Soybean Phytoalexins: Rates of Synthesis Are Not Regulated by Activation of Initial Enzymes in Flavonoid Biosynthesis

J. E. Partridge and N. T. Keen

Graduate Research Associate and Associate Professor, respectively, Department of Plant Pathology, University of California, Riverside, CA 92502. Present address of the senior author is the Department of Plant Pathology, University of Nebraska, Lincoln, NB 68503.

The research was supported by National Science Foundation Research Grants GB-35531 and BMS-75-03319 and by National Institute of Health Training Grant No. ES00084 from the National Institute of Environmental Health Sciences.

The authors acknowledge the assistance of D. C. Erwin and J. J. Sims with certain aspects of the work and are indebted to E. Wong, J. Bailey, and R. Lyne for counsel.

Accepted for publication 6 July 1976.

ABSTRACT

PARTRIDGE, J. E., and N. T. KEEN. 1977. Soybean phytoalexins: rates of synthesis are not regulated by activation of initial enzymes in flavonoid biosynthesis. Phytopathology 67: 50-55.

Soybean cultivars monogenically resistant or susceptible to *Phytophthora megasperma* var. *sojae* both showed activation of phenylalanine ammonia-lyase, chalcone-flavonone isomerase (CFI), and peroxidase after wounding or inoculation with the fungus. The similarity of the enzymatic responses in all wounded control and inoculated plants indicated that the activity increases were caused primarily by wounding and have no causal role in determining the rapid production of the pterocarpanoid phytoalexin, glyceollin, that occurs specifically in the resistant, inoculated plants. In agreement with the findings of others, two isozymes of soybean peroxidase both catalyzed the appearance of new products from 2′, 4, 4′-trihydroxy-chalcone. One peroxidase metabolite was positively identified as 4′, 7-dihydroxyflavon-3-ol and the other as a *spiro*-dienone which has been described by other workers. Isoenzymes of CFI were not found in soybeans.

Additional key words: phenylalanine ammonia-lyase, chalcone-flavonone isomerase, peroxidase, *Phytophthora megasperma* var. *sojae*, glyceollin.

The relation of antibiotic phytoalexins to disease resistance in higher plants has stimulated interest in understanding the biochemical mechanisms responsible for rapid phytoalexin production in genetically resistant plant cultivars and the corresponding slower production that occurs in near-isogenic susceptible cultivars. Soybean [*Glycine max* (L.) Merr.] plants that are monogenically resistant to race 1 of the pathogen *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb. exhibit rapid phytoalexin production after inoculation, but near-isogenic susceptible plants produce them at only 1-4% of that rate (11). There are at least four compounds in soybeans that may function as phytoalexins (12, 28). Keen et al. (12) isolated a pterocarpanoid phytoalexin and demonstrated by mass spectrometry and gas-liquid chromatography that it exists as three isomers. The tentative chemical structure of one of the isomers has been revised by Burden and Bailey (4) from the previous formulation, 6a-hydroxyphaseollin (26), to the isomeric structure shown in Fig. 1. Lyne et al. (16) recently formulated structures for the other two isomers (Fig. 1). Accordingly, we herewith propose renaming the pterocarpanoid soybean phytoalexin "glyceollin", a change endorsed by Burden and Bailey *(personal communication)*. We further suggest that the isomers be referred to as glyceollin I, II, and III (Fig. 1).

Previous studies with ^{14}C-labeled intermediates suggested that the production of glyceollin and several related isoflavonoids such as daidzein, coumestrol, and sojagol may involve activation of enzymes in the isoflavonoid biosynthetic pathway (13). This hypothesis

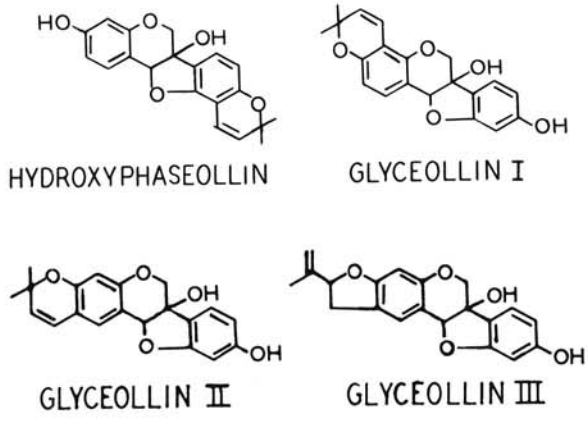


Fig. 1. Structures of 6a-hydroxyphaseollin and isomers I, II, and III of glyceollin, a soybean phytoalexin.

first was tested by studying (in near-isogenic resistant and susceptible soybean plants inoculated with *P. megasperma* var. *sojae*) the activities of several enzymes believed to be involved in the early steps of isoflavonoid biosynthesis. These were phenylalanine ammonia-lyase (PAL), chalcone-flavonone isomerase (CFI), and peroxidase. An abstract of part of this work was published previously (19).

MATERIALS AND METHODS

Six-day-old hypocotyls of susceptible Harosoy and near-isogenic resistant Harosoy 63 (H63) soybeans were wounded or inoculated with race 1 (P900) of *Phytophthora megasperma* var. *sojae* as previously described (12). Hypocotyl segments 1.5- to 2.0-cm long, encompassing the wounded areas, were harvested and acetone powders were prepared from them by a modification of the method of Rahe et al. (22). Powders were prepared in acetone at −78 C, lyophilized, suspended in sodium borate buffer (50 mM, pH 8.8), and centrifuged at 15,000 *g* for 20 minutes. The supernatant fluids were used as enzyme preparations.

Enzyme assays.—All assays were performed by monitoring absorbance changes with a Beckman Kintrac VII kinetics spectrophotometer with the cell compartment maintained at 30 C. Appropriate controls with substrate deleted and heat inactivated enzymes were included.

Phenylalanine ammonia-lyase (PAL) [E.C. 4.1.1.5] (14) was assayed by continuous recording of the absorption increase at 290 nm for 25 minutes. The increase was linear with time. The reaction mixture consisted of 400 μliters of enzyme preparation, 1.6 ml of sodium borate buffer (pH 8.8, 50 mM), and 1.0 ml of 15 mM phenylalanine.

Chalcone-flavonone isomerase (CFI) [E.C. 5.5.1.6] (17, 33) was assayed by continuously recording the loss of chalcone absorbance at 382 nm for 4 minutes; the rate was linear. Monitoring loss of absorbance was necessitated by the overlapping absorbances of substrate and product. Assays initially were performed at pH 8.6, but the rate curves for chalcone utilization suggested the possibility of more than one enzyme activity. It subsequently was found that, indeed, a second type of enzyme, peroxidase, was active in the crude extracts. Since the peroxidases were not appreciably active at pH 7.6, but CFI was, assays for the latter enzyme routinely were run at that pH. The extinction coefficient of the substrate under these conditions was (log ϵ = 4.56). The reaction mixture contained: 25 μliters of enzyme preparation, 3 ml of Tris-HCl buffer (50 mM, pH 7.6), and 50 μg of 2′, 4, 4′-trihydroxychalcone added in 10 μliters of absolute ethanol. When necessary, appropriate enzyme dilutions were made.

Peroxidase [E.C. 1.11.1.7] activity was monitored continuously by absorbance loss of 2′, 4, 4′-trihydroxychalcone at its absorption maximum ($\lambda_{max}^{pH\ 9.6}$ = 428 nm, log ϵ = 4.47) for 4 minutes. The reaction mixture consisted of 25 μliters enzyme preparation, 3 ml of glycine-NaOH buffer (pH 9.6, 50 mM), and 50 μg 2′, 4, 4′-trihydroxychalcone in 10 μliters of absolute ethanol. The rate of this reaction was linear after the first minute and very little CFI activity was observed at that pH. Peroxidase activity also was monitored by measuring absorbance increase at 460 nm as described by Shannon et al. (25) who used *o*-dianisidine as the substrate.

Lipoxygenase [E.C. 1.99.2.1] activity was monitored by recording absorbance increase at 234 nm (27) in a reaction of 1.6 μliters linoleic acid and 25 μliters enzyme preparation in 2 ml of 0.12 M sodium borate-HCl buffer, pH 9.0.

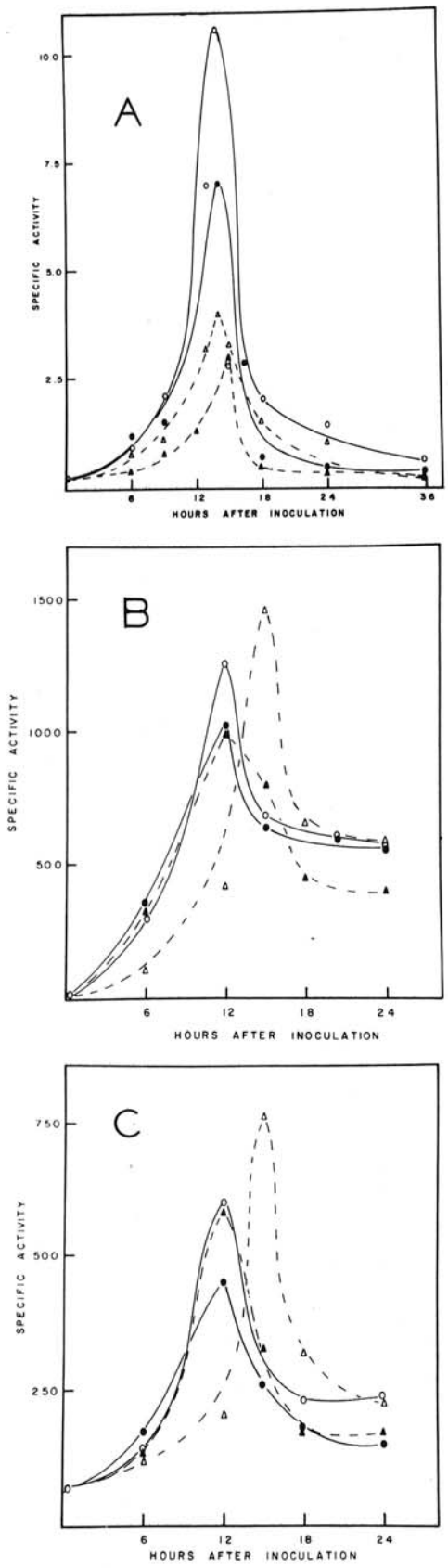
Enzyme activities were expressed in terms of μmoles product formed (PAL) or substrate utilized (CFI and peroxidase) per minute per mg protein. The protein content of enzyme preparations was determined by the Lowry method (15).

Purification of chalcone-metabolizing enzymes.—Healthy Harosoy 63 soybean hypocotyls were harvested and extracted in 50 mM sodium borate buffer, pH 8.0, by Moustafa and Wong's method (17). After the ammonium sulfate cut, the final supernatant was dialyzed against 1.25 mM Tris-HCl, pH 7.5, for 18 hours. The dialyzed enzyme preparation was centrifuged and the supernatant fluids placed on an LKB Model 8100 isoelectric focusing column. The gradients used were: sucrose (0-50%), Ampholine, pH 3-10 (LKB), and the dialyzed protein solution. The anode was at the bottom of the column. The column was run at 5 C for 120 hours at 300 V and 7.5 mA or less. The longer than usual run times gave superior resolution and good reproducibility. At the end of each run, the column was eluted and 1.5- to 2.0-ml fractions were collected. Fractions were assayed for peroxidase and CFI.

Isolation of peroxidase-catalyzed products from 2′, 4, 4′-trihydroxychalcone.—Two methods were used. A batch method was employed in which 2′, 4, 4′-trihydroxychalcone in ethanol (5 mg/ml) was added to several liters of pH 9.6 0.05 M glycine-NaOH buffer so that the final chalcone concentration was 33 mg/liter. Either soybean peroxidase or horseradish peroxidase (HRP) was added to the mixture and allowed to react until further decoloration did not occur. Alternatively, purified HRP was immobilized on Sepharose 4B (21) and a 2.5-cm × 4-cm column prepared in 0.05 M glycine-NaOH, pH 9.6. Then the chalcone in the same buffer (33 mg/liter) was passed through the column at approximately 100 ml/hour. The products from both methods were adjusted to pH 4 with HCl, concentrated at 45 C, and extracted several times with ethyl acetate. The extract was dried, dissolved in ethanol, and chromatographed on 0.375-mm layers of silica gel GF_{254} with a hexane, ethyl acetate, methanol (50:50:5, v/v) solvent system. Bands were eluted with acetone and rechromatographed on silica gel GF_{254} with $CHCl_3$, acetone, NH_4OH (60:40:5, v/v). After a second elution, the isolated compounds were found to chromatograph as single spots in TLC system using several solvents.

Synthesis of compounds.—*Isoliquiritigenin (2′, 4, 4′-trihydroxychalcone).*—This compound was prepared by Geissman and Clinton's cold condensation method (8). The product was recrystallized several times from 40% aqueous ethanol; m.p. 203-203.5 C [lit. 202-203 C (8)].

4′, 7-dihydroxyflavonone.—One g of 2′, 4, 4′-trihydroxychalcone was dissolved in 100 ml of methanol and saturated aqueous sodium acetate added (20); the final product was recrystallized several times from



aqueous ethanol; m.p. 205-208 C [lit. 204-208 C (33)].

4', 7-dihydroxyflavon-3-ol.—This was synthesized by Wong's method (32) and recrystallized several times from aqueous ethanol; m.p. 304-305 C [lit. 304-306 C (32)].

RESULTS

Enzyme activities.—Phenylalanine ammonia-lyase increased in inoculated Harosoy 63 hypocotyls after 6 hours (Fig. 2-A) and the increase continued until 15 hours, reaching a specific activity 55 times greater than at zero time (basal level). This was followed by a subsequent decrease, and the basal enzyme level was again attained within 36 hours. Specific PAL activity curves for wounded but noninoculated Harosoy 63 hypocotyls were of the same shape but of a lower magnitude, reaching a 35-fold greater specific activity than at zero time (Fig. 2-A). Both wounded and inoculated Harosoy hypocotyls also showed activity increases, reaching maxima of 15- to 20-fold the initial specific activity at 15-17 hours and then declining (Fig. 2-A).

Chalcone-metabolizing activity, later found to be a mixture of CFI and peroxidase, showed curves similar to the PAL data, except that the most dramatic increase was observed in the susceptible, inoculated Harosoy hypocotyls.

When CFI was assayed under conditions (pH 7.6) that did not favor peroxidase (Fig. 2-B) increased specific activity was seen after 6 hours in all treatments. Inoculated resistant hypocotyls reached their maximum of 125-fold the basal activity by 12 hours and then declined to a 55-fold greater activity than the zero- time controls. Changes in CFI activity were nearly identical in both wounded controls and were similar to the resistant inoculated plants (Fig. 2-B). Although CFI increased in inoculated Harosoy hypocotyls, the increase was slower than in the resistant inoculated hypocotyls (Fig. 2-B).

Similar to the curves for CFI, peroxidase specific activity (Fig. 2-C) reached its maximum of 8-10 times the basal level in Harosoy wounded and H63 wounded and inoculated plants after 12 hours. Activity in inoculated Harosoy plants again lagged by approximately 3 hours behind these other treatments, reaching a maximum at 12-13 hours. Peroxidase in all treatments decreased to the basal activity within 24 hours.

Purification of chalcone-metabolizing enzymes.—The enzyme kinetic curve of activity versus time initially raised the possibility that chalcone-metabolism assays at pH 8.6 reflect the activity of more than one enzyme in crude soybean extracts (Fig. 3). Three chalcone-metabolizing enzymes were subsequently recovered from isoelectric focusing columns (Fig. 4), with isoelectric points at pH 5.99 for A, 4.71 for B_1, and 8.53 for B_2. The

Fig. 2-(A to C). Specific activity of **A)** phenylalanine ammonia-lyase. **B)** chalcone-flavonone isomerase, and **C)** peroxidase extracted from Harosoy and Harosoy 63 soybean hypocotyls at various times after wounding and/or inoculation with race 1 of *Phytophthora megasperma* var. *sojae*. Legend: open circles = Harosoy 63 inoculated; closed circles = Harosoy 63 wounded only; open triangles = Harosoy inoculated; and closed triangles = Harosoy wounded only.

pH optima for chalcone metabolism were pH 7.9 for peak A and pH 9.6 for both peaks B_1 and B_2. Only peak A catalyzed the formation of 4′, 7-dihydroxyflavonone (II, Fig. 5) as determined by isolation of the product. This indicated that only peak A contained CFI activity. Rerunning the protein in peak A on the electrofocusing column with a narrower (pH 3-6) gradient failed to disclose the presence of isoenzymes. The UV spectrum of the major product from peaks B_1 and B_2 gave an absorption maximum of 385 nm at pH 9.6. Isolation of this reaction product from peaks B_1 and B_2 showed a parent ion at $M^+ = 270$, therefore indicating that an oxidation had occurred. Lipoxygenase and peroxidase, two known oxygenases from soybeans, were accordingly examined for ability to metabolize the chalcone. Lipoxygenase proved to be inactive, but purified horseradish peroxidase produced the same m/e 270 metabolite as the soybean enzymes B_1 and B_2 from the electrofocusing column and also gave rise to a second minor product. Peroxidase assays using *o*-dianisidine also showed that peaks B_1 and B_2 contained peroxidase activity. Neither B_1 nor B_2 was active on 4′, 7-dihydroxyflavonone, the product of CFI. Typical inhibitors of peroxidase confirmed the identifications of B_1 and B_2 as this enzyme. No oxidation of the chalcone occurred under N_2, but the reaction proceeded when O_2 was then bubbled into the reaction mixture. Sodium azide and hydrogen peroxide treatments were not informative since, as previously reported by Rathmell and Bendall (24), these compounds reacted with the chalcone substrate in the absence of enzyme. Potassium cyanide, however, inhibited the reaction as expected.

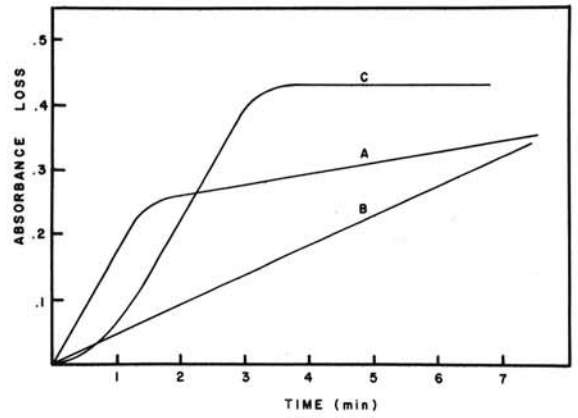


Fig. 3. Kinetic curves for loss of absorbance of 2′, 4, 4′-trihydroxychalcone catalyzed by: (curve A) crude extract from soybean hypocotyls; (curve B) purified chalcone-flavonone isomerase (CFI); and (curve C) purified soybean peroxidase.

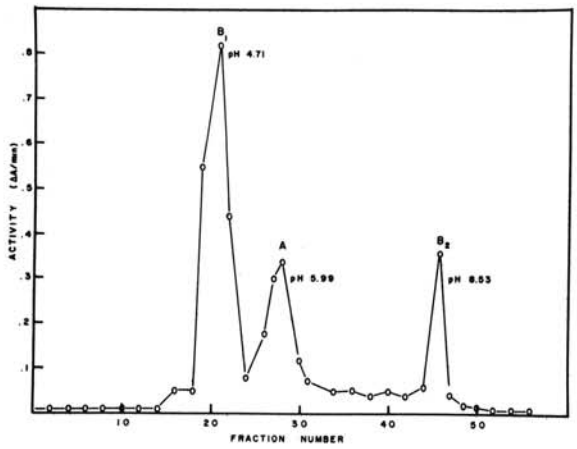


Fig. 4. Elution profile of isoelectric focusing column run of soybean hypocotyl proteins containing chalcone-metabolizing activity. Run conditions: pH 3-10 Ampholine gradient, 1-50% sucrose gradient, 300 V, 120 hours at 5 C, anode at the bottom of the column. Fractions (42 drops = 1.6 to 2.0 ml) were collected and assayed for chalcone-metabolizing activity. Peak "A" was found to contain chalcone-flavonone isomerase activity and "B_1" and "B_2" contained peroxidase activity.

Isolation of peroxidase products from 2′, 4, 4′-trihydroxychalcone.—Three products were detected in reaction mixtures containing peroxidase and 2′, 4, 4′-trihydroxychalcone (I, Fig. 5). One appeared to be 4′, 7-dihydroxyflavonone, (II, Fig. 5) formed nonenzymatically, and tentatively identified by comparison of its UV- and mass spectra with a synthetic sample. Another tentatively was identified as 4′, 7-dihydroxyflavon-3-ol (V, Fig. 5) by spectral data and synthesis. The other compound was weakly yellow-colored on TLC plates and fluoresced weakly blue at 360 nm and absorbed at 254 nm. It gave a more pronounced yellow color with NH_3 vapors or when sprayed with 50% aqueous H_2SO_4. This purified chalcone metabolite was a solid but could not be crystallized. It had an absorption maximum at 330 nm at neutral pH and 385 nm in alkali. The IR spectrum ($CHCl_3$) indicated the presence of hydroxyl groups and possibly more than one species of carbonyl. The mass spectrum showed a parent ion and base peak at m/e 270, and other prominent peaks at m/e 177, 148, 137, 121, 120, 110, and 94. The NMR spectrum ($CDCl_3$) of the compound after D_2O exchange disclosed the presence of seven aromatic protons, but no

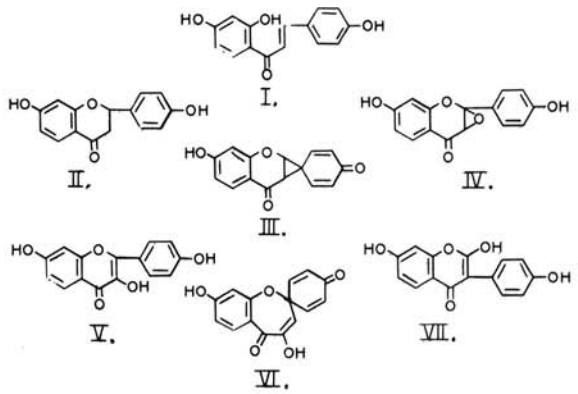


Fig. 5. Structures discussed in the text and possible intermediates in the peroxidase-mediated oxidation of isoliquiritigenin. I = 2′, 4, 4′-trihydroxychalcone (isoliquiritigenin); II = 4′, 7-dihydroxyflavonone; III = Proposed *spiro*-dienone intermediate of chalcone oxidation (6); IV = 4′, 7-dihydroxy-2,3-epoxyflavonone; V = 4′, 7-dihydroxyflavon-3-ol; VI = dihydroxy-*spiro*-dienone (35); VII = 2, 4′, 7-trihydroxyisoflavone.

other protons were detected. Several structural possibilities were considered, among which were a 4′, 7-dihydroxy-2,3-epoxyflavonone (IV, Fig. 5) and 2, 4′, 7-trihydroxyisoflavone (VII, Fig. 5). The former structure was not supported by the IR data and 2-hydroxyisoflavones have never been observed. Wong and Wilson reported a peroxidase metabolite from isoliquiritigenin that appeared to be identical to our $M^+ = 270$ metabolite and formulated it as the *spiro*-dienone (VI, Fig. 5), (34, 35 and E. Wong, *personal communication*). We tentatively suggest this structure for our peroxidase-mediated oxidation product of 2′, 4, 4′-trihydroxychalcone.

DISCUSSION

Wounding and/or inoculation of soybean hypocotyls with *P. megasperma* var. *sojae* increased PAL activity, similar to other stimuli in various plants (1, 9, 29, 30). The similarity of PAL activity curves in wounded and inoculated Harosoy and Harosoy 63 plants indicates that PAL activation is either a simple wound response and/or a nonspecific response to the fungus. Ebel et al. (7) have recently shown that suspension-cell cultures of soybeans underwent PAL activation when exposed to a high molecular weight phytoalexin elicitor. Since no cell-wounding should have occurred in their experiments, the PAL activation must have been a direct consequence of elicitor activity. The suspension-culture experiments also showed an activation of PAL before the production of glyceollin, again similar to our data with intact plants. This further suggests that PAL activation has no essential role in glyceollin biosynthesis. If the elevated PAL levels represented the control mechanism of phytoalexin synthesis, then PAL activity would be expected to remain high during rapid glyceollin synthesis. Instead it returned to the basal level at 30 hours, a time when rapid glyceollin biosynthesis occurs (11). As such, PAL activation clearly would seem to have no determinative relationship to the rapid isoflavonoid biosynthesis seen in inoculated H63 soybean plants. These conclusions are similar to those reached by Munn and Drysdale (18) for PAL activities in cowpea plants.

The kinetics of CFI and peroxidase activities (Fig. 2-B and 2-C) indicate that, irrespective of the resistance genotype of the host plant, wounded and inoculated plants exhibit activated levels of these enzymes. One difference was the consistently slower (approximately 3 hours) activation of peroxidase and CFI in inoculated Harosoy plants.

Despite the small differences in production of peroxidase and CFI, there is, however, little basis on which to suggest that they are determinatively linked to the basis for rapid glyceollin production in inoculated Harosoy 63 soybeans and the slower production that occurs in Harosoy. We conclude therefore, that the responses of all three enzymes studied are most likely caused by wounding and have no apparent role in the determination of resistance and susceptibility. It is likely that the determinative enzymatic events occur later in the metabolic pathway to isoflavonoids (13, 31); unfortunately, no enzymes catalyzing these latter biosynthetic steps have yet been demonstrated in cell-free preparations.

Purification of the chalcone-metabolizing enzymes revealed three distinct enzymes with catalytic activity toward 2′, 4, 4′-trihydroxychalcone. One of the enzymes was chalcone-flavonone isomerase, but the other two were clearly peroxidases. Therefore, like Boland and Wong (3), we could not confirm the report for CFI isozymes made by Hahlbrock et al. (10) for related plants. Curtis and Barnett (5) recently obtained (by electrofocusing techniques) two peaks of peroxidase activity from soybean hypocotyl cell walls. The isoelectric points for the two peroxidases observed in our work (pH 4.7 and 8.5) agree closely with their findings.

Purified soybean peroxidase and horseradish peroxidase produced two products from 2′, 4, 4′-trihydroxychalcone. These tentatively were identified as 4′, 7-dihydroxyflavon-3-ol (V, Fig. 5) (23, 24) and a new unknown metabolite to which structure VI was recently assigned by Wong and Wilson (34, 35). We did not find evidence to support the hypothesis that the peroxidase metabolite was a 2-hydroxyisoflavone (VII, Fig. 5) produced by an aryl migration of an isoliquiritigenin intermediate. Although peroxidase has been shown to catalyze these migrations in other systems (2), no evidence for such activity on chalcones thus far has been obtained. Nevertheless, it is apparent that oxidation of the hypothetical *spiro*-dienone intermediate III (Fig. 5) (6, 20) by peroxidase could lead to the 2-hydroxyisoflavone as well as to 4′, 7-dihydroxyflavon-3-ol (V, Fig. 5).

Wong and Wilson (36) have published more complete data on the peroxidase metabolite (VI, Fig. 5).

LITERATURE CITED

1. AMRHEIN, N., and M. H. ZENK. 1970. Concomitant induction of phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase during illumination of excised buckwheat hypocotyls. Naturwissenschaften 57:312.
2. BAKER, P. B., and B. C. SAUNDERS. 1974. Studies in peroxidase action. XXIV. The preparation and peroxidase oxidation of 4-phenyl-2,6-dimethyl-aniline. Tetrahedron 30:3303-3308.
3. BOLAND, M. J., and E. WONG. 1975. Purification and kinetic properties of chalcone-flavanone isomerase from soya bean. Eur. J. Biochem. 50:383-389.
4. BURDEN, R. S., and J. A. BAILEY. 1975. Structure of the phytoalexin from soybean. Phytochemistry 14:1389-1390.
5. CURTIS, C. R., and N. M. BARNETT. 1974. Isoelectric focusing of peroxidases from soybean hypocotyl cell walls by Sclerotium rolfsii culture filtrate. Can. J. Bot. 52:2037-2040.
6. DEWICK, P. M. 1972. Biosynthesis of rotenoids and coumestans. Hoppe Seyler's, Z. Physiol. Chem. 353:132-133.
7. EBEL, J., A. R. AYERS, and P. ALBERSHEIM. 1976. Host-pathogen interactions. XII. Response of suspension-cultured soybean cells to the elicitor isolated from Phytophthora megasperma var. sojae, a fungal pathogen of soybeans. Plant Physiol. 57:775-779.
8. GEISSMAN, T. A., and R. W. CLINTON. 1946. Flavanones and related compounds. I. The preparation of polyhydroxychalcones and flavanones. J. Am. Chem. Soc. 68:697-700.
9. HADWIGER, L. A. 1972. Increased levels of pisatin and phenylalanine ammonia lyase activity in Pisum sativum treated with antihistaminic, antiviral, antimalarial,

tranquilizing, or other drugs. Biochem. Biophys. Res. Commun. 46:71-79.
10. HAHLBROCK, K., E. WONG, L. SCHILL, and H. GRISEBACH. 1970. Comparison of chalcone-flavanone isomerase heteroenzymes and isoenzymes. Phytochemistry 9:949-958.
11. KEEN, N. T. 1971. Hydroxyphaseollin production by soybeans resistant and susceptible to Phytophthora megasperma var. sojae. Physiol. Plant Pathol. 1:265-275.
12. KEEN, N. T., J. J. SIMS, D. C. ERWIN, E. RICE, and J. E. PARTRIDGE. 1971. 6a-hydroxyphaseollin: an antifungal chemical induced in soybean hypocotyls by Phytophthora megasperma var. sojae. Phytopathology 61:1084-1089.
13. KEEN, N. T., A. I. ZAKI, and J. J. SIMS. 1972. Biosynthesis of hydroxyphaseollin and related isoflavanoids in disease-resistant soybean hypocotyls. Phytochemistry 11:1031-1039.
14. KOUKOL, J., and E. E. CONN. 1961. The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of Hordeum vulgare. J. Biol. Chem. 236:2692-2698.
15. LOWRY, O. H., N. J. ROSEBROUGH, L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
16. LYNE, R. L., L. J. MULHEIRN, and D. P. LEWORTHY. 1976. New pterocarpinoid phytoalexins of soybean. Chem. Commun. (J. Chem. Soc., Sect. D). (In press).
17. MOUSTAFA, E., and E. WONG. 1967. Purification and properties of chalcone-flavanone isomerase from soya bean seed. Phytochemistry 6:625-632.
18. MUNN, C. B., and R. B. DRYSDALE. 1975. Kievitone production and phenylalanine ammonia-lyase activity in cowpea. Phytochemistry 14:1303-1307.
19. PARTRIDGE, J. E., and N. T. KEEN. 1972. Enzymatic responses of soybean hypocotyls to wounding and inoculation with Phytophthora megasperma var. sojae. Phytopathology 62:781-782 (Abstr.).
20. PELTER, A., J. BRADSHAW, and R. F. WARREN. 1971. Oxidation experiments with flavonoids. Phytochemistry 10:835-850.
21. PORATH, J., K. ASPBERG, H. DREVIN, and R. AXEN. 1973. Preparation of cyanogen bromide-activated Agarose gels. J. Chromatogr. 86:53-56.
22. RAHE, J. E., J. KUC, and C. CHUANG. 1970. Cinnamic acid production as a method of assay for phenylalanine ammonia-lyase in acetone powders of Phaseolus vulgaris. Phytochemistry 9:1009-1015.
23. RATHMELL, W. G., and D. S. BENDALL. 1972. The oxidation of a chalcone to a dihydroflavonol by crystalline horse-radish peroxidase. Phytochemistry 11:873.
24. RATHMELL, W. G., and D. S. BENDALL. 1972. The peroxidase-catalysed oxidation of a chalcone and its possible physiological significance. Biochem. J. 127:125-132.
25. SHANNON, L. M., E. KAY, and J. Y. LEW. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. 241:2166-2172.
26. SIMS, J. J., N. T. KEEN, and V. K. HONWAD. 1972. Hydroxyphaseollin, an induced antifungal compound from soybeans. Phytochemistry 11:827-828.
27. STEVENS, F. C., D. M. BROWN, and E. L. SMITH. 1970. Some properties of soybean lipoxygenase. Arch. Biochem. Biophys. 136:413-421.
28. SVOBODA, W. E., and J. D. PAXTON. 1972. Phytoalexin production in locally cross-protected Harosoy and Harosoy 63 soybeans. Phytopathology 62:1457-1460.
29. WALTON, D. C. 1968. L-phenylalanine ammonia-lyase activity during germination of Phaseolus vulgaris. Plant Physiol. 43:1120-1124.
30. WALTON, D. C., and E. SONDHEIMER. 1968. Effects of abscisin II on phenylalanine ammonia-lyase in excised bean axes. Plant Physiol 43:467-469.
31. WONG, E. 1970. Structural and biogenetic relationships of isoflavonoids. Fort. Chem. Organ. Natur. 28:1-73.
32. WONG, E., P. I. MORTIMER, and T. C. GEISSMAN. 1965. Flavonoid constituents of Cicer arietinum. Phytochemistry 4:89-95.
33. WONG, E., and E. MOUSTAFA. 1966. Flavanone biosynthesis. Tetrahedron Lett. p. 3021-3022.
34. WONG, E., and T. M. WILSON. 1972. The oxidation of chalcones catalyzed by peroxidase. Phytochemistry 11:875.
35. WONG, E., and T. M. WILSON. 1972. The oxidation of chalcones by peroxidase. Hoppe Seyler's Z. Physiol. Chem. 353:132.
36. WONG, E., and J. M. WILSON. 1976. Products of the peroxidase-catalysed oxidation of 4, 2', 4'-trihydroxy-chalcone. Phytochemistry 15:1325-1332.