Characterization of a Host-Specific Protein Toxin (Ptr ToxB) from *Pyrenophora tritici-repentis*

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Pyrenophora tritici-repentis, the causal agent of tan spot of wheat, differentially induces tan necrosis and/or chlorosis in wheat. A chlorosis-inducing, host-specific toxin, termed Ptr ToxB (formerly Ptr chlorosis toxin), was purified from the culture filtrates of a race 5 isolate of *P. tritici-repentis*. Partial purification was performed by 25 to 80% ammonium sulfate precipitation and passage through a carboxymethyl-Sephadex C-25 cation exchange column. Final purification was performed by fast performance liquid chromatography, with a Mono S HR 5/5 cation exchanger, followed by size fractionation on a Superose 12 HR 10/30 column. The toxin was shown to be proteinaceous in nature, and purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular mass of Ptr ToxB was determined to be 6.61 kDa. The amino acid composition and partial N terminus amino acid sequence of the toxin were also obtained. Ptr ToxB was found to be heat stable, maintaining full toxic activity after 1 h at 55°C. Infiltration of toxin concentrations as low as 14 nM produced chlorosis on susceptible cultivars.

Additional keywords: Ptr necrosis toxin, Ptr ToxA, *Triticum aestivum* L.

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoemaker, is an important foliar disease of wheat (*Triticum aestivum* L.) worldwide. The incidence of this disease has been increasing in recent years, as a consequence of the shift by farmers toward soil conservation practices such as minimum and zero tillage (Hosford 1982). Tan spot consists of two distinct symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier 1989a, 1989b). Five races of the fungus have been described so far, producing necrosis and/or chlorosis on differential wheat cultivars (Lamari et al. 1995). Race 1 isolates are capable of producing both necrosis and chlorosis, race 2 isolates can only produce the necrotic symptom, race 3 and race 5 isolates both cause chlorosis, but on different cultivars, and race 4 isolates are avirulent, producing neither symptom (Lamari et al. 1995).

The necrotic symptom was shown to be associated with a host-specific toxin, termed Ptr ToxA (formerly Ptr necrosis toxin) (Lamari and Bernier 1989c). Ptr ToxA, a 13.2-kDa protein, has been characterized by several independent research groups and its encoding gene was recently cloned

(Ballance et al. 1996; Ciuffetti et al. 1997). This toxin exhibits the same host specificity as the isolates from which it is obtained, and has been recognized as an important factor in the development of the tan spot disease (Tomás and Bockus 1987; Ballance et al. 1989; Lamari and Bernier 1991).

A second host-specific toxin, termed Ptr ToxB (formerly Ptr chlorosis toxin), was identified from a race 5 isolate of *P. tritici-repentis* (Orolaza et al. 1995). The toxin is a pathogenicity factor, and preliminary characterization of Ptr ToxB suggested that it was a small, hydrophilic protein, stable to exposure to organics (Orolaza et al. 1995; Strelkov et al. 1998). Although partial results on the purification of Ptr ToxB were presented previously (Strelkov et al. 1998), the present work describes the purification and characterization of the toxin from culture filtrate of race 5 isolate Alg. 3-24 of *P. tritici-repentis*.

RESULTS

Purification.

Toxic activity in the culture filtrate was associated with protein precipitated at 25 to 80% ammonium sulfate saturation. The precipitate was redissolved, dialyzed, and loaded onto a carboxymethyl-Sephadex (CM-S) C-25 column. The unbound fraction was strongly UV-absorbing and possessed no toxic activity. The elution profile indicated that toxic activity was eluted at approximately 300 mM NaCl (results not shown). Electrophoretic analysis of the toxic fractions showed the presence of three major and several minor proteins (Fig. 1). The toxic fractions were concentrated, desalted, and fractionated on a fast performance liquid chromatography (FPLC) Mono S HR 5/5 column by gradient elution, as described in Materials and Methods. The elution profile (Fig. 2) indicated four major peaks (labeled I, II, III, and IV) but toxic activity was only associated with peaks II and III, with peak II possessing the strongest activity. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of peaks I to IV (Fig. 3) revealed that both peaks II and III contained strong bands of less than 6.5 kDa, plus a weaker band of approximately 27 kDa. However, the lower molecular mass band was more highly enriched in peak II than in peak III. Peaks II and III were pooled, desalted, concentrated, and partitioned with an FPLC Superose 12 HR 10/30 column (Fig. 4). The toxin, corresponding to a single UV-280 absorbing peak, was separated from two visible contaminants. SDS-PAGE of the pooled fraction revealed only one Coomassie blue stained band of approximately 6 kDa (Fig. 5). Symptoms produced by

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the purified toxin on sensitive (Katepwa) and insensitive (Glenlea) wheat leaves are shown in Figure 6. The protein recovery during the various purification steps is shown in Table 1. The total Ptr ToxB recovered accounted for 0.0123% of the total soluble protein present in the crude culture filtrate.

Molecular mass determination, amino acid composition, and sequence.

The molecular mass of Ptr ToxB was determined to be $6,612 \pm 7$ Da (0.1% error) by mass spectrometry. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-

TOF) mass spectrum of the toxin is shown in Figure 7. The amino acid composition of Ptr ToxB is presented in Table 2. After correction for cysteine and methionine underestimation, and glycine overestimation, Ptr ToxB appeared to have 63 residues and a molecular mass of 6,609 Da. The partial amino acid sequence of the N terminus of Ptr ToxB was determined to be NXVANILNINEAVIATGSPVAGGELRIFVG.

Thermal stability.

The thermal stability of the toxic activity was determined after several heat-period treatments. Activity was fully present after heating for 60 min at 30, 40, and 55°C. Slightly weaker activity was still present after 30 min at 80°C, and a low level

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of toxic fractions eluted from CM-S C-25 chromatography with a linear gradient of NaCl (0 to 400 mM). Lane 1: molecular weight markers (10 µg of total protein). Lanes 2 to 5: toxic fractions corresponding to fractions 20 to 23 from CM-S C-25 chromatography (1.5, 13.8, 8.4, and 0.93 µg of protein loaded, respectively). The fractions in lanes 3 and 4 had the strongest toxic activity.

Fig. 2. Mono S chromatography of Ptr ToxB. Fractions exhibiting toxic activity after CM-S C-25 chromatography were pooled and loaded onto a Mono S HR 5/5 column. Column was eluted with a linear gradient of 0 to 400 mM NaCl in 20 mM NaAc (pH 4.6) over 25 column volumes. Eluate was monitored at 280 nm and fractions were bioassayed for toxic activity. Peaks were labeled as I, II, III, and IV. Only II and III contained toxic activity.

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of fractions from Mono S chromatography. Lane 1: 5.8 µg of protein from peak IV; lane 2: 3.8 µg of protein from peak III; lane 3: 3.5 µg of protein from peak II; lane 4: 0.25 µg of protein from peak I; lane 5: molecular weight markers (10 µg of total protein). Only fractions in lanes 2 and 3 possessed toxic activity.

Fig. 4. Superose 12 chromatography of Ptr ToxB. Peaks II and III from MonoS HR 5/5 chromatography were pooled, concentrated, and loaded onto a Superose 12 HR 10/30 column. Column was eluted with 20 mM NaAc buffer, pH 4.6, at 0.5 ml/min. Eluate was monitored at 280 nm and fractions were bioassayed for toxic activity. The toxic fraction, corresponding to Ptr ToxB, is indicated with the solid bar.

Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of purified Ptr ToxB. Lane 1: molecular weight markers (10 µg of total protein); lane 2: empty; lane 3: purified Ptr ToxB (0.8 µg) obtained from Superose 12 HR 10/30 chromatography.

Fig. 6. Symptoms produced by Ptr ToxB. Leaves from toxin-sensitive cv. Katepwa (left) and toxin-insensitive cv. Glenlea (right), shown 72 h after infiltration with purified Ptr ToxB (at a concentration of 9.5 µg/ml).

of chlorosis was detectable after 60 min at 80°C. A trace level of chlorosis was still detectable after 30 and 60 min of heating at 100°C.

Effect of toxin concentration on toxic activity.

The activity of the purified toxin was detectable by the bioassay procedure, described in Materials and Methods, at a toxin concentration of 95 ng/ml (14 nM). At this concentration, chlorosis, although weak, was visible within 72 h post infiltration. Symptoms of chlorosis were readily apparent within 48 h post infiltration when the toxin was applied at 10, 100, and 1,000 times this concentration. By 96 h post infiltration, tissue treated with toxin solutions of 95 or 9.5 µg/ml had begun to die at the center of the infiltrated area; tissue infiltrated with 0.95 µg/ml, although severely chlorotic, had not developed any necrosis. Toxin applied at 9.5 ng/ml failed to produce chlorosis at any time. The toxin-insensitive cv. Glenlea was tested with the same range of toxin concentrations, but symptoms did not develop at any of the concentrations tested.

DISCUSSION

Earlier work indicated that Ptr ToxB was a hydrophilic molecule, stable to exposure to organics, with a molecular mass of at least 3.5 kDa (Orolaza et al. 1995). The present investigation conclusively showed that the toxin is a protein with a molecular mass of 6.61 kDa. Ptr ToxB stained and bound to chromatogra-

Table 1. Protein content at various steps of the purification procedure developed for Ptr ToxB

Step	Protein content (mg)	Total soluble protein (%)
Concentrated culture filtrate	1.51×10^3	100
25 to 80% $(\text{NH}_4)_2\text{SO}_4$ ppt	2.16×10^2	14.3
CM-S C-25 toxic fractions	9.04	0.598
Mono S 5/5 toxic fractions	0.359	0.0237
Superose 12 toxic fractions	0.186	0.0123

Fig. 7. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of Ptr ToxB. Spectrum was obtained from a sample of toxin deposited onto a polyurethane membrane. The peak corresponds to a molecular mass of $6,612 \pm 7$ Da.

phy columns in a manner consistent with a protein; the toxin was purified to homogeneity by conventional protein purification techniques. Most importantly, an amino acid composition (Table 2) and partial amino acid sequence were obtained for Ptr ToxB, further confirming its protein nature.

Attempts to estimate the concentration of Ptr ToxB based on the absorbance of toxin solutions at 280 nm invariably resulted in underestimations of its concentration, compared with values obtained with the Lowry assay and with band intensity on SDS-polyacrylamide gels. This suggested that Ptr ToxB may not contain any tryptophan residues, since 280 nm is the absorption maximum of tryptophan (Haurowitz 1963). Furthermore, the partial amino acid sequence of the toxin protein revealed no tryptophan residues among the 30 residues successfully sequenced. Hence, even though tryptophan is completely destroyed during acid hydrolysis prior to running on an amino acid analyzer, there appear to be no tryptophan residues in Ptr ToxB. Consequently, tryptophan was not incorporated into the corrected amino acid composition of the toxin (Table 2).

Ptr ToxB appears to be heat stable. There was no reduction in toxic activity after heating for 1 h at 55°C, conditions that resulted in the irreversible denaturation of Ptr ToxA (Ballance et al. 1989). After 1 h at 100°C, some toxic activity was still present. Since we did not have a way to monitor the change of structure of the toxin following heating, it is not clear whether this thermal stability was due to an inability of the heat to disrupt the functional site, or if the small nature of the protein made it difficult to denature. It is also possible that the toxin renatured after cooling. In addition, Ptr ToxB had a very high level of toxic activity, with a minimum active concentration of 14 nM.

An unusual aspect of *P. tritici-repentis* is its production of host-specific toxins that are proteinaceous in nature; Ptr ToxA is a 13.2-kDa protein (Tuori et al. 1995) and Ptr ToxB is a 6.61-kDa protein. All other known host-specific toxins are secondary metabolites with diverse structures and low molecular masses (Walton 1996). Aside from the fact that Ptr ToxA and Ptr ToxB are proteins and are host specific, they

have very little in common: they induce different types of symptoms, and a screening of genomic DNA from the Alg. 3-24 isolate with the full-length cDNA clone of Ptr ToxA yielded no complementary matches (Ballance et al. 1996). This difference in sequences was confirmed by partial N terminus amino acid sequencing of Ptr ToxB, which revealed no sequence homology with Ptr ToxA. The mode of action of Ptr ToxB remains the focus of other studies (Strelkov et al. 1998).

MATERIALS AND METHODS

Isolates and fungal cultures.

Race 5 isolate Alg. 3-24 of *P. tritici-repentis* used in this investigation is a laboratory stock that was previously collected from Algeria (Lamari et al. 1995). Cultures of this isolate were grown on V8 potato dextrose agar (Lamari and Bernier 1989a) until they were 4 to 5 cm in diameter. Five plugs (1 cm in diameter) were cut from each colony and transferred to 1-liter Roux bottles, each containing 250 ml of Fries medium amended with 0.1% yeast extract (Dhingra and Sinclair 1986). The cultures were incubated without agitation at 20°C for 21 days. The filtrates were removed from the mycelial mat by filtering through Whatman No. 1 filter paper and 0.45-µm cellulose nitrate filters. Culture filtrates were freeze dried and stored at -20°C until processed.

Plant materials and bioassays.

Wheat cultivars Katepwa (sensitive to Ptr ToxB) and Glenlea (insensitive to Ptr ToxB) were used throughout the study. The seedlings were grown in plastic pots (12 cm in diameter) filled with a 1:1 soil/peat mix at a density of five to six seedlings per pot. The plants were maintained in a growth room at 22/18°C (day/night) with a 16-h photoperiod at a light intensity of 250 µE s⁻¹m⁻². Seedlings were watered and fertilized as required. Bioassays were conducted by infiltrating seedlings at the two- to four-leaf stage with approximately 20 µl of sample, using a Hagborg device (Hagborg 1970).

Toxin purification.

Approximately 2 liters of culture filtrate was freeze dried to give approximately 20 g of dry matter. Freeze-dried culture filtrate was resuspended in 40 ml of 20 mM sodium acetate buffer (pH 4.6) containing 100 µM phenylmethylsulfonyl fluoride (PMSF). The resuspended culture filtrate was centrifuged at 17,400 × g for 10 min and the supernatant retained. Sequential salt fractionation of the protein in the supernatant was conducted with ammonium sulfate in two steps. Material precipitating out at 0 to 25% salt saturation was discarded, and that precipitating out at 25 to 80% saturation was retained. The precipitate was redissolved in 30 ml of 20 mM sodium acetate buffer (pH 4.6) containing 100 µM PMSF, and dialyzed against the same buffer. The dialyzed sample was loaded onto a CM-Sephadex C-25 column (19 × 2.5 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.6) containing 100 µM PMSF. The unbound fraction was eluted by washing the column with 7 bed volumes of equilibration buffer. The bound fraction was then eluted with a 0 to 400 mM NaCl gradient (250 ml of total volume) in equilibration buffer at a flow rate of 30 ml/h. The eluate was collected in 9-ml fractions while monitored at 280 nm. Toxic activity of the fractions was assessed following a 1/5 dilution with water by the bioassay procedure described above. Toxic fractions were

Table 2. Amino acid composition of Ptr ToxB isolated from culture filtrate of *Pyrenophora tritici-repentis* isolate Alg. 3-24

Amino acid	Actual amount (pmol)	Corrected amount (pmol) ^a	Predicted residue composition ^b
Asp + Asn	288	288	5
Glu + Gln	276	276	5
Ser	345	400	8
Gly	411	250	5
His	51	51	1
Arg	104	104	2
Thr	212	212	4
Ala	320	320	6
Pro	131	131	3
Tyr	74	100	2
Val	260	260	5
Met	20	40	1
Cys	65	130	2
Ile	234	250	5
Leu	273	273	5
Phe	119	119	2
Lys	99	99	2
Total	3,282	3,303	63
Molecular mass			6,609 Da

^a Corrected for amino acids that were over- or underestimated.

^b Predicted on the basis of a molecular mass of 6,612 Da.

pooled, concentrated by freeze drying, and dialyzed against 20 mM sodium acetate buffer (pH 4.6). The dialysate was loaded onto an FPLC Mono S HR 5/5 column (Pharmacia, Baie d'Urfe, Quebec, Canada; column volume = 1 ml) equilibrated with 20 mM sodium acetate buffer (pH 4.6). The bound proteins were fractionated with a linear gradient of 0 to 400 mM NaCl (in equilibration buffer) over 25 column volumes at 0.5 ml/min. Toxic fractions were again pooled, dialyzed, freeze dried, and fractionated on an FPLC Superose 12 HR 10/30 column (Pharmacia; column volume = 24 ml) equilibrated with 20 mM sodium acetate buffer (pH 4.6) and eluted with the same buffer at 0.5 ml/min. The purity of the toxic fraction was evaluated by SDS-PAGE.

Protein estimation and electrophoresis.

Protein concentration was determined by the method of Lowry et al. (1951). PAGE was run under denaturing conditions with SDS. This was carried out with the buffer system of Fling and Gregerson (1986) at pH 8.5 with a 10% separating gel and 5% stacking gel. Samples of the toxin and protein molecular weight markers (Bio-Rad Canada, Mississauga, Canada) were prepared in sample buffer (10 µl) consisting of 0.2 M Tris-HCl, pH 8.8, 0.5 M sucrose, 0.01% bromophenol blue, and 10 mM EDTA. To this were added 2.5 µl of 10% SDS and 1 µl of 0.25 M dithiothreitol (DTT). Samples were heated for 5 min at 100°C and cooled on ice. After cooling, 2.5 µl of 0.5 M iodoacetamide was added and the samples allowed to stand for 15 min before loading onto a 0.75-mm-thick mini-gel. After running (1 h at 100 V), the mini-gel was fixed for 15 min in methanol-acetic acid-water (5:1:5), stained with Coomassie brilliant blue (0.05% wt/vol in fix solution) for 1 h, and destained in methanol-acetic acid-water (2:0.8:7.2, vol:vol:vol).

Molecular mass determination, amino acid composition, and sequence.

The molecular mass of Ptr ToxB was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). Spectra were obtained from samples deposited onto non-porous, ether-type polyurethane membranes as described by McComb et al. (1997). Amino acid analysis was conducted on an Applied Biosystems (Foster City, CA) model 420A amino acid derivatizer-analyzer, after manual hydrolysis. Amino acid sequencing was carried out on an Applied Biosystems model 475A gas-phase sequencer.

Thermal stability.

The heat stability of Ptr ToxB was determined by incubating 500-µl samples of toxin (9.5 µg of protein per ml) at 30, 40, 55, 80, and 100°C for 30- and 60-min periods. After heat treatment, the samples were allowed to cool to room temperature and then bioassayed for toxic activity.

Effect of toxin concentration on toxic activity.

A stock solution of the purified toxin (0.95 mg of protein per ml) was serially diluted with distilled water to give dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . The various dilutions were then bioassayed for toxic activity.

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