Detection of Coronatine Toxin of *Pseudomonas syringae* pv. *glycinea* with an Enzyme-Linked Immunosorbent Assay

J. V. Leary, S. Roberts, and J. W. Willis

Department of Plant Pathology, University of California, Riverside 92521.
Present address of third author: EniChem, Monmouth Junction, NJ 08852.
Accepted for publication 19 July 1988 (submitted for electronic processing).

ABSTRACT

Leary, J. V., Roberts, S., and Willis, J. W. 1988. Detection of coronatine toxin of *Pseudomonas syringae* pv. *glycinea* with an enzyme-linked immunosorbent assay. Phytopathology 78:1498-1500.

A rapid, reliable method for detecting coronatine toxin produced by *Pseudomonas syringae* pv. *glycinea* employed antibodies raised to a bovine serum albumin-coronatine conjugate. The antiserum was used in an enzyme-linked immunosorbent assay to detect coronatine in culture filtrates and partially purified toxin preparations.

Coronatine, a phytotoxin produced by *Pseudomonas syringae* pv. *glycinea* (5), *Pseudomonas syringae* pv. *atropurpurea* (6), and *Pseudomonas syringae* pv. *tomato* (6), causes chlorosis in the respective hosts of the bacteria: soybean (*Glycine max* (L) Merr.), Italian ryegrass (*Lolium multiflorum* Lam.), and tomato (*Lycopersicon esculentum* Mill.). As little as 25 ng of coronatine causes detectable chlorosis when applied to soybean leaves (4). Although production of coronatine does not appear to be essential for pathogenicity (1), it is important in the development of typical disease symptoms in bacterial blight of soybeans.

Methods for detection of coronatine include induction of chlorosis in soybean leaves (1), induction of hypertrophy in potato tuber disks (8), and thin-layer chromatography of partially purified culture fluid extracts (4,5). However, none of these methods is sufficiently rapid and simple for screening the large number of bacterial isolates required for studies of the genetics of coronatine production. Therefore, antibodies to a bovine serum albumin (BSA)-coronatine conjugate were produced and employed in an enzyme-linked immunosorbent assay (ELISA) for the rapid and efficient detection of coronatine in culture filtrates and partially purified toxin preparations.

MATERIALS AND METHODS

Preparation of BSA-coronatine conjugate. Ten mg of coronatine, purified according to Mitchell's procedure (4), was dissolved in 5 ml of carbonate-bicarbonate buffer (pH 8.4). One hundred mg of BSA was dissolved in 5 ml of the same buffer, and the two solutions were mixed and stirred overnight (18 hr) at 4 C in the dark. The BSA-coronatine solution was then dialyzed overnight against sterile deionized-distilled water.

Confirmation of conjugation of coronatine to BSA was tested by the determination of free amino groups by trinitrobenzenesulfonic acid (2) and by applying a sample of the dialysate to an isoelectric focusing SERVALYT-precote plate (Serva Fine Biochemicals, Haake Buchler Saddle Brook, NJ) (pH 3–10) and focusing on a Desaga Brinkman double chamber model 171D isoelectric focusing apparatus (Brinkman Instrument Co., Westbury, NY), cooled by a Savant model RWC50A condenser to 0 C. Current was applied by a Savant HV 5000A high-voltage power supply (Savant Instruments, Farmingdale, NY). Voltage was started at 200 V and increased to 2,000 V over 2 hr. BSA suspended in buffer and dialyzed against water served as the control. Proteins were visualized by staining with Serva Blue W.

Production and purification of antibodies. Ascitic fluid was induced in Balb-C mice by one intraperitoneal injection with

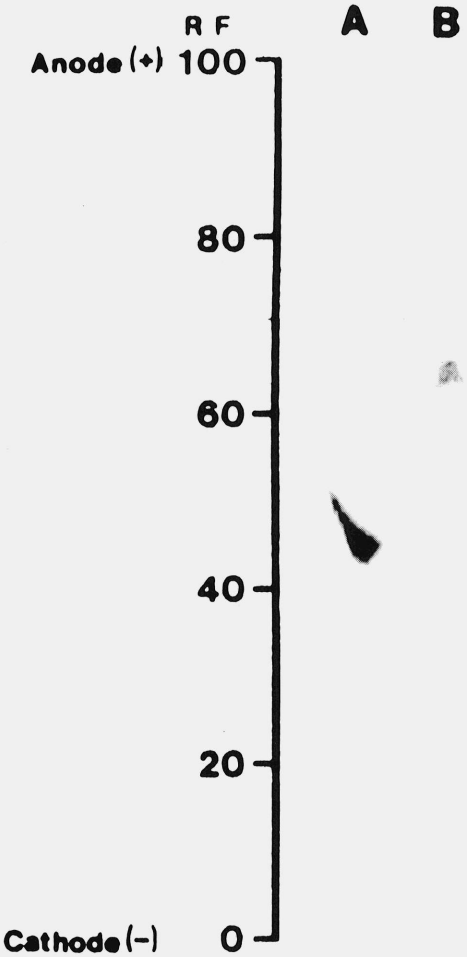

Fig. 1. Isoelectric focusing gel of bovine serum albumin (BSA) (**A**) and BSA-coronatine conjugate (**B**). A sample of the coronatine-BSA conjugate was applied to an isoelectric focusing SERVALYT-precote plate (Serva Fine Biochemicals, Inc. Dist., Haake Buchler Saddle Brook, NJ) (pH 3–10) and focused on a Desaga Brinkman double chamber model 171D isoelectric focusing apparatus (Brinkman Instrument Co., Westbury, NY) cooled by a Savant model RWC50A condenser to 0 C. Current was applied by a Savant HV 5000A high-voltage power supply (Savant Instruments, Farmingdale, NY). Voltage was started at 200 V and increased to 2,000 V over 2 hr. BSA suspended in buffer and dialyzed against water served as the control. Proteins were visualized by staining with Serva Blue W.

Pristane, followed by intraperitoneal injections with 0.2 ml of a 9:1 mixture of Freunds Complete Adjuvant and BSA-coronatine conjugate (10 mg/ml) (10). Two wk was allowed between the first and second injections, followed by weekly injections for the next 3 wk. Fluids were collected from the abdominal cavity by inserting a 18-gauge needle and allowing the fluid to drain. Ascitic fluid was collected whenever appreciable amounts of fluid became visibly evident, and was then frozen immediately. Upon subsequent thawing, fibrin clots were removed by centrifugation at 17,000 *g* at 5 C. IgG was purified by the method of Steinbuch and Audran (9).

ELISA reagents. The following were used for a coating buffer: Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; NaN_3, 0.2 g per liter of H_2O (pH 9.6). PBST buffer consisted of: NaCl, 8 g; KH_2PO_4, 0.2 g; NaH_2PO_4, 1.16 g; KCl, 0.2 g; 0.5 ml Tween 20 per liter of H_2O (pH 7.4); 10% diethanolamine (DEA), 9.7 ml; H_2O, 80 ml, adjusted to pH 8.6 with HCl, bringing final volume to 100 ml. The substrate was p-nitrophenolphosphate, 1 mg per milliliter of 10% DEA.

Coating. Two hundred μl of coronatine, diluted appropriately, and/or culture filtrates diluted 1:20 were added to wells of Immulon II Flat Bottom Microelisa plates (Dynatech, Chantilly, VA) by drying overnight with circulating air at 37 C.

Assay. Plates were washed three times for 3 min each with PBST at room temperature after coating and between additions of 200 μl of antiserum (IgG) diluted 100× with PBST; and 200 μl of goat anti-mouse alkaline phosphatase (Miles Laboratories) diluted 1:2,000 with PBST. Each addition was incubated for 2 hr. Two hundred μl of p-nitrophenolphosphate was then added and the reaction stopped after 45 min with 50 μl of 3 N NaOH.

TABLE 1. Sensitivity of ELISA for detecting coronatine

Coronatine concentration (μg/ml)[a]	ELISA A_{405}[b]
0.0	0.053 ± 0.003
0.006	0.266 ± 0.0160
0.06	0.639 ± 0.0491
0.60	0.940 ± 0.0092
6.0	0.785 ± 0.0214

[a] Antiserum at a 1/100 dilution.
[b] Mean ± standard deviation of four replications.

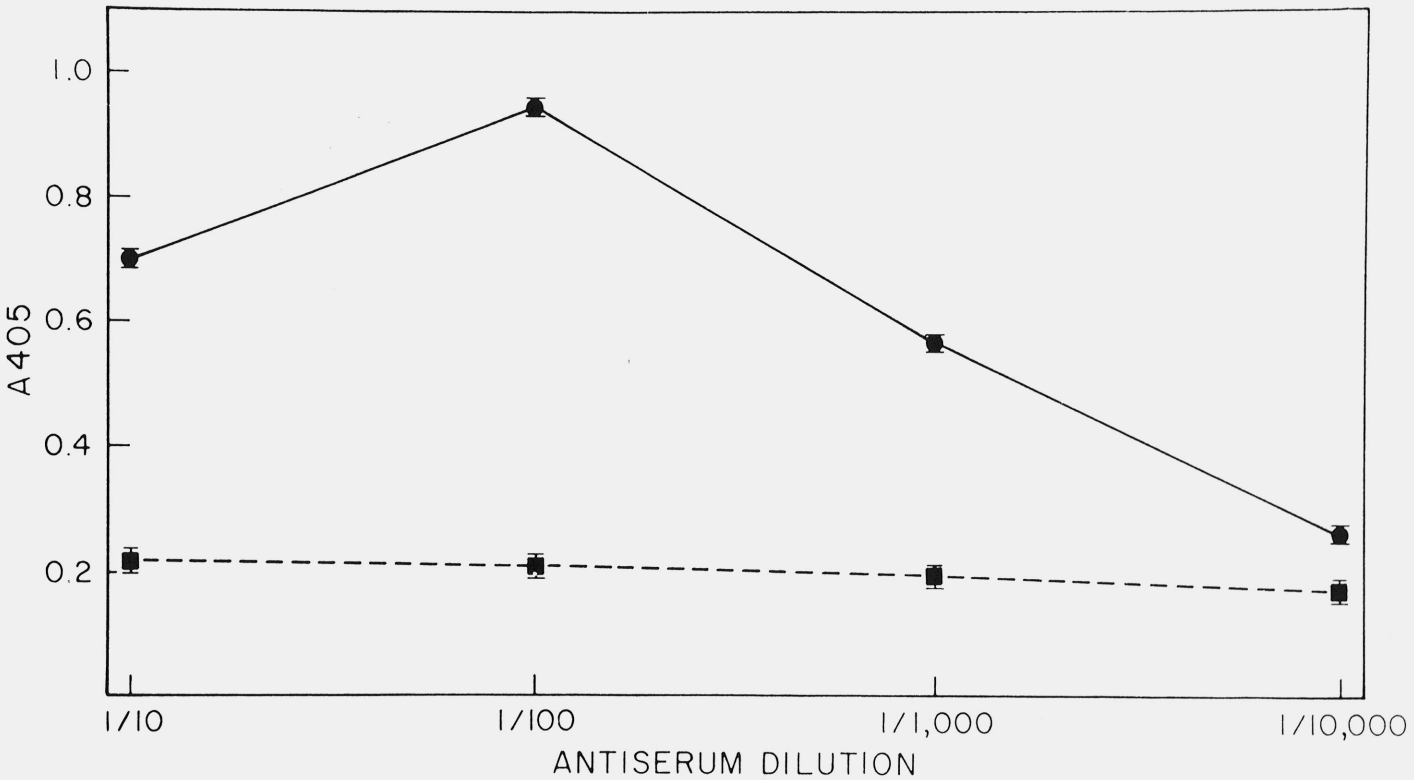

Fig. 2. Enzyme-linked immunosorbent assay results with coronatine diluted to 200 ng/ml to coat the wells. Antisera dilutions were made as indicated. Each point represents three repetitions, and the bars represent the standard deviation of three replicates. Control wells containing all reactants except coronatine were used to zero the instrument. ——- = rabbit antiserum after four injections of coronatine-BSA conjugate; - - - - = preimmune control.

TABLE 2. Detection of coronatine in *Pseudomonas syringae* culture filtrates by ELISA

Isolate	Source	Phenotype (4)	Plant response[a]	ELISA A_{405}[b] Normal (IgG)	ELISA A_{405}[b] Immune (IgG)
P. s. glycinea	R. W. Mitchell				
4180		Tox^+	+	0.130 ± 0.018	0.620 ± 0.026
4333		Tox^+	+	0.137 ± 0.009	0.553 ± 0.023
4182		Tox^+	+	0.131 ± 0.011	0.643 ± 0.028
4327		Tox^-	−	0.139 ± 0.016	0.203 ± 0.019
P. s. morsprunorum	R. W. Mitchell				
567		Tox^+	ND	0.078 ± 0.01	0.619 ± 0.054
3976		Tox^-	ND	0.070± 0.004	0.219 ± 0.054

[a] + = chlorosis produced by culture filtrate; − = no chlorosis; ND = not determined.
[b] Mean ± standard deviation of 12 replications. Antiserum (IgG) was used at a 1:100 dilution. Control wells containing all reactants except coronatine were used to zero the instrument.

TABLE 3. Specificity of the antibody for coronatine in the ELISA assay

HPLC fraction[a]	ELISA A_{405}[b]
Coronfacic acid (peak 1)	.048 ± .005
Unknown (peak 2)	.052 ± .002
Coronatine (peak 3)	.404 ± .008

[a] Equal amounts of substance per well.
[b] Mean ± standard deviation of four replications.

Screening of bacteria for coronatine production. Colonies were inoculated into 10 ml of minimal medium and grown for 5 days at 18–24 C. The bacteria were removed by centrifugation, and the culture filtrates were diluted 1:20 with coating buffer and used to coat the wells as described above. Culture filtrates were simultaneously infiltrated into primary leaves of 7-day-old soybean plants by using the Hagborg device (3) and incubated at 18 C. Symptoms were recorded 2 days postinoculation. The bacterial strains utilized were provided by R. Mitchell. The Tox^+ or Tox^- phenotype was determined by Mitchell (4). The phenotype of the *P. s. glycinea* strains was confirmed by inoculation of 10^6 colony-forming units per milliliter into soybean leaves.

RESULTS AND DISCUSSION

Preliminary experiments tested the assumption that the keto-oxygen of coronatine would conjugate to the free amino groups on BSA to form a molecule with an altered isoelectric point. Cyclohexanone, which also contains a cyclic keto-oxygen, was conjugated to BSA in buffers ranging in pH from 4.0 to 11.0. The optimum pH for conjugation was determined to be about 8.4, which was subsequently used in the conjugation of BSA to coronatine.

The reaction of BSA with coronatine under these conditions yielded a product with an isoelectric focusing Rf of 0.70, whereas BSA alone had an Rf of 0.59 (Fig. 1). These results indicated that the coronatine was conjugated to the BSA. This was confirmed by the failure to detect a significant number of free amino groups.

A 1:100 dilution of antiserum (IgG) raised against the coronatine-BSA conjugate gave the maximum absorbance in the ELISA reaction (Fig. 2). ELISA detected as little as 6 ng of coronatine (Table 1). The *P. s. glycinea* strains examined by Mitchell (4) produced between 0.5 and 6.7 $\mu g/\mu l$ of coronatine, substantially more than enough toxin to be detected by the ELISA assay described here. The ELISA results from the culture filtrates (Table 2) indicate that the antibodies can be routinely used to screen for coronatine production by *P. s. glycinea* isolates. When coronatine, purified by the procedures described above, is analyzed by high-pressure liquid chromatography (HPLC), three peaks are observed. The three compounds are coronafacic acid (peak 1), coronatine (peak 3), and an unknown compound (peak 2). To determine the specificity of the antibody for coronatine, the HPLC fractions were collected and reacted against a 1:100 dilution of the antibody. Only coronatine produced a positive ELISA reaction (Table 3).

This is the first report of an antiserum made to such a phytotoxic organic molecule and of the use of the antiserum in ELISA. The ELISA is more sensitive than whole plant assays in detecting coronatine, with the advantage that ELISA also allows rapid determination of coronatine production. Plant inoculation required higher concentrations of inoculum and the maintenance of plants. The ELISA assay can be readily adapted for the detection of mutants, spontaneous or induced, that no longer produce coronatine, or for the detection of coronatine produced by other bacterial species. The successful use of the ELISA assay for coronatine also indicates its possible value in the assay for the production of other phytotoxins.

LITERATURE CITED

1. Gnanamancickman, S. S., Staratt, A. N., and Ward, E. W. B. 1982. Coronatine production in vitro and in vivo and its relation to symptom development in bacterial blight of soybean. Can. J. Bot. 60:645-650.
2. Habeeb, A. F. S. A. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14:328-336.
3. Hagborg, W. 1970. A device for injecting solutions and suspensions into thin leaves of plants. Can. J. Bot. 48:1135-1136.
4. Mitchell, R. E. 1982. Coronatine production by some phytopathogenic pseudomonads. Physiol. Plant Pathol. 20:83-89.
5. Mitchell, R. E., and Young, H. 1978. Identification of a chlorosis-inducing toxin of *Pseudomonas glycinea* as coronatine. Phytochemistry 17:2028-2029.
6. Mitchell, R. E., Hale, C. N., and Shanks, J. C. 1983. Production of different pathogenic symptoms and different toxins by strains of *Pseudomonas syringae* pv. *tomato* not distinguishable by gel-immuno-diffusion assay. Physiol. Plant Pathol. 23:315-322.
7. Ichihara, A., Shiraishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R., Furusaki, A., and Matusmoto, T. 1977. The structure of coronatine. J. Amer. Chem. Soc. 99:636-637.
8. Sakai, R., Nishiyama, K., Ichihara, A., Shiraishi, K., and Sakamura, S. 1979. The relation between bacterial toxic action and plant growth regulation. Pages 165-179 in: Recognition and Specificity in Plant Host-Parasite Interactions. J. M. Daly and I. Uritani, eds. University Park Press, Baltimore.
9. Steinbuch, M., and Audran, R. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys. 134:279-284.
10. Tung, A. S., Ju, S.-T., Sato, S., and Nisonoff, A. 1976. Production of large amounts of antibodies in individual mice. J. Immunol. 116:676-681.