Simultaneous Detoxification of Phytoalexins by *Fusarium solani* f. sp. *phaseoli*

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ABSTRACT

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The isoflavonoid phytoalexins, kievitone and phaseollidin, added to liquid cultures of *Fusarium solani* f. sp. *phaseoli*, were simultaneously detoxified to kievitone hydrate and phaseollidin hydrate, respectively. Both phytoalexins (each at 20 μg/ml) were transformed within 24 hr after being added to fungal cultures. No increase in mycelial dry weight of *F. solani* f. sp. *phaseoli* occurred while detoxification was proceeding. A purified preparation of kievitone hydratase, the enzyme responsible for kievitone transformation, did not accomplish simultaneous detoxification; kievitone was detoxified, but phaseollidin was not.

Additional key words: phytoalexin metabolism, *Phaseolus vulgaris*.

Detoxification of phytoalexins by phytopathogenic fungi has been widely reported (1,2,4,11,14). *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. and Hans., an aggressive pathogen in French bean (*Phaseolus vulgaris* L.) hypocotyls (15), will separately detoxify three host phytoalexins, phaseollin (11,12), kievitone (3–6), and phaseollidin (3,5,8). Phaseollinisoflavan also may be metabolized by this fungus (15). Kievitone hydratase is an extracellular enzyme produced by *F. solani* f. sp. *phaseoli* and is responsible for detoxification of kievitone to kievitone hydrate (5). The similarity of the kievitone→kievitone hydrate (6) and phaseollidin→phaseollidin hydrate (8) transformations suggested that the same enzyme system might mediate both reactions (3,5).

Phytoalexin detoxification may be a useful attribute for certain pathogenic fungi (2,5,13). Since many plants respond to fungal pathogens by producing multiple phytoalexins (1), it seems likely that a fungal pathogen might have to accomplish simultaneous detoxification of more than one phytoalexin while colonizing host tissues.

This investigation was undertaken to determine if *F. solani* f. sp. *phaseoli* could detoxify kievitone and phaseollidin when simultaneously treated with these phytoalexins. In addition, the ability of kievitone hydratase to accomplish comparable dual transformation also was assessed.

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MATERIALS AND METHODS

Fungal culture. The isolate of *F. solani* f. sp. *phaseoli* employed in this investigation was the one used in a previous study (5).

Phytoalexins. Kievitone was isolated from rotting cowpea seed fragments as described earlier (5). Phaseollidin was the gift of J. A. Bailey, Long Ashton Research Station, Bristol, U.K. It was further purified by thin-layer chromatography (tlc) on silica gel (hexane:acetone, 2:1, v/v [$R_f = 0.41$]) prior to use. Quantitation of the phytoalexins and their detoxified products was accomplished by ultraviolet absorbance (5,8).

Liquid culture assays. For solitary detoxification of phaseollidin by *F. solani* f. sp. *phaseoli* (Fig. 1), the liquid medium and procedures reported previously (3,8) were used.

Simultaneous detoxification of kievitone and phaseollidin was determined by procedures similar to those described earlier for the individual phytoalexins (3–5,8); however, the liquid medium was changed to avoid the inconvenience of filter-sterilization. It contained (per liter of medium): 20 g D-glucose, 4.6 g casein hydrolysate, 1.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.01 g thiamine hydrochloride, and 10 ml of a minor element solution (9). The pH of the medium after autoclaving was 6.5.

Liquid medium (4 ml), contained in 25 ml Erlenmeyer flasks, was inoculated with mycelial pieces of *F. solani* f. sp. *phaseoli* taken from agar cultures (7). The flasks were incubated on a reciprocal shaker at 25 ± 2 C or 27 ± 2 C for 24 hr to allow mycelial growth to become established. Then solutions of kievitone and phaseollidin in ethanol were added to all treatment flasks to give initial phytoalexin concentrations of 20–25 μg/ml. The ethanol

concentration in the flasks was 1%. Control flasks received only ethanol. Incubation was continued in the same conditions.

After various periods of time, the flask contents were harvested. Mycelial dry weight (7) and/or phytoalexin (and detoxification product) concentrations were determined. For isoflavonoid analyses, the fungus was separated from the residual liquid medium by vacuum-filtration through Whatman No. 1 paper. Water washes (~2.5 ml) of the mycelium were collected with the medium. One drop of 1N HCl was added to the combined, clarified solution. Kievitone, kievitone hydrate, phaseollidin, and/or phaseollidin hydrate were removed by partitioning twice with diethyl ether (three volumes and two volumes). The ether fractions were combined, taken to dryness under air or N_2 and redissolved in a small volume of ethanol. The four isoflavonoids were separated by tlc on silica gel G-precoated (0.25 mm) glass plates (Analtech Inc., Newark, DE 19711) developed with chloroform:methanol (50:3, v/v). Freshly prepared diazotized *p*-nitroaniline reagent (10) was used to detect standards and/or the edges of separated bands. The R_f values were as follows: phaseollidin (0.63), phaseollidin hydrate (0.31), kievitone (0.27), and kievitone hydrate (0.13). Appropriate, unsprayed portions of the dry gel were scraped into centrifuge tubes. Known volumes of ethanol were mixed with the gel to elute the compounds. The mixtures were subjected to low-speed centrifugation and the clear supernatants were analyzed spectrophotometrically for isoflavonoid content.

Isoflavonoid recoveries from individual extractions were variable, which is probably to be expected from a multistep procedure. However, all compounds were extracted with comparable efficiencies; mean percentage recovery efficiencies were 41, 45, 45, and 41 for kievitone, kievitone hydrate, phaseollidin, and phaseollidin hydrate, respectively. Data presented are not corrected for extraction efficiencies. Even when the compounds were extracted from medium lacking fungus, the procedure was only ~50% efficient.

Kievitone hydratase assays. Kievitone hydratase was isolated from kievitone-induced *F. solani* f. sp. *phaseoli* cultures as described elsewhere (5, and T. E. Cleveland and D. A. Smith [*unpublished*]). The steps included filtration through 0.22-μm filters, lyophilization, dialysis, ultrafiltration, ion exchange chromatography (Sephadex DEAE A-50) and gel filtration (Sephadex G-200). The purified enzyme preparation, maintained in 50 mM potassium phosphate buffer (pH 6.0) at 27 C, was capable of converting 50 nmoles of kievitone to kievitone hydrate per minute per milligram of protein.

One milliliter of this kievitone hydratase preparation (27.5 μg protein) was diluted to 2 ml with the same buffer. Kievitone and phaseollidin were added in ethanol to the enzyme preparation; phytoalexin concentrations were each 20 μg/ml and the total ethanol concentration was 1%. As with the intact fungus, the samples were incubated at 27 ± 2 C for various periods of time prior to extraction of the isoflavonoids in diethyl ether (10 volumes). Subsequent separation and quantitation of the isoflavonoids were as presented above.

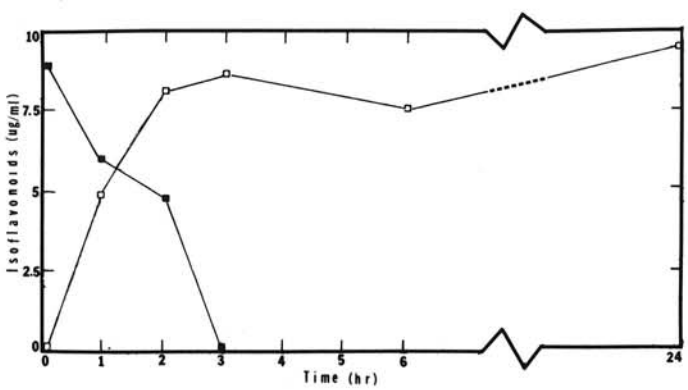

Fig. 1. Detoxification of phaseollidin by *Fusarium solani* f. sp. *phaseoli*. Phaseollidin in ethanol (ethanol concentration in the medium, 0.5%) was added to 24-hr-old liquid mycelial cultures of *F. solani* f. sp. *phaseoli*. Eighty μg of phytoalexin (20 μg/ml) was added to each flask. The flasks were again incubated on a reciprocal shaker at 25 C. At various time intervals thereafter, the concentrations of phaseollidin (■——■) and phaseollidin hydrate (□——□) were determined. The isoflavonoids were extracted in diethyl ether, separated by thin-layer chromatography, and quantified by ultraviolet spectrophotometry; each value is the mean of two replicates.

RESULTS

Phaseollidin detoxification. The time course of kievitone detoxification by *F. solani* f. sp. *phaseoli* is well documented (3,4). This information is not well established for phaseollidin. Consequently, prior to considering the simultaneous metabolism of the two phytoalexins, the time course of the metabolism of phaseollidin by *F. solani* f. sp. *phaseoli* was determined (Fig. 1). The phaseollidin apparent at time zero had been completely transformed into phaseollidin hydrate after 3 hr.

Simultaneous detoxification of kievitone and phaseollidin by *F. solani* f. sp. *phaseoli*. Transformation of kievitone and phaseollidin by *F. solani* f. sp. *phaseoli* occurred simultaneously, as illustrated by a representative tlc plate from a time course analysis (Fig. 2) and by quantitative assay (Fig. 3A). Neither hydrate declined appreciably after it was formed. The mycelial dry weight analysis (Fig. 3B) contrasts the steady growth in the control with the lack of growth in those that received the phytoalexin treatment.

Selective detoxification of kievitone by kievitone hydratase. A purified preparation of kievitone hydratase removed about two-thirds of the added kievitone after 4 hr (Fig. 4). A parallel increase in the level of kievitone hydrate was evident. The phaseollidin present in the reaction mixture, however, remained unchanged at about 10 μg/ml throughout the time period. Prolonged incubation, up to 24 hr, resulted in no decline in the phaseollidin level. Heat-treated enzyme (boiling water bath for ~2 min) transformed neither kievitone nor phaseollidin.

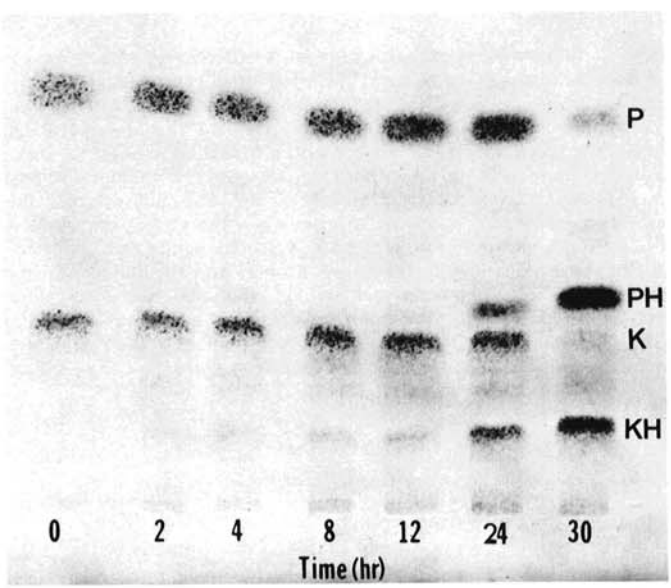

Fig. 2. Thin-layer chromatogram demonstrating simultaneous detoxification of kievitone and phaseollidin by *Fusarium solani* f. sp. *phaseoli*. Kievitone and phaseollidin in ethanol (ethanol concentration in the medium, 1%) were added to 24-hr-old liquid mycelial cultures of *F. solani* f. sp. *phaseoli*. One hundred μg of each phytoalexin was added to treatment flasks. The flasks were again incubated on a reciprocal shaker at 25 C. At intervals thereafter, isoflavonoids were extracted from the liquid medium by partitioning into diethyl ether. Ten percent samples of each concentrated extract were applied as short bands to a 20 × 20-cm silica gel-coated glass thin-layer chromatography plate, which was developed with chloroform:methanol (50:3, v/v). Phaseollidin (P), phaseollidin hydrate (PH), kievitone (K), and kievitone hydrate (KH) were resolved by spraying the developed and dried plate with diazotized *p*-nitroaniline.

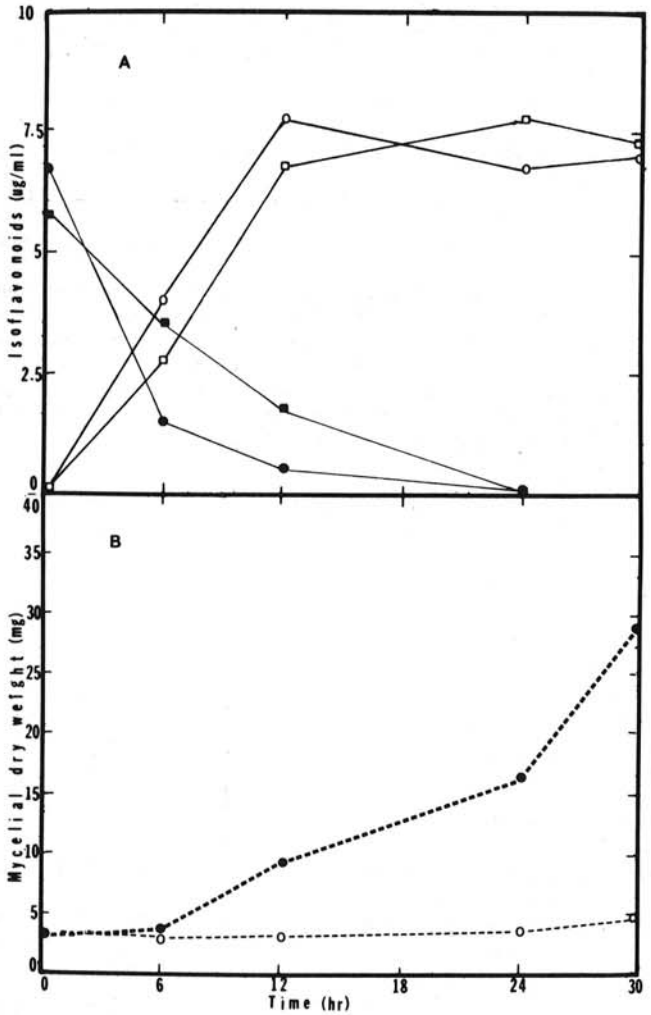

Fig. 3. Simultaneous detoxification of kievitone and phaseollidin by *Fusarium solani* f. sp. *phaseoli*. Kievitone and phaseollidin in ethanol (ethanol concentration in the medium, 1%) were added to 24-hr-old liquid mycelial cultures of *F. solani* f. sp. *phaseoli*. Eighty μg of each phytoalexin was added to treatment flasks, providing initial concentrations of 20 μg/ml for both kievitone and phaseollidin. The flasks were again incubated on a reciprocal shaker at 27 C. At intervals thereafter, isoflavonoid concentrations and mycelial dry weights were determined. All values are the means of four replicates. **A,** Isoflavonoids were extracted in diethyl ether, separated by thin-layer chromatography, and quantified by ultraviolet spectrophotometry. Kievitone (●——●), kievitone hydrate (O——O); phaseollidin (■——■), and phaseollidin hydrate (□——□). **B,** Phytoalexin-treated (O-----O) and control (1% ethanol) (●-----●) mycelia were harvested and their dry weights were determined.

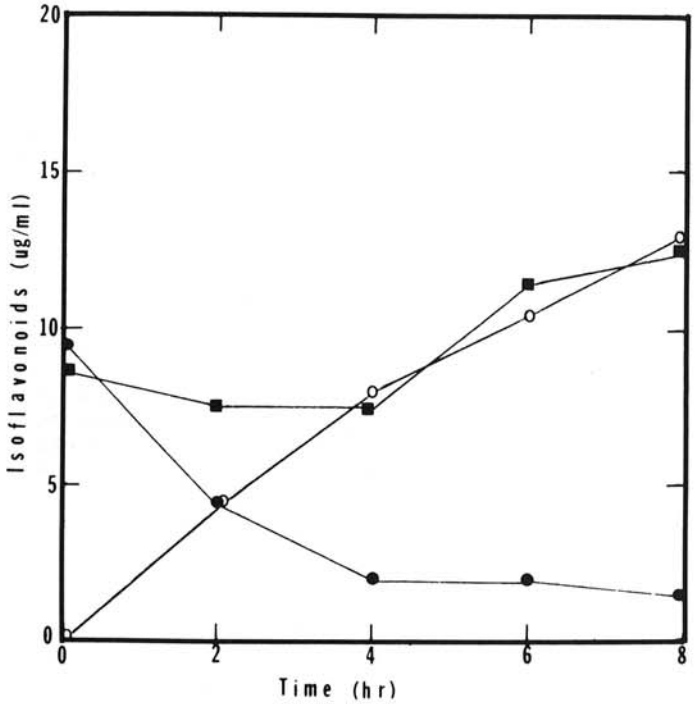

Fig. 4. Selective detoxification of kievitone by kievitone hydratase. Kievitone (●——●) and phaseollidin (■——■) in ethanol (ethanol concentration in the enzyme preparation, 1%) were added to 2 ml aliquots of purified kievitone hydratase in 50 mM phosphate buffer (pH 6.0). Forty μg of each phytoalexin was added to each 2-ml enzyme sample, providing initial concentrations of 20 μg/ml for both kievitone and phaseollidin. The phytoalexin-enzyme mixtures were incubated on a reciprocal shaker at 27 C for various periods of time. Thereafter, isoflavonoids were extracted from the reaction mixtures in diethyl ether, separated by thin-layer chromatography, and quantified by ultraviolet spectrophotometry. Kievitone hydrate (O——O) was the only new product. Each value is the mean of two replicates.

DISCUSSION

Phytoalexin metabolism by *F. solani* f. sp. *phaseoli* in a heat-sterilized medium was comparable to that in filter-sterilized medium employed previously (3–6,8). This point is important in view of reports (2,13) that media composition may appreciably affect the metabolic breakdown of phytoalexins; the type and amount of carbohydrate source seem particularly influential. Perhaps little effect was apparent in this investigation because the same concentration of glucose was present in each medium.

F. solani f. sp. *phaseoli* readily detoxifies kievitone (3–5) and phaseollidin (3,8, and Fig. 1) when separately exposed to the individual phytoalexins. Furthermore, simultaneous detoxification of kievitone and phaseollidin was accomplished by this fungus (Figs. 2 and 3A). Not unreasonably, the process took longer than for the individual transformations reported previously (3,4, and Fig. 1), because the combined concentration used in the dual treatment exceeded those in individual experiments and, presumably, presented a more toxic environment to the fungus.

This demonstration of dual phytoalexin detoxification corroborates the proposition that the ability to detoxify phytoalexins may be a useful property of pathogens (5). Kievitone and phaseollin also disappear simultaneously in the presence of *F. solani* f. sp. *phaseoli* (R. Longden and D. A. Smith, *unpublished.* Similar experiments can be undertaken with three or more phytoalexins from bean to determine whether there is a limit to the number of host phytoalexins with which this fungus can cope.

The difficulty of isolating phytoalexin-metabolizing enzymes has been discussed elsewhere (14). Kievitone hydratase is a major exception (5). The present findings with this enzyme seem particularly relevant to the problem of isolating active enzymes. Cellfree culture filtrates of *F. solani* f. sp. *phaseoli* will detoxify kievitone and phaseollidin upon separate treatment with these compounds (3,5,8). Both phytoalexins are detoxified by identical hydration reactions (6,8). If the same enzyme is involved, its ability to catalyze kievitone transformation in vitro greatly exceeds that for phaseollidin when simultaneously exposed to the phytoalexins (Fig. 4). This finding is at odds with the similar rates of transformation of kievitone and phaseollidin by the intact fungus (Fig. 3A). Additional research is needed to resolve these questions.

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