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# Metabolism of Phloridzin by *Erwinia herbicola*: Nature of the Degradation Products, and the Purification and Properties of Phloretin Hydrolase

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*Erwinia herbicola* Y46 degrades phloridzin to yield phloretin, phloroglucinol, and phloretic acid, when grown on defined medium containing phloridzin as the sole source of carbon. The identities of the intermediates isolated from culture filtrates were established by co-chromatography and by ultraviolet absorption spectra. Only 3 of 11 strains of this species, and none of the 12 species of bacterial phytopathogens tested could effect this breakdown. Some of the latter organisms possessed β-glucosidase activity which liberated D-glucose from phloridzin. The enzyme phloretin hydrolase was purified from cells of *E. herbicola* Y46 grown on Yeast Beef Broth, by treatment of crude extracts with protamine sulfate, ammonium sulfate precipitation, elution from calcium phosphate gel, elution from diethylaminoethyl-cellulose, and concentration by ultrafiltration. The final preparation was free of β-glucosidase, had a specific activity of 213 units per mg of protein, and represented a 142-fold purification over the crude extract. The enzyme had a *p*H optimum of 6.7 to 6.8, and produced only phloroglucinol and phloretic acid as products of phloretin breakdown, there being an equimolar relationship between the cleavage of phloretin and the formation of the products. The Michaelis constant ($K_m$) for the enzyme with phloretin as substrate was $3.8 \times 10^{-5}$ M, and the enzyme was sensitive to $Hg^{2+}$ and $Cu^{2+}$ ions. Phloroglucinol, phloretic acid, *p*-chloromercuribenzoate and iodoacetamide were without effect on the activity. The enzyme did not react with phloridzin, naringin, or naringenin. The physiological significance of the results is discussed.

Yellow saprophytic bacteria are known to be associated with the lesions of the "fire-blight" disease of applies and pears (1, 5, 11; A. T. Hendry, M.Sc. Thesis, Univ. of Guelph, Ontario, Canada, 1966). There has been conjecture as to the possible physiological interactions of the "fire-blight" pathogen, *Erwinia amylovora* (Burrill) Winslow et al., the host plant, and the yellow saprophytes (6, 8). The yellow bacteria which were isolated from the diseased apple and pear trees, which have been identified as *E. herbicola* (Dugelli) Dye (A. K. Chatterjee, M.Sc. Thesis, Univ. of Guelph, 1968), possess β-glucosidase activity which will, in vitro, cleave arbutin, the principle β-glucoside of pear trees, and *p*-nitrophenyl-β-D-glucoside (*p*-NPG). *E. amylovora* has only feeble β-glucosidase activity.

All 13 strains of *E. herbicola* tested in the current study exhibited β-glucosidase activity toward arbutin when grown on arbutin broth for 24 to 48 hr, but only 5 possessed sufficient activity to be detected in crude extracts by the quantitative assay described below. Of these five, three degraded phloridzin [4,6-dihydroxy-2-β-D-glucosido-(*p*-hydroxyphenyl)propiophenone], the predominant β-glucoside of apple trees, including strain Y46, the principle organism used in the present study. As the hydrolysis of arbutin has been invoked as a possible defense mechanism of the pear tree in response to tissue damage and the occurrence of the opportunity for microbial invasion of the plant via the lesion (12, 14), it was of interest to determine the possible contribution that the saprophytic *E. herbicola* may be making towards the resistance of the diseased plant to the pathogenic *E. amylovora*. Since the aglycone of arbutin, namely hydroquinone, was implicated as an antibiotic against *E. amylovora* in damaged pear tree tissue (14), it became of significance to determine

whether an analogous situation obtained in the apple tree. Arbutin does not occur in the apple tree, the principle β-glucoside being phloridzin (28). Preliminary experiments in vitro in this laboratory indicated that the products of phloridzin degradation by *E. herbicola* are inhibitory to the growth of *E. amylovora*. To investigate this inhibition and to assess the possible significance in the association between *E. herbicola* and *E. amylovora* in the "fire-blight" syndrome, it was necessary to determine the manner in which phloridzin was degraded by *E. herbicola* and to identify the intermediate compounds involved in the metabolic sequence. This paper describes the metabolic sequence and reports on the enzyme activities involved in its execution. The purification and properties of phloretin (*p*-hydroxyphenyl-2,4,6-trihydroxypropiophenone) hydrolase are reported for the first time, and the distribution of the enzymes β-glucosidase and phloretin hydrolase among bacterial phytopathogens is discussed. A preliminary report of some of this work has been presented (A. K. Chatterjee and L. N. Gibbins, Proc. Can. Soc. Microbiol., June 1969).

## MATERIALS AND METHODS

**Organisms and cultural conditions.** *E. herbicola* strain Y46, which had been isolated from apple trees and characterized by numerical taxonomy (A. K. Chatterjee, M.Sc. Thesis, Univ. of Guelph, 1968), was selected because it was representative of the species and had the capacity to degrade phloridzin. This and other isolates referred to in this paper are deposited in the Culture Collection of the Department of Microbiology, University of Guelph. The organisms were maintained on Yeast Beef Agar (Difco), incubated at 30 C, and transferred routinely at intervals of 4 weeks.

For the production of intermediates in the degradation of phloridzin, *E. herbicola* was grown on a liquid medium containing: phloridzin, 0.5%; $NH_4H_2PO_4$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.02%; KCl, 0.02%; final *p*H 7.2. Cells grown with D-glucose as the sole source of carbon and energy were harvested from the above medium in which phloridzin had been replaced by 0.1% D-glucose. For the production of larger quantities of cells for enzyme purification purposes, the organism was grown on Yeast Beef Broth (Difco). Medium was distributed either in 100-ml amounts in 500-ml Erlenmeyer flasks or in 300-ml amounts in 1-liter flasks. The incubation temperature was 30 C, and the cultures were agitated on reciprocal shakers throughout the growth period, which varied from 16 to 72 hr, depending on the purpose for which the cells were being grown.

**Sources of materials.** Phloridzin and phloretin were obtained from J. T. Baker Chemical Co., Phillipsburg, N.Y., and a second batch of phloridzin was obtained from the Sigma Chemical Co., St. Louis, Mo. Phloroglucinol (1,3,5-trihydroxybenzene), arbutin, naringin, naringenin, *p*-NPG, protamine sulfate, and diethylaminoethyl (DEAE)-cellulose were purchased from Sigma. Ethylenediaminetetraacetic acid (EDTA) was supplied by the British Drug Houses (Canada) Ltd., Toronto. Phloretic acid (*p*-hydroxyphenylpropionic acid), *p*-chloromercuribenzoate (*p*-CMB), and iodoacetamide were supplied by the Aldrich Chemical Co., Milwaukee, Wis. Crystalline bovine serum albumin was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. All the phenolic compounds were tested for purity by thin-layer chromatography in two solvent systems. Phloridzin was recrystallized from water as the dihydrate for analytical purposes. Naringin was recrystallized from water and naringenin from 25% ethyl alcohol twice to remove contaminants. All other reagents and solvents were of analytical reagent or equivalent grade. Deionized, glass-distilled water was used throughout this work.

**Analytical methods.** Phloroglucinol was determined by the method of Zaprometov (29), as modified by Chatterjee and Gibbins (Anal. Biochem., *in press*). Protein was determined by the method of Lowry et al. (20), or Groves, Davies, and Sells (9). Bovine serum albumin was used as the standard in both methods. The two procedures agreed well in the determination of protein derived from *E. herbicola*, except that in crude extracts of this organism the spectrophotometric method (9) gave values approximately double those of the Lowry method. Hydroquinone was determined by the method of Barnett, Ingram, and Swain (3), modified in that the phloroglucinol reagent was reduced from 0.5 to 0.1%, and the incubation time for the chromogenic reaction was reduced to 15 min. D-Glucose was assayed by the method of Nelson (22).

**Thin-layer chromatography.** Phenolic compounds were chromatographed on 0.25-mm layers of silica gel (Absorbosil 5; Applied Scientific Laboratories, Inc., State College, Pa.). The plates were activated before use by heating at 100 C for 30 min. Four solvent systems were used: (A) chloroform-acetic acid-water (75:10:15, v/v), modified according to Van Sumere et al. (27); (B) chloroform-ethyl acetate-formic acid (50:40:10, v/v; *see* reference 25); (C) toluene-ethyl formate-formic acid (50:40:10, v/v; *see* reference 27); and (D) methanol-benzene (30:70, v/v; *see* reference 4). Solvent A resolved phloretic acid from mixtures of phloridzin, phloretin, phloroglucinol, and phloretic acid. Solvent B resolved phloretin, phloroglucinol, and phloridzin. The phenolic compounds were located on the plates by using the sulfanilic acid spray reagent (24), or by irradiating the plates with ultraviolet light.

**Isolation of phenolic degradation products of phloridzin.** *E. herbicola* Y46 was grown on the defined phloridzin medium described above for 72 hr, at which time optimal yield of intermediates was obtained. The cells were removed by centrifugation for 10 min at 12,100 × *g* and 5 C in a Sorvall RC2 refrigerated centrifuge. The supernatant fluid (1,000 ml) was concentrated in a flash evaporator at 37 C to approximately 100 ml, and the concentrate was extracted successively with six portions, each of

approximately 150 ml, of diethyl ether. The extracts were pooled and evaporated to dryness under reduced pressure at laboratory temperature. The residue was dissolved in the minimal volume of methanol (approximately 2.0 ml). This solution was applied as a band at the origin of a number of thin-layer plates and irrigated with solvent A. The areas of the gel containing the phenolic compounds were scraped from the plates and eluted exhaustively with methanol; the methanolic solutions were evaporated in vacuo to crystallize the solute. The material remaining at the origin was eluted in similar fashion, concentrated, and applied as a band to a second series of plates, which were then irrigated with solvent B. The resultant phenolic zones were eluted and crystallized as before. The samples were subjected to co-chromatography with authentic samples of the suspected intermediates, in solvents B and C for phloretin and phloroglucinol, and solvents A and D for phloretic acid. Ultraviolet absorption spectra were measured with a spectrophotometer (Unicam SP800). Solutions of the sample and reference compounds were prepared in methanol at a concentration of 5 mg/100 ml. Silica cells, of optical pathlength 1.0 cm, were used throughout. To trace the spectra in the region 200 to 250 nm, the solutions were diluted 10-fold with methanol.

**Enzyme assay systems.** β-Glucosidase was assayed by using either *p*-NPG, arbutin, or phloridzin as substrate in 0.05 M potassium phosphate buffer, *p*H 6.5. In the case of *p*-NPG, the reaction mixture consisted of 0.1 ml of 0.01 M *p*-NPG, 0.8 ml of 0.05 M potassium phosphate buffer (*p*H 6.5), and 0.1 ml of enzyme preparation. The mixture was incubated at 30 C for 10 min, and the reaction was terminated by the addition of 4.0 ml of 1.0 M $Na_2CO_3$. The intensity of the yellow color produced was measured at 400 nm, with a Spectronic 20 spectrophotometer (Bausch & Lomb). When arbutin or phloridzin was used as substrate, the mixture consisted of 1.0 ml of 0.01 M substrate, 1.8 ml of 0.05 M potassium phosphate buffer (*p*H 6.5), and 0.2 ml of enzyme preparation. The digests were incubated as before, and the enzyme reaction was terminated by the addition of 0.5 ml of 1.0 M $Na_2CO_3$. The liberated hydroquinone and D-glucose, from arbutin and phloridzin, respectively, were assayed by the analytical methods cited above.

Two methods were used to determine phloretin hydrolase activity. In the first, the phloroglucinol released from phloretin was assayed as described previously (A. K. Chatterjee and L. N. Gibbins, Anal. Biochem., *in press*), the unit of activity being defined as the amount of enzyme causing the formation of 1 μmole of phloroglucinol in 1 min under the conditions of the assay. In the second method, the disappearance of phloretin from the assay mixture was followed by monitoring the decrease in absorbancy at 300 nm, at which wavelength neither phloroglucinol nor phloretic acid absorbs. The assay mixture consisted of 1.0 ml of freshly prepared 0.24 mM phloretin dissolved in 1.0 mM NaOH, 1.9 ml of 0.05 M potassium phosphate buffer (*p*H 6.8), and 0.1 ml of enzyme preparation. The assays were run in a visible and ultraviolet recording spectrophotometer (Unicam SP 800), fitted with a temperature-controlled cell housing operating at 30 C. The molar extinction coefficient for phloretin at 300 nm was found to be $5.8 \times 10^5$ liters per mole per cm, and activities were recorded as units (micromoles of phloretin utilized per minute under the conditions of the assay). Specific activity was defined as units per milligram of protein. For investigation of the effect of various adjuncts to the assay system, they were first dissolved in the assay buffer and then added to the cuvette in the appropriate amounts. In the case of *p*-CMB and iodoacetamide, the enzyme preparation was preincubated with the reagent for 10 min at 4 C prior to dilution for assay purposes. In all cases, the reaction was started by the addition of enzyme to the mixture. Boiled enzyme controls were carried out for all assays.

**Purification of phloretin hydrolase.** Although the presence of phloridzin in the growth medium resulted in increased specific activity of phloretin hydrolase (*unpublished data*), the amount of growth and the total yield of enzyme per unit volume of medium was reduced. The initial purification of the enzyme was therefore made from cells grown in the absence of phloridzin. Unless otherwise stated, all operations were carried out at 0 to 4 C, and within the *p*H range 6.7 to 7.5. Cells of *E. herbicola* Y46 were grown in shake culture in 1-liter Erlenmeyer flasks containing 300 ml of Yeast Beef Broth each, at 30 C for 22 hr. Cells from 20 flasks were harvested with a Sharples continuous centrifuge at laboratory temperature, and washed twice with 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer, *p*H 7.0, by centrifugation. After resuspension in approximately 200 ml of the same buffer, they were subjected in 50-ml portions to ultrasonic disruption in a magnetorestrictive oscillator (Raytheon) operating at 9 kc for 15 min. The disrupted cells were centrifuged in a refrigerated centrifuge (Sorvall RC2) at 12,100 × *g* for 20 min, and the resulting supernatant fluid was used as the crude extract for the subsequent purification.

To the crude extract (260 ml, 16.8 mg of protein per ml) was added 31.2 ml of 2.0% protamine sulfate, *p*H 5.0. The mixture was stirred for 10 min, and the precipitate was removed by centrifugation and discarded. The supernatant fluid (271 ml, 7.8 mg of protein per ml) was treated with 65.6 g of ammonium sulfate to give 40% saturation with respect to ammonium sulfate, and allowed to stand for 30 min. The precipitate was removed by centrifugation and discarded. To the supernatant fluid was added 38.2 g of ammonium sulfate to bring the concentration up to 60% saturation. The mixture was allowed to stand for 2 hr and was then centrifuged at 12,100 × *g* for 20 min. The pellet was dissolved in 10 to 15 ml of 0.01 M Tris-hydrochloride buffer, *p*H 7.0. The dialyzed preparation (36 ml, 18.0 mg of protein per ml) was treated with calcium phosphate gel suspension (23 mg, dry weight, per ml; reference 7), at the rate of 2.0 ml of gel for each ml of enzyme preparation. The gel, to which the enzyme was adsorbed, was washed with 0.001 M potassium phosphate buffer, *p*H 7.0. The activity was eluted from the gel with several portions of 0.007 M potassium phosphate buffer, *p*H 7.4, and the eluted fractions were pooled (200 ml, 0.72 mg of

protein per ml). Without further treatment, the eluate was mixed with 10 g of DEAE-cellulose, which was pretreated according to Leggett-Bailey (18), and then equilibrated with 0.007 M potassium phosphate buffer, *p*H 7.4. After standing for 10 min, the DEAE-cellulose was filtered as dry as possible, and the activity was eluted with several portions of 0.02 M potassium phosphate buffer, *p*H 7.0. This eluate (400 ml, 0.03 mg of protein per ml) was concentrated by ultrafiltration through a Diaflo filtration unit fitted with a UM-10 membrane (Amicon Corp., Inc., Lexington, Mass.). The final concentrated preparation (20 ml, 0.30 mg of protein per ml) had a specific activity of 213 units per mg of protein and represented a 140-fold purification over the crude extract. This preparation, which was free of β-glucosidase activity, was used in all experiments described in this paper. A summary of the purification details is given in Table 1.

**Nature of the products of phloretin hydrolase activity.** The identities of the products of reaction of phloretin hydrolase with phloretin as substrate were established by incubating 20.0 mg of phloretin with 5.0 ml of 0.01 M potassium phosphate buffer (*p*H 6.7) and 0.3 ml of enzyme, purified as described above. In the control experiment, the enzyme was inactivated by heating in boiling water for 10 min. The reaction mixtures were incubated at 30 C for 6 hr. After evaporation of the entire mixture to dryness in a flash evaporator at 35 C, the residue was taken up in methanol and chromatographed, eluted, crystallized, and analyzed as described above for the isolation and identification of phenolic intermediates from culture filtrates.

**Stoichiometry of the reaction.** To establish the stoichiometry of the reaction, 0.4 ml of purified phloretin hydrolase was incubated with 20.0 mg of phloretin suspended in 5.0 ml of 0.01 M potassium phosphate buffer (*p*H 6.70) at 30 C for 6 hr. A boiled enzyme control experiment was performed simultaneously. The digests were evaporated to dryness at 30 C, and the residue was extracted with 2.0 ml of methanol to dissolve the phenolic constituents. Of this extract, 200 μliters was applied with a Hamilton syringe as a 12-cm strip at the origin of a silica gel thin-layer plate. Marker spots were applied at each side of the main strip. The plates were irrigated with solvent A to separate phloretic acid, or with solvent B to yield phloroglucinol in one band and a mixture of phloretic acid and phloretin in the second. The phenolic bands were located by spraying the marker spots with the sulfanilic acid reagent (24). The bands were scraped from the plates, transferred to miniature columns, and eluted exhaustively with methanol. The eluate was collected in a volumetric flask of appropriate size (usually 50 ml). The individual components were determined in the respective eluates by spectrophotometric assay, with the use of a Unicam SP 500 spectrophotometer. Standard absorbancy curves were prepared for phloretin (over the range 0 to 0.05 μmoles/ml), phloroglucinol (0 to 1.5 μmoles/ml), and phloretic acid (0 to 0.5 μmole/ml), by using methanolic solutions and silica cells with a 1.0-cm light path. Phloretin was measured at 300 nm when in mixture with phloretic acid (which does not absorb at this wavelength), or at its absorption maximum of 287 nm when in pure solution. Phloroglucinol and phloretic acid were determined at 268 and 278 nm, respectively. The balance sheet for phloretin utilized and phloroglucinol and phloretic acid produced was drawn up from the data obtained, allowances being made for the appropriate control determinations.

TABLE 1. *Purification of phloretin hydrolase from E. herbicola Y46*

| Treatment | Total units | Specific activity | Purification | Recovery (%) |
|---|---|---|---|---|
| Crude extract | 6,707 | 1.50 | 1.0 | 100 |
| Protamine sulphate | 5,745 | 2.70 | 1.8 | 85 |
| Ammonium sulphate, 40–60% | 5,791 | 8.40 | 5.6 | 86 |
| Calcium phosphate gel eluate | 2,652 | 18.0 | 12.0 | 40 |
| DEAE-cellulose eluate | 1,944 | 162.0 | 108.0 | 29 |
| Ultrafiltration concentrate | 1,236 | 213 | 142 | 19 |

**Specificity of the enzyme.** The activity of the enzyme preparation in the presence of phloretin, phloridzin, naringin, and naringenin was tested qualitatively by mixing 0.9 ml of 0.01 M substrate suspended in 0.05 M Tris-hydrochloride buffer (*p*H 6.7) with 0.2 ml of enzyme preparation. A control experiment with boiled enzyme was performed simultaneously. After incubation at 30 C for 2 hr, samples (20 μliters) were subjected to thin-layer chromatography in both solvents A and B. The products of the reaction were located as described above.

## RESULTS

**Nature of the intermediates of phloridzin degradation.** Thin-layer chromatography demonstrated the presence of three major products of phloridzin breakdown in culture filtrates of *E. herbicola* Y46 grown on the defined medium containing phloridzin as sole source of carbon. Three other trace components of this mixture were noted but were not investigated further. The main components were tentatively identified as phloretin, phloroglucinol, and phloretic acid on the basis of their chromatographic behavior in two solvent systems. This identification was confirmed by comparison of the ultraviolet absorption spectra of the unknown compounds with those of authentic samples of the suspected intermediates. The chromatographic and spectral data are summarized in Table 2.

**β-Glucosidase activity.** The results of the survey of β-glucosidase and phloretin hydrolase activities in strains of *E. herbicola* and in some phytopathogenic bacteria are presented in Table

TABLE 2. *Identification of the products of phloridzin degradation by Erwinia herbicola Y46 by thin-layer chromatography and by inspection of ultraviolet absorption spectra*

| Compound | $R_F$ values in two solvents[a] | | Absorbancy maxima (nm) |
|---|---|---|---|
| Standards | | | |
| Phloretin | 0.82(B) | 0.58(C) | 205, 225, 287 |
| Phloroglucinol | 0.54(B) | 0.43(C) | 218, 267.5, 270, 274 |
| Phloretic acid | 0.32(A) | 0.51(D) | 203.5, 224, 278.5 |
| Unknowns | | | |
| 1 | 0.85(B) | 0.58(C) | 205, 227, 287 |
| 2 | 0.54(B) | 0.43(C) | 220, 267.5, 270, 274 |
| 3 | 0.32(A) | 0.53(D) | 203.5, 224, 278.5 |

[a] Capital letters in parentheses refer to the solvent systems described in Materials and Methods.

TABLE 3. *Distribution of β-glucosidase and phloretin hydrolase activities among strains of E. herbicola and some phytopathogenic bacteria*[a]

| Organism | No. of strains tested | β-Glucosidase activity in substrate[b] | | | Phloretin hydrolase |
|---|---|---|---|---|---|
| | | *p*-NPG | Arbutin | Phloridzin | |
| *Erwinia herbicola* | 11 | 5+ | 5+ | 3+ | 3+ |
| *E. amylovora* | 6 | − | − | − | − |
| *E. carotovora* | 2 | 2− | 2− | 2− | 2− |
| *Xanthomonas vesicatoria* | 1 | − | + | + | − |
| *Pseudomonas pisi* | 1 | + | + | + | − |
| *P. syringae* | 1 | + | + | − | − |
| *Agrobacterium tumefaciens* | 2 | 2+ | 2+ | 2+ | 2− |
| *A. rhizogenes* | 1 | + | + | − | − |
| *A. rubi* | 1 | + | + | + | − |
| *Corynebacterium insidiosum* | 1 | − | − | − | − |
| *C. sepedonicum* | 1 | + | + | − | − |
| *C. michiganense* | 1 | + | + | − | − |
| *C. fascians* | 1 | − | − | − | − |

[a] Although these enzymes were determined quantitatively in crude extracts of these organisms, the results are summarized qualitatively in this table in the interests of clarity.

[b] Activity detected (+), no activity (−). Figures show number of strains tested that showed indicated characteristic. Activities were determined in crude extracts of the organisms, as described.

3. Of the 13 species tested, only 5 showed β-glucosidase activity towards phloridzin; of these, only 3 strains of *E. herbicola* also possessed phloretin hydrolase. Among the strains of *E. herbicola*, only 5 exhibited β-glucosidase towards *p*-NPG; of these, 3 also possessed activity towards phloretin and phloridzin. Although the assay system used in the present work gave no indication of β-glucosidase activity after 10 min of incubation of crude extracts of some *E. herbicola* strains in the quantitative assay, the application of the qualitative method of Harda (10) to whole cultures, with an incubation time of 24 hr, gave positive β-glucosidase tests in all cases, as indicated by the liberation of *p*-nitrophenol from *p*-NPG (6).

**Properties of phloretin hydrolase.** The optimum *p*H for activity lies at 6.7 to 6.8 as determined by the assay of the release of phloroglucinol. The *p*H/activity curve followed a symmetrical rise and fall, being zero at *p*H 3.9 and approximately 9.5.

The purified preparation of the enzyme, when incubated with phloretin as substrate, yielded phloroglucinol and phloretic acid as the only products. The identity of these compounds was confirmed in a similar manner to that employed for the intermediates from culture filtrates, and the co-chromatographic and absorption spectrum data were identical with those reported in Table 2.

It was found that, in the experiment to determine the stoichiometry of the reaction, simultaneous with the disappearance of 30.7 μmoles of phloretin, 32.1 μmoles of phloretic acid and 25.8 μmoles of phloroglucinol were formed. These data support the hypothesis that phloretin hydrolase converts one equivalent of phloretin

to yield one equivalent each of phloroglucinol and phloretic acid.

The initial velocity, v, for the reaction, phloretin + water → phloroglucinol + phloretic acid, at different concentrations of phloretin, was determined in the spectrophotometric assay, and the Lineweaver and Burk (19) transformation was applied to the data. From this plot, the Michaelis constant ($K_m$) for the enzyme and phloretin as substrate was calculated to be $3.8 \times 10^{-5}$ M. This figure is the mean of three separate determinations. Efforts to demonstrate the reverse reaction in the presence of phloroglucinol and phloretic acid were unsuccessful.

The effects of various compounds on the activity of phloretin hydrolase are given in Table 4. The enzyme is very susceptible to $Hg^{2+}$ ions, moderately so to $Cu^{2+}$ ions, but is not affected by $Mg^{2+}$, $Ca^{2+}$, *p*-CMB, iodoacetamide, phloroglucinol, or phloretic acid.

Of four possible substrates tested, namely phloretin, phloridzin, naringin, and naringenin, only phloretin was degraded.

TABLE 4. *Effect of various compounds and cations on the activity of phloretin hydrolase from E. herbicola Y46*

| Adjunct | Concn | Maximum activity |
|---|---|---|
| | M | % |
| $Mg^{2+}$ | $1.0 \times 10^{-3}$ | 98 |
| | $1.0 \times 10^{-4}$ | 99 |
| | $1.0 \times 10^{-5}$ | 99 |
| $Ca^{2+}$ | $1.1 \times 10^{-3}$ | 87 |
| | $1.1 \times 10^{-5}$ | 94 |
| $Cu^{2+}$ | $2.4 \times 10^{-4}$ | 63 |
| | $2.4 \times 10^{-5}$ | 97 |
| $Hg^{2+}$ | $1.0 \times 10^{-6}$ | 50 |
| | $1.0 \times 10^{-7}$ | 82 |
| Phloroglucinol | $6.6 \times 10^{-4}$ | 97 |
| Phloretic acid | $6.6 \times 10^{-4}$ | 97 |
| *p*-Chloromercuribenzoate | $1.0 \times 10^{-3}$ | 96 |
| Iodoacetamide | $1.0 \times 10^{-3}$ | 92 |
| No addition | | 100 |

## DISCUSSION

On the basis of the evidence presented, it is postulated that *E. herbicola* Y46 degrades phloridzin by initial cleavage to phloretin, followed by further hydrolytic cleavage of phloretin to phloroglucinol and phloretic acid. The subsequent degradation of phloroglucinol and phloretic acid has not been elucidated, although three other phenolic compounds have been detected in trace amounts as products of phloridzin breakdown. Preliminary evidence suggests that one of these compounds is *p*-hydroxyphenyl acetic acid (Chatterjee and Gibbins, *unpublished data*). If this is confirmed, it will indicate that phloretic acid may be further degraded by the elimination of a single carbon fragment from the side chain of the molecule. In any event, this appears to be only a minor pathway, as the minor intermediates have been detected only in trace amounts, whereas phloroglucinol and phloretic acid accumulate in the culture medium in large quantities.

Although the degradation of phloridzin and the concomitant formation of phloroglucinol and phloretic acid have been noted in fungi (2, 15–17), the present work represents, as far as we are aware, the first example of bacterial degradation of phloridzin and the first documented report of the enzyme phloretin hydrolase. It is not clear what the physiological role of this enzyme is in *E. herbicola*. The likelihood of the aglycone providing a significant carbon or energy source for the organism appears remote in view of the very limited degradation of phloroglucinol or phloretic acid. Neither of these compounds will support the growth of *E. herbicola* Y46 when presented as sole source of carbon. It remains to be demonstrated whether the accumulation of these two compounds occurs in vivo, e.g., in wounded plant tissue or in the "fire-blight"-damaged areas caused by infection with *E. amylovora*, and whether these degradation products of phloridzin have any effect on the metabolism of either the host plant or the pathogen. Although it has been reported (21) that phloroglucinol stimulated the growth of carrot tissue in vitro, conflicting reports have appeared describing the effect of phloretic acid on apple tissues (15, 23). Phloretin has been shown to have a complex effect on wheat roots in that, while growth is stimulated, the absorption of sugars is inhibited and oxidative phosphorylation is both inhibited and uncoupled (26). It is not yet clear what effect, if any, these compounds have on the metabolic or phytopathogenic activities of *E. amylovora*.

It is possible that the cleavage of phloretin by strains of *E. herbicola* represents a detoxification mechanism whereby phloretin is eliminated from the cell after the D-glucose moiety has been removed from phloridzin. It is interesting to compare the fungal and bacterial contexts at this juncture. Jayasankar, Bandoni, and Towers (17) noted that, of 54 species of fungi studied, 31 produced phloroglucinol and phloretic acid from phloridzin, whereas only 1 of 13 species of bacteria tested in the present study possess phloretin hydrolase activity, and no such ac-

tivity was detected in any bacterial phytopathogen when grown on D-glucose as the main source of carbon and energy. However, it is possible that the enzyme may be inducible in some of these organisms when grown on phloridzin or other suitable inducer. The possession of β-glucosidase activity towards phloridzin appears to be closely associated with that of phloretin hydrolase in *E. herbicola*. The spectra of β-glucosidase activities in this species and among bacterial phytopathogens (13) will be the subject of further study in this laboratory.

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