mats were washed free of culture fluids at least thrice with sterile distilled water and suspended in 50 ml of distilled water or buffer (0·05 M citric acid–phosphate buffer, pH 5·0) containing either phloretin or phloretic acid at a concentration of 0·5 mg per ml. Because of its insolubility, phloretin was dissolved in a minimal volume of ethanol prior to its addition to the buffer solution. At periodic intervals aliquots were withdrawn and examined spectrophotometrically. In order to follow the disappearance of phloretin in the replacement studies, 2·5-ml aliquots were extracted twice with 10 ml of ethyl acetate (after adjusting the pH to 2·0). The ethyl acetate residue was made up to 0·5 ml with ethanol and 25 μl of this sample applied on the thin-layer plates. Phloretin was located on the chromatogram with a u.v. lamp (long-wave model SL 3660). The layer was removed carefully with a scalpel and transferred to a centrifuge tube. The sample was extracted with 3 ml of ethanol and the spectrum recorded.

Cell-Free Phloretin Hydrolase

A heavy suspension of spores was inoculated into minimal media containing phlorodizin as sole source of carbon and the mycelial mats were collected after 6 days of growth by filtering under suction. The organism sporulated heavily from the third day of growth onward and consequently the mycelial felts along with the spores were employed in further studies. Ten g of frozen mycelia was macerated to a uniform paste in a chilled mortar with an equal weight of aluminum oxide. At this stage, it was extracted with 50 ml of 0·05 M phosphate buffer (pH 7·0) and centrifuged at 5000 *g* for 15 min. To this extract was added, with thorough mixing, 6 ml of protamine sulfate (4 per cent) dissolved in 0·05 M phosphate buffer, pH 7·0. The stirring was continued for 30 min and the precipitated nucleic acids were removed by centrifuging at 20,000 *g* for 15 min. The resultant clear supernatant was used as the crude enzyme. Reaction mixture consisted of 1·5 ml citric acid (0·1 M)–disodium phosphate (0·2 M) buffer pH 6·0; 0·5 ml substrate containing 2 μmoles of phloretin and 1 ml of this enzyme preparation. At the end of 2 hr incubation at 30° the reaction was checked by the addition of 1N HCl to pH 2. An ethyl acetate extract was chromatographed as indicated earlier.

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