MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 4, pp. 221-224, 1990

Purification of a Cultivar-Specific Toxin from *Pyrenophora tritici-repentis*, Causal Agent of Tan Spot of Wheat

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Received 26 October 1989. Accepted 29 January 1990.

***Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, produces a toxin in culture that induces necrosis only on wheat cultivars susceptible to the pathogen. The toxic compound, named Ptr toxin, was purified by gel filtration chromatography and ion exchange chromatography. Unlike most other fungal phytotoxins, Ptr toxin is a low molecular weight protein of 14,700. It has a high content of aspartate/asparagine, serine, and glycine, and a low content of histidine, methionine, and lysine. Reduction with dithiothreitol abolished toxic activity indicating that cysteine is present and that a disulfide bond is required for activity. Concentrations of 90 nM Ptr toxin caused symptoms in leaves of a susceptible, but not a resistant wheat cultivar using the leaf infiltration bioassay described in this study.**

Additional keywords: fungal toxin, *Helminthosporium tritici-repentis*, protein toxin.

Tan spot, a foliar disease of wheat (*Triticum aestivum* L.), is caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. Infected susceptible plants develop light brown necrotic blotches, often surrounded by rapidly expanding yellow halos. Resistant plants develop small lesions that remain restricted until leaf senescence. Leaves of highly resistant plants show only minute brown flecks, even after senescence. In a previous study, we found that isolates of *P. tritici-repentis* produced a toxic activity which induced typical tan spot symptoms after infiltration into susceptible plants (Tomas and Bockus 1987). In the same study, we compared 10 wheat cultivars, ranging from highly susceptible to resistant, for their reactions to the pathogen and to toxic culture filtrates. We found a strong correlation between susceptibility to the pathogen and sensitivity to the toxic activity. This finding suggested that a toxin(s) was involved in symptom development and that disease resistance was due, at least in part, to insensitivity to the toxin(s).

The toxic activity from culture filtrates of *P. tritici-repentis* was heat stable (10 min at 100° C), but was lost after treatment with proteinase K (Tomas *et al.* 1988). In addition, the activity was retained by membranes with a molecular weight cutoff of 6,000–8,000. Therefore, unlike most fungal phytotoxins (Macko 1983), the toxin(s) from *P. tritici-repentis* had a high molecular weight and was probably a protein(s). We now describe a procedure for the purification of the cultivar-specific toxin from *P. tritici-repentis*.

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MATERIALS AND METHODS

Culture conditions. Stationary liquid cultures of isolate Pt-1c of *P. tritici-repentis* were grown for 16–22 days at 24 ± 1° C under constant light (46 $\mu E \cdot m^{-2} \cdot s^{-1}$, fluorescent). Cultures were grown in 500-ml flasks containing 100 ml of modified Fries medium (Tomas and Bockus 1987).

Toxin purification. All steps were performed at 4° C unless otherwise indicated. Filtrate was collected by passing cultures through a coarse filter. The filtrate was then brought to 5% polyvinylpolypyrrolidone (w/v) and gently stirred for 20 min. After centrifugation (15,000 × *g* for 20 min), the clarified supernatant was concentrated 20-fold by ultrafiltration with an Amicon PM10 membrane (10,000 molecular weight cutoff). The 20× supernatant was applied to a Sephadex G-50 column (1.3 × 95 cm) equilibrated with 20 mM 2-(*N*-morpholino)ethanesulfonic acid adjusted to pH 6.0 with KOH (buffer A) and eluted with the same buffer at a flow rate of 0.5 ml/min. Toxic fractions were pooled and loaded onto a CM-cellulose column (0.5 × 20 cm) equilibrated with buffer A. The column was eluted with a 0–0.25 M KCl linear gradient (five bed volumes) at a flow rate of 0.20 ml/min. The CM-cellulose peak associated with toxicity was resolved by ion exchange HPLC using a cation exchange Mono S column (Pharmacia LKB, Piscataway, NJ). Alternatively, toxic fractions of the Sephadex G-50 column were pooled, lyophilized, dialyzed against buffer A, and applied directly to the Mono S column. In either case, the Mono S column was washed with buffer A and eluted with a 0–0.25 M KCl gradient in 40 min at 1 ml/min. HPLC analysis was performed at room temperature. Protein concentration in crude preparations was measured by the Bio-Rad microassay (Bio-Rad, Richmond, CA) based on the method of Bradford (1976). After gel permeation through Sephadex G-50, protein concentration was estimated by A_{280}, assuming $A_{280}^{1\ mg/ml} = 1$. All purification protocols reported in this study were performed at least five times.

Amino acid analysis and reduction. Amino acid content was determined with the PicoTag Waters technique

(Bidlingmeyer *et al.* 1984). Duplicate samples were hydrolyzed at 105° C for 24 hr in 6 N HCl containing 1% (v/v) phenol. For determining the content of cysteine, samples were oxidized with performic acid before acid hydrolysis. The content of tryptophan was determined spectrophotometrically by the method of Edelhoch (1967) using 6 M guanidine hydrochloride in 0.1 M potassium phosphate buffer, pH 6.5. To test activity under reduced conditions, HPLC-purified Ptr toxin (14 μg) was inactivated at 37° C for 30 min in 400 μl of 50 mM Tris-HCl, pH 7.5, containing 25 mM dithiothreitol (DTT). Samples were then infiltrated into susceptible and resistant plants. DTT alone and Ptr toxin without DTT were included as controls, and the experiment was performed three times.

Gel electrophoresis. Sodium dodecyl sulfate-gel electrophoresis (Vertical Slab SE200, Hoefer Scientific, San Francisco, CA) was performed in 15% acrylamide gels using the high-molarity Tris buffer system of Fling and Gregerson (1986). This method provides high resolution for low molecular weight peptides. Gels were stained for protein in 40% methanol, 12% acetic acid, 0.1% Coomassie Brilliant Blue R 250, and destained in 20% ethanol, 10% acetic acid. Gels were stained for carbohydrate as follows: 15% acetic acid, overnight; 1% periodic acid, 30 min; 15% acetic acid, 30 min; 10% Schiff reagent in 7% acetic acid, 30 min; and 0.1% sodium metabisulfite in 0.01 N HCl.

For experiments in which the toxin was extracted from gels, HPLC-purified Ptr toxin was dialyzed against water and electrophoresed (5 μg per lane, 1.5-mm gels). Sample buffer did not contain β-mercaptoethanol and the sample was not boiled. The lane with the standard was stained with Coomassie blue after electrophoresis. To remove sodium dodecyl sulfate, the lane containing Ptr toxin was washed in 2.5% Triton X-100 at room temperature for 1 hr and then rinsed twice with distilled water and once with buffer A. From this lane, the region corresponding to the toxin band was excised and incubated with buffer A at 4° C for 3 hr. The extract was then tested for activity in susceptible and resistant wheat plants.

Assay for toxic activity. Fractions to be tested for toxicity were infiltrated with a Hagborg device (Hagborg 1970) into leaves of susceptible cultivar TAM 105 and resistant cultivar Auburn as described previously (Tomas and Bockus 1987). Each fraction was infiltrated at four random sites using the third and fourth leaves of plants grown to the five-leaf stage. To quantify toxic activity, serial dilutions of fractions were prepared in buffer A. In all cases, approximately 10 μl was infiltrated per site, with infiltrated areas averaging 27 mm^2. Plants were left at room temperature and under constant light until symptoms developed (2 to 4 days).

RESULTS AND DISCUSSION

Culture conditions used in this study for the purification of the toxic activity differed from those previously reported (Tomas and Bockus 1987). Conditions were changed after the production of toxic activity in modified Fries medium was characterized (Tomas 1989). These studies established that filtrates from cultures grown in constant light had as much as 30 times more activity than filtrates of cultures grown in total darkness and that toxic activity peaked at 16–22 days. These findings also explained the low activity present in cultures used in our first study, where cultures were grown for 14 days at low light intensities with 10- to 12-hr photoperiods (Tomas and Bockus 1987).

Gel permeation chromatography separated toxic activity from colored material. Toxicity was not associated with any peak of absorbance but eluted consistently at one-half of the bed volume (Fig. 1). After chromatography of active fractions on CM-cellulose, toxicity was found to be associated with a major peak of absorbance (Fig. 2). Electrophoretic analysis of this peak, however, revealed two protein bands of 12.8 and 14.7 kDa in approximately a 3:1 ratio. These proteins could not be completely separated on CM-cellulose, but they were resolved by cation exchange HPLC. The protein corresponding to the 14.7-kDa band eluted isocratically whereas that corresponding to the 12.8-kDa band eluted later with a salt gradient (Fig. 3). Cultivar-specific toxic activity was associated only with the peak containing the 14.7-kDa protein. This protein was named Ptr toxin.

Electrophoretic analysis of the HPLC-purified Ptr toxin revealed no contaminants (Fig. 4A), even when loading 6 μg per lane (1.5-mm gels). Ptr toxin extracted from gels induced typical symptoms in susceptible but not in resistant plants (Fig. 4B, site 1). Gel extracts from above and below (Fig. 4B, site 2) the toxin band did not induce symptoms.

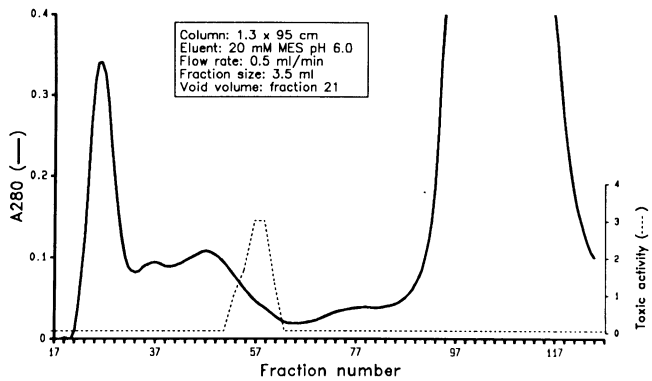

Fig. 1. Gel permeation on Sephadex G-50 of concentrated culture filtrates from *Pyrenophora tritici-repentis*.

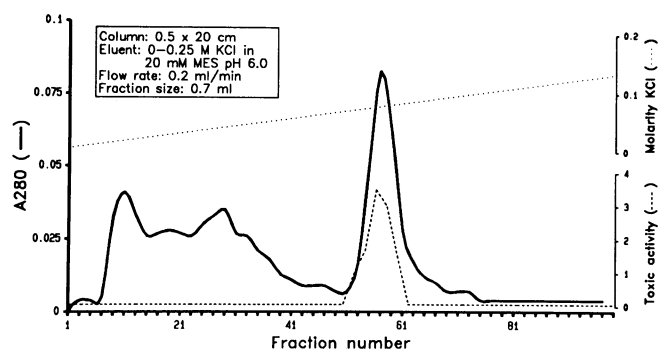

Fig. 2. Ion exchange chromatography on CM-cellulose of a pool of toxic fractions eluted from Sephadex G-50. The peak associated with toxic activity contained at least two proteins.

The band did not stain with Schiff-periodate (data not shown), thus, we determined that Ptr toxin probably does not have a high carbohydrate content. Amino acid analysis of HPLC-purified Ptr toxin revealed a high content of aspartate/asparagine, serine, and glycine; very low levels of histidine, lysine, and methionine; and no cysteine (Table 1). Although we did not detect cysteine, reduction with DTT abolished the toxic activity indicating that there is cysteine in the molecule and that a disulfide bond(s) is important for activity. Toxin incubated alone caused necrosis in susceptible cultivars while DTT alone induced no symptoms.

Average recoveries were approximately 60% of the total activity in the crude filtrate with the protocol described above. Comparable recoveries were obtained in an alternate procedure in which filtrates were concentrated at 40° C under reduced pressure and precipitated with methanol (final concentration of 40% v/v, assuming additive volumes) before chromatography. Because chromatography on CM-cellulose resulted in a loss of protein (Table 2), that step was later omitted from routine purifications. Ptr toxin was recovered at approximately 100–200 μg per 100 ml of filtrate.

In the qualitative bioassay used in this study, the average minimum active concentration of Ptr toxin was 1.3 μg/ml (90 nM). Minimum active concentrations ranged from 0.7 μg/ml to 1.5 μg/ml. These concentrations are comparable to those reported for lower molecular weight fungal phytotoxins (Bach *et al.* 1979; Danko *et al.* 1984; Duvick *et al.* 1984; Kim *et al.* 1987; Rasmussen and Scheffer 1988; Stevenson *et al.* 1979; Wolpert *et al.* 1988). More sensitive, quantitative biochemical assays, such as CO_2 fixation (Duvick *et al.* 1984), if applicable, are likely to detect Ptr toxin activity at lower concentrations.

While this manuscript was in preparation, a study was published on the purification of a toxin from *P. tritici-repentis* (Ballance *et al.* 1989). The toxin was named Ptr necrosis toxin based on symptoms produced on susceptible wheat. Ptr necrosis toxin and Ptr toxin are similar in that they are both proteins with low molecular weights (13,900 and 14,700, respectively). The amino acid compositions of the two toxins show only minor differences.

Some characteristics of the two toxins differ. The activity of Ptr necrosis toxin was lost when culture filtrate was precipitated with methanol (Ballance *et al.* 1989). However, the concentration of methanol was not reported. Our results indicated that 40% methanol (v/v final concentration) did not affect activity. In fact, toxic activity precipitated at 50–70% methanol but was recovered when the precipitate was resuspended in water (data not shown). Another difference resides in the values for minimum active concentrations found for the two toxins. Ptr necrosis toxin was active at 0.2 nM (2.4 ng/ml, Ballance *et al.* 1989), about 450 times more active than Ptr toxin (90 nM). These activities, however, are difficult to compare due to the qualitative character of the bioassay as well as differences in assay conditions (for example, volume infiltrated, plant growth stage, and cultivar susceptibility). It is also possible that the cultivar-specific toxin of *P. tritici-repentis* exists as a family of molecular forms, as is the case for other fungal toxins (Duvick *et al.* 1984; Scheffer *et al.* 1987; Wolpert *et al.* 1988). Ptr toxin and Ptr necrosis toxin could be

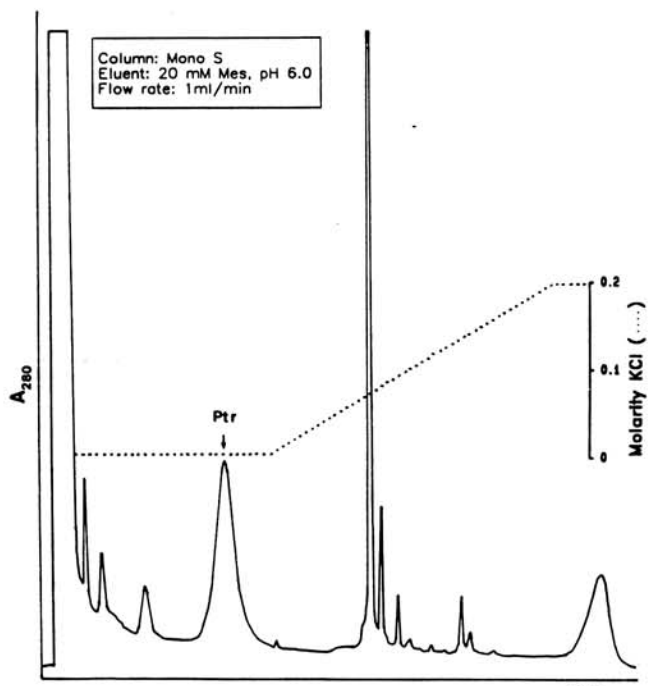

Fig. 3. Purification of Ptr toxin by ion exchange HPLC. Toxic fractions eluted from Sephadex G-50 were pooled, lyophilized, dialyzed against water, and applied to a Mono S column. The toxin (Ptr) was eluted isocratically, while a 12.8-kDa contaminating protein was eluted with a 0–0.25 M KCl gradient over 40 min.

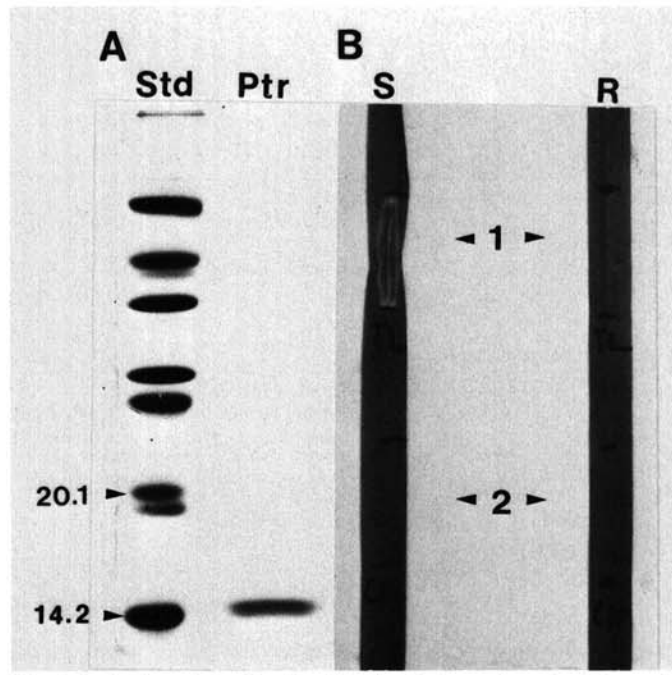

Fig. 4. A, Sodium dodecyl sulfate (SDS)-gel electrophoresis of Ptr toxin (2 μg) purified by cation exchange HPLC. **B**, Reactions of susceptible (S) and resistant (R) wheat leaves to infiltration of fractions extracted from an SDS gel. Site 1, extract from the band containing Ptr toxin. Site 2, extract from the area immediately below (2 mm × 6 mm) the band containing Ptr toxin.

mixtures of isomers with different specific activities at ratios determined by the isolates used or the purification methodology employed. Alternatively, some Ptr toxin activity could have been lost by reduction during purification. In spite of the differences observed, Ptr toxin and Ptr necrosis toxin are probably the same or related compounds.

In our study, a 12.8-kDa protein copurified with Ptr toxin through chromatography on CM-cellulose. We were unable to reliably separate these two proteins by varying column conditions. It is interesting that although Ballance *et al.* (1989) also used gel permeation and chromatography on CM-cellulose to purify Ptr necrosis toxin, they did not observe a 12.8-kDa protein. This difference could be accounted for by variables such as isolates used, culture conditions, and methodology.

In summary, we have purified a cultivar-specific toxin, Ptr toxin, produced by *P. tritici-repentis*. Ptr toxin (and Ptr necrosis toxin, if the same) is only the second fungal toxin described as being a protein, the first being cerato-ulmin, a toxin produced by *Ceratocystis ulmi* (Buisman) C. Moreau. The amino acid compositions of cerato-ulmin (Stevenson *et al.* 1979) and Ptr toxin suggest that the proteins are unrelated. The fact that Ptr toxin is a protein opens exciting possibilities for the study of host-pathogen interactions. For example, the cloning of a gene(s) encoding Ptr toxin is likely to be less complicated than the cloning of genes involved in the biosynthesis of toxins that are secondary metabolites.

Table 1. Amino acid composition of Ptr toxin

Amino acid	Mole % Ptr toxin[a]
Aspartic acid/asparagine	18.7
Glutamic acid/glutamine	9.2
Serine	10.7
Glycine	10.5
Histidine	0.7
Arginine	9.2
Threonine	7.0
Alanine	4.3
Proline	8.4
Tyrosine	2.4
Valine	5.5
Methionine	0.7
Cysteine	N.D.[b]
Isoleucine	4.3
Leucine	4.9
Phenylalanine	2.6
Lysine	1.1
Tryptophan	2[c]

[a] Based on two independent determinations.
[b] N.D., not detected after performic acid oxidation. Reduction with dithiothreitol, however, destroyed toxic activity.
[c] Residues per molecule, after the method of Edelhoch (1967).

Table 2. Purification of Ptr toxin

Step	Protein concentration (μg/ml)	Total protein (mg)	Minimum active concentration (μg/ml)	Purification factor[a]
Filtrate	45[b]	16	6.8	–
Polyvinyl-polypyrrolidone	37[b]	13	4.9	–
Sephadex G-50	56[c]	1.8	7.5	1
CM-Cellulose	73[c]	0.26	2.3	3.3
HPLC	50[c]	0.02[d]		
		0.16[e]	1.3	6.3

[a] Because different methods were used to estimate protein concentration, purification factors were calculated starting with Sephadex G-50 chromatography.
[b] Measured using the Bio-Rad microassay with acetylated bovine serum albumin as standard.
[c] Measured by A_{280}, assuming $A_{280}^{1\ mg/ml} = 1$.
[d] Value derived from one experiment when CM-cellulose step was included.
[e] Total protein obtained when CM-cellulose step was omitted.

ACKNOWLEDGMENTS

Contribution 89-530-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan.

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