Techniques

An Indirect Enzyme-Linked Immunosorbent Assay for Measurement of Abscisic Acid in Soybean Inoculated with *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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An indirect enzyme-linked immunosorbent assay (ELISA) was developed to measure the concentration of abscisic acid (ABA) in soybean hypocotyl tissues after inoculation with *Phytophthora megasperma* f. sp. *glycinea*. The assay, which uses monoclonal antibodies, has a measuring range of 5–250 pg of ABA. Validation of the assay with high-performance liquid chromatography showed that the assay is a sensitive and reliable method for the determination of ABA in crude tissue extracts. In an incompatible interaction the concentration of ABA in lesions was reduced to 32% of uninoculated control levels by 6 hr after inoculation.

The phytohormone abscisic acid (ABA) has been shown to regulate several biochemical and physiological processes within plants (1,23). It has primary roles at the whole plant level, especially in the response to stress, but is also involved at the cellular level in membrane transport and integrity (7,15) and in protein and RNA synthesis (2,3).

There is a wealth of information concerning the metabolism and physiological effects of ABA within healthy plants, but few studies of the involvement of ABA in plant disease. Whenham et al (21,22) found that in tobacco infected with tobacco mosaic virus there were localized changes in the concentrations of ABA. In this host-virus interaction, visible symptoms of infection included that of decreased growth, and an increase in ABA concentration was thought to contribute to this decrease. Others have found that ABA may suppress disease resistance or influence phytoalexin accumulation (6,8,9). We have shown recently that the addition of ABA to soybean hypocotyls suppressed production of a key enzyme in the phenylpropanoid pathway, phenylalanine ammonia lyase, and its mRNA (18). As a result, levels of the phytoalexin, glyceollin, were reduced and an incompatible interaction changed to one that was phenotypically compatible.

To date there have been no attempts to follow the course of changes in endogenous ABA concentrations after phytoalexin-elicitor application or after inoculation, especially during the early critical hours of the host-pathogen interaction. This may have been due in part to the lack of sensitivity or availability of physico-

chemical methods for ABA measurement.

Immunoassays are being used increasingly to determine the concentrations of phytohormones in plants (20). The great advantage of immunoassays over conventional bioassays and physico-chemical methods such as HPLC and GC-MS is that it is possible to analyze many samples quickly and without the loss of material experienced in most multi-step extraction procedures. In addition, due to the sensitivity of the assays (routinely to the picomole level) only small amounts of plant tissue are required for analysis. Immunoassays have been useful in plant pathology in the identification and quantitative measurement of disease-causing organisms, particularly viruses, but have not been used to determine the effects of disease on phytohormone concentration. In this paper we describe the development of an enzyme-linked immunoassay for the phytohormone abscisic acid and demonstrate its use in measuring ABA in soybean (*Glycine max* (L.) Herr.) hypocotyl tissue inoculated with *Phytophthora megasperma* (Drechs) f. sp. *glycinea* (Hildeb.) Kuan and Erwin.

MATERIALS AND METHODS

Chemicals and reagents. The following reagents were from Sigma Chemical Co.: bovine serum albumin (Fraction V); anti-mouse immunoglobulin G conjugated to alkaline phosphatase; *p*-nitrophenyl phosphate (Sigma 104); (±)-2-*cis,trans*-abscisic acid; (±)-2-*cis,trans*-abscisic acid methyl ester. (±)-2-*cis,trans*-[2^{14}C]-Abscisic acid was from Amersham, and (±)-2-*cis*-abscisic acid monoclonal antibodies were from Idetek Inc., San Bruno, CA.

Soybean cultivars, growth conditions, and inoculation. Seedlings of soybean cultivar Harosoy 63 (supplied by R. I. Buzzell, Agriculture Canada, Research Station, Harrow, Ontario), which is resistant to race 1 of *P. m. glycinea*, were grown in the dark as described previously (19). After 6 days' growth, seedlings were arranged in glass trays (19) and each hypocotyl inoculated by placing two 10-μl drops of a zoospore suspension (10^5 zoospores m^{-1}) 3 mm apart, 2–3 cm below the cotyledons. Control plants were treated similarly except that water was used in place of inoculum. The trays containing the inoculated seedlings were sealed with plastic film and placed in the dark at 25 C.

At 6 hr after inoculation two types of samples were collected: 1) sections 1 cm in length containing the inoculation sites, and 2) lesions alone. These were excised from each of 10 hypocotyls and the samples placed in small aluminum foil packets and immediately frozen in liquid nitrogen. Samples were then lyophilized and stored at −70 C until analyzed. To keep isomerization of naturally occurring (+)ABA to a minimum, the inoculation of plants and the collection and processing of tissue samples were carried out as rapidly as possible in dim light.

Extraction procedure. Lyophilized hypocotyl sections (each sample weighed approximately 200 mg) were ground to a powder at 4 C. The powdered sample was then extracted in 3 ml of ice-cold acidified methanol (methanol/acetic acid, 99:1) with 10 mg of butylated hydroxytoluene per liter added as an antioxidant. Tissues in the lesion samples (approximately 30 mg) were not ground but extracted by diffusion by placing them directly into 1 ml of acidified methanol. Samples were extracted for 16 hr at 4 C on an orbital shaker at 250 oscillations min^{-1}. The extract was centrifuged at 7,600 *g* for 10 min at 4 C, the supernatant was collected and dried down in vacuo at 35 C in a Speed-vac concentrator (Savant). The residue was dissolved in 1 ml of 10% methanol in water, centrifuged at 15,600 *g* for 2 min and filtered (Millipore HV Type, 0.45 μm). Dilutions of the filtrate were used directly in the ELISA or further purified for HPLC quantification of ABA. In extracts analyzed by HPLC, an internal standard of (±)-2-*cis,trans*-[2^{14}C] ABA (0.10 nCi, 20.8 μCi mg^{-1}) or unlabeled (±)-2-*cis,trans*-ABA was added to extracts before the first centrifugation.

High-performance liquid chromatography. Extracts of both infected and control hypocotyl tissues were purified partially before HPLC, by passage through a small C_{18} reverse-phase column (Sep-Pak, Waters Chromatography Division, Millipore Corporation, Milford, MA.), preequilibrated with 10 ml of water. The sample, in 10% methanol, was then applied to the column and abscisic acid eluted with 5 ml of 50% methanol in water. The methanol in the eluate was removed on a rotary evaporator at 35 C, the remaining aqueous solution made to 4 ml with water, the pH adjusted to 2.8 with acetic acid, and the solution partitioned twice against equal volumes of diethyl ether. The ether phases were combined and the ether carefully removed under a stream of nitrogen. The residue was dissolved in 200 μl of methanol:water:acetic acid (50:49:1) and filtered (0.45 μm).

Concentrations of ABA were determined using a reverse-phase C_{18} column (Nova-Pak, Waters; 3.9 mm × 15 cm, 5-μm particle size) in combination with a C_{18} precolumn (Guard-Pak, Waters). The initial solvent was 50% methanol in water acidified with 1% acetic acid run isocratically for 11 min, followed by a linear gradient to 100% methanol over 3 min then 100% methanol isocratically for 6 min, for a total run time of 20 min. Under these conditions [^{14}C] ABA eluted at a retention time of 6–8 min. To measure immunoreactivity in the column eluate, fractions were dried under reduced pressure at 35 C and the residue dissolved in 100 μl of 10% methanol in water. An aliquot taken from each fraction and suitably diluted was used in the indirect ELISA.

Fractions with retention times corresponding to that of the [^{14}C] ABA standard were pooled and dried under reduced pressure at 35 C. The residue was dissolved in a small volume of methanol and methylated by the addition of several drops of cold ethereal diazomethane until the yellow color persisted. The methylated sample was dried carefully under a stream of nitrogen, redissolved in 200 μl of the initial running solvent and injected onto the reverse-phase column. The solvent and running conditions were the same as those used for the first HPLC separation. The retention time of a [^{14}C] ABA standard, methylated in the same manner as the sample, was 15–17 min. Methylation efficiency was usually 100%.

The column eluate was monitored at 260 nm with an on-line UV detector (Lambda Max model 481, Waters) and the concentration of ABA was determined from a calibration curve constructed from peak heights for known amounts of methylated ABA standard. Amount of ABA was corrected for losses (up to 35% in some samples) against the internal [^{14}C] ABA standard and adjusted assuming that the (±) enantiomers occurred in equal proportion in the (±) ABA purchased.

Enzyme-linked immunosorbent assay. The indirect ELISA was based on an assay developed for cytokinins (4) and used 96-well flat-bottomed polystyrene microtiter plates (Costar, Bio-Rad Laboratories). All washing steps were performed using an automated plate washer (Biotek Instruments). For analysis of ABA, samples were diluted (routinely at least two dilutions were run) in assay buffer and assayed in triplicate. The outer rows of wells on each microtiter plate were not used as these gave inconsistent results.

Buffers: 1) Coating buffer: 0.05 M bicarbonate buffer, pH 9.6. (Na_2CO_3, 5.3 g L^{-1}; $NaHCO_3$, 4.2 g L^{-1}).

2) Assay buffer: 1.5 mM KH_2PO_4; 8 mM Na_2HPO_4; 0.7 M NaCl; 40 mM KCl; 0.05% Tween 20, pH 7.4.

3) Substrate buffer: diethanolamine 9.7% in H_2O; pH 9.8 with 6N HCl.

Standards: Dilutions of standard (±)-2-*cis,trans*-ABA from 5–500 pg per well were made in assay buffer and assayed in triplicate on each microtiter plate.

ABA-BSA Conjugate: Bovine serum albumin (BSA) was coupled to (±)-2-*cis,trans*-ABA through the carbon in position 4 by the method of Daie and Wyse (5). To coat the wells of the microtiter plate, the conjugate was suitably diluted in coating buffer.

Monoclonal antibodies: The monoclonal antibodies to (+)-2-*cis*-ABA were purchased commercially. The lyophilized antibody (2 mg) was dissolved in 2 ml of assay buffer and diluted to a total volume of 135 ml with assay buffer. This solution was divided into 3-ml aliquots and stored at −20 C. One aliquot suitably diluted with assay buffer contained sufficient antibody for two microtiter plates.

The stepwise procedure for the indirect ELISA of ABA was

as follows. Each well of a microtiter plate was coated with 200 μl of ABA-BSA conjugate diluted in coating buffer, and incubated at 4 C for 16 hr. The plates then were washed three times for 3 min each with assay buffer. To each well, 200 μl of 0.2% BSA in assay buffer were added and the plates were incubated for 2 hr at 4 C. The plates were washed as above and to each well were added 25 μl of standard ABA or sample in assay buffer followed by 175 μl of ABA antibody diluted in assay buffer. The plates were incubated for 3 hr at room temperature and then washed as above. Anti-mouse IgG coupled to alkaline phosphatase (200 μl of a 1:1,000 dilution in assay buffer) was added to each well and the plates incubated for 16 hr at 4 C. The plates were washed as above, and then 200 μl of a 1 mg ml^{-1} solution of *p*-nitrophenyl phosphate substrate in substrate buffer was added to each well. The plates were incubated for 1 hr at 37 C and the absorbance read at 405 nm with a Bio-Rad Model 2550 EIA Reader.

The concentration of ABA in a sample was calculated from Logit B/Bo transformed standard curve data (13) where B and Bo are the absorbance values in the presence and absence of competing antigen, respectively. The concentration of ABA in a sample is expressed in terms of ng $gram^{-1}$ dry weight. All experiments were repeated at least twice and the mean concentration of ABA and standard error of the mean calculated.

RESULTS AND DISCUSSION

The standard curve for the specific indirect ELISA of ABA is shown in Figure 1. The effective range of the assay was 0.02–1.0

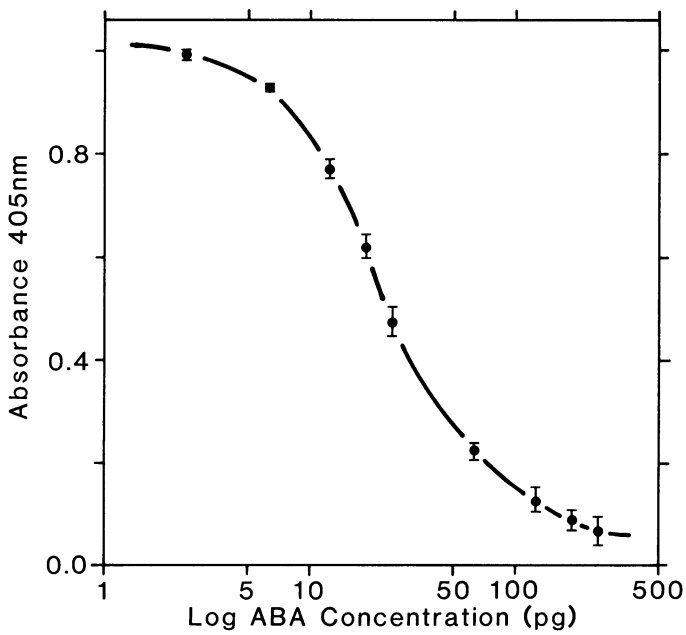

Fig. 1. Standard curve for the indirect enzyme-linked immunosorbent assay of abscisic acid. Each data point represents the mean and standard error of three determinations.

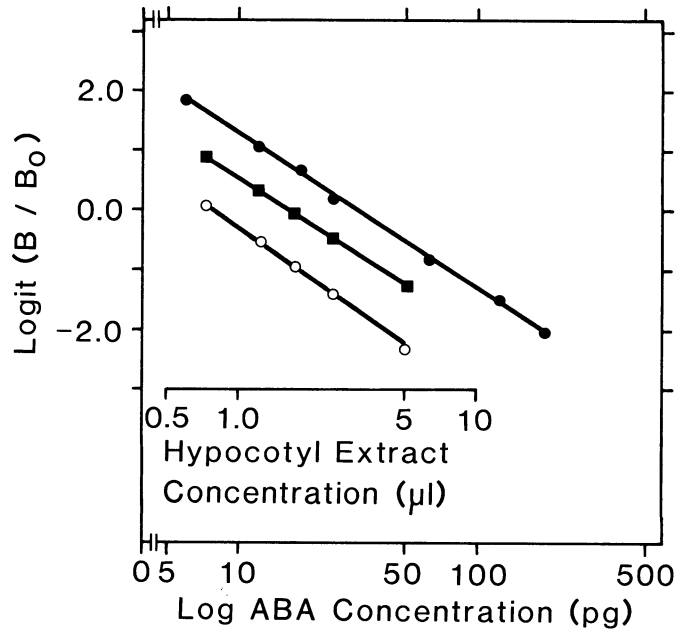

Fig. 2. Logit transformed standard curve for indirect enzyme-linked immunosorbent assay of abscisic acid standards (●) and serial dilutions of crude extracts of soybean hypocotyl sections inoculated with *Phytophthora megasperma* f. sp. *glycinea* (■) or with water (○).

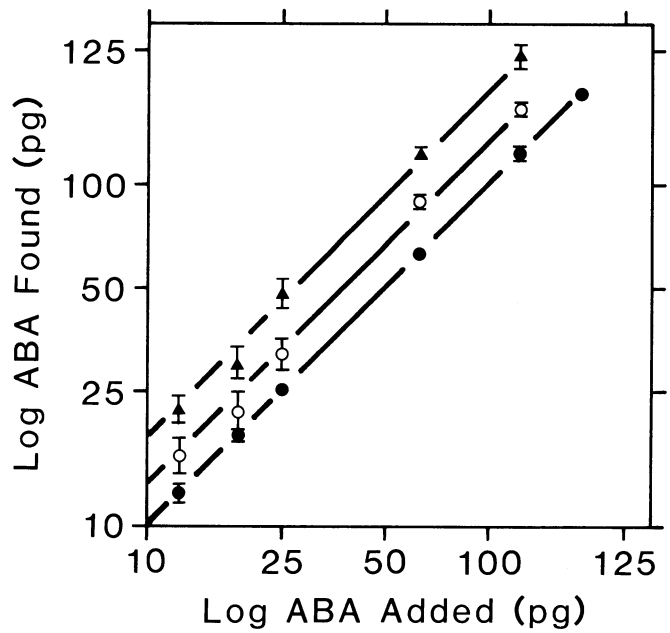

Fig. 3. Indirect enzyme-linked immunosorbent assay for abscisic acid (ABA) concentrations in dilutions of crude soybean hypocotyl extract supplemented with known quantities of ABA. Abscisic acid standard curve (●), ABA plus 10 μl (O) and 20 μl (▲) of hypocotyl extract.

TABLE 1. Concentrations of abscisic acid in hypocotyl sections of soybean cultivar Harosoy 63[a] determined by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC)

	ABA concentration (ng g^{-1} dry weight)							
	Uninoculated				Inoculated			
Sample purification stage	Sample 1	%[b]	Sample 2	%	Sample 1	%	Sample 2	%
1. Initial crude extract	45.0 ± 1.4[c]	100	43.8 ± 2.1	100	28.7 ± 0.4	100	26.5 ± 2.4	100
2. C_{18} Sep-Pak	47.6 ± 0.8[c]	107	46.4 ± 1.1	108	27.9 ± 1.6	97	27.8 ± 2.3	105
3. C_{18} Reverse-phase HPLC	44.8 ± 1.3[c]	99	45.1 ± 0.8	104	29.6 ± 1.5	103	26.5 ± 0.6	100
4. C_{18} Reverse-phase HPLC of methylated samples	46.9 ± 0.4[d]	105	42.0 ± 2.3	95	29.3 ± 0.9	102	27.1 ± 1.7	102

[a]Cultivar Harosoy 63 hypocotyls were either inoculated with zoospores of race 1 of *Phytophthora megasperma* f. sp. *glycinea* or treated with water only; tissue sections containing the lesions were sampled 6 hr after inoculation.
[b]Percentages are amounts of ABA found at each purification stage relative to that of the initial extract.
[c]Values determined by ELISA. All ELISA data are the mean ± the standard error of at least two sample dilutions.
[d]Values determined by HPLC. HPLC data are the mean ± standard error of two replicate injections.

picomole (5–250 pg) of (+) ABA (Fig. 2). Evidence that the method can be applied reliably to the measurement of ABA in crude tissue extracts was provided by assay of a series of dilutions of extracts from soybean hypocotyls (Fig. 2) and from a similar series of extracts spiked with ABA (Fig. 3). In both series, curves parallel to the standard curve were obtained. From the spiked samples, it is evident that the efficiency of the assay is virtually 100% (Fig. 3).

Independent evidence for the validity of the method was provided by HPLC. Abscisic acid (retention time 6–8 min) in crude hypocotyl extracts could not be adequately distinguished from other compounds absorbing at 260 nm in the eluate from the first C_{18} HPLC column. However, in extracts spiked with [^{14}C] ABA, all immunoreactivity was associated with radioactive fractions that eluted in a single peak at the retention time of ABA (Fig. 4A). Efficiency of recovery at: this stage, calculated from the loss of [^{14}C] ABA and confirmed by ELISA, was 80%. The radioactive fractions from the first C_{18} HPLC column purification were methylated and reapplied to the same C_{18} column. Methylated ABA, detected by absorption at 260 nm, eluted in 100% methanol as a single peak (Fig. 4B). This coincided with a single peak of radioactivity in [^{14}C] spiked samples that had the same retention time as that found for a methylated ABA standard. Therefore, only ABA was quantified in the second reverse phase purification and possibly small amounts of cochromatographing compounds, which also were methylated and eluted at the retention time of the methylated ABA. Hence, quantification with the methylated derivative of ABA provided a further check on the first HPLC reverse phase purification of ABA. Methylated ABA could not be measured with the ELISA.

These results indicate that the ELISA provides a specific and reliable method for determining ABA in crude soybean tissue extracts. The ELISA assay is especially suited to material that needs a minimum of prepurification (11,12,16). It is highly efficient

TABLE 2. Enzyme-linked immunosorbent assay of abscisic acid (ABA) in lesions of soybeans (cv. Harosoy 63) in an incompatible interaction with *P. megasperma* f. sp. *glycinea* race 1[a]

	ABA (ng g^{-1} dry weight)	
Sample[b]	Uninoculated control	Inoculated
1	25.41	5.22
2	18.97	7.20
3	28.33	7.25
4	23.39	8.70
5	16.88	6.95
Mean ± S.E.	22.60 ± 4.18	7.06 ± 1.11

[a]The concentration of ABA was determined in tissue of lesions excised 6 hr after inoculation.

[b]Each sample consisted of 20 lesions, i.e., two lesions excised from each of 10 hypocotyls.

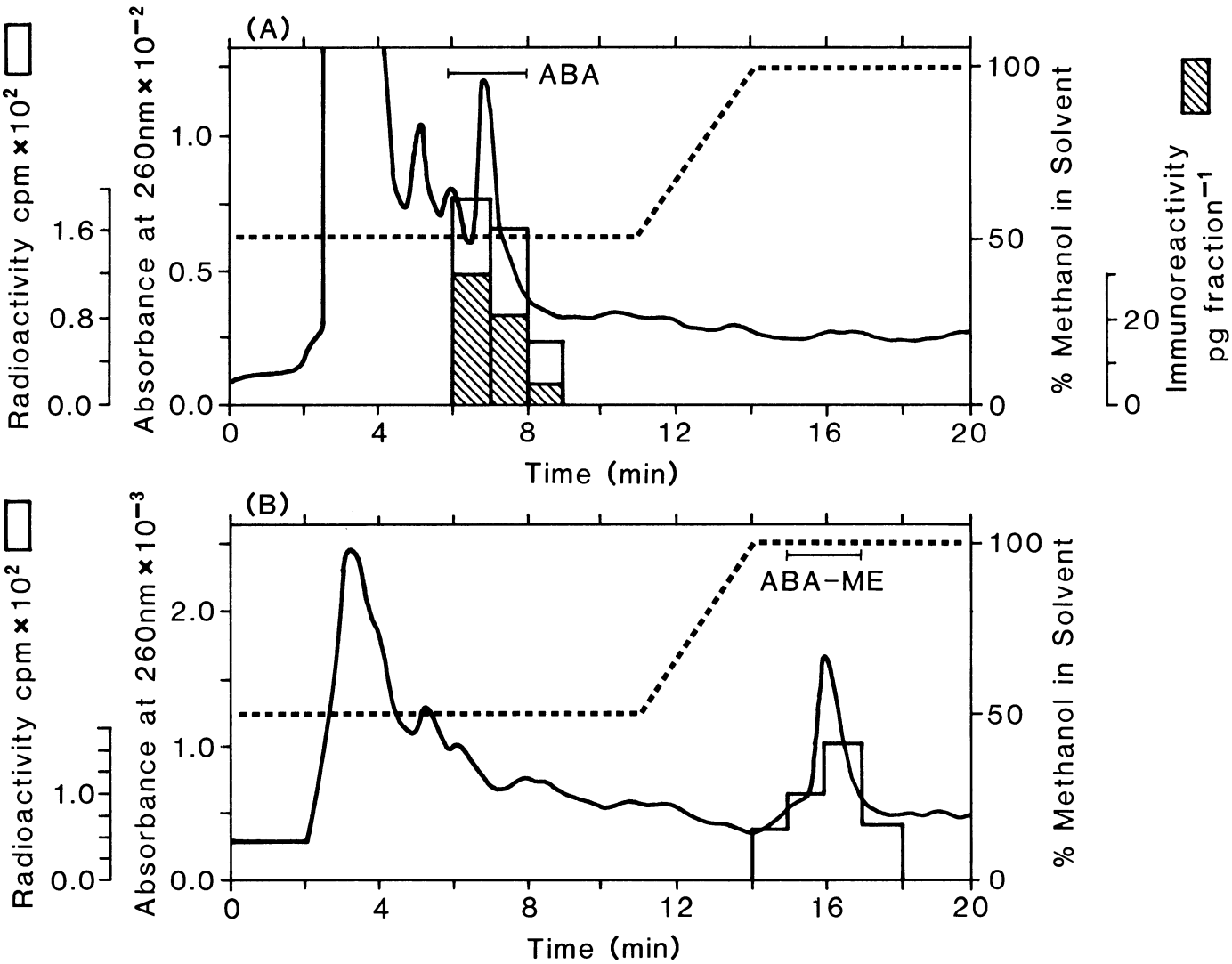

Fig. 4. C_{18} reverse phase high-performance liquid chromatographic purification of crude extracts from lesions of soybean hypocotyls inoculated with *Phytophthora megasperma* f. sp. *glycinea*. **A,** Elution profile of an extract after first high-performance liquid chromatography (HPLC) purification. **B,** Elution profile of pooled and methylated fractions with the same retention time as authentic abscisic acid after the first HPLC purification. Absorbance at 260 nm represented by solid line (—) and methanol in solvent indcated by dashed line (- - -).

especially compared with other procedures such as HPLC. The use of (+)-2-*cis*-ABA monoclonal antibodies from the same source has been verified also in immunoassays in other systems by CC-MS (10,14,17).

The data of Table 1 are for extracts of hypocotyl sections inoculated either with spore suspensions of *P. m. glycinea* or with drops of water. They indicate that after infection there are changes in ABA concentration that do not occur in control hypocotyls. Similar differences were measured also in extracts of tissue from excised lesions (Table 2) indicating that the method could be used for the determination of localized concentrations at and around infection sites. The data of Table 2 are based on extracts of 20 excised lesions, but the method is of sufficient sensitivity that ABA concentrations in a single lesion (approximately 0.15 mg of tissue dry weight) could be measured.

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