Purification and Partial Characterization of a Host-Specific Pathotoxin from Culture Filtrates of *Septoria glycines*

H. S. Song, S. M. Lim, and J. M. Clark, Jr.

Graduate research assistant, Department of Plant Pathology; plant pathologist and professor, Crop Protection Unit, Agricultural Research Service, U. S. Department of Agriculture, and Department of Plant Pathology; and professor, Department of Biochemistry, University of Illinois, Urbana 61801.

Present address of S. M. Lim: Department of Plant Pathology, University of Arkansas, Fayetteville 72701.

This work was supported in part by a grant from the Illinois Soybean Program Operating Board.

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture or imply approval to the exclusion of other products that also may be suitable.

Accepted for publication 17 February 1993.

ABSTRACT

Song, H. S., Lim, S. M., and Clark, J. M., Jr. 1993. Purification and partial characterization of a host-specific pathotoxin from culture filtrates of *Septoria glycines*. Phytopathology 83:659-661.

A host-specific pathotoxin isolated from culture filtrate of *Septoria glycines* caused typical symptoms of brown spot disease on cotyledons and leaves of soybean. This toxin was stable until autoclaving, but drying the toxin under flash-evaporation at 46 C in vacuo destroyed over 99% of the toxin activity. The toxin was purified approximately 16,000-fold by sequential carboxymethyl (CM)-cellulose treatment, diethylaminoethyl (DEAE)-cellulose chromatography, dialysis, gel filtration, and 5% charcoal treatment. The purified toxin appeared to be a polysaccharide because it was resistant to proteinase K, but oxidation with 35 mM IO_4^- and incubation with α-mannosidase, β-galactosidase, or β-glucosidase markedly reduced toxin activity. The partial characterization of the purified toxin as a polysaccharide was supported by positive results with phenol-H_2SO_4 and carbazole tests and increased generation of reducing center analysis (detected by reduction with NaB^3H_4) after acid hydrolysis. The combined results indicated that the toxin is an approximately 20,000-Da polysaccharide with a high content of uronic acids and perhaps low levels of mannose, galactose, and glucose.

Septoria glycines Hemmi causes brown spot disease on soybeans expressed as initial reddish-brown leaf spots surrounded by yellow halos (12). Progression of the disease yields rapidly spreading chlorotic lesions, premature defoliation, and a yield reduction of up to 10% in naturally infected soybeans (20,21). These disease symptoms suggest that a pathotoxin may be a primary determinant of brown spot development (17).

The relationships between host-specific toxins and pathogenesis have been investigated. Various host-specific toxins have been purified from culture filtrates and generally, are low molecular-weight compounds (13–16,19,23,26). Among *Septoria* spp., *Septoria nodorum* (Berk.) Berk. has been reported to produce a nonhost-specific phytotoxin that is a melleine-containing acidic phenol (4,5). This phytotoxin has been used in a series of physiological studies (4,5). The purification and characterization of pathotoxins of *S. glycines*, however, have not been reported. Here we describe the purification and partial characterization of a host-specific pathotoxin produced by *S. glycines*.

MATERIALS AND METHODS

Pathogen and pathotoxin production. An Illinois isolate of *S. glycines* (ATCC 38699) was cultured on potato-dextrose agar (PDA) for 21 days. A 50-ml portion of the conidial suspension (5×10^5 conidia/ml of sterile distilled water) of *S. glycines* was prepared from PDA cultures, added to 500 ml of Septoria medium (the MS medium of Murashige and Skoog [18] modified to include 3 g of KNO_3, 0.25 amounts of micronutrients, 2 g of yeast extract per liter, and pH 4.5), and incubated without agitation at room temperature for 21 days. Culture filtrates were obtained by successive filtration through four layers of cheesecloth, two layers of Whatman No. 1 filter paper, and four separate Millipore filters (5.0-, 1.2-, 0.8-, and 0.45-μm pore sizes). The culture filtrates were stored at 4 C for 12 h before precipitates were removed by centrifugation ($800 \times g$ for 15 min).

Bioassay methods. Toxin toxicity on soybean cotyledons was assayed by placing a drop (20 μl) of culture filtrate on the detached cotyledons previously wounded by pricking gently with a sterilized needle. The inoculated cotyledons were incubated in the growth chamber at 25 $\pm$2 C for 1 wk prior to measurement of pathotoxicity (11).

Toxin toxicity on plant calli was assayed by culturing 14-day-old calli of host plants (soybean [*Glycine max* (L.) Merr.]) and nonhost plants (carrot [*Daucus carota* L.], corn [*Zea mays* L.], cotton [*Gossypium hirsutum* L.], *Datura innoxia* Mill., and tobacco [*Nicotiana tabacum* L.]) on 2 volumes of filter-sterilized pathotoxic culture filtrate diluted with 1 volume of sterile 40 μM 2,4-dichlorophenoxyacetic acid in MS medium for 4 wk at 25 $\pm$2 C in the dark. Viability of the calli was measured 7 days after incubation by 2,3,5-triphenyltetrazolium chloride assay (25).

Toxin toxicity on soybean leaves was assayed by spraying (20 ml per five leaves) the pathotoxic culture filtrate onto the surface of soybean (trifoliate seedlings) leaves previously wounded by rubbing gently with a dry sponge and incubating (25 ±2 C) the inoculated seedlings in a saturated moist chamber for 2 days, followed by further incubation (25 ±2 C) in a greenhouse. Disease symptoms were quantified by the method of Pataky and Sim (20).

Toxin purification. All steps were carried out at 0–4 C. Contaminating cationic components initially were removed from 500 ml of centrifuged clarified culture filtrate by stirring for 3 h with 20 g of preequilibrated CM-cellulose suspended in 250 ml of 30 mM sodium acetate, pH 4.5, followed by vacuum filtration. Toxin in this filtrate was concentrated by passage over a 2.5- × 17-cm column of DEAE-cellulose preequilibrated with 10 mM Tris-HCl, pH 8.0, and subsequent elution with 100 ml of a linear gradient of 0.01–2.0 M NaCl in 10 mM Tris-HCl, pH 8.0, while collecting 1.5-ml fractions. The three to four fractions containing toxin activity were combined, dialyzed free of salts (3 h, molecular weight-cutoff 6,000–8,000, against water), lyophilized to dryness, and dissolved in 1 ml of distilled water, prior to passage over a 1.5- × 40-cm column of Sephadex G-75 preequilibrated with distilled water. The one or two fractions (1 ml each) containing toxin activity were mixed with 50 mg of activated charcoal and clarified by passage through a Millipore filter (0.45 μm) before lyophilization. Toxin purification was assessed by comparing the concentration (milligrams per milliliter, dry weight per volume) of the sample from each step with the minimum dilution required to induce typical brown spot symptoms in the cotyledon bioassay. Relative toxin activity was expressed as the proportion of pathotoxicity present at each step relative to pathotoxicity of the first step. No significant activity was obtained when biologically inactive fractions from each step were pooled and concentrated.

Characterization of pathotoxin. Heat, enzymatic, acid, and IO_4^- sensitivity were assayed with partially purified toxin (after gel filtration). These assays employed either an autoclave (120 C for 20 min), incubations with 5% proteinase K (Sigma P6556, Sigma Chemical Company, St. Louis, MO; 30 min at 37 C), α-mannosidase (38 U/ml; Sigma M1266; 6 h at 25 C), β-galactosidase (1 U/ml; Sigma G1875; 5 h at 37 C), and β-glucosidase (16 U/ml; Sigma G4511; 5 h at 37 G), treatment with a final concentration of 1 N HCl for 3 h at 100 C, or treatment with 35 mM $NaIO_4^-$ for 48 h at 25 C.

More specific chemical tests of carbohydrate character employed the final purified toxin. The phenol-H_2SO_4 assay employed 1- to 5-μg samples of acid-hydrolyzed (1 N HCl; 100 C for 6 h) and unhydrolyzed standards (D-glucuronic acid, D-mannuronic acid lactone, polygalacturonic acid, and bovine serum albumin) and purified toxin in 200 μl of water. These samples were analyzed by the phenol-H_2SO_4 test (1). The carbazole test (3) employed 5- to 25-μg samples of galacturonic or glucuronic acid, mannuronic acid lactone, or protein standards and the purified toxin. Reducing sugars were determined by isotopic microanalysis of carbohydrate mixture according to the method of Clark and Switzer (2), with toxin, polygalacturonic acid, and maltose standards and D-sorbitol as a background control.

RESULTS

Purification of toxin. Preliminary results indicated that the pathotoxin was anionic at pH 4.5 because it did not adsorb to the CM-cellulose but did adsorb to DEAE-cellulose at pH 8.0. Therefore, we employed pH 4.5 treatment of the initial culture filtrate of *S. glycines* with CM-cellulose to remove contaminating cations and a subsequent pH 8.0 DEAE-cellulose-column chromatography step in the presence of a NaCl gradient to generate an approximately 300-fold purification of the toxin (Table 1). We noted that the toxin was macromolecular: It would not dialyze through a 6,000- to 8,000-Da dialysis membrane. Therefore, we employed gel filtration chromatography (Sephadex G-75) to collect the approximately 20,000-Da toxin (gel filtration size characterization relative to DNP-glycine, cytochrome c, and blue dextran standards) and obtained, overall, an approximately 10,000-fold purification (Table 1). This highly purified toxin finally was freed of colored contaminants by treatment with charcoal and subsequent filtration to obtain a clear solution of approximately 16,000-fold purified toxin (Table 1).

Characterization of the pathotoxin. The pathotoxin in the culture filtrate of *S. glycines* is host specific. Specifically, the pathotoxic culture filtrate produced by *S. glycines* induced typical symptoms of brown spot on soybean leaves and cotyledons and inhibited soybean callus growth (and eventually killed soybean calli). In contrast, the pathotoxic culture filtrate did not affect other nonhost calli, such as carrot, corn, cotton, *Datura innoxia*, and tobacco.

The results indicated that the purified pathotoxin of *S. glycines* was a large polysaccharide (approximately 20,000 Da) rich in uronic acid(s). First, the steps used to purify the pathotoxin indicated it was a large polyanion because the pathotoxin did not adsorb to CM-cellulose at pH 4.5 but did adsorb to DEAE-cellulose at pH 8.0 and was resolved on gel filtration as a 20,000-Da component. Second, the toxin was a macromolecule: It was undialyzable yet was inactivated by high surface tensions associated with flash evaporation and was not inactivated by less stressful lyophilization. Third, the pathotoxin was not a protein because the pathotoxin was autoclavable, resistant to treatment with the broad-spectrum protease proteinase K (5%), and relatively stable after acid hydrolysis with 1 N HCl for 3 h at 100 C. Fourth, the pathotoxin showed characteristics of a carbohydrate: The pathotoxin yielded a weak, but positive, response in the carbohydrate-specific phenol-H_2SO_4 assay; the pathotoxin was totally destroyed after treatment with partially purified glycosidases (α-mannosidase, β-galactosidase, and β-glucosidase); and, most significantly, the pathotoxin was totally destroyed after mild oxidation by the glycol-specific reagent IO_4^- (35 mM). Fifth, several lines of evidence indicated that the pathotoxin was a carbohydrate rich in uronic acid(s). Specifically, the purification showed that the pathotoxin was a polyanion adsorbed by DEAE-cellulose at pH 8.0 but not by CM-cellulose at pH 4.5. Most importantly, the pathotoxin yielded a positive response in the uronic acid-specific carbazole test (3). This response was only slightly enhanced by prolonged hydrolysis in 1 N HCl, a result that agreed with the well-known (3) resistance of polyuronic acids to acid hydroly-

TABLE 1. Relative activity of toxin from culture filtrate *Septoria glycines* recovered during purification

Step[a]	Dry weight (mg)	Dilution end point	Minimum conc.[b] (mg/ml)	Relative activity[c] (fold purification)
CM-cellulose treatment	11.66	1:80	1.4×10^{-1}	1
DEAE-cellulose chromatography	0.47	1:1,000	4.6×10^{-4}	304
Sephadex G-75 gel filtration	0.14	1:10,000	1.38×10^{-5}	10,145
5% Charcoal treatment	0.07	1:8,000	8.6×10^{-6}	16,279

[a] Carboxymethyl (CM)-cellulose supernatant fluid was obtained by filtration of CM-cellulose after culture filtrate was mixed with CM-cellulose for 3 h at 4 C; diethylaminoethyl (DEAE)-cellulose chromatography.
[b] Minimum concentration of samples exhibiting toxin activity was determined measuring dilution end point.
[c] Relative activity was expressed as the proportion of the original toxin activity of each step relative to toxin activity of the first step.

TABLE 2. Comparison of the incorporation of NaB^3H_4 into acid hydrolyzed and unhydrolyzed D-sorbitol, maltose, toxin produced by *Septoria glycines*, and polygalacturonic acid

Treatment	Total amount (mg/ml)	Radioactivity in samples[a] (dpm)	Specific activity (dpm/mmole)
Acid hydrolyzed[b]			
D-sorbitol	3.6	76,904	1.2×10^7
maltose	7.2	164,275	2.6×10^7
polygalacturonic acid	4.2	213,908	3.3×10^7
toxin	4.2	384,992	6.1×10^7
Nonhydrolyzed			
D-sorbitol	3.6	70,625	1.1×10^7
maltose	7.2	84,463	1.3×10^7
polygalacturonic acid	4.2	82,426	1.3×10^7
toxin	4.2	224,943	6.1×10^7

[a] Counting efficiency was 63%.
[b] Samples was hydrolyzed with 1 N HCl at 100 C for 6 h. Each treatment had two replicates.

sis, as confirmed by the similar response of polygalacturonic acid. Finally, the limited hydrolysis with acid, as detected by subsequent reduction with NaB^3H_4 (2), indicated that the pathotoxin was a polyuronic acid resistant to acid hydrolysis (Table 2).

DISCUSSION

We report that *S. glycines* produces a host-specific, macromolecular, uronic acid-rich, polysaccharide pathotoxin that causes typical brown spot symptoms on soybeans. Although purification and characterization of the toxin produced by *S. glycines* has not been reported until now, a toxin produced by *S. nodorum* has been studied chemically and physiologically (4,5). The *S. nodorum* toxin was characterized as a low molecular-weight compound (melleine-containing acidic phenol) and nonhost specific. There is, therefore, no similarity between the toxins of *S. glycines* and *S. nodorum* with respect to chemical structure and host specificity.

The *S. glycines* toxin can be purified 16,000-fold through successive use of CM-cellulose treatment, DEAE-cellulose chromatography, Sephadex G-75 gel filtration, and charcoal treatment and is a polysaccharide macromolecule. Most of the other macromolecular toxins purified to date are either polysaccharide or glycopeptide in character. These other toxins (13,22,24,27) are similar to the *S. glycines* toxin because they exhibit high viscosity in solution, are nondialyzable, and can be purified through ion-exchange chromatography (6–10). These macromolecular pathotoxins, however, differ from the pathotoxin of *S. glycines* because they are nonhost specific and appear to function by causing vascular disease. MacNeil and Zalasky examined the host-specific morphological and potential physiological role of *S. glycines* on soybeans (17). Using light microscopy, they found that plasmolysis occurred in soybean epidermal cells prior to *S. glycines* penetration and assumed that a toxin activity was required for pathogenesis (17). This finding suggests a more specific pathotoxic function (other than vascular disease) for the host-specific pathotoxin produced by *S. glycines* but does not rule out a vascular-disease role for the *S. glycines* pathotoxin.

Demonstration of *S. glycines* toxin as a uronic acid-rich polysaccharide suggests that further characterization of the structure of the pathotoxin will be difficult. Acidic sugar derivatives or aminosugars are much more resistant to acid hydrolysis than are neutral sugars (3). Therefore, acid-mediated hydrolytic cleavage of this pathotoxin into specific subunits will be difficult. This point is supported by our observation (Table 2) that the *S. glycines* toxin is not extensively hydrolyzed during prolonged acid hydrolysis (1 N HCl at 100 C for 6 h) (i.e., it yielded only a two-fold increase in reducing sugars compared to the nonhydrolyzed sample). Therefore, one must explore the potential of specific polysaccharidases or polyuronidases to aid the further chemical characterization of the most specific pathotoxin produced by *S. glycines*.

LITERATURE CITED

1. Ashwell, G. 1966. New colorimetric methods of sugar analysis. Methods Enzymol. 8:85-94.
2. Clark, J. M., Jr., and Switzer, R. L. 1977. Microanalysis of carbohydrate mixture by isotopic, enzymic, and colorimetric methods. Pages 153-158 in: Experimental Biochemistry. 2nd ed. W. H. Freeman & Co., New York.
3. Davidson, E. A. 1966. Analysis of sugars found in mucopolysaccharides. Methods Enzymol. 8:52-59.
4. Devys, M., Bousquet, J. F., Kollmann, A., and Barbier, M. 1980. Dihydroiso-coumarines et acid mycophenolique du milieu de culture du champignon phytopathogene *Septoria nodorum*. Phytochemistry 19:2221-2222.
5. Devys, M., Bousquet, J. F., Skajennikoff, M., and Barbier, M. 1974. L'ochracine (melleine), phytotoxine isole du milieu de culture de *Septoria nodorum* Berk. Phytopathol. Z. 81:92-95.
6. Dow, J. M., and Callow, J. A. 1979. Leakage of electrolytes from isolated leaf mesophyll cells of tomato induced by glycopeptides from culture filtrates of *Fulvia fulva* (Cooke) Ciferri (syn. *Cladosporium fulvum*). Physiol. Plant Pathol. 15:27-34.
7. Dow, J. M., and Callow, J. A. 1979. Partial characterization of glycopeptides from culture filtrates of *Fulvia fulva* (Cooke) Ciferri (syn. *Cladosporium fulvum*), the tomato leaf mould pathogen. J. Gen Microbiol. 113:57-66.
8. Dow, J. M., and Rubery, P. H. 1977. Chemical fractionation of the cell walls of mycelial and yeast-like forms of *Mucor rouxii*. A comparative study of the polysaccharide and glycoprotein components. J. Gen. Microbiol. 99:29-41.
9. Goodman, R. N. 1960. Observation on the production, physiological activity and chemical nature of colletotin, a toxin from *Colletotrichum fascum* Laub. Phytopathol. Z. 37:187-194.
10. Goodman, R. N. 1960. Colletotin, a toxin produced by *Colletotrichum fascum*. Phytopathology 50:325-327.
11. Hwang, I. Y., Lim, S. M., and Shaw, P. D. 1992. Use of detached soybean cotyledons for testing pathogenicity of *Xanthomonas campestris* pv. *glycines*. Plant Dis. 76:182-183.
12. Kamicker, T. A., and Lim, S. M. 1985. Field evaluation of pathogenic variability in isolates of *Septoria glycines*. Plant Dis. 69:744-746.
13. Kern, H. 1972. Phytotoxins produced by *Fusaria*. Pages 35-45 in: Phytotoxin in Plant Disease. R. K. S. Wood, A. Ballio, and A. Graniti, eds. Academic Press, Inc., London.
14. Kohmoto, K., and Otani, H. 1991. Host recognition by toxigenic plant pathogen. Experientia 47:755-764.
15. Kono, Y., and Daly, J. M. 1979. Characterization of the host-specific pathotoxin produced by *Helminthosporium maydis* race T, affecting corn with Texas male-sterile cytoplasm. Biol. Chem. 8:391-397.
16. Kuo, M. S., and Scheffer, R. P. 1970. Comparative effects of host-specific toxins and *Helminthosporium* infections on respiration and carboxylation by host tissue. Phytopathology 60:1391-1394.
17. MacNeil, B. H., and Zalasky, H. 1957. Histological study of host-parasite relationships between *Septoria glycines* Hemmi and soybean leaves and pods. Can. J. Bot. 35:501-505.
18. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
19. Nishimura, S., and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. Annu. Rev. Phytopathol. 21:87-116.
20. Pataky, J. K., and Lim, S. M. 1980. Effects of Septoria brown spot on the yield components of soybeans. Plant Dis. 65:588-590.
21. Pataky, J. K., and Lim, S. M. 1981. Effects of row width and plant growth habit on Septoria brown spot development and soybean yield. Phytopathology 71:1051-1056.
22. Salemink, C. A., Rebel, H., Kerling, L. C. P., and Tchernoff, V. 1965. Phytotoxin isolated from liquid cultures of *Ceratocystis ulmi*. Phytopathol. A. 38:201-202.
23. Scheffer, R. P., and Livingston, R. S. 1984. Host-selective toxins and their role in plant disease. Science 223:17-21.
24. Thomas, C. A. 1949. A wilt-inducing polysaccharide from *Fusarium solani F. eumartii*. Phytopathology 39:572-579.
25. Towill, L. E., and Mazur, P. 1975. Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can. J. Bot. 53:1097-1102.
26. Wolpert, T. J., Macko, V., Acklin, W., Jaun, B., Seible, J., Meili, J., and Arigoni, D. 1985. Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*. Experientia 41:1524-1529.
27. Woodward, J. R., Keane, P. S., and Stone, B. A. 1980. Structures and properties of wilt-inducing polysaccharides from *Phytophthora* spp. Physiol. Plant Pathol. 16:439-454.