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Isolation and Purification of a Factor from *Pseudomonas solanacearum* That Induces a Hypersensitive-like Response in Potato Cells

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Callus tissues derived from bacterial wilt-resistant or -susceptible clones of *Solanum phureja* responded differentially to inoculation with strains of *Pseudomonas solanacearum*. Calli from resistant clones exhibited rapid browning followed by death of the cells, whereas calli from susceptible clones retained their normal appearance for 48 hr after inoculation. Similarly, leaves from resistant clones exhibited a typical hypersensitive response (HR), whereas those from susceptible clones remained symptomless for 48 hr after inoculation. When inoculated with one particular mutant strain (B1), death of callus cells from the potato clone C-3 appeared to be caused by a bacterial protein that is excreted in increased amounts when the bacteria are in contact with plant tissues. This protein was purified by ion exchange, gel permeation, and affinity chromatography. It has a high proline and glycine content, is highly basic (pI = 9.15), and has a mass of approximately 60 kDa. The protein causes rapid browning of callus tissue, rapid death of suspension-cultured cells, and a typical HR in leaves of potato clone C-3.

Additional keywords: disease resistance, incompatibility.

Bacterial wilt caused by *Pseudomonas solanacearum* E. F. Smith is one of the most important diseases of potato in tropical and subtropical areas of the world. Most commercial potato cultivars are highly susceptible to one or more of the many strains of *P. solanacearum* that occur worldwide. However, certain clones of the cultivated diploid *Solanum phureja* Juz. et Buk. have a high degree of resistance to bacterial wilt, and this resistance has been transferred into commercial cultivars of *S. tuberosum* L. (Sequeira and Rowe 1969).

Analyses of segregating progenies of crosses between resistant and susceptible clones of *S. phureja* have shown that resistance to two strains of the pathogen, K60 and S123, is controlled by three separate, dominant genes in each instance (Rowe and Sequeira 1970; Rowe *et al.* 1972). Leaves from resistant plants exhibit a typical hypersensitive response (HR) when inoculated with incompatible strains of the bacterium (Y. Huang, unpublished data). Thus, the differential response (resistant or susceptible) of clones of *S. phureja* to different strains of the pathogen suggested that this host-parasite system could be very useful for the study of the genetics and physiology of the bacterially induced HR. The value of this system became evident when it was determined that callus tissues from individual clones of *S. phureja* exhibited responses to inoculation with different strains of *P. solanacearum* that corresponded very closely to those of intact plants (Huang *et al.* 1986). We now report that a protein produced by strain B1, when placed in contact with plant tissues, appears to be responsible for inducing a hypersensitive-like response in clone C-3 of *S. phureja*.

MATERIALS AND METHODS

Bacterial cultures. The six strains of *P. solanacearum* used in this study (Table 1) were obtained from lyophilized stocks in the culture collection maintained in the Department of Plant Pathology, University of Wisconsin-Madison. Bacterial stocks were resuspended in sterile deionized water, streaked on casamino acids-peptone-glucose (CPG) medium (Hendrick and Sequeira 1984), and incubated for 48 hr at 28° C. Individual colonies were collected and resuspended in sterile deionized water to reach A_{600} nm values corresponding to 5×10^8 cfu/ml (for plant inoculations) or to 5×10^7 cfu/ml (for callus inoculations).

Potato clones. Hybrid (F1) seed from the cross of *S. phureja* accessions CCC 1339 (susceptible) × CCC 1386 (resistant) was obtained from the Inter-Regional Potato Introduction Project IR-1, Sturgeon Bay, Wisconsin. Seeds were sown in Jiffy Mix (Jiffy Products of America, Chicago, Illinois); seedlings were grown in a growth room for 3 wk at 24° C under fluorescent lighting ($60\ \mu Em^{-}/sec^{-1}$) and a 16-hr photoperiod. Stem cuttings from individual seedlings were rooted and grown for an additional 4 wk, and these plants were used as sources of material for tissue culture or for inoculation experiments.

Callus cultures. Calli from each selected clone of *S. phureja* were derived from tissue excised from the middle

Table 1. Origin of the strains of *Pseudomonas solanacearum* used in this work

Strain	Race	Host of origin	Location
S123	1	*Eupatorium odoratum*	Coto, Costa Rica
S206	3	Potato	Las Palmas, Colombia
S207	3	Potato	Popayan, Colombia
K56	1	Potato	Israel
K60	1	Tomato	North Carolina, U.S.A.
B1	1	Tomato (variant of K60)	North Carolina, U.S.A.

portion of the stem. Explants were sterilized by immersion in 95% (v/v) ethanol for 45 sec, followed by 5 min in 30% (v/v) sodium hypochlorite and three rinses in sterile deionized water. Sterilized explants were trimmed to 1.5-cm-long pieces, which were then placed on Murashige and Skoog's (1962) medium amended with 5 mg/L of naphthalene acetic acid (NAA). Explants were incubated at 24° C and 60 $\mu Em^{-2}/sec^{-1}$ from fluorescent lights on a 16-hr photoperiod. After 3-4 wk growth, callus tissues growing at the edges of the explants were transferred to fresh medium. The newly transferred calli were grown for 3 wk before they were used for inoculation experiments.

Inoculations. Intact plants were inoculated by placing a drop of bacterial suspension on the axil of the third fully developed leaf and puncturing the stem with a sterile needle thrust through the drop. Five plants of each of five different clones were inoculated with six different strains of *P. solanacearum*. Inoculated plants were maintained in the greenhouse at 28° C (±5° C) and a 16-hr photoperiod and scored for wilt symptoms 10–15 days after inoculation.

Leaves of three selected clones (C-3, C-6, and C-15) were infiltrated (abaxial surface) with bacterial suspensions by means of a hypodermic syringe fitted with a 30-gauge needle (Klement *et al.* 1964). After infiltration, plants were maintained in the greenhouse, as described above, and rated for presence or absence of the HR 48 hr after infiltration.

Calli were inoculated by placing 10 μl of bacterial suspension on the surface of each piece. At least five callus pieces from each of six clones were inoculated with each bacterial strain. Presence or absence of browning of calli was determined after 48–60 hr of incubation under the conditions described previously for callus cultures. Browning of tissues from resistant clones was evident only in the area immediately adjacent to the inoculation site after 36 hr but had spread throughout the entire callus piece by 48–60 hr, closely following the pattern of bacterial growth. Tissues from susceptible clones retained their normal color (light green) during the same period.

Cell suspension cultures and cocultivation. Potato cell suspension cultures were prepared by placing 5 g of friable C-3 callus tissue in 30 ml of 10% CPG broth in a 250-ml Erlenmeyer flask. The flask was placed in a shaker-incubator at 160 rpm at 26° C (±1° C) under fluorescent lighting. Individual cells or clumps of cells were present in large numbers in the medium after 12 hr of continuous shaking. To determine the viability of the suspension-cultured cells, samples (0.5 ml each) were removed at 12-hr intervals for 120 hr, and the cells were collected by filtering through Miracloth. After rinsing three times with sterile deionized water, viability of the potato cells was determined by fluorescence microscopy (Withers 1985). For this purpose, potato cells were resuspended in one drop of 10% CPG broth and mixed with one drop of dilute (0.5 μg/ml) fluorescein diacetate on a microscope slide. Total number of cells per field was determined by ordinary light microscopy; the number of live (fluorescent) cells was determined by ultraviolet light microscopy of the same slide preparation. Counts from five separate fields were used to calculate the ratio of living to total number of cells.

In cocultivation experiments, bacteria, grown as described previously, were added to the potato cell suspension culture to an initial concentration of 5×10^7 cfu/ml. Bacterial populations were determined by standard plate-dilution techniques of samples removed at 12-hr intervals for 72 hr after inoculation.

Purification of the browning factor. Cell suspension cultures from approximately 5 g of C-3 friable tissues were cocultivated with an initial population of 5×10^7 cfu/ml of strain B1 of *P. solanacearum*. After 12 hr of continuous shaking, the suspension was sonicated for 30 sec and then centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was lyophilized and resuspended in 50 mM phosphate buffer at one-tenth the original volume. This crude fraction (50 ml) was applied to a 4 cm × 1 cm column of DEAE-cellulose (Whatman) pre-equilibrated in 50 mM phosphate buffer and was eluted with the same buffer at a flow rate of 15 ml/hr. The eluate was collected, concentrated fivefold by ultrafiltration, and applied to a 60 cm × 2.5 cm column of Bio-Gel P200 (Bio-Rad, Richmond, CA). After eluting with the same buffer at a flow rate of 6 ml/hr, each fraction (5 ml) was tested for browning activity as described below. The active fractions were pooled and concentrated fivefold by ultrafiltration. This concentrate was applied to a 5 cm × 1 cm column of Affi-Gel Blue (Bio-Rad) and eluted with the same buffer at a flow rate of 10 ml/hr. The unbound fraction, which retained the biological activity, was pooled, concentrated by ultrafiltration, and stored at −70° C.

The purified fraction was separated by PAGE according to Laemmli (1970) in a Protean double-slab apparatus (Bio-Rad) or in a minigel apparatus (Hoefer Scientific Instruments). Proteins were detected by staining with either Coomassie Blue R-250 or silver nitrate, according to Oakley *et al.* (1980). Protein content was estimated by the Bradford (1976) procedure with bovine serum albumin as the standard. To determine the amino acid composition of the purified, biologically active protein, a 10-μg sample was dialyzed against distilled water for 2 days to remove buffer salts and then was hydrolyzed under vacuum with 6 N HCl and 0.2% phenol (v/v) for 48 hr. The concentration of amino acids in the hydrolysate was determined with an automatic amino acid analyzer at the facility for protein analysis of the Biotechnology Center, University of Wisconsin-Madison.

Isoelectric focusing of the purified protein was performed on a model 2117 Multiphor Apparatus (LKB) essentially as described by Ried and Collmer (1985), except that a 0.75-mm-thick gel was used. Gels were pre-electrofocused at 5.0 W for 30 min. After samples were applied, gels were electrofocused at 10.0 W for 60 min.

To verify that the browning factor was produced by strain B1 of *P. solanacearum* rather than by potato cells, a dialysis membrane with a 3,500 M_r cut-off was used to separate bacterial cells from potato cells. For this purpose, a suspension (50 ml) of strain B1 (10^8 cfu/ml) in 10% CPG medium was sealed in a dialysis bag, which was then placed in a 500-ml Erlenmeyer flask containing a suspension (100 ml) of 5 g of friable C-3 callus tissues in 10% CPG. The flask was placed in a shaker-incubator at 160 rpm at 26° C (±1° C) under fluorescent lighting. After 12 hr, the dialysis bag was retrieved and the bacteria in suspension within the bag were removed by centrifugation followed by ultrafiltration. The culture supernatants from both bacterial and plant cell cultures then were assayed for biological activity on potato (C-3) callus as described below.

Bioassays. Browning activity of column fractions was assayed directly on callus tissue. For this purpose, 20-μl samples were placed in the center of rapidly growing callus

tissues of clone C-3. Browning was detected visually after 24-hr incubation at 25° C.

The ability of various fractions to cause rapid death of potato cells was tested by adding samples (20 μl each) to cell suspension cultures prepared as described before but distributed in wells (150 μl per well) of microtiter test plates (Falcon). The mixtures were incubated with shaking at 70 rpm for 12 hr. Viability of the potato cells was determined after staining them with fluorescein diacetate, as described above.

RESULTS

There was an almost absolute correspondence between the resistance or susceptibility of clones of *S. phureja* to bacterial wilt and the response of callus tissues or intact leaves of the same clones to inoculation with various strains of *P. solanacearum* (Table 2). The data summarized in Table 2 describes the responses of three clones only, C-3, C-6, and C-15, to inoculation with six different strains of the pathogen. Similar results were obtained with inoculations of two additional clones (not described here). The resistant response of whole plants consisted of no wilting at all or of epinasty limited to the inoculated leaf, as previously described (Sequeira and Rowe 1969). Susceptible responses of inoculated plants ranged from complete wilting and death of the plant in 21 days to wilting of the inoculated leaf and epinasty of the leaves immediately above or below the inoculated leaf. There were clear differences in degree and rapidity of wilting caused by different strains on different clones, but all plants exhibiting wilting responses were classified as susceptible.

In incompatible combinations with bacteria, calli exhibited rapid browning next to the point of inoculation within 24 hr; by 48–60 hr, browning had spread throughout each individual callus piece. In compatible combinations, calli retained their normal color (light green) during the same period after inoculation (Fig. 1).

With one exception, calli that gave a browning response always came from plants that exhibited the HR upon inoculation with the same bacterial strain. The HR obtained in attached potato leaves was similar to that described for tobacco leaves infiltrated with *P. solanacearum* (Lozano and Sequeira 1970). There was rapid (24 hr) collapse of the infiltrated tissue, which became desiccated and paper thin.

Table 2. Responses of clones of *S. phureja* and of calli derived from these clones to inoculation with various strains of *P. solanacearum*

	Responses of whole plants (R or S)[a] or calli (+ or −)[a] to inoculation with strains of *P. solanacearum*:					
Clone	**S123**	**S206**	**S207**	**K56**	**K60**	**B1**
C-3	R (+)	R (+)	S (−)	S (−)	R (+)	R (+)
C-6	R (+)	R (+)	S (−)	S (−)	S (−)	R (+)
C-15	S (−)[b]	R (+)	S (−)	S (−)	R (+)	R (+)

[a] Plants: R = resistant (HR positive); S = susceptible (HR negative). Calli: (+) = rapid browning; (−) = no browning.

[b] In this combination only, plants were HR positive although susceptible to the pathogen.

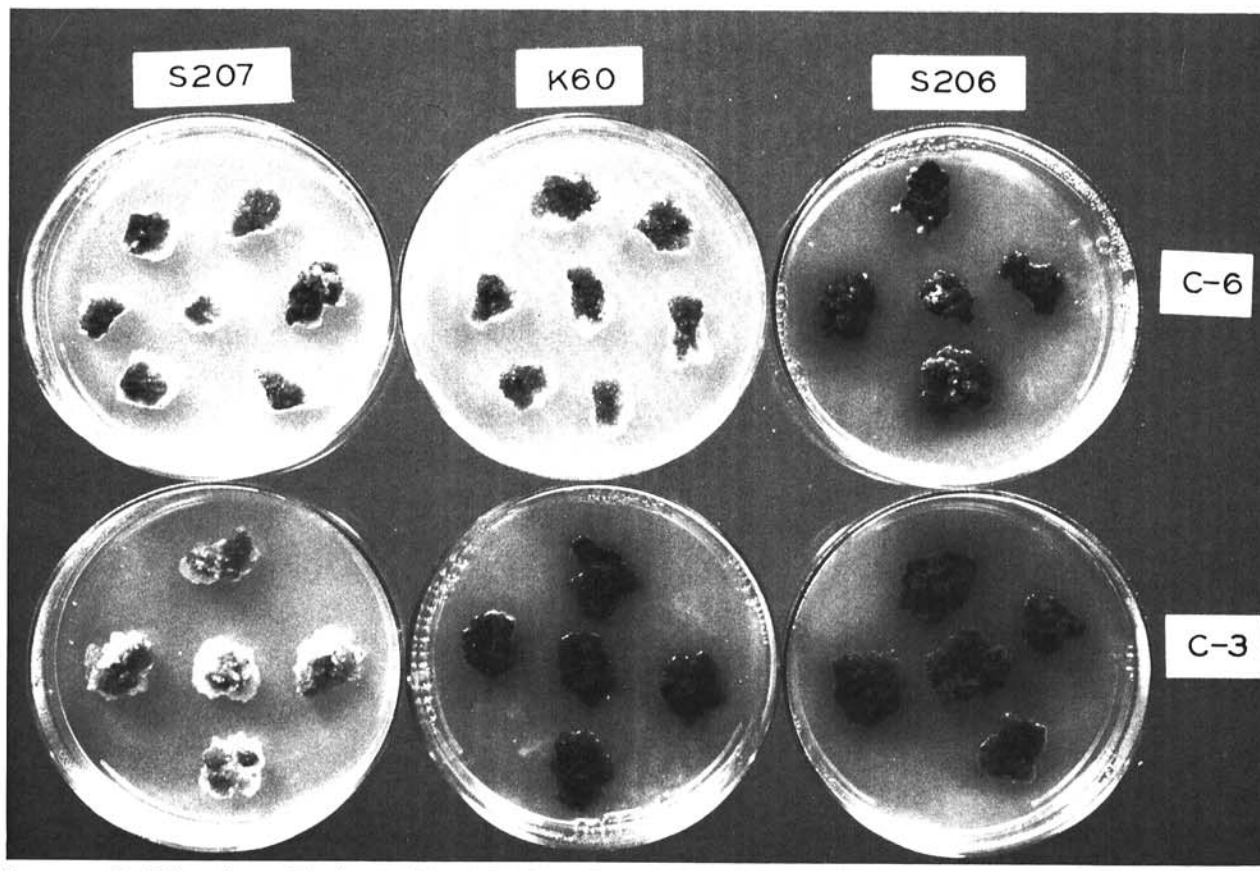

Fig. 1. Responses of calli from clones of *S. phureja* (C-3 and C-6) 60 hr after inoculation with different strains (S207, K60, and S206) of *P. solanacearum*. Compatible (susceptible) calli (upper row, left and center plates; lower row, left plate) remain light green in color; incompatible (resistant) calli (upper row, right plate; lower row, center and right plates) turn dark brown.

The only exception was clone C-15 infiltrated with strain S123; in this instance, the HR was induced in leaves, although the plants wilted after stem inoculation and there was no browning of inoculated calli. S123, however, is a peculiar strain in that it also causes the HR in tobacco leaves, although it is highly pathogenic to that host (Granada and Sequeira 1975).

The differential response of the calli from clones of *S. phureja* to bacterial inoculation was also obtained in suspension-cultured cells. When cocultivated with incompatible strains of *P. solanacearum*, potato cells died very rapidly (Fig. 2). About 80% of the cells were dead (nonfluorescent) by 12 hr after exposure to the bacteria. In contrast, only 25% of the cells were dead in the compatible combinations, a proportion that was not significantly different (t test) from that of control cells that were not exposed to bacteria for 12 hr.

There were no significant differences in growth of bacteria grown in cocultivation with either resistant or susceptible clones of *S. phureja* (Fig. 3). Although there was a slight decline in population sizes of the two incompatible bacteria after 60 hr, this change was not significant when compared with the population of the compatible bacterium after the same incubation period. Maximum population sizes in the controls (bacteria alone) were, as expected, considerably lower than those of bacteria grown in association with plant cells. Presumably, plant cells provide nutrients that the bacteria can utilize for growth.

When supernatants from cultures involving incompatible and compatible combinations of bacteria with potato cells (Fig. 3) were tested for biological activity in the callus assay, only those from incompatible combinations caused rapid browning of the tissues. No biological activity was detected in the supernatant of bacteria grown in the absence of plant tissues. That the browning factor was produced by bacteria and not by plant cells was demonstrated by separating the two culture components by means of a dialysis membrane. Browning activity was detected only in the supernatant of the B1 culture contained within the dialysis bag. The highest level of biological activity (rapid browning) was detected in the supernatant of the combination of strain B1 with clone C-3 after 12 hr of incubation; activity declined slowly thereafter (data not shown).

The factor responsible for the biological activity of the concentrated supernatant of B1:C-3 cultures was not retained by the DEAE-cellulose column. When the eluate of this column was separated by gel permeation, biological activity was associated with a single peak (Fig. 4), and when these fractions (numbers 23–28) were separated by SDS-PAGE, all contained a protein band of approximately 60 kDa. This protein was purified by affinity chromatography (Fig. 5, Table 3). Amino acid analysis showed that the protein consists of 17 different amino acids, the most abundant being proline and glycine (Table 4). The protein is highly basic, with an isoelectric point of 9.15.

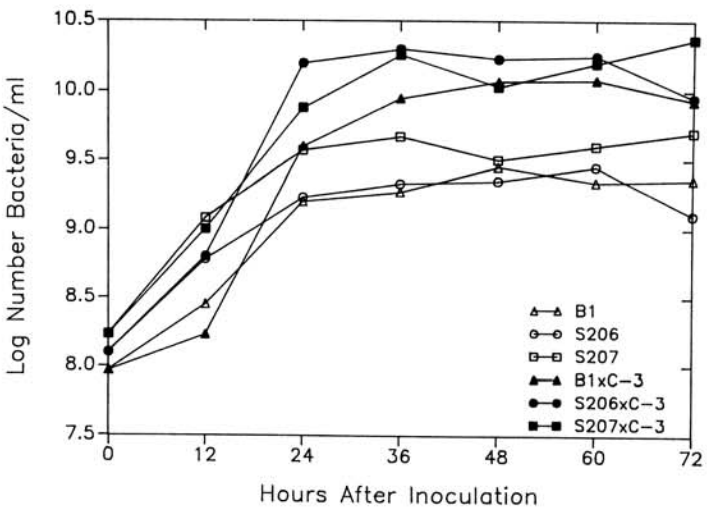

Fig. 3. Multiplication of different strains of *P. solanacearum* in 10% CPG medium with and without potato (C-3) cells. S207 is a compatible strain, B1 and S206 are incompatible. Potato cells from 1 g of callus tissues per flask were grown in 30 ml of medium; bacteria were added at 5×10^8 cfu/ml. Each point is the average of three replications.

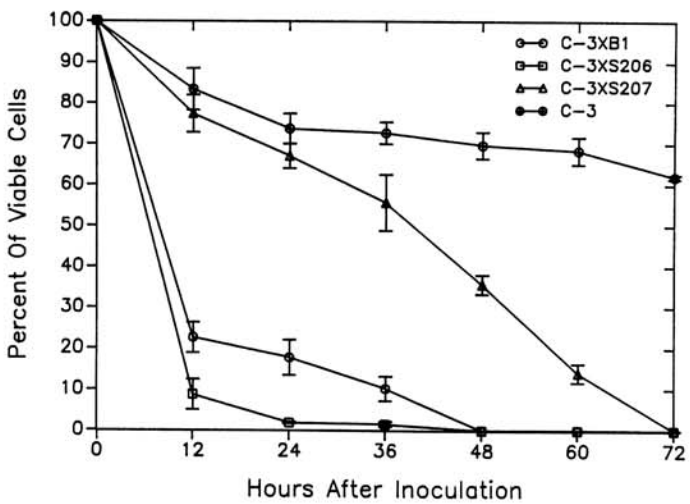

Fig. 2. Viability of potato clone C-3 cells in suspension culture. C-3 × B1 and C-3 × S206 are incompatible combinations; C-3 × S207 is a compatible combination; C-3 cells without bacteria are the control. Potato cells from 1 g of callus tissues per flask were grown in 30 ml of 10% CPG medium; bacteria were added at 5×10^8 cfu/ml. Data represent the average and standard deviation of five replications.

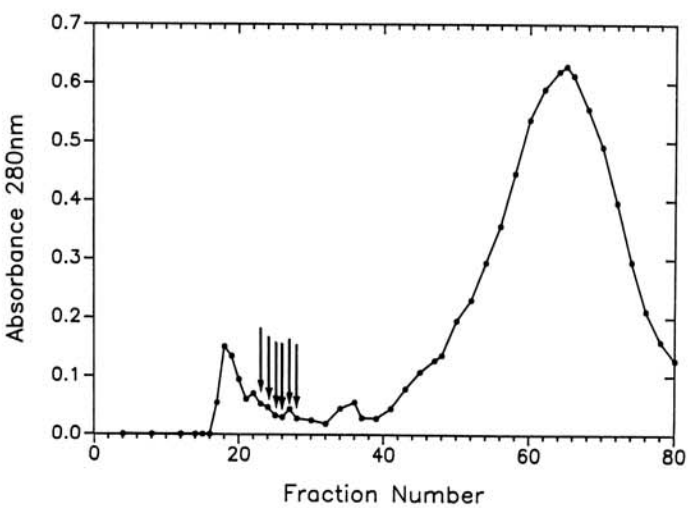

Fig. 4. Gel filtration profile of crude extracts of potato (C-3) tissue cultures inoculated with strain B1 of *P. solanacearum*, after elution from a DEAE-cellulose column. Fractions 23–28 (indicated by arrows) contained all browning factor activity on C-3 callus. Five-milliliter fractions; flow rate: 6 ml/hr.

When tested by the direct assay on C-3 tissue cultures, the purified protein caused a detectable browning response at concentrations as low as 0.05 μg per 2 g of callus tissue. Heating the 60-kDa protein at 60° C for 1 min or treating it with Pronase (data not shown) completely eliminated biological activity. When infiltrated into leaves of all three clones of *S. phureja*, the purified 60-kDa protein caused collapse of the tissues within 24 hr. Tissues became desiccated, bleached, and paper thin, as is typical of the HR induced by live B1 bacteria.

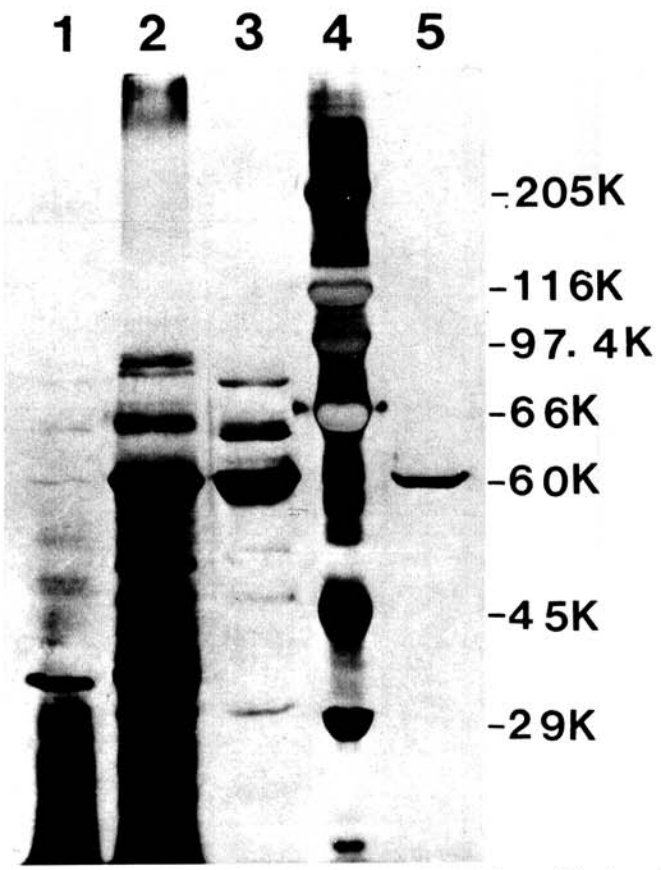

Fig. 5. SDS-PAGE of different fractions obtained during purification of the callus browning factor from strain B1 of *P. solanacearum*. Lane 1, fraction from the culture supernatant of strain B1 grown in 10% CPG medium only; lane 2, fraction from the culture supernatant of strain B1 grown in the presence of potato (C-3) cells and after DEAE-cellulose chromatography; lane 3, fraction as in (2) after gel filtration; lane 4, protein molecular weight standards; lane 5, fraction as in (3) after Affi-Gel Blue chromatography. The gel was stained first with Coomassie Blue and then purposely overstained with silver reagent.

Table 3. Biological activities[a] of different fractions obtained during purification of the callus browning factor produced by strain B1 of *Pseudomonas solanacearum* in the presence of potato (C-3) cells

Step in purification	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purity factor
Crude extract	30	11.5	6,000	522	100	···
DEAE cellulose	20	7.7	5,000	649	83	12
Gel permeation	10	1.9	3,000	1,563	50	30
Affi-Gel Blue	5	0.3	2,500	7,500	42	14

[a] One unit of activity was the minimum amount required to cause visible browning of a rapidly growing callus in culture.

DISCUSSION

The very strong correlations between the resistance of whole plants, the HR of attached leaves, and the browning response of tissue cultures derived from the same plants indicate that the *S. phureja–P. solanacearum* system may be a useful model for the study of disease resistance mechanisms. The facts that *S. phureja* is a diploid potato and is readily intercrossed by sib mating and that there is substantial information on the genetics of resistance to bacterial wilt (Sequeira and Rowe 1969; Rowe and Sequeira 1970; Rowe *et al.* 1972) make this an attractive system, because it allows both traditional and modern genetic approaches to the problem of hypersensitivity in plants.

The rapid browning response of callus tissues to inoculation with incompatible strains of *P. solanacearum* was distinct from the lack of a visible response to compatible strains within 48 hr after inoculation. Browning responses of callus tissues to other pathogens have been reported (Atkinson *et al.* 1985; Haberlach *et al.* 1978; Miller *et al.* 1984). With *Phytophthora parasitica* var. *nicotianae*, however, differences in rates of colonization of tobacco callus by hyphae of compatible and incompatible races provided a more reliable indication of resistance or susceptibility than the color of the tissue (deZoeten *et al.* 1982; Helgeson *et al.* 1972).

The growth conditions that allow a differential response of potato tissues to compatible and incompatible strains of *P. solanacearum* were established after extensive experimentation. It is well known that environmental and nutritional conditions greatly influence the expression of disease resistance in tissue culture (Haberlach *et al.* 1978; Ingram 1976; McComb *et al.* 1987; Miller *et al.* 1984). In our system, a medium containing 5 mg NAA/L and no cytokinins provided the type of friable tissue that gave a differential response. Addition of cytokinins to the medium, even at low concentrations, eliminated this differential response.

The rapid death of mesophyll cells that occurs when incompatible bacteria are present in the intercellular spaces is diagnostic of hypersensitive-type resistance in plants

Table 4. Amino acid analysis of the 60-kDa protein produced by strain B1 of *P. solanacearum* in the presence of potato (C-3) cells

Amino acid	Residues/mole
ASK	42
GLX	48
SER	32
GLY	146
HIS	6
ARG	17
THR	24
ALA	58
PRO	110
TYR	5
VAL	28
MET	4
CYS	3
ILE	22
LEU	34
PHE	18
LYS	24
	621
Estimated molecular weight	59,364
Hydrophobic amino acids	44%

(Klement *et al.* 1964; Klement 1982). Although tobacco has been the plant most commonly used for HR studies, we report here that leaves of *S. phureja* respond in a similar manner, although, because of their small size, they are more difficult to infiltrate by conventional means. Unlike tobacco, however, different cultivars (clones) of *S. phureja* exhibit differential responses to the same strain of the bacterium, thus providing a useful system for the study of the genetics of host-parasite interactions.

Although the browning response of potato calli to incompatible strains of *P. solanacearum* apparently is due to rapid death of host cells, as is characteristic of the HR in attached leaves, the growth pattern of the bacterium in the cocultivation experiments (Fig. 3) was unlike that in leaves. In leaves, bacterial populations decline rapidly in incompatible combinations leading to the HR, but multiply exponentially in compatible combinations (Lozano and Sequeira 1970). In potato cell suspension cultures, however, bacteria grew exponentially in both combinations (Fig. 3). Whether this difference is due to the fact that desiccation occurs on leaves but not in culture or that compounds released from dying leaf cells do not reach toxic concentrations in culture is not known.

Although rapid browning and death of potato cells in contact with strains of *P. solanacearum* are thought to be intimately related, there is no proof that there is a causal relationship. Death was determined by staining with a dye that, presumably, allows detection of living cells by their fluorescence (Huang and Van Dyke 1978; Withers 1985). Whether the same cells that do not fluoresce are the same cells that undergo discoloration could not be determined by microscopy.

Many attempts have been made to isolate bacterial products that induce the HR in plants (Klement 1982). Most of these attempts have failed because of the difficulty of distinguishing the HR from nonspecific toxic products produced by bacteria in culture. The 60-kDa protein we have isolated may have more potential as the putative HR-inducing agent of strain B1 of *P. solanacearum* because: it is produced at very low levels when the bacteria are grown in ordinary media, but at high levels (detectable by bioassay) when bacteria are placed in contact with host cells or their products; it is produced early in the interaction of bacteria with host cells; and it is active at very low concentrations, that is, within the physiological range of compounds that may be significant *in planta*. We have preliminary evidence that production of this protein is induced by low molecular weight compounds of host origin, based on application of methods similar to those employed by Stachel *et al.* (1986) for the study of plant products that induce virulence genes in *Agrobacterium tumefaciens* (data not shown).

The 60-kDa protein from strain B1 caused a hypersensitive-type reaction in all three potato clones, as expected, for this strain is incompatible with all of them. These results suggest that putative receptors for this compound exist in all clones of *S. phureja*. The more critical question, however, is whether browning factor production is repressed in strains that do not induce the HR in certain potato clones. Alternatively, in compatible interactions the bacterium may produce a similar, but physiologically inactive form of the protein we have isolated from strain B1. Work in progress in our laboratory is designed to answer these questions.

Genetic studies designed to determine whether the 60-kDa protein is the structural protein responsible for the HR induced by *P. solanacearum* in potato are being completed. We have used Tn*5* mutagenesis to isolate a series of mutants that lack the ability to cause the HR and also lack the 60-kDa protein (Huang *et al.* 1987). The results obtained thus far provide support for the concept that this protein is responsible for the HR.

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