Isolation of Phaseollin from Rhizoctonia-Infected Bean Tissue

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Cruickshank & Perrin (2) demonstrated that a conidial suspension of *Monilinia fructicola* added to the seed cavities of detached pods of French bean (*Phaseolus vulgaris*) induced production of an antifungal compound. The induced compound is a phenol containing the chromanocoumaran ring system (2, 4). It has been classified as a phytoalexin and given the trivial name, phaseollin (1, 2, 4).

Pierre & Bateman (5) demonstrated that antifungal compounds are produced in bean in response to infection by *Rhizoctonia solani* Kühn, and that such compounds are associated with the delimitation of lesions. They tentatively identified one of the antifungal substances as phaseollin. In this report we confirm this identification and describe an extraction procedure which results in the isolation of phaseollin in crystalline form from *R. solani*-infected bean (*P. vulgaris* L. 'Red Kidney') hypocotyls.

One-week-old bean plants were inoculated with *R. solani* (isolate RB) as previously described (6). *Rhizoctonia* lesions, under our experimental conditions, are generally delimited within 36 hr after inoculation (6), and considerable phaseollin has accumulated in the lesions by this time (5). Phaseollin continues to accumulate in infected tissues for at least 12 days after the lesions have become delimited. Therefore, bean hypocotyl sections bearing 12-day-old *R. solani* lesions were used as a phaseollin source in this study. Diseased plant material was harvested and stored at −14°C.

Extraction procedure.—One thousand g of *R. solani*-infected bean hypocotyl were ground in 4,000 ml of 95% ethanol for 1-2 min in a Waring Blendor. The extract was filtered through several layers of cheesecloth and the residue washed with 500 ml of 95% ethanol. These liquid fractions were combined and centrifuged at 12,000 *g* for 20 min at 4°C. The supernantant was saved. Approximately 2.5 liters of water were added to the supernatant, and the ethanol was removed by evaporation at about 40°C under reduced pressure. The aqueous fraction was adjusted to about pH 3.0 with 6 N HCl, and partitioned twice with 4 volumes of petroleum ether (85% hexane). Partitioning was accomplished by mixing the two solvents in a Waring Blendor for 0.5-1 min, then separating the two phases with separatory funnels. The petroleum ether fractions were combined, evaporated under reduced pressure at 40°C to one-fourth their original volume, and then partitioned once with cold 0.2 N NaOH (1:1, v/v). The 0.2 N NaOH fraction was adjusted to pH 6.0 with cold 6 N HCl. The acidified solution was partitioned once with petroleum ether (1:1, v/v). The petroleum ether fraction was taken to dryness under reduced pressure at about 45°C. The residue was taken up in a few ml of 95% ethanol. Phaseollin was crystalized, using ethanol and water as the solvent pair. Crystallization was accomplished by the stepwise addition of small quantities of water to an ethanolic solution of the compound and exposure to cold temperatures. Chloroform and heptane were also found to be a good solvent pair for crystallization. After recrystallization twice, 168 mg of phaseollin crystals were obtained from 1,000 g fresh wt of *R. solani*-infected hypoctyl tissue.

Analysis.—The white, needle-shaped crystals of phaseollin, when dissolved in ethanol, had an ultraviolet absorption spectrum with λ max at 279 nm, a shoulder at 284-286 nm, and a minor peak at 315 nm. The ratios of the absorbance at 286 nm and 315 nm to the absorbance at 279 nm were 0.87 and 0.24, respectively. This compound has a molecular wt of 322 based on mass spectrum analysis. These characteristics are the same as those reported for phaseollin (2, 4), and R. H. Shapiro's interpretation (*personal communication*) of the mass spectrum is in agreement with the structure proposed for phaseollin by Perrin (4). The melting point of the crystals was 177-178; when our compound was mixed with an authentic sample of phaseollin, melting points of 176.5-177°C and 177-178.5°C were obtained. This compound exhibited an ED_{50} value (mycelial inhibition) of approximately 30 μg/ml for *R. solani* (isolate RB).

The thin-layer chromatographic system developed by Hedin et al. (3) for phenols is an effective means for the isolation of phaseollin from small amounts of crude extracts from diseased plant tissues. Silica gel (250 μ thick) is used as a support, and pentane:ethyl ether:acetic acid (75:25:1) as the developing solvent. In this system, phaseollin has an R_F of about 0.34, and can be located with the usual phenol-detecting reagents or by use of short wave ultraviolet light. When recovering phaseollin from thin-layer plates, the time of exposure to ultraviolet light as well as the time this compound is left on dried silica gel should be kept at a minimum, since both result in some decomposition.

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