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Host-Pathogen Interactions[1]

XXI. EXTRACTION OF A HEAT-LABILE ELICITOR OF PHYTOALEXIN ACCUMULATION FROM FROZEN SOYBEAN STEMS

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ABSTRACT

An extract of frozen and thawed soybean (*Glycine max* L. Merr. cv. Wayne) stems is active, in wounded soybean cotyledons, as a heat-labile elicitor of phytoalexins. The elicitor activity of the extract is destroyed by heating to 95°C for 10 minutes. The fraction that contains heat-labile elicitor activity releases heat-stable elicitor-active molecules from purified soybean cell walls. Heat-labile elicitor activity voids a Bio-Gel P-6 column and can be absorbed onto and eluted from a DEAE Sephadex ion exchange column. Using the cotyledon phytoalexin elicitor assay, maximum heat-labile elicitor activity was obtained when soybean stems were extracted with acetate buffer at pH 6.0. Addition of 1 millimolar $CaCl_2$ increased apparent heat-labile elicitor activity. The heat-labile elicitor stimulated maximum phytoalexin accumulation when applied to cotyledons immediately after the cotyledons were cut. Partially purified stem extracts lost heat-labile elicitor activity during storage for several days at 3°C. The possible role of a heat-labile elicitor in stimulation of phytoalexin accumulation by both abiotic and biotic elicitors is discussed.

Phytoalexins are antimicrobial compounds of low mol wt that are synthesized by and accumulate in plants after exposure to microorganisms (12). Phytoalexins also accumulate in plants after exposure to compounds termed elicitors (9, 10). These include biotic elicitors from microorganisms (3) and plants (5–8) as well as such abiotic elicitors as detergents, heavy metals (16), and UV Light (15). Hargreaves and Bailey (7) have found in plants a "constitutive" heat-stable elicitor that can be released by a freeze-thaw technique. Hahn *et al.* (6) have recently shown that an endogenous elicitor can be released from purified cell walls of plants either by partial acid hydrolysis or by autoclaving; this endogenous elicitor could be similar to the "constitutive elicitor" found by Hargreaves, Bailey, and Selby (7, 8). We report here the extraction and partial characterization of HL[4]-elicitor activity from soybean stems, which releases HS-elicitor activity from purified soybean cell walls.

MATERIALS AND METHODS

Plant Growth and Elicitor Bioassay. Soybean (*Glycine max* [L.] Merr., cv. Wayne) seedlings were grown under previously established conditions (14, 15). The soybean cotyledon elicitor assay used 90-μl droplets of test solutions applied to the cut abaxial surface of each of 10 soybean cotyledons (2, 5, 6). The assay was modified by adding 200 μg streptomycin/ml to all test solutions to inhibit microbial growth on the acetate buffer used.

Purification of Soybean Cell Walls. Cell walls were purified from 8-d-old soybeans, as described (5, 6).

Extraction of Tissue. Stems (hypocotyls plus epicotyls) from 8-d-old soybean plants were collected, weighed, and then frozen at −26°C to cause cell disruption. After 1 h at that temperature, the frozen stems were removed and incubated at 25°C for 1 h. After extraction, the stems (7 g) were macerated by pestle in a mortar at 5°C with 25 ml 50 mM sodium acetate (pH 6.0) containing 10 mM 2-mercaptoethanol (30 g stems/100 ml buffer for large-scale experiments) and then further comminuted in a motor-driven Potter-Elvehjem homogenator. The extract was centrifuged for 10 min at 12,000*g*, and the supernatant solution was passed through a 1.2 μm Millipore filter.

Bio-Gel P-2 or P-6 Chromatography. Stem extracts from small-scale experiments (5 ml) were applied to either a Bio-Gel P-2 (100–200 mesh) or a Bio-Gel P-6 (100–200 mesh) column (24 × 1.4 cm), which was then eluted with 50 mM acetate buffer (pH 6.0) at room temperature. A 4-ml void fraction containing HL-elicitor activity was collected and diluted for bioassay by the addition of 1 ml H_2O, thereby reducing the buffer concentration to 40 mM.

Assay of HL Elicitor. Test solutions were divided into two parts. One was heated to 95°C in a boiling water bath for 10 min, and the other was left unheated. After cooling, streptomycin (200 μg/ml) was added to all samples, and the solutions were tested for elicitor activity using the soybean cotyledon bioassay. After a 20-h incubation at 26°C, the 10 cotyledons from each assay plate were transferred to 20 ml of distilled H_2O, and the A at 286 nm of this wound-droplet solution was measured. This A is proportional to the phytoalexin content of the wound-droplet solution (3, 6). The absorbance of a water control was subtracted from each value. All assay plates were replicated. The A at 286 nm of the 20-ml wound droplet solution is converted by the curve in Figure 1 to μg of endogenous elicitor equivalents applied to each cotyledon. This is necessary because the amount of elicitor required to stimulate increasing amounts of phytoalexin accumulation increases in a logarithmic fashion as the amount of phytoalexin accumulation, in the wound-droplet solutions on the cotyledons, approaches a maximum (Fig. 1).

The difference in elicitor activity between solutions that had been heated at 95°C for 10 min and those that were unheated was regarded as HL-elicitor activity. HS-elicitor activity is calculated by subtracting the A_{286} due to buffer alone from the A_{286} elicitation resulting from the HS elicitor. HL- and HS-elicitor activities are expressed in arbitrary units: one unit of HL- or HS-elicitor activity causes the same increase in the A_{286} of the wound-droplet solution

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[2] This work was carried out while G. D. L. was on sabbatical leave from the Scottish Crop Research Institute, Invergowrie, Dundee, Scotland.

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[4] Abbreviations: HL, heat labile; HS, heat stable.

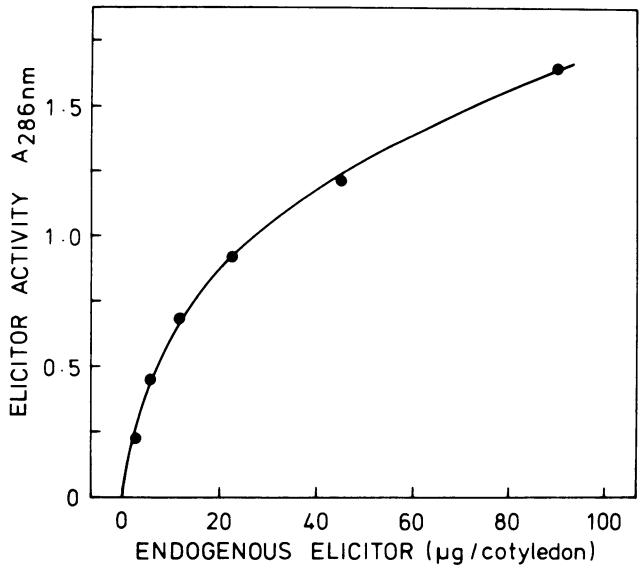

FIG. 1. Response of soybean cotyledons to increasing amounts of QAE-purified endogenous elicitor (6). Elicitor in 90 µl sterile distilled H_2O was applied to sets of 10 wounded cotyledons. The treated cotyledons were incubated at 100% humidity for 20 h at 26°C. The droplets from each set of cotyledons were rinsed into 20 ml H_2O and the A at 286 nm was measured. (Data kindly supplied by Dr. M. Hahn [see Fig. 1 of Ref. 6]).

as is caused by 1 µg of Hahn and Albersheim's QAE-purified endogenous elicitor (6).

RESULTS

Partial Purification of the HL Elicitor.

Bio-Gel P-2 or P-6 Chromatography. Preliminary experiments showed that HL-elicitor activity could not be detected unless extracts were partially purified by the removal of low-mol-wt compounds. This partial purification was accomplished with large-scale HL-elicitor preparations by applying 100 ml extract to a Bio-Gel P-6 (100–200 mesh) column (355 × 6 cm) that was equilibrated and eluted with 50 mM acetate buffer (pH 6.0) at 1°C. The HL-elicitor activity eluted at or near the void volume of the column (Fig. 2). The column effluent was monitored by measuring the $A_{280\ nm}$ as a guide to solute concentrations. The HL-elicitor activity was highest in the void fractions of the column. In the cotyledon bioassay, the 280 nm-absorbing compounds in the HL-elicitor-active void fractions account for less than 5% of the absorbance at 286 nm.

The HL elicitor caused both phytoalexin and red pigment accumulation in the cotyledon wound droplets. The amount of red pigment that accumulates is roughly proportional to the amount of phytoalexins. This is not surprising because the red pigment has been shown to be a catabolite of the phytoalexins (16).

DEAE-Sephadex Chromatography. Fractions 29 through 44 from the Bio-Gel P-6 column depicted in Figure 2 were combined (volume 150 ml), and 140 ml was applied to a DEAE-Sephadex A-25-120 column (10 × 2 cm). The column was washed with 75 ml 50 mM acetate buffer (pH 6.0) followed by three sequential 60 ml washes with 50 mM acetate buffer (pH 6.0) containing 0.2, 0.5, and 1.0 M NaCl, respectively. Five-ml fractions were collected. Before bioassay, the fractions were dialyzed for 24 h at 5°C against several changes of 30 mM acetate buffer using Spectra-Por 2,000 mol wt cut-off dialysis tubing. HL-elicitor activity was found in the fraction that was eluted from the DEAE-Sephadex with 0.5 M NaCl in the 50 mM acetate buffer (Fig. 3).

Other experiments showed that HL elicitor also binds to CM Sephadex in 100 mM acetate buffer, pH 5.2, and to QAE-Sephadex

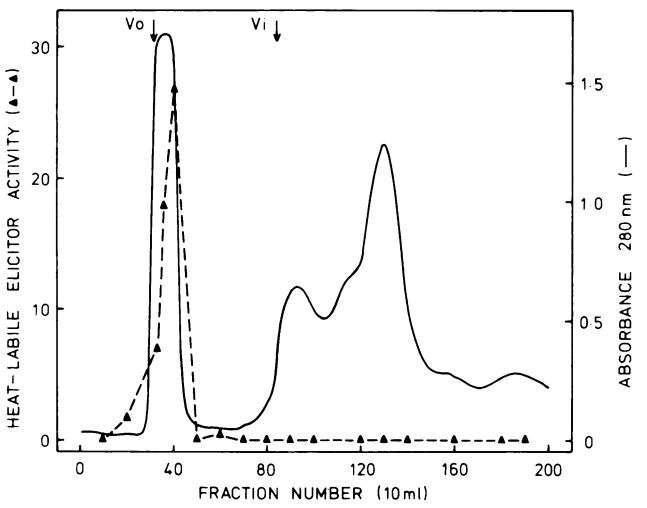

FIG. 2. Bio-Gel P-6 chromatography of 100 ml of stem extract. The column (35 × 6 cm) was eluted with 50 mM acetate buffer (pH 6.0). Fractions were 10 ml. The void and included volumes are indicated by V_0 and V_i, respectively. Fractions were diluted (1 ml of the fraction + 0.25 ml of H_2O) before bioassay. HL-elicitor activity was determined as described under "Materials and Methods."

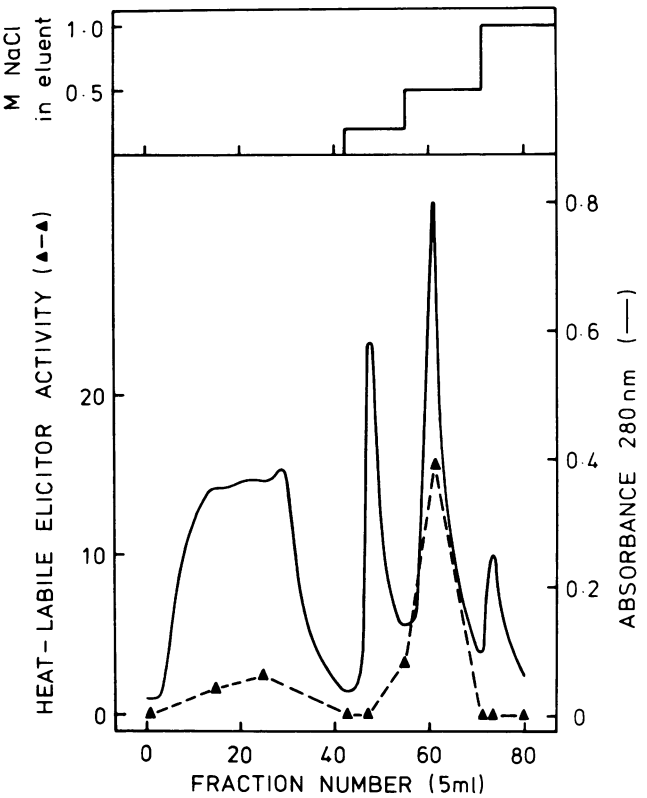

FIG. 3. DEAE-Sephadex A-25-120 column chromatography of the Bio-Gel P-6 void fraction in Figure 2. The column (10 × 2 cm) was eluted with 50 mM acetate buffer (pH 6.0) containing 0, 0.2, 0.5, or 1.0 M NaCl as described in the text. The volume of the fractions was 5 ml. HL-elicitor activity was determined as described under "Materials and Methods."

in 100 mM Tris-HCl buffer, pH 8.0.

Optimum Conditions for HL-Elicitor Activity.

pH. Maximum phytoalexin accumulation in the wound-droplet solutions occurred when the pH of the HL-elicitor-containing Bio-Gel P-2 or P-6 void fractions (Fractions 36–44 in Fig. 2) was approximately 6.0, as shown in Figure 4.

Effect of Ca^{2+}. Addition of 1 mM $CaCl_2$ to the Bio-Gel P-2 or P-

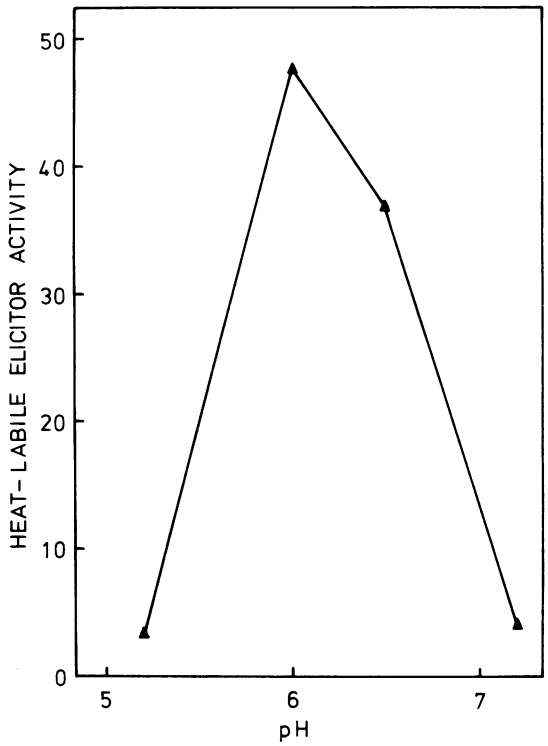

FIG. 4. Effect of pH on the HL-elicitor activity of Bio-Gel P-6 void fractions. The extracts were at pH 5.2, 6.0, and 6.5 in 40 mM acetate buffer, or at pH 7.2 in 40 mM Tris buffer. Data are the mean of three experiments. The HL-elicitor activity was determined as described in "Materials and Methods."

Table I. *Effect of Ca^{2+} or Mg^{2+} on the Activities of HL and HS Elicitors in Extracts of Frozen Soybean Stems*

Extract[a]	Elicitor Activity[b]	
	HL[c]	HS[d]
Bio-Gel P-2 void	4.3	3.3
Bio-Gel P-2 void in 0.8 mM $CaCl_2$	13.1	12.8
Bio-Gel P-2 void in 0.8 mM $MgCl_2$	6.1	5.4

[a] From a single extract of soybean stems (7 g stems/25 ml buffer), 5 ml was applied to each of 3 Bio-Gel P-2 (100–200 mesh) columns (24 × 1.4 cm). The columns were developed with 50 mM acetate buffer (pH 6.5) with the buffer plus 1.0 mM $CaCl_2$, or with the buffer plus 1.0 mM $MgCl_2$. Void fractions (4 ml) were collected from each column and diluted by addition of 1 ml of H_2O before bioassay.

[b] Data are the mean of two experiments.

[c] Units of HL elicitor calculated as described under "Materials and Methods." SD = 5.92 (17 *df*).

[d] Units of HS elicitor in the stem extract calculated as described under "Materials and Methods" by subtracting elicitation due to buffer alone from heat stable elicitation. SD = 4.22 (17 *df*).

6 void fractions increased the apparent HL-elicitor activity (Table I). Addition of 1 mM $MgCl_2$ resulted in little stimulation.

Choice of Buffer. Bio-Gel P-2 or P-6 void fractions in phosphate or Mes (2[*N*-morpholino]ethane sulfonic acid) buffers showed lower HL-elicitor activity than similar extracts in acetate or Tris buffer (Table II). Increasing the acetate buffer (pH 6.5) concentration from 10 to 50 mM increased the amount of phytoalexin accumulated in the wound droplets in the absence of exogenously applied elicitor. Buffer concentrations higher than 50 mM were not tested.

Instability of the HL-Elicitor Activity. HL-elicitor activity was recovered in greater amounts from stems extracted within 1 d of freezing; little activity was recovered from stems stored for 2 to 3

Table II. *Effect of Buffer on the Activities of HL and HS Elicitors in Extracts of Frozen Soybean Stems*

Extract[a]	Elicitor Activity	
	HL[b]	HS[c]
Bio-Gel P-2 void in acetate	27.7	16.6
Bio-Gel P-2 void in Tris	13.7	2.3
Bio-Gel P-2 void in phosphate	5.1	2.2
Bio-Gel P-2 void in Mes	4.4	1.5

[a] From a single extract of soybean stems (7 g stems/25 ml buffer), 5 ml was applied to each of 4 Bio-Gel P-2 (100-200 mesh) columns (24 × 1.4 cm). The columns were developed in either acetate, Tris, phosphate, or Mes buffer (50 mM [pH 6.5] containing 1.0 mM $CaCl_2$). Void fractions (4 ml) were collected from each column and diluted by addition of 1 ml of H_2O before bioassay.

[b] Units of HL elicitor calculated as described under "Materials and Methods." SD = 23.18 (7 *df*).

[c] Units of HS elicitor in the stem extract calculated as described under "Materials and Methods" by subtracting elicitation due to buffer alone from heat stable elicitation. SD = 16.39 (7 *df*).

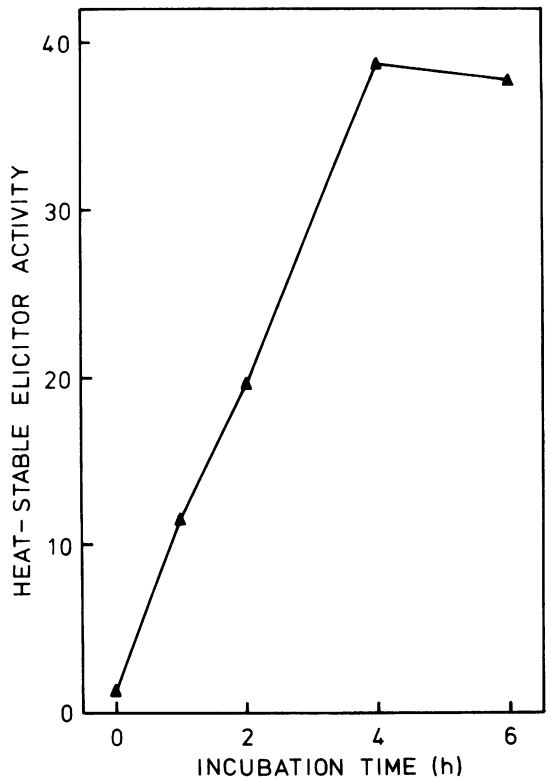

FIG. 5. Release of HS elicitor from purified soybean cell walls by the action of the HL elicitor. The HL elicitor, which was the Bio-Gel P-6 void (4 ml) in 25 mM actetate buffer (pH 6.0), containing 0.5 mM $CaCl_2$ (1 mM $CaCl_2$ in the 2nd experiment), was incubated with 30 mg purified soybean cell walls. The reaction mixtures were incubated at 30°C for up to 6 h, passed through a 1.2 μm Millipore filter to remove residual walls, heated at 95°C for 10 min to destroy HL-elicitor activity, and then assayed for elicitor activity. Bio-Gel P-6 void fractions heated at 95°C for 10 min before incubating with cell walls were used as controls, and the elicitor activity of these samples was subtracted from unheated treatments to give the amount of HS-elicitor activity released by the HL elicitor. Data are the mean of two experiments.

weeks at −26°C. HL-elicitor activity in Bio-Gel P-2 or P-6 void fractions was not stable at 3°C. For example, a Bio-Gel P-2 void fraction assayed as soon as possible after extraction contained 470 units of HL elicitor activity/ml, whereas the sample preparation

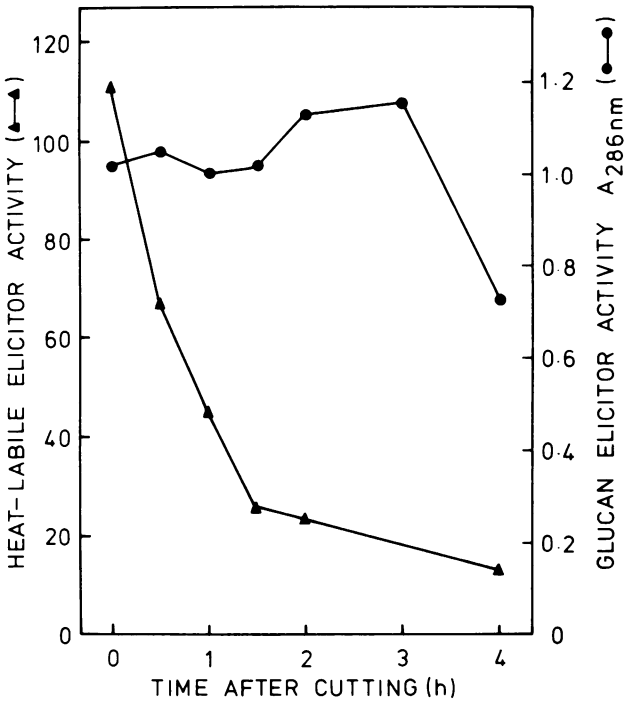

FIG. 6. The effect of exposing cut cotyledons to air before applying either the HL elicitor or the *Phytophthora* glucan elicitor. The HL elicitor was a Bio-Gel void fraction in 40 mM acetate buffer (pH 6.5) containing 0.8 mM $CaCl_2$, in the 1st experiment and 40 mM acetate buffer (pH 6.0) without $CaCl_2$, in the 2nd experiment. The data are the mean of two experiments and represent the difference between elicitation in heat-inactivated extracts (95°C for 10 min) and unheated extracts. Elicitation by the glucan elicitor (33 ng/cotyledon) was determined as described (6). The glucan was the "Fraction I" of Ayers *et al.* (4) and was prepared by J. Sharp and B. Valent in this laboratory.

assayed after storage at 3°C for 48 h contained 170 units of HL elicitor/ml. The stability of the HL elicitor was not improved by purification on DEAE-Sephadex with either step or gradient elution (data not presented).

Action of the HL Elicitor on Purified Cell Walls. The ability of the HL elicitor to release HS elicitor from purified cell walls is illustrated in Figure 5. The absence of a further release of HS elicitor after a 4 h incubation period may be due to the instability of the HL elicitor (see above).

Inactivation of the HL Elicitor on Cotyledons. The accumulation of phytoalexins was greatest when the cut surface of the cotyledon was exposed to HL-elicitor activity immediately after cutting. If the cut surface was exposed to the air for 30 min before applying HL elicitor, the response was reduced by approximately 40% (Fig. 6); long periods of preincubation reduced the response still further. The response of cotyledons to glucan phytoalexin elicitor (Fig. 6), extracted from walls of *Phytophthora megasperma* f. sp. glycinea, was not similarly decreased even when the glucan was applied up to 3 h after cutting (data provided by B. Hodgson of this laboratory).

DISCUSSION

A heat-labile component of freeze-thawed soybean stems, extracted in acetate or Tris buffer, elicits the accumulation of phytoalexins when applied to the cut surface of cotyledons. The HL elicitor is probably an enzyme because of its physical and chromatographic properties and its ability to catalyze the release of a HS elicitor from purified soybean cell walls. The wall-released HS elicitor might be similar to the endogenous elicitor described by Hahn *et al.* (6). Extracts containing HL elicitor, when heated to 95°C for 10 min, not only do not elicit phytoalexin accumulation but do not release HS elicitor from cell walls.

The optimum pH of the extraction buffer for HL-elicitor activity is approximately 6.0 (Fig. 4), but this pH should not be interpreted as the optimum for a single enzyme. It is more likely that this is the pH optimum for a combination of enzymes, *e.g.* The HL elicitor together with enzymes involved in biosynthesis of the phytoalexins.

Soybean cell walls can be partially hydrolyzed by 2 N trifluoroacetic acid to release pectic polysaccharide fragments, some of which elicit phytoalexins in the soybean cotyledon assay (6). These wall fragments are referred to as the endogenous elicitor. We have not determined whether the HS elicitor solubilized from isolated cell walls by the HL elicitor is similar to the endogenous elicitor described by Hahn *et al.* (6).

The extraction of HL-elicitor activity from frozen and thawed soybean stems suggests that the elicitor may be involved in stimulating phytoalexin accumulation in response to freezing damage (7, 13). Heat-labile elicitor may also be involved in the observed accumulation of phytoalexins when cells are damaged by UV light (15), heavy metals, and detergents (16). We have not examined HL-elicitor involvement in host-pathogen interactions, although we believe that it exists (1, 11). Evidence for such involvement must await further purification and characterization of the HL elicitor.

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