Partial purification of an endogenous elicitor from suspension-cultured cells of red bean, *Vigna angularis*

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Abstract. Brief irradiation with a germicidal UV lamp of cells of red bean, *Vigna angularis*, cultured in suspension in a quartz flask caused the release into the culture medium of an endogenous substance with elicitor activity, as well as the accumulation of isoflavone glucoside stress metabolites in the cells. The active compound was fractionated using phenylalanine ammonia lyase (PAL)-inducing activity in fresh cells as a marker. The elicitor active principle appears to be a low molecular weight (< 2000 MW) water-soluble acidic oligosaccharide.
Key words. Suspension cells; *Vigna angularis*; UV irradiation; endogenous elicitor.

The induction of phytoalexin synthesis and accumulation in plants is a localized defense response against invading pathogens. This response is currently understood to be triggered by the action of biotic elicitors produced by the invading pathogens[1], and also by endogenous elicitors released from the host plant cell walls by the action of extracellular hydrolytic enzymes secreted by the microorganisms[2–4].

The induction of phytoalexin formation can conveniently be modeled in suspension-cultured plant cells treated with various abiotic elicitors such as autoclaved RNase A, heavy metal salts, detergents, hydrogen peroxide, or UV light. As these abiotic elicitors of diverse chemical nature can stimulate similar inducible defense mechanisms in plant cells, great attention has been focused on the mode of action of these stimuli. One possibility is that some endogenous elicitors are formed or released by the plant cells when exposed to such stimuli[5]. This endogenous elicitor of treated cell origin would then serve as the primary signal to activate phytoalexin biosynthesis in non-treated cells[6,7].

Early studies by Hargreaves and Bailey[8] and Hargreaves and Selby[9] showed that bean hypocotyls damaged by freezing or autoclaving released dialyzable molecules which could induce phytoalexin accumulation in suspension-cultured cells of the same plant. Dixon et al. demonstrated the transmission of endogenous elicitors from bean cells treated with macromolecular abiotic elicitor (autoclaved RNase A) across a dialysis membrane to equivalent untreated cells, in which phytoalexin biosynthetic enzymes were also induced[10]. The chemical structures of the endogenous elicitor(s) reported by these workers have not yet been elucidated.

In the present study, we examined the release of the endogenous elicitor(s) from UV-irradiated suspension cells of red bean, *Vigna angularis*. As reported previously, treatment of this cell line with various abiotic elicitors such as autoclaved RNase A, nigeran, or sodium vanadate caused the accumulation of isoflavone glucosides in parallel with phenylalanine ammonia lyase (PAL) activation[11,12]. Brief irradiation of cultured cells with UV light was expected to have the advantage of eliminating the ambiguity regarding the origin of the active compound: whether it originates from the cells themselves or is derived from the administered stimulus, e.g., autoclaved RNase A.

Preliminary results suggested that a low molecular weight, water soluble factor released into the culture medium was responsible for the activity. Fractionation and preliminary characterization of this compound will be described.

Materials and methods

Distilled water was prepared from deionized H_2O with a Corning Mega-Pure automatic distillation apparatus. HPLC grade H_2O and MeOH were obtained from Nakarai Tesque, Ltd.

Nigeran and RNase A (Type II-A) were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Water-soluble polygalacturonate (< 10,000 MW) was a gift from Professor H. Konno, Okayama University.

Cell culture. The principal suspension culture of red bean, *Vigna angularis* Ohwi et Ohashi, used in these studies had been maintained for over 7 years by subculturing at 14-day intervals in Murashige-Skoog medium[13] containing 3% sucrose and 1 μg/ml of 2,4-dichlorophenoxyacetic acid. The culture was grown at 25 °C in 200-ml Erlenmeyer flasks containing 100 ml of the medium on a rotary shaker (100 rpm) under continuous light (300 ft-c).

UV irradiation of the cells. The cell suspension culture of red bean (250 ml) which had been grown for 6 days after inoculation was transferred to a sterile, 500-ml quartz flask and placed on a rotary shaker. An UV lamp (National GL-15 germicidal lamp, 253.7 nm) fitted with aluminum foil shields was hung approximately 8 cm above the rim of the flask. After a brief period of UV irradiation (15–30 min), the culture sample was transferred to a sterile glass flask and incubated on the rotary shaker for a suitable period of time. A control culture of the same age was incubated in a glass flask during the same period. For the time course studies, 20-ml aliquots of the culture were removed from the flasks and divided into portions for measurement of dry weight (1 ml culture), PAL activity (3 ml culture), isoflavone glucoside production (3 ml

culture), and endogenous elicitor activity (3 ml medium). The samples of medium to be assayed for endogenous elicitor activity were lyophilized to dryness and then redissolved in 0.2 ml H_2O. The concentrated solutions were assayed for their ability to induce PAL activity in fresh cell suspension cultures of the same plant as described below.

Measurement of PAL activity and analysis of isoflavone glucosides. Extraction and measurement of PAL activity were carried out according to the method reported previously[12]. For analysis of isoflavone glucosides, the cells were collected by vacuum filtration, washed with H_2O, transferred to test tubes, and extracted with 3 ml of boiling MeOH. The methanolic extract was passed through a Sep-pak C_{18} cartridge (Waters Associates, Inc.). The eluate was evaporated to dryness, redissolved in 2 ml 70% MeOH and analyzed by HPLC. Chromatography was performed using a Jasco Finepak ODS column with H_2O/15%CH_3CN as solvent at a flow rate of 1 ml/min. Absorbance was monitored at 250 nm and peaks for daidzein 7-O-β-D-monoglucoside, daidzein 7,4′-di-O-β-D-glucoside, and 2′-hydroxy-daidzein 7,4′-di-O-β-D-glucoside were integrated and compared with a standard solution[12].

Assays of elicitor activity. Assays were conducted by monitoring the increase in PAL activity induced in fresh suspension cultures of red bean which had been grown for 6 or 7 days after inoculation. 3-ml portions of the suspension culture were transferred to test tubes (13 mm in diameter) containing 50–200 µl of sample solutions. Distilled water was added instead of the sample solutions in the control experiment. The test tubes were incubated at 25 °C on an angled rotator (50 rpm) for 3–4 h, after which cells were processed for PAL activity measurement. Because of the short incubation periods, experiments were conducted unter non-sterile conditions. Solutions of Nigeran and RNase A used in these studies were autoclaved at 121 °C for 15 min prior to use.

Isolation of endogenous elicitor. All the isolation experiments were repeated at least three times. In spite of the attempt to carry out the chromatographic separation under as uniform conditions as possible, fluctuation in bed volume, gel activities, or flow rates resulted in recovering the active principle in different positions in each repeated experiment. Thus, we describe the results of a representative isolation experiment to avoid confusion.

(a) Preparation of active culture medium. The cell culture transferred into a quartz flask was UV-irradiated for 15 min as described above and incubated in a glass flask for 12–44 h until endogenous elicitor activity in the culture medium neared its maximum level. At the appropriate time, cells were removed by vacuum filtration, and the culture medium was stored at −20 °C. A total of 5 l of medium from repeated equivalent preparations was pooled and a typical isolation procedure was started by concentrating 1 l of the medium in vacuo (< 28 °C) to 30 ml.

(b) Dialysis. The concentrated medium sample (30 ml) was dialyzed against distilled H_2O (4 × 200 ml) at 4 °C (Spectrapor Membrane Tubing, Spectrum Medical Industries, Inc, 2,000 MW cutoff). The pooled dialysate was concentrated in vacuo (< 28 °C) to 500 ml.

(c) HP-20 column chromatography. The concentrated dialysate (500 ml) was slowly applied to a column of HP-20 gel (Diaion HP-20, Nippon Rensui Co.) equilibrated in H_2O. The column was then washed with an additional 500 ml of H_2O to produce fraction 1, followed by 1 l H_2O (fraction 2), 1 l H_2O/50% MeOH (fraction 3), and 1 l MeOH (fraction 4). The first two fractions were concentrated in vacuo to a fixed volume (30 ml). Fractions 3 and 4 were each evaporated to dryness, redissolved in a small volume of H_2O, filtered through Millex GS 0.22-µm filters, and then made up to a fixed volume of 30 ml with H_2O for assay of the activity.

(d) RP-8 column chromatography. Combined HP-20-fractions 3 and 4 were dissolved in a small volume of H_2O, and loaded onto a 30 × 2.5 cm Lobar Lichroprep RP-8 column equilibrated in H_2O. The column was washed first with 100 ml H_2O = (two 50-ml fractions) at a flow rate of 2 ml/min, then with a 500-ml linear gradient from H_2O to 75% MeOH, and finally with 200 ml MeOH.

(e) Polyvinylpyrrolidone (PVP) column chromatography. A column of PVP (Nakarai Tesque, Ltd) was prepared according to Glenn et al.[14]. The second fraction from RP-8 chromatography dissolved in 1 ml H_2O was loaded onto the PVP column and eluted with H_2O at a flow rate of 1 ml/min. The eluate was monitored with UV (Shimadzu SPD-6A detector, 200 nm). Fractions (3 ml each) were lyophilized and redissolved in 1 ml H_2O for bioassay.

(f) DEAE cellulose column chromatography. Whatman DE52 diethylaminoethyl cellulose anion exchange gel (Whatman Chemical Separation, Ltd.) was precycled by washing with 0.5 M HCl, 0.5 M NaOH, 0.5 M NH_4HCO_3, and H_2O. PVP column fractions 10 and 11 were combined, dissolved in 10 ml H_2O and applied slowly to the equilibrated DE52 column. The column was washed first with H_2O (32 ml), followed by a 100-ml linear gradient to 0.5 M NH_4HCO_3.

Fractions (4 ml each) were lyophilized and redissolved in H_2O for bioassay. The activity was also examined in pooled fractions 16–19 after desalting with cation exchange resin (Diaion SK-1B).

Results

Stress with UV irradiation. A relatively short irradiation with germicidal UV light of 254 nm was sufficient to induce production of isoflavone glucoside stress metabolites in *Vigna angularis* cell suspension in a quartz flask. Table 1A shows the amount of isoflavone glucosides accumulating in the cells irradiated for 15–30 min and

Table 1 A. Effects of UV-irradiation periods on production of isoflavone glucosides

Irradiation period (min)	Isoflavone glucosides in cells (μg/mg d.wt)*		% of control
	Control	Irradiated	
15	11.23	21.44	191
20	9.78	26.57	272
25	2.29	3.23	141
30	5.94	3.06	51

*Amount of isoflavone glucosides accumulated in the cells incubated for 45 h after UV-irradiation for designated periods

Table 1 B. Effects of UV-irradiation periods on release of endogenous elicitor activity into culture medium

Irradiation period (min)	Endogenous elicitor activity PAL (nmol cinnamic acid/min/ml)		% of control
	Control	Irradiated	
15	0.274	0.514	188
20	0.274	0.549	200
25	0.309	0.617	200
30	1.120	3.118	278

Endogenous elicitor activity was determined by monitoring PAL activity induced in fresh cells of *V. angularis* treated with medium from the control culture (control) or UV-irradiated culture (irradiated).

incubated for 45 h in an ordinary glass flask. Approximately 2–3-fold higher amounts of the glucosides were produced by the cells exposed to UV for 15 or 20 min compared to non-irradiated cells. Longer irradiation was deleterious to the cells and led to reduced or no production of those compounds.

Endogenous elicitor activity of the medium was assayed by determining PAL activity induced in fresh cells of red bean (see 'Materials and methods'). The PAL-inducing activity in the medium increased upon irradiation of the cells with UV light compared to the non-irradiated cells. However, this activity was fairly similar in cultures exposed to different UV dosages (table 1 B), and an UV-irradiation period of 15 min was chosen for further experiments.

In order to establish optimum conditions for production of 'active' culture medium for isolation work, the time course of elicitation was studied in detail. PAL activity in the UV-irradiated cells started to increase within 10 h after irradiation and was followed by accumulation of isoflavone glucosides (fig. 1 B and C). As shown in figure 1 A, the release of endogenous elicitor activity into the medium preceded the PAL activation (fig. 1 B) and isoflavone glucoside production (fig. 1 C) in the treated cells. The release started within 3 h and reached a maximum at around 12 h after the irradiation. This suggested that the released active substance activated the metabolic pathway leading to formation of isoflavone stress metabolites in the treated cells.

Isolation of an endogenous elicitor. As illustrated in table 1 B and fig. 1 A, UV irradiation for 15 min generally produced suspension culture medium with roughly twice the elicitor activity of untreated controll medium.

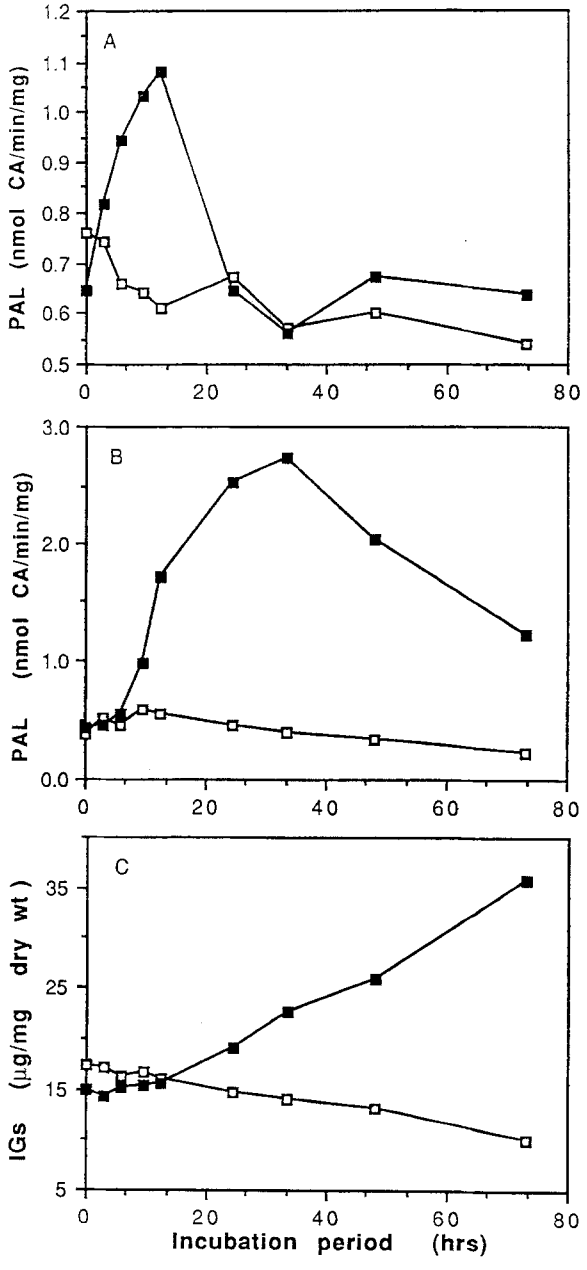

Figure 1. Time course of release of endogenous elicitor active compounds into the culture medium determined by its PAL-inducing activity (A), changes in PAL activity of the cells (B), and accumulation of isoflavone glucosides (C) in suspension-cultured cells of *V. angularis* after irradiation with UV (254 nm) for 15–20 min (—□—, control; —■—, UV-irradiated). Endogenous elicitor activity was assayed as described in 'Materials and methods'. CA is cinnamic acid. IG is isoflavone glucoside.

For isolation work, the suspension culture medium was first concentrated in vacuo. Dialysis of the concentrated medium demonstrated that most of the elicitor activity could pass through 2000 MW cut off tubing, suggesting that the active principle was a relatively small molecule (table 2).

The large amount of mineral salts and sucrose present in the dialysate was a difficult matrix from which to isolate water-soluble bioactive compounds, presumably present in low concentrations. After experimenting with several

Table 2. Endogenous elicitor activity in dialysate and sequential chromatographic fractions

Dialysis fr.	PAL*	HP-20 No.	PAL	RP-8 No.	PAL	PVP No.	PAL
Control	0.206	Control	0.479	Control	1.117	Control	1.331
Dialysate	1.063	1	0.365	1	1.331	1–2	1.117
Retentate	0.583	2	0.651	2	3.780	3–4	1.160
		3	1.132	3	1.546	5–6	1.117
		4	0.724	4	1.761	7–8	1.117
				5	1.632	9–10	3.651
				6	1.890	11–12	4.896
				7	2.534	13–14	1.718
				8	1.933	15–16	1.246
				9	1.890	17–18	1.288
				10	1.761	19–20	1.374
				11	2.190	21–22	1.331
				12	3.006	23–24	1.374
				13	2.479	25–26	1.288
				14	2.577	27–28	1.331
						29–30	1.374

For experimental details, see the text. Fractions underlined in each column were pooled and subjected to further purification.
*nmol cinnamic acid/min/ml.

initial fractionation steps, a crude separation of the sample on a column of HP-20 polystyrene reverse phase adsorption resin was found to give the best results. Most of the elicitor activity in the dialysate could be adsorbed from aqueous solution onto HP-20 resin, and then eluted from the resin with H_2O/50% MeOH (fraction 3) and 100% MeOH (fraction 4). Combined fractions 3 and 4 yielded a total of 216 mg of material from 5 l of 'active' medium. The HP-20 sample (149 mg) was further fractionated on RP-8 columns, eluted first with H_2O, then with a linear gradient to 70% MeOH, and finally with a MeOH flush. Greatest elicitor activity was found in fraction 2 from RP-8 (17.1 mg) eluted with H_2O (table 2). A total of 13.8 mg of this material was applied to medium pressure columns of PVP and eluted with H_2O. Elicitor activity was associated with the first peaks (fractions 9 through 12, 6.3 mg) from this column (fig. 2 and table 2). Further fractionation of 4.4 mg of the PVP sample on a column of DEAE anion exchange resin eluted with a linear gradient from H_2O to 0.5 M NH_4HCO_3 (fig. 3) produced one major peak of an acidic material which exhibited elicitor activity (fractions 16–19, 2.8 mg), suggesting the acidic nature of the active principle. Attempts to obtain a complete bioassay profile for the lyophilized DEAE column fractions were unsuccessful due to high levels of background activity, presumably due to non-volatile residues in the salt gradient employed. The acidic sample from this column was therefore desalted on SK-1B cation exchange resin in the H^+ form and assayed for activity (table 3).

In all of the fractionation steps described, fractions were effective at doses of < 100 µg/ml. This compares reasonably well with some of the common abiotic elicitors tested for comparison purposes (table 3). However, the greatest problem in this series of experiments was the gradual loss of elicitor activity inevitably accompanying

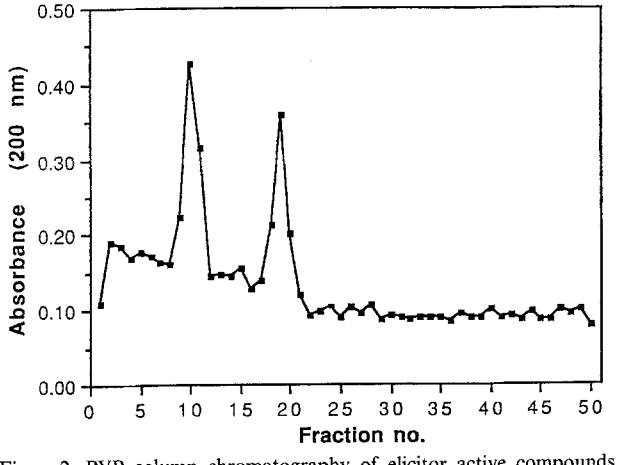

Figure 2. PVP column chromatography of elicitor active compounds previously fractionated through a RP-8 column. The column was washed with H_2O at a flow rat of 1 ml/min and the eluate monitored by UV absorbance at 200 nm.

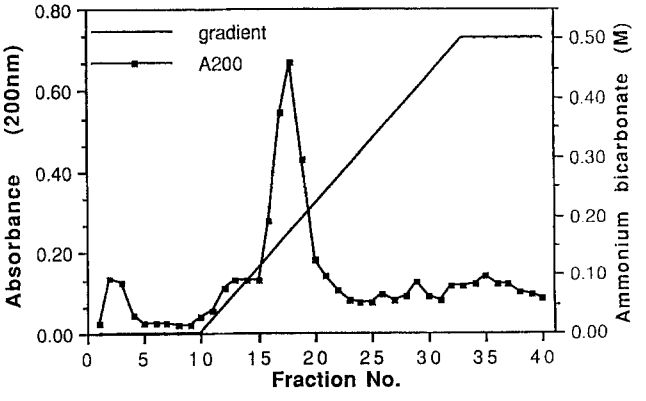

Figure 3. Anion exchange column chromatography on DEAE cellulose. The elicitor active fractions from the PVP column were pooled and further purified on a DEAE cellulose column, which had been preequilibrated with water. The column was washed first with H_2O, followed by a linear gradient from H_2O to to 0.5 M NH_4HCO_3. Fractions were lyophilized and redissolved in H_2O for measurement of UV absorbance at 200 nm.

Table 3. Comparison of elicitor activities

Sample	PAL*	% of Control
PolyGalUA	3.307	943
Nigeran	1.900	537
RNase A	0.644	182
DEAE 16–19	0.623	176
Desalted DEAE 16–19	0.977	276
Control(H_2O)	0.354	100

For experimental details, see the text. Final concentrations (μg/ml) were polyGalUA : 24, nigeran : 100, RNase A : 1000, DEAE fractions 16–19 : 50, desalted DEAE fractions 16–19 : 50
*nmol cinnamic acid/min/ml

the isolation procedure. This problem had already been encountered in a previous study (Kobayashi, private communication).

Examination of the 500 MHz ^{1}H NMR spectrum of the final DEAE acidic fraction in D_2O revealed that the sample was complex oligosaccharide and that, at the same time, it was not a simple homoglycan of galacturonic acid when compared with a published ^{1}H NMR data for synthetic oligogalacturonide[15]. Various attempts to analyze the underivatized sample by liquid secondary ion mass spectrometry failed to produce clear cut results. However, presence of uronic in our sample was further suggested by its IR spectrum. The FTIR spectrum of the desalted acidic sample provided evidence for carboxylate (1654 cm^{-1}) and free acid (1724 cm^{-1}) moieties in the molecule. In situ treatment of the sample film with a drop of 1 N HCl produced more of the free acid, which could then be converted to the ammonium salt (ca 1600 cm^{-1}) with a drop of 1 N NH_4OH.

Discussion

Elicitation is a complex systemic process resulting in activation of enzymes responsible for the synthesis of stress-related metabolites. Elucidation of the mechanisms involved in elicitation by abiotic elicitors will help to promote secondary metabolite production by cultured plant cells. Our present interest lies in identifying the primary signal produced by abiotic elicitors of diverse chemical nature.

UV irradiation has been used in several studies to activate metabolic pathways leading to flavonoid glycoside formation in cells cultured in suspension in a variety of light conditions[16–18]. In these studies the cells were irradiated with UV light of 280 nm or longer wavelengths for several hours.

The release of material with elicitor activity into the culture medium was a relatively rapid response of suspension cells of *Vigna angularis* exposed to short wavelength UV light, and was observed to occur before PAL activation and isoflavone glucoside production in the cells. We have collected culture medium near the point where this activity was greatest and have fractionated the activity to isolate an active principle. Indirect evidence links the acidic oligosaccharide we isolated with the released activity. The release of compounds of similar nature has been reported to occur from hypocotyls of *Phaseolus vulgaris* damaged by freezing[8]. While final proof that the appearance of the compound in the medium correlates with the time course of elicitor activity requires a more rigorous quantitative analysis, it is likely that this acidic oligosaccharide acts as an endogenous elicitor in our experimental system.

In the interaction between pathogenic microorganisms and host plants, oligomers of galacturonic acid (which are released from plant cell walls by hydrolytic enzymes introduced by invading pathogens) apparently serve as messengers to trigger a series of processes leading to phytoalexin biosynthesis[3,19]. Bishop and co-workers observed the release of oligosaccharides, acting as endogenous proteinase inhibitor-inducing factors, from tomato leaves digested by tomato endopolygalacturonases, enzymes known to be released from wounded tissues[20].

In our experiments the endogenous acidic oligosaccharide with elicitor activity might be released from the irradiated cells by the action of hydrolytic enzymes. The hydrolytic enzymes are presumably synthesized or activated to repair the cell walls damaged by UV irradiation. It will be interesting to see whether the other abiotic elicitors which induce the same response in red bean cells operate through the same mechanism observed for the UV irradiation. At this stage, we can only conclude that the elicitor is a heterogeneous acidic oligosaccharide(s), probably with a molecular weight of less than 2000. Further experiments are required to determine the purity of the active fraction and identify monosaccharide components of the elicitor active species.

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