An Inducible Hydrolase from *Aspergillus niger*, Acting on Carbon–Carbon Bonds, for Phlorrhizin and other C-Acylated Phenols

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(Received 8 September 1969)

1. An inducible enzyme catalysing the hydrolysis of phloretin to form phloroglucinol and phloretic acid has been extracted from the acetone-dried powders of the mycelial felts of an *Aspergillus niger* strain grown in the presence of phlorrhizin. The enzyme was partially purified by treatment with protamine sulphate, ammonium sulphate fractionation, negative adsorption on tricalcium phosphate gel, and DEAE-cellulose column chromatography. 2. The hydrolytic activity on phloretin appeared to be maximal at about pH 9.6. However, the characteristics of the enzyme were studied at pH 7.2, because of the lability of the product, phloroglucinol, under alkaline conditions. 3. The apparent K_m value at pH 7.2 was about 0.3–0.4 mM for phloretin and 0.15 mM for 3'-methylphloracetophenone. 4. Maximum activity of the enzyme was obtained without the addition of any cofactor or metal ion. The involvement of thiol groups in the reaction was demonstrated by the potent inhibitory action of both heavy-metal ions and *p*-chloromercuribenzoate. 5. The enzyme showed a rather broad substrate specificity, and some other C-acylated phenols related to phloretin were hydrolysed. It was found that 3'-methylphloracetophenone, phloracetophenone and 2',4,4'-trihydroxydihydrochalcone were attacked more efficiently than phloretin. We propose the systematic name C-acylphenol acylhydrolase for the enzyme. This enzyme belongs to EC group 3.7.1.

Phlorrhizin (2',4,4',6'-tetrahydroxydihydrochalcone 2'- β -glucoside), a well-known constituent of *Malus* species (De Koninck, 1835), is known to be degraded to phloroglucinol, phloretic acid [3-(*p*-hydroxyphenyl)propionic acid] and *p*-hydroxybenzoic acid by non-sterile soil (Börner, 1959), races of the fungus *Venturia inaequalis* (Holowczak, Kuc & Williams, 1960) and species of *Aspergillus* and *Penicillium* (Towers, 1964). Previous investigations (Jayasankar, Bandoni & Towers, 1969) in our laboratory have involved screening a variety of fungi for their ability to metabolize phlorrhizin, and the results of a survey show that this ability to degrade phlorrhizin is a common feature of fungi. Moreover, cell-free preparations from *Aspergillus niger* were shown to catalyse the hydrolysis of phloretin (2',4,4',6'-tetrahydroxydihydrochalcone) to phloroglucinol and phloretic acid, and this enzyme was tentatively named phloretin hydrolase. In addition to providing information about fungal degradation of a naturally occurring dihydrochalcone, this hydrolytic reaction of phloretin is also

noteworthy from an enzymological viewpoint, since hydrolytic enzymes that cleave carbon–carbon bonds are not common.

The present paper describes the partial purification and properties of a phloretin-hydrolysing enzyme from the mycelial felts of *A. niger* grown on a synthetic medium containing phlorrhizin. A wide range of compounds has been examined as potential substrates of the enzyme, and it appears to catalyse the hydrolysis of several other C-acylated phenols structurally related to phloretin. Although phloretin has been used as the substrate of the reaction in routine analyses, we have found that 3'-methylphloracetophenone, phloracetophenone and 2',4,4'-trihydroxydihydrochalcone are more susceptible substrates than phloretin. This study should provide a basis for further investigations into the fungal metabolism of naturally occurring phenylketonic substances in general.

MATERIALS AND METHODS

Chemicals. Phlorrhizin dihydrate (m.p. 115–118°C, uncorr.) was isolated from apple roots or purchased from Calbiochem, Los Angeles, Calif., U.S.A. Phloretin [m.p.

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Phlorrhizin (2',4,4',6'-tetrahydroxydihydrochalcone
2'-β-glucoside)

2',4,4'-Trihydroxychalcone

2',4,6'-Trihydroxy-3'-methylacetophenone
(3'-methylphloracetophenone)

267–268°C (decomp.), uncorr.] was prepared by acid hydrolysis of phlorrhizin. Phloretic acid (m.p. 126–127°C, uncorr.) was obtained by hydrogenation of *p*-coumaric acid. Methylphloroglucinol and 3'-methylphloracetophenone were supplied by Dr J. P. Kutney, Department of Chemistry, University of British Columbia. Naringenin, 4',5,7-trihydroxy-3-methoxyflavanone and 2',3,4,4'-tetrahydroxychalcone (butein) were gifts from Dr T. A. Geissman, Department of Chemistry, University of California. Six derivatives of 2',4,6'-trihydroxydihydrochalcone 4'-β-neohesperidoside were gifts from Dr L. Krbeček, International Minerals and Chemical Corp., Growth Science Center, Libertyville, Ill., U.S.A. 2',3,4,4',6'-Pentahydroxydihydrochalcone 4'-β-glucoside (3-hydroxyphlorrhizin) was supplied by Dr A. H. Williams, Long Ashton Research Station, University of Bristol, U.K. 2',3,4,4',6'-Pentahydroxydihydrochalcone was obtained by emulsin hydrolysis of 3-hydroxyphlorrhizin. Phloracetophenone 4'-β-neohesperidoside was prepared from naringin by the method of Krbeček *et al.* (1968). 2',6-Dihydroxy-4,4'-dimethoxy-, 2',3,4',6'-tetrahydroxy- and 2',3,4',6'-tetrahydroxy-4-methoxy-dihydrochalcones were obtained from the corresponding neohesperidosides by acid hydrolysis. Quercetin, rhamnetin and naringin were obtained from Fluka A.-G., Buchs, Switzerland. 2-Hydroxy- and 2-methoxy-1,4-naphthaquinones were synthesized. Methyl vanilloyl ketone was a gift from Dr J. Pepper, Saskatoon, Sask., Canada.

The syntheses of the following compounds are outlined below: 4-hydroxy-, 2'-hydroxy-, 2',4,4'-trihydroxy-3-methoxy-, 2',4-dihydroxy-, 2',4,5'-trihydroxy-, 2',4-dihydroxy-4'-methoxy-, 2'-hydroxy-4,4'-methoxy-, 2',4,4'-trihydroxy- and 2',4'-dihydroxy-chalcones; 2',4,4'-trihydroxy- and 2',4'-dihydroxy-4-methoxy-dihydrochal-

cones and 6-hydroxy- and 4,6-dihydroxy-coumaranones. The chalcones were synthesized by condensing equimolar amounts of acetophenone and benzaldehyde derivatives in the presence of an excess of aq. 60% KOH. Methoxylated reactants were dissolved with the aid of ethanol. The reaction mixtures were heated for several hours at about 80°C, cooled to room temperature and poured into crushed ice-HCl. The solids thus obtained were air-dried and recrystallized from methanol. For 2'-hydroxychalcone crystallization from methanol was followed by sublimation at a pressure of 0.1 mmHg at 100°C. Dihydrochalcones were synthesized by hydrogenation of chalcones in ethanol at room temperature with 10% (w/w) Pd-charcoal as catalyst. The dihydrochalcones were recrystallized from aq. ethanol. Coumaranones were synthesized by treating equimolar quantities of the appropriate dried phenol and chloroacetonitrile in anhydrous ether with anhydrous HCl for 2 h. The precipitated imine hydrochloride was collected by filtration, washed with ether, dried briefly and dissolved in water. For 0.1 mol 400 ml of water was sufficient. The volume of this solution was halved by gentle boiling. After cooling, the *o*-chloroacetylphenol was collected by filtration. Conversion into the coumaranone was accomplished by gently refluxing the above product with an equal weight of sodium acetate in water for 1 h. Recrystallization was from ethanol.

The other special chemicals were obtained as follows: 2'-hydroxy-, 4'-hydroxy-, 4'-methoxy-, 2',4'-dihydroxy-, 2',4',6'-trihydroxy- and 2'-hydroxy-4'-methoxy-acetophenones, 4'-hydroxy- and 2',4'-dihydroxypropionophenones, phloracetophenone, acacetin, chrysin, phloroglucinol dihydrate, 2,5-dihydroxybenzaldehyde from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; *p*-dimethylaminobenzaldehyde and 2,4- and 2,5-dihydroxybenzoic acids from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; sodium arsenite, L-cysteine, *o*-phenanthroline and sodium diethyldithiocarbamate from British Drug Houses Ltd., Poole, Dorset, U.K.; emulsin, iodoacetic acid, 2,3-dimercaptopropanol, *p*-hydroxyphenylpyruvic acid and DEAE-cellulose from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; 3,5-dihydroxybenzoic acid from L. Light and Co. Ltd., Colnbrook, Bucks., U.K.; 3,4-dihydroxybenzaldehyde from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.; acetanilide and acetophenetidin from Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.; 2',6'-dihydroxyacetophenone from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 3',4'-dihydroxypropionophenone from Chemicals Procurement Laboratories Inc., College Point, N.Y., U.S.A.; sodium pyruvate and 2-mercaptoethanol from Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.; *p*-chloromercuribenzoate, GSH, $\alpha\alpha'$ -bipyridyl, EDTA, protamine sulphate and tricalcium phosphate gel (A grade) from Calbiochem; crystalline bovine serum albumin from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; cellulose powder MN300 from Macherey, Nagel and Co., Düren, Germany.

Organism and cultivation conditions. *A. niger* (U.B.C. 814) was obtained from the culture collection of the University of British Columbia. The stock cultures were maintained on slants of malt-yeast-peptone agar. The culture medium contained 5 g of KNO₃, 2.5 g of KH₂PO₄, 1 g of MgSO₄·7H₂O, 1 g of Na₂SO₄, 20 mg of FeCl₃·6H₂O, 10 mg of ZnSO₄·7H₂O, 3 mg of MnSO₄·4H₂O, 1.5 mg of

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 20 g of glucose and water to 1 litre. The basal mineral solution was sterilized at 120°C for 20 min, and phlorrhizin was added aseptically to a final concentration of 0.1%. Roux culture flasks each containing 100 ml of the medium were inoculated with a suspension of *A. niger* spores and the fungi were cultivated as still cultures at room temperature (approx. 25°C). Cultures were harvested before sporulation started (52–70 h). The mycelial felts were thoroughly washed with water and collected by filtering under suction, through a Buchner funnel.

Enzyme purification. All manipulations were carried out at 0 – 4°C , and glass-distilled water was used for preparing all solutions.

A 50 g portion of chilled mycelial felts was macerated with 500 ml of cold acetone (-20°C) in a Waring Blendor for 90 s. The acetone was rapidly filtered by suction, and the residue was again blended with cold acetone. This procedure was repeated twice more and the final residue washed with cold acetone. After being dried thoroughly *in vacuo*, the powder was stored at -20°C .

A 15 g portion of the acetone-dried powder, corresponding to approx. 230 g of fresh mycelia, was suspended in 300 ml of 0.04 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 0.5 mM-GSH and gently stirred for 2 h. The suspension was squeezed through cheesecloth and centrifuged at 10000g for 10 min to obtain the crude enzyme solution (step 1). To the crude extract (271 ml) was added 30 ml of 2% protamine sulphate dissolved in 0.04 M-potassium phosphate buffer, pH 7.0. After being stirred for 20 min the precipitate was removed by centrifuging at 20000g for 15 min (step 2). Finely powdered $(\text{NH}_4)_2\text{SO}_4$ (61 g) was slowly added, with constant stirring, to the resulting yellowish supernatant (294 ml) to bring it to 35% saturation. The solution was neutralized by dropwise addition of 10% K_2CO_3 solution. After 30 min, the suspension was centrifuged at 20000g for 15 min, and the precipitate discarded. Solid $(\text{NH}_4)_2\text{SO}_4$ (42 g) was added to the clear supernatant (325 ml) to bring it to 55% saturation. After being stirred for 30 min the precipitate was collected by centrifugation as above, dissolved in a minimum volume of 0.02 M-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA and 0.5 mM-GSH and dialysed for 8 h against two changes of the same buffered solution. The precipitate formed during dialysis was removed by centrifugation (step 3). The clarified dialysis residue (20.5 ml) was treated with 500 mg of tricalcium phosphate gel. After being stirred for 15 min, the suspension was centrifuged and the precipitate discarded (step 4). The supernatant (20 ml) from the previous step was added to a column (1.5 cm $\times$ 20 cm) of DEAE-cellulose, which was previously equilibrated with 0.02 M-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA and 0.5 mM-GSH. The loaded column was first washed with 100 ml of the same buffer and subsequently 100 ml each of 0.05 M- and 0.1 M-KCl in the buffer were applied to the column. The eluate was collected in 5.5 ml fractions at a flow rate of 35–40 ml/h. The enzyme was eluted with the 0.05 M-KCl solution (Fig. 1). The fractions containing enzymic activity (fractions 33–36) were combined and desalted by dialysis against 0.01 M-potassium phosphate buffer, pH 7.0 (step 5).

Polyacrylamide-gel electrophoresis. The eluate from the

DEAE-cellulose column was freeze-dried and purified further by the method of Ornstein & Davis (1962) as modified by Fox, Thurman & Boulter (1964). Approx. 200 μg of protein was applied to each gel column. After electrophoresis, discs containing enzyme activity (mobility 0.60–0.66 with respect to Bromophenol Blue) were homogenized in 0.02 M-potassium phosphate buffer, pH 6.5 (2 ml/disc). The homogenate was filtered and the gel residue washed four times with the same volume of buffer. The pooled filtrates were concentrated to 5 ml by freeze-drying and dialysed with several changes against water for 32 h at 4°C . Any residue was removed by centrifugation and the supernatant was freeze-dried.

N-Terminal group identification. Protein, purified as described above, was treated with DNS chloride (Gray, 1967). Performic acid-oxidized protein (Hirs, 1956) was also treated with DNS chloride (Gray & Hartley, 1963). The reaction mixtures were dialysed against water for 48 h, freeze-dried and hydrolysed in 5.7 M-HCl at 110°C for 18 h. DNS-amino acids were identified by chromatography on polyamide sheets (Woods & Wang, 1967).

Enzyme assays. Except where otherwise stated, the standard reaction mixture consisted of 1 mM-substrate, 0.1 M-potassium phosphate buffer, pH 7.2, and an appropriate dilution of the enzyme in a total volume of 1.0 ml. The reaction was usually allowed to proceed for 60 min at 30°C and was terminated by adding 0.15 ml of M-HCl. Consumption of the substrate or formation of the products was then determined as follows. (a) For routine assay, the disappearance of phloretin was measured spectrophotometrically. The acidified reaction mixture was extracted with 4 ml of ethyl acetate with the aid of a cyclomixer and a 2 ml portion of the ethyl acetate layer was diluted with 3 ml of ethanol. The control reaction mixture was treated in the same manner except that the enzyme solution was added to the mixture after the reaction had been stopped. The extinction due to phloretin was measured in a Unicam SP.800A recording spectrophotometer. The molar extinction coefficient of phloretin in the above solution is 2.00×10^4 at 287 nm. The contribution to E_{287} of phloretic acid produced in the reaction was found to be 6%, and this was taken into account in calculating the substrate consumption; the contribution of phloroglucinol was negligible. Formation of phloretic acid was determined as follows. The acidified reaction mixture was extracted twice with 5 ml of diethyl ether. The ether was evaporated and the residue was applied as a thin band to a cellulose plate 0.5 mm thick. After being developed in aq. 2% (v/v) formic acid, the band corresponding to phloretic acid was detected by its absorption of u.v. light and was removed carefully with a scalpel. The cellulose powder containing phloretic acid was packed on a 2 mm glass filter and extracted with 80% ethanol. To 5 ml of the ethanolic extract was added 0.2 ml of M-NaOH, and E_{243} was recorded against a control run. The amount of phloretic acid was calculated from a standard curve obtained by treating known amounts of phloretic acid in the same way. This method was used especially in studying the effect of pH on phloretin hydrolysis because of the stability of phloretic acid under alkaline conditions. In some experiments phloroglucinol formation was determined similarly; the corresponding band on the cellulose plate was removed and the amount of phloroglucinol was determined by the

method of Jayasankar & Bhat (1966). (b) Hydrolytic activity on 3'-methylphloracetophenone was assayed by measuring methylphloroglucinol formation. Methylphloroglucinol was determined by a slightly modified version of the micro-method for phloroglucinol determination (Jayasankar & Bhat, 1966). A 2 ml volume of 100% (w/v) trichloroacetic acid solution was added to 1 ml of reaction mixture, followed by the addition of 2 ml of 2% *p*-dimethylaminobenzaldehyde solution in acetic acid. After exactly 1 min, the E_{544} value of the coloured solution was measured against the reagent blank in a Beckman DU spectrophotometer (model 2400). Under these conditions the substrate, 3'-methylphloracetophenone, does not react with *p*-dimethylaminobenzaldehyde and does not show any significant influence on the colour development of methylphloroglucinol. (c) For experiments designed to study the possibility of hydrolytic activity on various compounds other than the above two substrates, the reactions were carried out under the usual conditions with substrates at a concentration of 1 mM. Concentrations of the enzyme were varied according to the susceptibility of the compounds to the activity in the range 0.13–0.65 unit. The reaction with phloretin as the substrate was always run simultaneously as a standard. Where necessary, the substrate was dissolved in water with the aid of a minimum amount of ethanol before being added to the reaction mixture. After the reaction had been stopped, the mixture was diluted with 50% ethanol and its absorption spectrum was compared with that of a control with enzyme added after reaction. If any consumption of the substrate was observed spectrophotometrically, a concentrated ethyl acetate extract from the acidified reaction mixture was subjected to t.l.c. on cellulose to detect the products formed. Formic acid (2%, v/v) or benzene-methanol-acetic acid (45:8:4, by vol.) was used as the solvent. The spots on the chromatograms were detected by spraying with diazotized *p*-nitroaniline-NaOH reagent (Bray, Thorpe & White, 1950) as well as by observation under u.v. light. For quantitative determination of the hydrolysis rates of the compounds that had been found to be successful as substrates, the bands corresponding to either of the products were removed from the cellulose plate and analysed in a similar way to the phloretic acid determination described above. When the aliphatic ketones were tested as potential substrates, the possible disappearance of the ketones was measured by the following methods: α -oxoglutaric acid and pyruvic acid (Friedemann, 1957), acetone (Lehninger, 1957) and dihydroxyacetone (Burton, 1957).

A unit of enzyme activity is defined as the hydrolysis of 1 μ mol of phloretin/h under the standard conditions given above. Specific activity was expressed in units/mg of protein. Protein concentration was determined as described by Lowry, Rosebrough, Farr & Randall (1951), with dry crystalline bovine serum albumin as standard.

RESULTS

Inducibility of enzyme. When grown on synthetic medium in the absence of phlorrhizin, buffer extracts from acetone-dried powders of *A. niger* showed no hydrolytic activity on phloretin. When grown in media containing any one of phlorrhizin, phloretin, phloracetophenone or methylphloracetophenone, to a final concentration of 2 mM, however, enzyme formation was induced.

Purification. The purification procedures for a preparation are summarized in Table 1. The elution profile of the protein through a DEAE-cellulose column is presented in Fig. 1. In three series of experiments, final enzyme preparations having specific activities of 2.5, 3.6 and 3.8 units/mg of protein were obtained with 32–45% recovery over the crude extracts. The enzyme preparations were reasonably stable during storage. When kept at 3°C for 24 h at a concentration of 0.1 mg of protein/ml of 0.01 M-potassium phosphate buffer, pH 7.0, the enzyme showed 22% loss of activity. When the diluted solution was frozen at –20°C for 4 weeks, 20% of the activity was lost. When freezing (–20°C) and thawing (5 min at 30°C) were repeated five times in 3 h, 10% loss of activity was observed. However, when the solution was maintained at 45°C for 15 min, the activity fell by 50%.

Homogeneity of enzyme. The eluate from the DEAE-cellulose column was resolved into five components by electrophoresis in tris-glycine buffer, pH 8.3. The major band (mobility 0.60–0.66 with respect to Bromophenol Blue) contained the enzyme activity. This ran as a single component with identical mobility when electrophoresis was repeated under identical conditions. The enzyme did not migrate to the cathode when subjected to electrophoresis at pH 4.5 (Reisfeld, Lewis & Williams, 1962). This highly purified preparation

Table 1. *Purification of the enzyme from Aspergillus niger that hydrolyses phloretin and related C-acylated phenols*

Step		Total volume (ml)	Total activity (units)	Specific activity (units/mg of protein)
1.	Crude extract from acetone-dried powder	271	129	0.114
2.	Protamine sulphate-treated supernatant solution	294	119	0.112
3.	Dialysis residue of 35–55%-satd.-(NH ₄) ₂ SO ₄ fraction	20.5	91	0.32
4.	Fraction not adsorbed on tricalcium phosphate gel	20	81	0.57
5.	Eluate from DEAE-cellulose column	22	58	3.6

Fig. 1. Elution pattern from a DEAE-cellulose column of the hydrolase preparation obtained by treatment of tricalcium phosphate gel. Elution was achieved by the addition of 0.02M-potassium phosphate buffer, pH 7.0, containing 0.1mM-EDTA and 0.5mM-GSH, followed by the stepwise addition of 0.05M- and 0.10M-KCl dissolved in the same buffer. Each fraction contained 5.5ml. Fractions 33-36 were pooled., Enzyme activity; —, E_{280} .

catalysed the hydrolyses of phloretin and methylphloracetophenone.

The DNS derivatives of glycine, alanine and lysine (ϵ -DNS-lysine) were the only DNS-amino acids present after 'dansylation' of native and performic acid-oxidized enzyme. DNS-glycine and DNS-alanine were present in approximately equal amounts. The yield from performic acid-oxidized enzyme was much greater.

Enzyme properties. The hydrolytic rate of phloretin was shown to be proportional to the enzyme concentration over a fourfold concentration range and to deviate from linearity only at high protein concentration because of the considerable depletion of the substrate. When the reaction proceeded to completion, 94% of the phloretin was consumed. With an appropriate enzyme concentration, the reaction rate was shown to be constant for at least 2h. The pH-activity curve indicated that the maximum hydrolytic activity on phloretin occurred at about pH 9.6 (Fig. 2), although the characteristics of the enzyme were studied at pH 7.2 because of the lability of the product, phloroglucinol, under alkaline conditions. Apparently the maximum rate of hydrolysis of 3'-methylphloracetophenone occurred at about pH 7.2. When the reaction was carried out under nitrogen gas the pH optimum was shown to be 7.6. The decline in activity on the alkaline side, however, is due in part to the extreme lability of the product, methylphloroglucinol, in alkaline solutions, and it was impossible to determine the optimum pH for activity under normal reaction conditions. The apparent K_m value for 3'-methylphloracetophenone at pH 7.2, determined from a Lineweaver-Burk plot, was 0.15mM. An accurate determination of the K_m value for phloretin was not possible because of the difficulties in measuring the small quantity

Fig. 2. Effect of pH on the hydrolysis of phloretin and 3-methylphloracetophenone. The buffer solutions used were (final concns.): $\circ$, 0.05M-citric acid-0.1M- KH_2PO_4 between pH 4.6 and 8.0; $\bullet$, 0.1M-diethanolamine-HCl between pH 8.0 and 10.0; $\square$, 0.1M-sodium carbonate-bicarbonate between pH 9.0 and 10.8. For the hydrolysis of phloretin, each reaction mixture (1.0ml) consisted of 1.0mM-phloretin, buffer solution of the pH indicated and enzyme solution (3.6 units/mg of protein) containing 72.4 μg of protein for pH values between 4.6 and 8.0, or 14.5 μg of protein for the pH range above 8.4. Phloretic acid formed was extracted with ether from the acidified reaction mixture and determined as described in the Materials and Methods section. For the hydrolysis of 3-methylphloracetophenone, the reaction mixture (1.0ml) containing 1.0mM-3-methylphloracetophenone and the buffer solution was incubated with enzyme solution (0.19 unit) (3.8 units/mg of protein). Methylphloroglucinol formed was determined by colorimetry after treatment with *p*-dimethylaminobenzaldehyde. For the hydrolysis under N_2 gas, the reaction mixture (2.0ml) consisting of 1.0mM-methylphloracetophenone and the buffer solution was incubated with enzyme solution (0.38 unit) in a Thunberg tube in which the air had been replaced by N_2 gas. The reaction was initiated by adding 0.5ml of 0.4mM-3-methylphloracetophenone solution from the side arm to the main tube containing the enzyme and buffer solution. After incubation for 30min the tube was put into acetone at -20°C to terminate the reaction, the cap removed, and 0.3ml of 1.5M-HCl was added to the frozen mixture. A 1ml portion of the reaction mixture was used for the determination of methylphloroglucinol. The results are expressed as μmol of the product formed/h per 10 μg of protein. —, In N_2 gas; ----, in air.

of phloroglucinol formed, but the value was estimated to be about 0.3-0.4mM. There was stoichiometry between disappearance of the substrate and formation of the products. A scaled-up experiment showed a consumption of 9.1 μmol of phloretin and the formation of 11.1 μmol of phloroglucinol and 9.8 μmol of phloretic acid. In another

Table 2. *Effect of metal ions on the rate of phloretin hydrolysis*

The reaction mixture consisted of 1.0mM-phloretin, 0.1M-tris-HCl buffer, pH 7.2, enzyme solution (0.26 unit) and the metal ions in a total volume of 1.0ml. The enzyme was preincubated for 6min with the metal ion before the addition of the substrate. The reaction was allowed to proceed in the presence of metal ion under standard conditions, except that tris-HCl buffer was used because of the insolubility of some metals in phosphate buffer. Consumption of phloretin was determined by measurement of E_{287} after extraction into ethyl acetate.

Metal salt added	Final concn. (mM)	Relative activity (%)
No addition	—	100
HgCl ₂	0.02	6
	0.005	15
AgNO ₃	0.02	21
	0.005	100
CdSO ₄	1.0	37
	0.2	80
CoSO ₄	1.0	86
CuCl ₂	1.0	21
ZnCl ₂	1.0	37
	0.2	63
NiSO ₄	1.0	89
MgCl ₂	1.0	94
MnCl ₂	1.0	103
FeSO ₄	1.0	94
FeCl ₃	1.0	63
Al ₂ (SO ₄) ₃	1.0	3
	0.2	75
Na ₂ MoO ₄	1.0	21
Na ₂ WO ₄	1.0	69

experiment the conversion of 0.43 μ mol of 3'-methylphloracetophenone corresponded to the formation of 0.47 μ mol of methylphloroglucinol.

Effect of various substances on the reaction. The effect of various metal ions on the rate of phloretin hydrolysis is presented in Table 2. Of the metal ions tested, Ag⁺ and Hg²⁺ at low concentrations strongly inhibited the reaction. Al³⁺ at 1mM concentration also showed a potent inhibition. Addition of Al³⁺ to an aqueous solution of phloretin produced a shift in the u.v. spectrum (from 288nm to 300nm for maximum absorption), indicating complex-formation between phloretin and the ion (Harborne, 1964). This complex may not be a substrate for the enzyme, and this would account for the apparent inhibition. All of the other metal ions tested, except Mg²⁺, Ca²⁺ and Mn²⁺, inhibited the reaction to some extent. As shown in Table 3, metal-chelating agents such as *o*-phenanthroline, diethyldithiocarbamate and $\alpha\alpha'$ -bipyridyl were ineffective. The reaction was very susceptible to *p*-chloromercuribenzoate, and this inhibition was

Table 3. *Effect of thiol inhibitors and metal-chelating agents on the rate of phloretin hydrolysis*

Each reaction mixture consisted of 1.0mM-phloretin, 0.1M-potassium phosphate buffer, pH 7.2, enzyme solution containing 72 μ g of protein and the test compound(s) in a total volume of 1.0ml. The enzyme was preincubated for 5min with the test compounds before the addition of the substrate. Consumption of phloretin was determined by measurement of E_{287} after extraction into ethyl acetate.

Compound added	Final concn. (mM)	Relative activity (%)
No addition	—	100
<i>p</i> -Chloromercuribenzoate	0.01	79
	0.05	42
Sodium arsenite	1.0	97
Iodoacetate	0.5	95
GSH	1.0	97
L-Cysteine	0.5	99
2,3-Dimercaptopropanol	0.5	93
2-Mercaptoethanol	0.5	95
<i>p</i> -Chloromercuribenzoate + GSH	0.05	90
<i>p</i> -Chloromercuribenzoate + L-cysteine	0.05	85
<i>o</i> -Phenanthroline	0.5	92
Diethyldithiocarbamate	0.5	97
$\alpha\alpha'$ -Bipyridyl	0.5	102
EDTA	1.0	91

partially reversed by the addition of GSH or L-cysteine to the reaction system. Arsenite and iodoacetate had no effect on the reaction. So far, no cofactor or activator for the reaction has been found.

Substrate specificity. The purified enzyme preparations were examined against a wide variety of compounds, of which 13 appeared to be hydrolysed, as listed in Table 4. Of the dihydrochalcones examined, 2',4,4'-trihydroxydihydrochalcone was hydrolysed more efficiently than phloretin. Phlorrhizin was shown to be as good a substrate as phloretin. The enzyme was also able to act on the other dihydrochalcones having a phloroglucinol nucleus in ring A. 2',6'-Dihydroxy-4,4'-dimethoxy-, 2',3,4,4',6'-pentahydroxy- and 2',4'-dihydroxy-4-methoxy-dihydrochalcones were hydrolysed very slowly. Phlorrhizin tetra-acetate was not a substrate. Of the substituted acetophenones and propiophenones, 3'-methylphloracetophenone and phloracetophenone were found to be good substrates, and the former compound was the most susceptible substrate of all compounds tested in this study. The enzyme hydrolysed 2',4'-dihydroxypropiophenone and 2',6'- and 2',4'-dihydroxyacetophenones at low rates. It was assumed, by the sizes of resorcinol spots revealed on chromatograms with diazotized *p*-nitroaniline reagent, that

Table 4. *Hydrolytic activity on different dihydrochalcones, acetophenones and propiophenones*

The reaction mixture contained 1.0mM substrate indicated, 0.1M-potassium phosphate buffer, pH7.2, and a suitable dilution of the purified enzyme preparation. Amounts of enzyme were varied in the range 0.13–0.65 unit according to the susceptibility of the substrate. The reaction proceeded under the standard conditions. After termination of the reaction, the acidified mixture was extracted with ether, and the ethereal extract was subjected to t.l.c. on cellulose. Bands corresponding to the products were eluted with 80% ethanol. Phloretic acid in alkaline 80% ethanol was determined by measuring E_{243} . Resorcinol in 80% ethanol was determined by measuring $E_{277.5}$. Phloroglucinol and methylphloroglucinol in aqueous extract were assayed by colorimetry after treatment with *p*-dimethylaminobenzaldehyde. When phlorrhizin was used as a substrate, the reaction mixture was treated with *m*-HCl at 100°C for 30 min before ether extraction in order to hydrolyse phloroglucinol β -D-glucoside formed, because the glucoside has a similar R_F value to that of phloretic acid on cellulose t.l.c. plates when developed in 2% formic acid as a solvent. Hydrolytic activity on each substrate is expressed as a relative reaction rate on the basis of activity on phloretin. The experiments were carried out in duplicate, and mean values are presented. +, Although the product formed was not quantitatively determined, the relative activity was judged to be less than one-tenth of the activity on phloretin on the basis of the colour reaction of spots on chromatograms.

Substrate tested	Relative reaction rate	Product determined or identified
2',4,4',6'-Tetrahydroxydihydrochalcone (phloretin)	1.0	Phloretic acid or phloroglucinol
2',4,4'-Trihydroxydihydrochalcone	1.7	Phloretic acid
2',4,4',6'-Tetrahydroxydihydrochalcone 2'- β -glucoside (phlorrhizin)	1.1	Phloretic acid
2',3,4',6'-Tetrahydroxy-4-methoxydihydrochalcone	0.8	Phloroglucinol
2',3,4',6'-Tetrahydroxydihydrochalcone	0.4	Phloroglucinol
2',3,4,4',6'-Pentahydroxydihydrochalcone	0.1	Phloroglucinol
2',4'-Dihydroxy-4-methoxydihydrochalcone	+	Resorcinol
2',6'-Dihydroxy-4,4'-dimethoxydihydrochalcone	+	3-Methoxyresorcinol
2',3,4,4',6'-Pentahydroxydihydrochalcone 2'- β -glucoside	+	Phloroglucinol β -glucoside
2',2,4',6'-Tetrahydroxydihydrochalcone 4'- β -neohesperidoside	-	
2',4,4',6'-Tetrahydroxydihydrochalcone 4'- β -neohesperidoside	-	
2',3,4',6'-Tetrahydroxydihydrochalcone 4'- β -neohesperidoside	-	
2',3,4',6'-Tetrahydroxy-4-methoxydihydrochalcone 4'- β -neohesperidoside	-	
2',3,4',6'-Tetrahydroxy-4-ethoxydihydrochalcone 4'- β -neohesperidoside	-	
2',3,4',6'-Tetrahydroxy-4- <i>n</i> -propoxydihydrochalcone 4- β -neohesperidoside	-	
Phlorrhizin tetra-acetate	-	
2',4',6'-Trihydroxy-3'-methylacetophenone (3'-methylphloracetophenone)	2.3	Methylphloroglucinol
2',4',6'-Trihydroxyacetophenone	1.1	Phloroglucinol
2',4'-Dihydroxyacetophenone	+	Resorcinol
2',6'-Dihydroxyacetophenone	+	Resorcinol
2'-Hydroxyacetophenone	-	
4'-Hydroxyacetophenone	-	
3'-Methoxyacetophenone	-	
2'-Hydroxy-4'-methoxyacetophenone	-	
Phloracetophenone 4'- β -neohesperidoside	-	
2',4'-Dihydroxypropiophenone	+	Resorcinol
4'-Hydroxypropiophenone	-	
3',4'-Dihydroxypropiophenone	-	
3',4'-Dimethoxypropiophenone	-	

the hydrolytic rates of these three phenyl ketones, in decreasing order, were: 2',4'-dihydroxypropiophenone, 2',6'-dihydroxyacetophenone, 2',4'-dihydroxyacetophenone. There was essentially no activity on 2'-hydroxy-, 4'-hydroxy-, 3'-methoxy- and 2'-hydroxy-4'-methoxy-acetophenones, or 4'-hydroxy-, 3',4'-dihydroxy- and 3',4'-dimethoxy-

propiophenones. The enzyme did not act on phloracetophenone 4'- β -neohesperidoside, or 2',4',6'-trihydroxydihydrochalcone 4'- β -neohesperidoside derivatives having 2-hydroxy-, 3-hydroxy-, 4-hydroxy, 3-hydroxy-4-methoxy, 3-hydroxy-4-*n*-propoxy or 3-hydroxy-4-ethoxy substituents.

In addition to the above-mentioned compounds, it was shown that the following hydroxyphenyl ketones are not substrates: 2'-hydroxy-, 4-hydroxy-, 2',4-dihydroxy-, 2',4'-dihydroxy-, 2',4,4'-trihydroxy-, 2',4,5'-trihydroxy-, 2',3,4,4'-tetrahydroxy-, 2',4-dihydroxy-4'-methoxy-, 2'-hydroxy-4,4'-dimethoxy- and 2',4,4'-trihydroxy-3-methoxy-chalcones, 3,3',4',5,7-pentahydroxyflavone (quercetin), 3,3',4',5-tetrahydroxy-7-methoxyflavone (rhamnetin), 5,7-dihydroxyflavone (chrysin), 5,7-dihydroxy-4'-methoxyflavone (acacetin), 4',5,7-trihydroxyflavanone (naringenin), naringenin 7-neohesperidoside (naringin), 4',5,7-trihydroxy-3-methoxyflavanone, 6-hydroxy- and 4,6-dihydroxycoumaranones, and methyl vanilloyl ketone. The inactivity on the chalcones might be partially due to their poor solubility in aqueous solution. Moreover, no activity was observed towards the following aliphatic ketones or quinones: α -oxoglutaric acid, pyruvic acid, acetone, dihydroxyacetone, *p*-hydroxyphenylpyruvic acid, and 2-hydroxy- and 2-methoxy-1,4-naphthaquinones.

The enzyme preparations did not show hydrolytic activity with the methyl esters of caffeic acid, *p*-hydroxybenzoic acid, sinapic acid, acetanilide, acetophenetidin, 2,5-, 3,5- and 2,4-dihydroxybenzoic acids or 2,5- and 3,4-dihydroxybenzaldehydes.

Phlorrhizin is a major phenolic compound of *Malus* species and its oxidative transformation has been demonstrated by using cell-free preparations of apple leaves (Noveroske, Kuc & Williams, 1964; Raa & Overeem, 1968). Grochowska (1967) has reported that crude extracts from the sap of apple spurs can hydrolyse phlorrhizin and phloretin to phloroglucinol and phloretic acid. We have applied the same procedures as with *A. niger* to obtain active extracts from young leaves of a variety of *Malus* species, but so far have been unsuccessful.

DISCUSSION

Previous work in our laboratory (Jayasankar *et al.* 1969) has shown that 37 out of 60 species of fungi examined, such as *Aspergillus* and *Penicillium*, attack phlorrhizin to produce phloretin, phloroglucinol and/or phloretic acid, and has also provided the first evidence for the solubilization of the phloretin-hydrolysing enzyme from a micro-organism. As shown by the present results, this hydrolytic enzyme is produced by *A. niger* when phlorrhizin is added to the growth medium. Experiments with a wide range of compounds and potential substrates have shown that the purified enzyme from this source is a new hydrolytic enzyme acting on carbon-carbon bonds in ketonic substrates. It appears to be specific in the hydrolysis of phloretin and related hydroxyphenyl ketones and

does not act on aliphatic ketones or quinones. The substrate specificity of this enzyme may be summarized as follows.

A hydroxyl substitution of the benzene nucleus at the position *ortho* to a ketone group is essential for the enzymic activity. Further, an additional hydroxyl group at the position *para* to the ketone group also appeared to be necessary for activity, although 2',6'-dihydroxyacetophenone was hydrolysed at a very low rate. If the free *p*-hydroxyl group is replaced by a glycosyloxy or methoxyl group, there is no activity. Therefore both hydroxyl groups on the benzene ring (ring A of dihydrochalcone) would appear to be involved in the formation of the enzyme-substrate complex. Addition of a third hydroxyl group to give the phloroglucinol configuration of hydroxyl groups is not an essential factor for the activity, but affects the reaction rate. 2',4,4'-Trihydroxydihydrochalcone, which differs from phloretin only in that the 6'-hydroxyl group is missing, is a more susceptible substrate than phloretin, and phlorrhizin, whose 6'-hydroxyl group is masked, is as good a substrate as phloretin. On the other hand, phloracetophenone was found to be better than 2',4'-dihydroxyacetophenone or 2',4'-dihydroxypropiofenone. It seems that the extent of hydrogen-bond formation between the carbonyl group and a vicinal hydroxyl group in each substrate may influence the reaction rate. The inactivity of naringenin, the corresponding flavanone of phloretin, may be partly due to stable intramolecular hydrogen-bonding. The ability of Al^{3+} to form a complex with phloretin through the ketone and *o*-hydroxyl groups and the potent inhibitory action of Al^{3+} on the activity support this view. Too little is known about the influence on the hydrolytic activity of other substitutions on the benzene ring of phenylketones, although it should be noted that 3'-methylphloracetophenone was hydrolysed at twice the rate of phloracetophenone.

The limited data suggest that the enzyme is less specific with regard to the nature of the other group attached to the carbonyl carbon atom. Phloracetophenone is as good a substrate as phloretin, but 2',4'-dihydroxyacetophenone is much poorer than the corresponding dihydrochalcone, 2',4'-dihydroxydihydrochalcone. Of a series of dihydrochalcones having a phloroglucinol nucleus as ring A, phloretin is the best substrate, and the hydrolytic rates are considerably influenced by the other substituents on ring B. In particular, 2',3,4,4',6'-pentahydroxydihydrochalcone with an additional hydroxyl group giving an *o*-diphenolic substitution in ring B is hydrolysed slowly in comparison with phloretin; this is also the case with their 4'- β -glucosides. The relative insolubility in buffered solutions of some of the substrates tested is probably

a significant factor in determining the extent of their hydrolysis.

No cofactor or activator for the hydrolytic reaction has been found so far. The experimental results with metal-chelating agents do not provide any evidence for the participation of Fe^{3+} or other metal ions in the activity. Most metal ions tested appeared to be inhibitory, and Mg^{2+} , Mn^{2+} and Ca^{2+} had no effect on the activity, whereas oxaloacetate acetylhydrolase (EC 3.7.1.1) from a strain of *A. niger*, one of the few known hydrolytic enzymes acting on carbon-carbon bonds in ketonic substances, is known to require Mn^{2+} for maximum activity (Hayaishi, Shimazono, Katagiri & Saito, 1956). We propose the systematic name C-acylphenol acylhydrolase for the enzyme. This enzyme belongs to EC group 3.7.1.

The enzyme appears to be homogeneous by polyacrylamide-gel electrophoresis at pH 8.3. The presence of two N-termini, glycine and alanine, indicate that enzymic activity resides either in a single protein with two polypeptide chains or in two separate proteins.

We thank the National Research Council of Canada for generous financial support. We are grateful also for the samples of dihydrochalcones and other phenolic compounds received from Professor T. A. Geissman, Professor J. P. Kutney and Dr L. Krbecek. The technical assistance of Mrs A. M. Elliott and Mr A. D. Mitchell is gratefully acknowledged.

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