

## Studies on Biosynthesis of Phaseollin in Excised Pods of *Phaseolus vulgaris*

Samuel L. Hess, Lee A. Hadwiger, and Martin E. Schwochau

NDEA Fellow, Associate Professor, and Research Associate, respectively, Department of Plant Pathology, Washington State University, Pullman 99163.

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### ABSTRACT

Based on the incorporation of phenylalanine- $ul-^{14}C$ , cinnamic acid-carboxyl- $^{14}C$ , acetate- $1-^{14}C$ , and daidzein- $ul-^3H$  into phaseollin, a general biosynthetic pathway for this isoflavonoid in *Phaseolus vulgaris* L. is suggested. Mevalonic acid- $2-^{14}C$  did not appear to be incorporated, suggesting that it is not a pre-

cursor of phaseollin. Stimulation of phenylalanine ammonia-lyase (PAL) activity occurs before phaseollin can be detected in the pod tissue. The level of increased PAL activity is not always proportional to the amount of phaseollin produced. *Phytopathology* 61:79-82.

*Additional key words:* phytoalexin, isoflavonoid, *Fusarium solani* f. sp. *phaseoli*, *F. solani* f. sp. *pisi*.

Information on the biosynthesis of phytoalexins is necessary to understand their role in disease resistance (1, 2). Phaseollin (7-hydroxy-3', 4'-dimethyl-chromenyl chromanocoumarane) is a phytoalexin produced in bean tissue (2, 3, 5, 8, 13). Phaseollin production and the increased activity of at least one enzyme in its biosynthetic pathway can be induced with various microorganisms, microbial metabolites, and chemical compounds (2, 3, 5, 8, 13). This paper identifies precursor compounds which can be incorporated into phaseollin. The biosynthetic pathway of phaseollin will be compared with those of other isoflavonoids.

**MATERIALS AND METHODS.**—L-phenylalanine- $ul-^{14}C$ , acetate- $1-^{14}C$ , and mevalonic acid- $2-^{14}C$  were obtained from New England Nuclear Corp.

Monospore cultures of *Fusarium solani* (Mart.) Appel & Wr. f. sp. *pisi* (F. R. Jones) Snyder & Hans. and *F. solani* f. sp. *phaseoli* (Burk.) Snyder & Hans. were transferred from water agar to potato-dextrose agar and maintained under diffuse light.

**Induction treatments.**—Three ml of  $CuCl_2$  ( $3 \times 10^{-3} M$ ) or 3 ml of spore suspensions of macroconidia with  $1 \times 10^6$  spores/ml of sterile distilled water were used to induce immature bean pods, *Phaseolus vulgaris* L. 'Topcrop', to produce phaseollin as previously described (5).

**Incorporation determinations.**—Two  $\mu c$  of radioactively labeled phenylalanine- $ul-^{14}C$ , cinnamic acid-carboxyl- $^{14}C$ , acetate- $1-^{14}C$ , mevalonic acid- $2-^{14}C$  or daidzein- $ul-^3H$  were added to 2 g split bean pods, immediately preceding or 16 hr after inducer application, spread evenly over the surface, and allowed to penetrate (concn of the isotopes used are given in Table 1). Forty-eight hr after induction with  $3 \times 10^{-3} M$   $CuCl_2$ , phaseollin was extracted and quantitated as described below. The per cent incorporation and isotopic dilution values for each labeled precursor were determined, and three separate thin layer chromatography (TLC) separations were used to establish purity and constant spe-

cific activity of radioactively labeled phaseollin. Benzene, 10% methanol in benzene, and chloroform were used as solvent systems, and the respective  $R_F$  values for phaseollin were 0.08, 0.52 and 0.22.

Phenylalanine-labeled phaseollin of known specific activity was combined with purified (mp 178-179°C) unlabeled phaseollin. The combined phaseollin was recrystallized in aqueous ethanol and the specific activity and melting point determined (14).

**Extraction and quantitation of phaseollin.**—Phaseollin was extracted 8, 16, 24, 48, and 84 hr after treatment with the abovementioned inducers. Two g of treated pods were frozen and pulverized in liquid nitrogen with a mortar and pestle. The powdered tissue was extracted with 15 ml 95% ethanol and centrifuged at 20,000 g for 10 min. The supernatant was removed and the pellet resuspended in an additional 15 ml of ethanol and recentrifuged as above. The two ethanol extracts were combined and evaporated in vacuo at 40°C. The residue was dissolved in 5 ml deionized water and phaseollin extracted 5 times with 10 ml petroleum ether, bp 30-60°C. The extracts were combined, and the residue remaining after volatilization of the petroleum ether was streaked on silica gel thin-layer plates (100  $\mu$  thick). The plates were developed in chloroform. Phaseollin was located by spraying a small transverse strip of a 10- $\times$ -20-cm TLC plate with 3% ferric chloride in methanol (a reagent which develops a red color in the presence of phaseollin after slight heating. This color reaction is specific for phaseollin). The silica gel was scraped from the untreated portion of the TLC plate, and phaseollin removed by extraction with ethanol. The presence of phaseollin was verified on the basis of the absorbance spectrum (340 nm-240 nm) (3, 12, 13). Phaseollin was quantitated on the basis of its absorbance at 279 nm (3).

**Phenylalanine ammonia-lyase extraction and assay.**—Phenylalanine ammonia-lyase (PAL) was assayed according to the procedure of Koukol & Conn (9) with

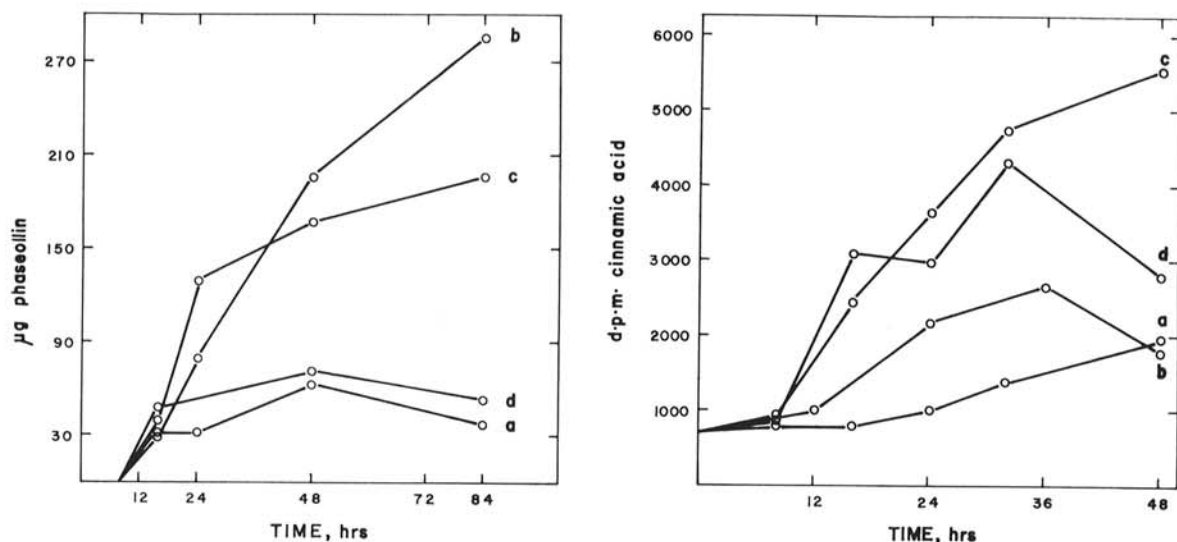


Fig. 1. (Left) Phaseollin production ( $\mu\text{g/g}$  fresh wt) in excised pods of *Phaseolus vulgaris* L. at varying times after induction. (Right) Phenylalanine ammonia-lyase activity, expressed as dpm cinnamic acid (1,000 dpm = 0.036 mmole cinnamic acid), produced in 1 hr by 1 g excised pods of *Phaseolus vulgaris* L. at varying times after induction. a: H<sub>2</sub>O; b: CuCl<sub>2</sub>; c: *Fusarium solani* f. sp. *phaseoli*; d: *F. solani* f. sp. *phaseoli*.

the modifications of Hadwiger et al. (5). The reaction mixture was incubated for 1 hr at 37 C.

**RESULTS.—Phaseollin production.**—Production of phaseollin in beans treated with sterile deionized water, CuCl<sub>2</sub>, *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *phaseoli* (Fig. 1, left), is not detectable until 8 hr after applying the inducer. CuCl<sub>2</sub> is the most effective inducer. At 24 hr, the amount of phaseollin extractable from bean pods induced with the bean pathogen, *F. solani* f. sp. *phaseoli*, was similar to that induced by the other treatments. Longer incubation periods did not increase the extractable phaseollin in pods treated with the pathogen. With the other two inducers, phaseollin content continued to increase as the period of induction increased. Phaseollin was not detected in extracts of untreated pods.

**Incorporation of radioactively labeled precursors.**—Phenylalanine-<sup>14</sup>C, cinnamic acid-<sup>14</sup>C, or acetate-<sup>14</sup>C were readily incorporated into phaseollin (Table 1). The isotopic dilution values at 48 hr were 835, 685, and

2,100, respectively, when the labeled precursors were applied immediately preceding induction. Incorporation efficiency improved when precursors were applied 16 hr after the inducer. This is a peak period for phaseollin synthesis. The resulting isotopic dilution values at 48 hr were reduced to 410, 110, and 626, respectively. The isoflavonoid daidzein was also incorporated into phaseollin. The isotopic dilution value was 369.

The isotopic dilution value obtained with mevalonic acid-<sup>14</sup>C was greater than 22,000, and it was not possible to purify the phaseollin produced to constant specific activity with subsequent chromatographic separations.

Isotopic dilution values (precursor specific activity per product specific activity, mc per mmole) provide an indication of the relative proximity of precursors to the end product in a given metabolic pathway. Isotopes of close proximity will have less isotopic dilution than those that are further removed from the end product.

Phenylalanine-<sup>14</sup>C-labeled phaseollin which had been

TABLE 1. Relative incorporation of precursors into phaseollin in excised bean pods

Labeled precursors	Precursor specific activity ( $\mu\text{c}/\mu\text{mole}$ )	% Incorporation <sup>a</sup>	Isotopic dilution values Time of precursor application <sup>b</sup>	
			0 hr	16 hr
Phenylalanine- <sup>14</sup> C	3.14	0.53	835	410
Cinnamic acid-carboxyl- <sup>14</sup> C	0.302	0.31	685	110
Acetate- <sup>14</sup> C	0.82	0.10	2,100	626
Mevalonic acid- <sup>14</sup> C	7.05		17,195	22,000
Daidzein- <sup>3</sup> H	9.00	0.63 <sup>c</sup>		396 <sup>c</sup>

<sup>a</sup> Total activity of phaseollin produced in 48 hr/total activity of precursor  $\times$  100. Precursor was applied 16 hr after inducer application.

<sup>b</sup> Two  $\mu\text{c}$  of precursor added immediately before (0 hr) or 16 hr after application of 3 ml inducer ( $3 \times 10^{-3}\text{M}$  CuCl<sub>2</sub>) to 2 g of *Phaseolus vulgaris* pods.

<sup>c</sup> Two and one-half  $\mu\text{c}$  of precursor were applied 14 hr after inducer application. Isotopic dilution and per cent incorporation calculations were based on the theoretical loss of the 4', 5' Tritium of daidzein when converted into phaseollin.

recrystallized to constant specific activity (specific activity  $0.73 \times 10^{-5} \mu\text{C}/\mu\text{g}$ ) was combined with an equal amt of unlabeled phaseollin and recrystallized. The resulting specific activity was  $0.35 \times 10^{-5} \mu\text{C}/\mu\text{g}$ , and the melting point of the combined sample was 172-174 C.

*Phenylalanine ammonia-lyase activity.*—The relative incorporation rates of phenylalanine and cinnamic acid, as evidenced by their isotopic dilution values, suggest that phenylalanine precedes cinnamic acid in the biosynthetic pathway of phaseollin. The activity of the enzyme PAL, responsible for converting phenylalanine to cinnamic acid, is increased in pods treated with the various inducers (Fig. 1, Right).

These and other studies (5) suggest that phaseollin production is associated with an increase in PAL activity which occurs 3-4 hr before detection of phaseollin is possible. This increase in PAL activity is not always proportional to the amount of phaseollin produced. For example,  $\text{CuCl}_2$  has a low capacity to stimulate PAL activity, but readily stimulates phaseollin production. PAL activity resulting from all treatments except *P. solani* f. sp. *pisi* is opt at about 36 hr after treatment, then declines.

**DISCUSSION.**—Several reports (2, 3, 4) indicate that bean cultivars moderately resistant to various pathogens accumulate more phaseollin than do susceptible cultivars. The level of phaseollin present at the infection site at any given time in the infection process is dependent on (i) the preformed quantity; (ii) the rate of synthesis; and/or (iii) the rate of degradation in the invaded host cell. The incorporation into phaseollin of radioactive precursors (Table 1) introduced subsequent to induction, and the observation that phaseollin was not detectable in bean tissue until 8 hr after induction, indicate that the phaseollin molecule is synthesized de novo and not formed by metabolic breakdown of a preformed complex.

Our data establish that at least part of the phaseollin molecule is assembled from small molecules such as phenylalanine, acetate, or cinnamic acid. The incorporation data are consistent with pathways proposed for other isoflavonoids (4, 6, 7, 10). The benzene ring of phenylalanine or cinnamic acid would logically form the B ring of phaseollin (Fig. 2). The carboxyl, alpha,

and beta carbons of the side chains would become the 4, 3, and 2 carbons, respectively, of the phaseollin molecule. Head to tail condensations of 3 acetate- $^{14}\text{C}$  molecules would form the A ring, as has been shown for quercetin and others (10, 15). Such a scheme involves at least two separate metabolic pathways; i.e., the acetate pathway and the shikimic acid pathway, in the biosynthesis of phaseollin.

The source of the D ring remains speculative. The 3',4'-dimethyl chromenyl moiety of phaseollin comprising the D ring could be derived from mevalonic acid. But the extremely poor incorporation of mevalonic acid-2- $^{14}\text{C}$  tends to eliminate this possibility. The limited incorporation of mevalonic acid-2- $^{14}\text{C}$  is not due to the inability of this molecule to enter the cell, as it is readily incorporated into other secondary metabolites (carotenoids).

Since the daidzein molecule is incorporated into phaseollin with an isotopic dilution value nearly equal to that of phenylalanine- $^{14}\text{C}$ , daidzein may be an intermediate in the biosynthesis of phaseollin. It may also be inferred that the formation of the ether bridge and alkylation leading to the building of ring D occurs after construction of the basic isoflavonoid ring system.

The isotopic dilution values for phenylalanine- $^{14}\text{C}$  and cinnamic acid- $^{14}\text{C}$  suggest that PAL participates in the biosynthetic pathway. The increased PAL activity is not always proportional to the amount of phaseollin produced. We conclude that conversion of phenylalanine to cinnamic acid is not always the rate-limiting step. The decrease of PAL activity following an initial increase could be indicative of a PAL-inactivating system, as suggested by Zucker (16).

Knowledge derived from the characterization of phaseollin precursors, and the time course of phaseollin production and of PAL activation, should increase the precision in evaluating the role of phaseollin in infected tissue and the induction mechanism of the disease resistance response.

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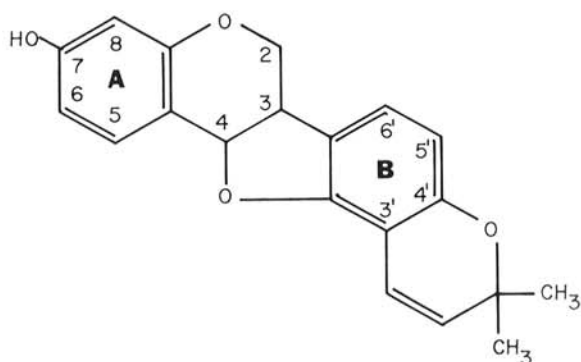


Fig. 2. Structure of phaseollin after Perrin (11).

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