Development of a Radioimmunoassay for the Soybean Phytoalexin Glyceollin I

PETER MOESTA, MICHAEL G. HAHN, HANS GRISEBACH
Biologisches Institut II der Universität, Schanzlestrasse 1, D-7800 Freiburg i.Br., Federal Republic of Germany

ABSTRACT
A radioimmunoassay for glyceollin I, the major phytoalexin produced by soybean (Glycine max [L.] Merr.), has been developed. Antibodies were raised in rabbits against a glyceollin I-bovine serum albumin conjugate. The antisera were used to establish a radioimmunoassay for glyceollin I using [125I]glyceollin I as the tracer. A logit plot of a standard concentration series yielded a straight line in the range of 1 to 100 picomoles (0.34–34 nanograms) of glyceollin I. The structurally related pterocarpan phytoalexins, glyceollin II and III, glyceollidin II and glycinol, which also accumulate in infected soybean tissue, show a low cross-reactivity in the radioimmunoassay (0.5–5% at 50% displacement of the tracer). Two related isoflavones present constitutively in soybean tissue, daidzein and genistein, have cross-reactivities of less than 0.84% and 1.1%, respectively. The radioimmunoassay permitted the quantitative determination of glyceollin I in 15-micrometer microscope sections of soybean hypocotyl tissue infected with zoospores of Phytophthora megasperma f. sp. glycinea.

Phytoalexins are low mol wt compounds with antimicrobial properties which accumulate post-infectionally in plants from various families (2, 5). Because of these characteristics, an important role in plant disease resistance has been suggested for phytoalexins, and considerable circumstantial evidence for such a role has accumulated (2). To evaluate the significance of phytoalexins in plant disease resistance, quantitative knowledge of their exact spatial and temporal distribution within plant tissue at and near infection structures of invading microorganisms is of great importance.

Fluorescence and UV microspectrophotometry have been used in several studies to show the localization of phytoalexins at infection sites (6, 9, 10). This technique is limited to systems in which characteristic fluorescence and/or absorption spectra for the phytoalexin(s) can be obtained from the tissue being examined. Furthermore, quantitation of the phytoalexins by this technique is difficult. Techniques such as TLC, GLC, and HPLC are not sufficiently sensitive to quantify phytoalexins in the extremely small amounts of tissue immediately surrounding infection sites. We have recently applied laser microprobe mass analysis (LAMMA) to detect the soybean phytoalexin, glyceollin, at the cellular level (14). This method permits an analysis of glyceollin content in infected tissue with high lateral resolution. However, LAMMA requires a very expensive instrument and elaborate tissue preparation. Furthermore, accurate quantitation of glyceollin in tissue by this technique is not yet possible.

The radioimmunoassay is a simple, highly selective and sensitive technique that has been used to quantitate compounds such as IAA (18), ABA (17), and cAMP (16), that occur in low concentrations in plant tissues. This suggested that this technique might be applicable to the quantitation of phytoalexins in the cell layers immediately surrounding infection sites. We report here the successful development of a radioimmunoassay for glyceollin I (Fig. 1), the major phytoalexin produced by soybeans (Glycine max).

MATERIALS AND METHODS

Soybean Seedlings. Seeds of soybean (Glycine max [L.] Merr., cv Harosoy 63) were obtained from R. I. Buzell (Harrow, Ontario, Canada). Seedlings were grown in vermiculite for 5 d as described previously (1).

Fungal Cultures. Phytophthora megasperma Drechs. f.sp. glycinea Kuan and Erwin races 1 and 3 were obtained from B. L. Keeling (Stoneville, MS) and were grown as described (1). Zoospores were obtained from 6-d-old cultures according to a published method (4).

Chemicals. BSA was purchased from Sigma (München, FRG); p-aminohippuric acid, chloramine T, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl from Merck (Darmstadt, FRG); sodium [125I]iodide from Amersham (Braunschweig, FRG). Normal rabbit serum was obtained from Paesel (Frankfurt, FRG) and Tissue Tek II O.C.T. Compound from Miles Labs. (Naperville, IL).

Pterocarps and Isoflavones. Glycinol and glyceollin isomers I, II, and III were purified by HPLC (13). Glyceollidin II was obtained from J. Leube of our laboratory. Daidzein and genistein were from our laboratory collection. Standard solutions of the pterocarps and isoflavones were prepared using the following extinction coefficients: glycinol, ε280 = 5,870 (19); glyceollin I, ε280 = 10,300 (1); glyceollin II, ε280 = 8,700 (8); glyceollin III, ε280 = 9,600 (8); daidzein, ε280 = 27,480 (3); genistein, ε280 = 44,100 (W. Barz, personal communication). An ε260 = 10,000 was estimated for glyceollidin II by analogy to the glyceollin isomers.

Buffers. The following buffers were used: buffer A, 0.01 M potassium phosphate (pH 7.0); buffer B, 0.01 M Na-phosphate (pH 7.4) containing 0.15 M NaCl and 0.1% (w/v) NaN3.

HPLC. HPLC was performed on a 9–x 250-mm silica gel column (Lichrosorb Si 60 5 μm) (Merck) with a solvent mixture of n-hexanciscopropanol, 95:5 (v/v), at a flow rate of 3 ml/min. Column effluent was monitored at 280 nm.

Synthesis of Glyceollin IBSA Conjugate. p-Aminohippuric
Acid was coupled to BSA with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl according to the procedure of Weiler (17). Ten mg of the conjugate were suspended in 5 ml H2O and the pH adjusted to 1.5 with 1 N HCl. This solution was cooled to 0°C, and a solution of 60 mg NaNO2 in 0.5 ml H2O was slowly added. After 5 min, excess nitrite was destroyed by addition of 30 mg ammonium sulfamate in 0.5 ml H2O. The diazotized protein was then added dropwise to a stirred solution of 8 mg glyceollin I in 10 ml of 50% (v/v) methanol in 0.1 M borate buffer (pH 9.0) at 0°C. After 30 min, the deep orange solution was dialyzed for 5 d against repeated changes of distilled H2O and subsequently lyophilized. The molar ratio of glyceollin I to BSA was determined using [125I]glyceollin I and found to be 42 and 53 in two experiments.

Preparation of Antiserum. Ten mg of the glyceollin-I-BSA conjugate were suspended in 5 ml of a 0.25% (w/v) NaHCO3 solution, and mixed in a 1:1 ratio with complete Freund’s adjuvant. Aliquots (2 ml) of this suspension were injected into the foot pads, and subcutaneously and intramuscularly at multiple sites of each of four rabbits (New Zealand White). Booster injections were given subcutaneously and intramuscularly 5 and 17 weeks later with 2 and 1 mg of conjugate per rabbit, respectively. Antisera were obtained 6, 7, 8, and 18 weeks after the first injection.

Synthesis of [125I]Glyceollin I Tracer. In a glass test tube, 0.5 μg glyceollin I (1.48 nmol) in 100 μl methanol were mixed with 0.56 μg chloramine T (2 nmol) dissolved in 100 μl buffer A. A solution of 17.5 ng Na125I (0.14 nmol) in 50 μl buffer A was then added. After incubation for 4 min at 0°C, the reaction was terminated by addition of 1 μg sodium metabisulfite (5 nmol) dissolved in buffer A. The solution was then extracted three times with 1 ml chloroform. The chloroform phase was dried over anhydrous sodium sulfate and applied to a SepPak silica cartridge (Waters, Königstein, FRG) and the cartridge eluted with 8 ml of chloroform. The eluate was evaporated to dryness and redissolved in 50% (v/v) methanol in buffer B. A yield of 36% was calculated based on the recovery of 125I in labeled product. The [125I]glyceollin I tracer was stored at -20°C until needed, and was diluted to about 2 × 10^4 cpm/ml with 50% (v/v) methanol in buffer B for use in the radioimmunoassay.

Radioimmunoassay. Radioimmunoassays were carried out in Eppendorf vials (1.5 ml capacity). Each vial contained: 100 μl buffer B, 100 μl of sample in 10% (v/v) methanol in H2O, 50 μl [125I]glyceollin I tracer (~10^6 cpm) in 50% (v/v) methanol in buffer B, and 100 μl antiserum diluted 1:1000 in buffer B, added in that order and then mixed. The final titer of the antibody in the immununoassay was therefore 1:3500. After incubation for 15 h at 8°C, 100 μl of normal rabbit serum diluted 1:10 in buffer B were added, followed by 0.5 ml of a saturated (NH4)2SO4 solution. The final solutions, after mixing, were 53% saturated with respect to (NH4)2SO4. The solutions were incubated for 30 min at room temperature and then centrifuged at 8800g for 4 min. The supernatants were carefully decanted and discarded. The pellets were washed once by suspension in 0.75 ml of a 50% saturated (NH4)2SO4 solution and centrifugation at 8800g. The supernatant was again discarded, and the vials containing the pellets were counted in a gamma counter. A complete standard curve with glyceollin I, as well as a blank control containing no antisera and a sample containing only tracer, were included in each assay.

Hypocotyl Inoculation Procedure. Five-d-old seedlings were fixed on glass plates and the roots covered with a layer of Zellstoff (C. A. Roth, Karlsruhe, FRG) soaked in distilled H2O. Ten μl of spore suspension (~10^6 spores/ml) was applied to the hypocotyl about 1.5 cm below the cotyledons. Subsequently, the seedlings were incubated at 25°C and 100% humidity in the dark for 24 h.

Quantitation of Glyceollin I in Infected Soybean Hypocotyls. Hypocotyl segments about 3 mm long centered on the infection site were excised and embedded in Tissue Tek II OCT compound, and were immersed in liquid N2 slowly to avoid cracking the embedding material. Imbedded tissues were stored at -70°C. Serial sections (15 μm) were cut parallel to the epidermis using a freeze microtome (R. Jung, Heidelberg, FRG) at -20°C. Every other section was extracted with 1 ml of 10% (v/v) methanol in H2O, and 0.1-ml aliquots of the extracts were analyzed for glyceollin I in the radioimmunoassay. The remaining tissue sections mounted on microscope slides were examined for fungal hyphae by means of an indirect immunofluorescent stain (15). The area of the sections was also determined under the microscope, thus allowing the volumes of the sections to be calculated.

Results and Discussion

Generation of Antiserum. Glyceollin I was coupled in good yield to a diazotized p-aminophenillic acid derivative of BSA. Injection of the conjugate into rabbits led to the production of antiserum against glyceollin I (Fig. 2). Sera obtained 1 week after the second injection were able to bind up to 60% of a [125I]-labeled glyceollin I tracer at an antibody dilution of 1:1000. The antibody titer was not increased following a second booster injection after 17 weeks (Fig. 2). The antibody titer is low when compared with antibodies generated to mammalian peptide hormones for example, but is not out of line with titers obtained against other small molecules such as IAA or ABA (17, 18). Although all four rabbits produced antiserum to glyceollin I, sera obtained from one rabbit were used throughout the present study.

Preparation of the [125I]Glyceollin I Tracer. In initial experiments, glyceollin I was iodinated with unlabeled sodium iodide and chloramine T, and the products analyzed by HPLC as described in "Materials and Methods." Two iodinated glyceollin derivatives were obtained with retention times of 9.5 min (product A) and 11.5 min (product B). The ratio of these two products depended on the molar ratio of glyceollin I to iodide in the reaction mixture. At a molar ratio of 1:1 the product A to product B ratio was 3 to 7. When a 10-fold excess of glyceollin I was used in the iodination reaction, formation of product A could be suppressed almost completely. Since only product B behaved similarly to unlabeled glyceollin I in the radioimmunoassay (see below), the latter conditions were chosen for the preparation of [125I]glyceollin I.

The [125I]glyceollin I tracer was not very water soluble, and significant amounts were absorbed from aqueous solutions onto the surface of the vials used for the radioimmunoassay. Several organic solvent additives (methanol, ethanol, acetone, Triton X-
FIG. 2. Formation of antisera against a glyceollin I-BSA conjugate in four immunized rabbits. Arrows indicate the times at which booster injections of the conjugate were given. The binding assay contained 100 µl of buffer B, 100 µl of 10% (v/v) methanol in H2O, 100 µl of antiserum diluted 1:1000 in buffer B, and 50 µl of [125I]glyceollin I (~10,000 cpm) in 50% (v/v) methanol in buffer B. The assay was incubated and the amount of binding determined as described for the radioimmunoassay in the text.

FIG. 3. Standard curve for the radioimmunoassay of glyceollin I. Each point represents the average, with SD, of seven independent assays with duplicates for each value. B0 = cpm of tracer bound in the absence of exogenous glyceollin I; B = cpm of tracer bound in the sample. Logit B/B0 = ln([B/B0]/(100-B/B0)), where B/B0 is expressed as a percentage.

100 µl (0.34–34 ng) of glyceollin I. The assay is therefore more than 1000-fold more sensitive than any other technique available for the quantitation of glyceollin I.

The assay blank was very sensitive to the protein used as the carrier to ensure complete precipitation of the antigen-antibody complex with (NH4)2SO4. For example, the use of normal bovine serum or rabbit gamma globulin as carrier resulted in unacceptably high blank values (as great as 33% of the total counts). Normal rabbit serum at a 1:10 dilution consistently yielded low blank values (<4% of the total counts).

The radioimmunoassay is very specific for glyceollin I (Table I). Glyceollin I and III, two structural isomers of glyceollin I (Fig. 1), show cross-reactivity only at much higher concentrations (20- to 100-fold). The same is true for two biosynthetic precursors of the glyceollins, glycinoI and glyceollid II. The related isoflavones, daidzein and genistein, showed no cross-reactivity even when assayed at 7.2 and 5.3 nmol, respectively. Thus, the presence of these structurally and biosynthetically related molecules will not interfere significantly with the detection of glyceollin I in tissue extracts using the radioimmunoassay.

The cross-reactivity of the two products obtained upon iodination of glyceollin I (see above) was also determined. Product A did not cross-react when assayed at 10 pmol. Product B showed a displacement curve that was indistinguishable from that of uniodinated glyceollin I. Inasmuch as synthesis of the radioactive tracer was carried out under conditions favoring the formation of product B, these results demonstrate that the iodination does not alter the binding of glyceollin I to the antibody.

The cross-reactivity data suggest that binding to the antibody is strongly influenced by the substituents on ring A of glyceollin I. The D ring is identical for all of the pterocarps tested. These results further imply that the diazo-coupling of glyceollin I to p-amino hippuric acid substituted BSA occurs ortho to the phenolic hydroxyl group in ring D. Iodination of glyceollin I to form product B probably occurs at the same location.

Quantitation of Glyceollin I in Infected Soybean Hypocotyl Tissue by Radioimmunoassay.

The applicability of the radioimmunoassay to the quantitation of phytoalexins in thin tissue sections was tested using soybean hypocotyl tissue (cv Harosoy 63) infected with zoospores of either race 1 (incompatible) or race 3 (compatible) of P. megasperma f. sp. glycinea. The amounts of glyceollin I present in extracts of 15 µm microtome sections of the infected hypocotyls were determined and the results are shown in Figure 4. Levels of glyceollin I that are inhibitory to P. megasperma in vitro (EC50 = 0.59 pmol/ml [7]) accumulate in soybean hypocotyl tissue in both the incompatible and the compatible interactions. Differences can be noted between the two interactions in the distribution of glyceollin I and the extent of fungal penetration. Whether these observed differences can be correlated with the expression of resistance must await a more detailed analysis of the distribution of glyceollin I vis-a-vis the extent of fungal penetration as a
function of time after infection. Still, these results clearly demonstrate that glyceollin I can be quantitated in very small amounts of soybean tissue using the radioimmunoassay.

The specificity of the radioimmunoassay for glyceollin I results in an underestimation of the total phytoalexin content of the hypocotyl sections. The predominant phytoalexins that accumulate in infected soybean hypocotyls are the glyceollin isomers I to III, which accumulate in a ratio of 8:1:1 (12). The amounts of the other pterocarpan phytoalexins produced in hypocotyl, glycinol, and the glyceollidins, are insignificant (12, 20). Thus, the radioimmunoassay gives a value for the total phytoalexin content of infected hypocotyl tissue that is about 20% too low.

The relative ratio of the various pterocarpan phytoalexins found in infected soybean tissue depends on the tissue used. For example, in soybean cotyledons, the glyceollin isomers accumulate in a ratio of 3:1:1 (14). Furthermore, glycinol, which does not accumulate in hypocotyl tissue, accounts for about half of the phytoalexins produced in the cotyledons (11, 19). Thus, one needs to determine the relative amounts of the various phytoalexins produced in a given tissue and then use antisera of the appropriate specificity if one wishes to obtain an accurate determination of the total phytoalexin content.

The results presented in this paper demonstrate that the use of a radioimmunoassay provides a significantly more sensitive technique for the quantitation of phytoalexins. This is the first application of this technique to the detection and quantitation of this important class of compounds in plants. The power of this technique is shown by the ability to quantitate phytoalexins in a few cell layers around sites of infection in a single hypocotyl. Together with the detection and quantitation of the hyphae of P. megasperma by indirect immunofluorescence (15), the radioimmunoassay will permit an exact correlation of phytoalexin accumulation with the degree of infection by different races of the fungus. Such studies could be complemented by LAMMA analyses of glyceollin content at the cellular level (14).

Acknowledgments—We thank Dr. D. K. Hammer for help in the preparation of antibodies and Dr. V. Speth for assistance in the preparation of the microtome sections.

LITERATURE CITED

18. Weiler EW 1981 Radioimmunoassay for p-methyl-quinoline-3-phenol-3-acetic acid for use with highly stable [3H]- and [3H]-IAA derivatives as radiotracers.

Fig. 4. Glyceollin I concentrations in soybean hypocotyls. After infection with zoospores of P. megasperma f.sp. glycinea race 1 (■) or race 3 (▲). Hypocotyl segments were sectioned parallel to the epidermis, the sections extracted, and glyceollin I quantitated in the extracts by radioimmunoassay, all as described in the text. The extent of hyphal penetration was determined using an indirect immunofluorescence stain (15). Heavy bars indicate regions in which significant numbers of fungal hyphae were observed for each race. Light bars indicate regions where single hyphae were observed.