[CONTRIBUTION BY THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Chlorogenic and Caffeic Acids as Fungistatic Agents Produced by Potatoes in Response to Inoculation with *Helminthosporium carbonium*[1,2]

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Two compounds, fungistatic for *Helminthosporium carbonum*, were separated chromatographically from extracts of potato peel and from extracts of pulp tissue on which the fungus had been able to make limited growth. The two compounds corresponded to chlorogenic and caffeic acid in their physical and chemical properties. It appears these acids are associated with the immunity of white potatoes from attack by *H. carbonum*.

It has been reported[3] that potato slices respond to inoculation with *H. carbonum* by producing a substance inhibitory to fungus growth. Potato peel was also reported to possess the inhibitory properties. The inhibitory substance in both the inoculated pulp and the peel was shown to be extractable with alcohol. The purpose of the present study was to identify the active components in the extracts.

Experimental

Preparation of Potato Tissue Extracts.—Netted Gem potatoes (Idaho-grown), free from visible surface defects, were washed with soap and water and surface-sterilized by immersion for 2 minutes in aqueous 2.5% sodium hypochlorite. After washing with sterile water five test materials were prepared; A, peel tissue approximately 1 mm. thick was removed; B, fresh pulp tissue was obtained after removal of the peel; C, inoculated pulp tissue was obtained by covering sterile fresh potato slices, cut approximately 1 cm. thick, with a heavy spore suspension of *H. carbonum*, incubating in sterile Petri dishes at 22° for 72 hours in a moist atmosphere, and removing a layer 1 mm. thick of inoculated slice surface; D, inoculated autoclaved pulp tissue was obtained by subjecting autoclaved slices to the same treatment as in C; and E, control pulp tissue was provided by holding sterile potato slices at 22° for 72 hours.

Extracts of these test materials were prepared by dropping 40 g. of tissue into 300 ml. of boiling alcohol and boiling for 2–5 minutes. After cooling the tissue was macerated in a Waring blendor for 5 minutes, the homogenate filtered through Whatman No. 2 paper filter on a Buchner funnel, and filtrate concentrated to 100 ml. under reduced pressure at 40°.

Chromatography of Potato Extracts.—Fifty *lambda* of each of the extracts was applied to sheets of Whatman No. 1 filter paper and chromatograms developed at 25° with mixtures of *n*-butanol, acetic acid and water (4:1:5 or 4:1:1) or 77% alcohol as descending developing solvents. The papers were dried for 12 hours, observed under ultraviolet radiation and then sprayed with either diazotized sulfanilic acid, ammoniacal silver nitrate or 1% aqueous ferric chloride.

Chromatographic separation using the above-mentioned solvents (Fig. 1) indicated the presence of two fluorescent compounds (I and II) in extracts A (peel) and C (inoculated pulp). Extract E (sterile pulp) showed a lower concentration of compound I and no II. Extracts B (fresh pulp) and D (inoculated after autoclaving) showed little or no evidence of compounds I and II.

Viewed under ultraviolet radiation, compound I appeared on chromatograms as a blue-white fluorescent spot; it fluoresced yellow-green after exposure to ammonia vapor. In daylight, exposure to ammonia vapor produced a yellow spot. After spraying with 1% aqueous ferric chloride the compound appeared as a green-grey spot. This behavior, typical of certain phenols, was suggestive of chlorogenic acid. A sample of chlorogenic acid, obtained commercially,[4] showed identical R_f values (Fig. 1), the same appearance under ultraviolet radiation, and the same color reactions as described above. When chromatograms were sprayed with 1% sodium nitrite in 10% acetic acid, compound I appeared as a yellow spot, then turned brick-red when subsequently sprayed with 5% potassium hydroxide. This reaction, according to Roberts and Wood[5] is specific for chlorogenic acid.

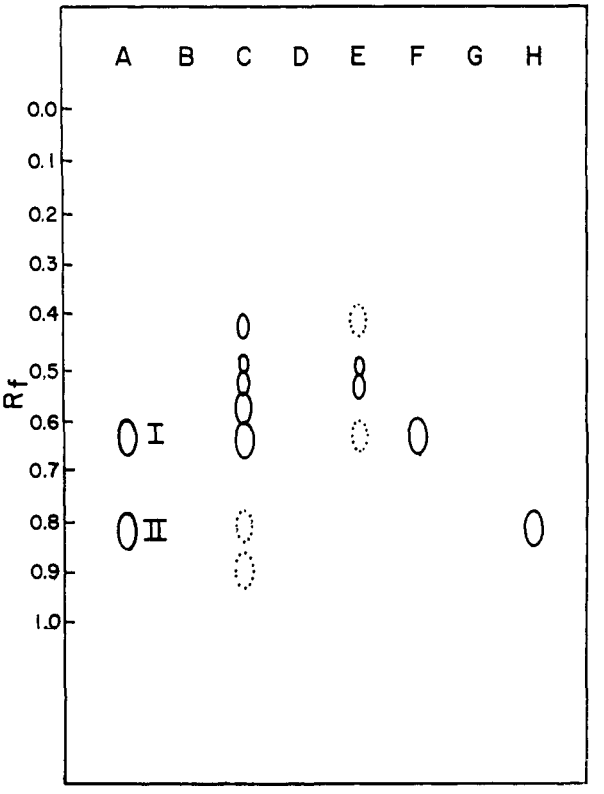

Fig. 1.—Chromatographic pattern of fluorescent compounds from potato tissue extracts: A, peel; B, fresh pulp; C, inoculated pulp (*H. carbonum*, 72 hours at 22°); D, inoculated autoclaved pulp (*H. carbonum*, 72 hours at 22°); E, pulp held for 72 hours at 22°; F, chlorogenic acid; G, protocatechuic acid; H, caffeic acid. Paper, Whatman No. 1; solvent, *n*-BuOH, HOAc, H_2O (4:1:5); faint spots or traces are outlined with dots.

Hydrolysis of compound I, by treatment with 13% potassium hydroxide for 15 minutes at 20°, followed by acidification to *p*H 4.0 with sulfuric acid, yielded chlorogenic, caffeic and quinic acids (as determined by separation on

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(2) Joint contribution from the Department of Biochemistry, the Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana, and Field Crops Research Branch, ARS, U. S. Department of Agriculture, Washington, D. C.

(3) J. Kuć, A. J. Ullstrup and F. W. Quackenbush, *Science*, **122**, 1186 (1955).

(4) Mann Research Laboratories, Inc., New York 8, N. Y.

(5) E. A. H. Roberts and D. J. Wood, *Arch. Biochem. Biophys.*, **33**, 299 (1951).

paper chromatograms and comparison of R_f values with those of authentic samples).

Compound II, separated from extracts A and C by paper chromatography, showed a characteristic brick-red color when sprayed with a 1% solution of sodium nitrite in 10% acetic acid, and a green-grey coloration after spraying with 1% aqueous ferric chloride. From these reactions, its R_f value and fluorescent properties it could not be differentiated from an authentic sample of caffeic acid.

Spectral Absorption of the Crude Extracts.—One-ml. portions of extracts A and C and 2-ml. portions of extracts B, D and E were each diluted with 18 ml. of alcohol and examined spectrophotometrically (Beckman DU) in the range 250 to 350 mμ. Extracts A, C and E showed substantial absorption in this range, and the contours of their absorption curves were essentially the same. However, extract E showed a lower absorbance than A or C even at a 2-fold concentration. Contour and position of maximum shown by the spectral curves of these extracts were essentially the same as those shown by commercial samples of chlorogenic and caffeic acid (Fig. 2). However, unlike solutions of commercial chlorogenic and caffeic acid which exhibited a shelf at 285–305 mμ, extracts A, B and C exhibited a definite absorption maximum at 285 mμ. Extracts B and D showed a slight absorption at 270 mμ, but lacked entirely the characteristic absorption properties of the fungistatic extracts.

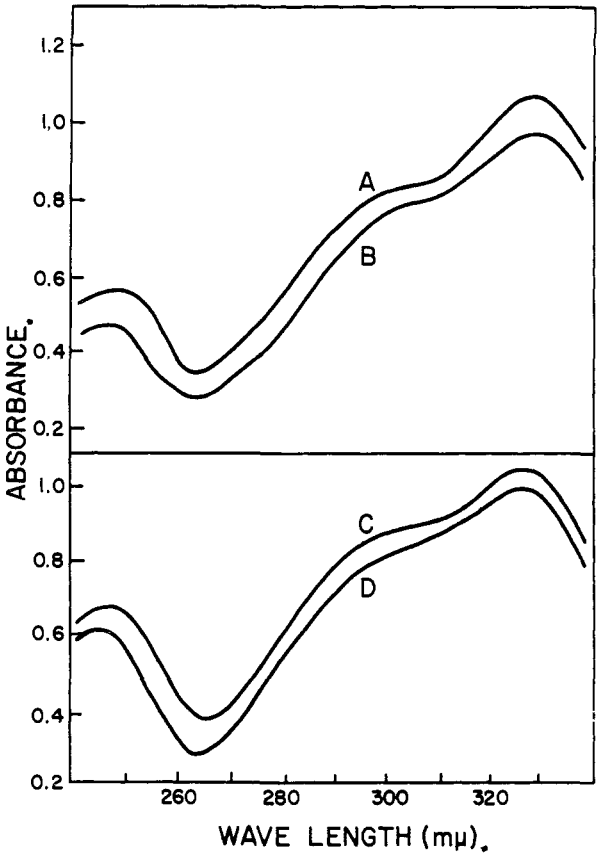

Fig. 2.—Spectral absorption curves of compounds I and II separated from potato tissues compared with commercial chlorogenic and caffeic acids: A, compound I from extracts A and C; B, chlorogenic acid; C, compound II from extracts A and C; D, caffeic acid.

Fractionation of Potato Extracts with Basic Lead Acetate.—Larger quantities of extracts A and C were prepared by extracting 100 g. of each of the corresponding tissues. Each was treated as follows. Fifty ml. of a 0.5 *N* basic lead acetate solution was added to 100 ml. of the extract in a centrifuge bottle; after 10 minutes the mixture was centrifuged and the clear supernatant liquid was collected. The residue was suspended in 50 ml. of 70% alcohol containing 5 ml. of 0.5 *N* basic lead acetate solution, the suspension again centrifuged and the supernatant collected. This was repeated two additional times to assure complete extraction of soluble material from the residue. The combined supernatant fractions (lead acetate-soluble) were treated with hydrogen sulfide, centrifuged, the residue discarded and the supernatant collected (fraction 1). The residue (lead acetate-insoluble) was suspended in 100 ml. of boiling water and heated on a steam-bath while a stream of hydrogen sulfide was passed through the suspension for approximately 30 minutes. The centrifuge bottle was stoppered, allowed to stand overnight, and the contents were then boiled for 5 minutes to expel hydrogen sulfide. After cooling to room temperature, the suspension was centrifuged and the supernatant was collected (fraction 2). A portion of fraction 2 was shaken with four successive half-volumes of ether. The aqueous phase was designated as fraction 3 and the combined ether extracts as fraction 4.

The Effect of Fractions 1–4 on the Growth of *H. Carbonum*.—Fractions 1–4 from each extract were concentrated to dryness under reduced pressure at 40° and the residue from each was redissolved in warm water and filtered. To fifty ml. of each solution, representing 40 g. of the corresponding tissue, two grams of dehydrated potato dextrose agar was added, and the solutions were autoclaved at 15 pounds pressure for 10 minutes. Petri dishes containing 16 ml. of the resultant media were seeded with a dilute spore suspension of *H. carbonum* and allowed to incubate at 24°.

H. carbonum made good growth on the potato dextrose agar medium and on the medium to which was added fraction 1 from either Extract A or C (Table I). Growth was strongly inhibited on the medium containing either of the extracts or fraction 2 therefrom, and also was inhibited by fractions 3 and 4. Inhibited growth generally exhibited red pigmentation.

Chlorogenic and Caffeic Acids in Fractions 3 and 4.—Fractions 3 and 4 from each of the extracts A and C, were chromatographed on Whatman No. 1 filter paper using *n*-butanol, acetic acid, water (4:1:5) as the descending developing solvent. No separation of components was detected on any of the chromatograms. Each showed a single blue-white fluorescent spot under ultraviolet radiation, those from fraction 3 corresponding in R_f value to compound I (chlorogenic acid) and those from fraction 4 corresponding to compound II (caffeic acid). The fluorescent areas cut from the sheets were eluted with alcohol and the spectral absorption curves were prepared from the eluates (Fig. 2). The curves could not be differentiated from those of authentic chlorogenic and caffeic acids. Chromatograms prepared similarly from fractions 3 and 4 and sprayed with ammoniacal silver nitrate, 1% sodium nitrite and 5% potassium hydroxide, or aqueous ferric chloride served to substantiate the homogeneity and the identity of the components as chlorogenic and caffeic acid, respectively.

To estimate the quantities of these compounds present, an assay based on the procedures of Wilkinson, *et al.*,[6a] Weiss,[6b] and Moores, *et al.*,[7] was employed. Absorbance of solutions was measured at 324 mμ, and absorptivity values of ($a^{1\%}_{1\text{ cm.}}$) of 526 for chlorogenic acid in water, and 930 for caffeic acid in alcohol were used for calculations. Twenty-fold to fifty-fold dilutions of the fractions were necessary for measurement in the optimal range. After analysis of fractions 3 and 4 by this method, extract A was calculated to contain 113 mg. of chlorogenic acid and 45 mg. of caffeic acid per 100 g. of tissue (peel), and extract C to contain 100 mg. of chlorogenic and 40 mg. of caffeic acid per 100 g. of tissue (inoculated).

Effects of Chlorogenic Acid, Caffeic Acid and Cysteine on the Growth of *H. carbonum*.—The potato dextrose agar basal medium containing 1×10^{-3} *M* to 1×10^{-2} *M* reagent chlorogenic or caffeic acid adjusted to *p*H 4.5 to 5.0 was inoculated with a dilute spore suspension of *H. carbonum*. Similarly, media containing 1×10^{-2} *M* cysteine, 1×10^{-2} *M* cysteine + 1×10^{-3} *M* chlorogenic acid, and 1×10^{-2} *M* cysteine + 1×10^{-3} *M* caffeic acid were prepared and inoculated.

After four days, chlorogenic acid had effected 0 to 49% inhibition, and caffeic acid had effected 20 to 100% inhibi-

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TABLE I

ACTIVITY OF FRACTIONS FROM FUNGISTATIC EXTRACTS

Test material	Colony diameter (mm.) and pigmentation[b] Time (hours) 24	48	72	96	120	144
Extract A (peel)	0	0	0	0	TW	TW
Fraction 1 ((lead filtrate)	5W	18WB	35WB	49WB	68WB	85WB
2 (lead ppt.)	0	TW	5W	10WR	18WR	28WR
3 (water-soluble)	TW	5WR	12WRB	22WRB	34WRB	45WRB
4 (ether-soluble)	TW	TW	8WRB	20WRB	30WRB	40WRB
Extract C (inoculated pulp)	0	0	TWR	TWR	TWR	10WR
Fraction 1 (lead filtrate)	5W	18WB	35WB	49WB	68WB	85WB
2 (lead ppt.)	0	TW	8W	15WR	23WR	32WR
3 (water-soluble)	TW	8WR	16WRB	26WRB	36WRB	47WRB
4 (ether-soluble)	TW	5WR	12WR	20WRB	30WRB	40WRB
Basal medium[a]	5W	18WB	35WB	51WB	70WB	.....

[a] Dehydrated potato dextrose agar to which all extracts and fractions were added. [b] Key to symbols: T = trace of growth; W = white growth; B = black pigmentation; R = red pigmentation.

tion over the same range of concentrations (Table II). Cysteine, although relatively ineffective alone, showed a synergistic action with each of the acids.

TABLE II

THE EFFECT OF REAGENT CHLOROGENIC ACID, CAFFEIC ACID AND CYSTEINE ON THE GROWTH OF *H. Carbonum*

Test substances	Concn., *M*	% Inhibition after 96 hr.
None	0	0[a]
Caffeic acid	1×10^{-3}	20
	2×10^{-3}	27
	4×10^{-3}	47
	6×10^{-3}	67
	8×10^{-3}	75
	1×10^{-2}	100
Chlorogenic acid	1×10^{-3}	0
	4×10^{-3}	23
	8×10^{-3}	40
	1×10^{-2}	43
Cysteine	1×10^{-2}	12
Chlorogenic acid +	1×10^{-3}	49
Cysteine	1×10^{-2}	
Caffeic acid +	1×10^{-3}	63
Cysteine	1×10^{-2}	

[a] Colony diameter of *H. carbonum* was 51 mm. on the basal medium, prepared by dissolving 2 g. of dehydrated potato dextrose agar in 50 ml. of water. Test substances were added to this medium and radial growth compared percentagewise.

Discussion

It appears that chlorogenic and caffeic acid are closely associated with the natural immunity of white potatoes from attack by *Helminthosporium carbonum*. Other workers[8-11] have recently reported the accumulation of chlorogenic acid, caffeic acid, methyl caffeate, umbelliferone, scopoletin, ascorbic acid and ipomearone in sound sweet potato tissue adjacent to that rotted by *Ceratostomella fimbriata*. It was postulated that these compounds are associated with the resistance of sweet potato to attack by *C. fimbriata*. Further investigations[12] have shown that respiratory enzymes in *C. fimbriata* are inactivated during the oxidation of sweet potato polyphenols.

Chlorogenic and caffeic acid, isolated from extracts of potato peel and inoculated pulp could not account for all the inhibition evident in crude potato extracts. This suggested a synergistic effect by a substance in the lead acetate-soluble fraction, which itself is not fungistatic. Cysteine added to solutions of reagent chlorogenic and caffeic acid appeared to possess this synergistic activity. Recent evidence[13] has shown a correlation between the resistance of apples to apple scab and the level of phenolic compounds and amino nitrogen in leaf tissue.

Phenolic compounds have often been associated with plant disease resistance and immunity. In most cases, however, the fungistatic phenols were present in plant tissue prior to infection.[14-20] It has been suggested that phenolic compounds are produced by plants in response to inoculation. The work described earlier with sweet potatoes[8-12] and the present data with white potatoes establish the validity of this suggestion.

Many writers have suggested that quinones formed during the oxidation of phenols by polyphenoloxidase are associated with plant disease resistance. Schaal and Johnson[21] have observed that autoxidation products of chlorogenic and caffeic acids were more inhibitory to *Streptomyces scabies in vitro* than the parent phenols. These autoxidation products were obtained in solutions at *p*H 8.0 to 8.5. Perhaps enzymatic action produces highly active oxidation products *in vivo* which are also effective in inhibiting the growth of *H. carbonum* on white potatoes.

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