Structural and Physical Properties of a Necrosis-Inducing Toxin from *Pyrenophora tritici-repentis*

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ABSTRACT

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Cultivar-specific toxic metabolites of *Pyrenophora tritici-repentis* are involved in the appearance of necrotic and chlorotic foliar lesions characteristic of tan spot. A *P. tritici-repentis* necrosis-inducing toxin, Ptr necrosis toxin, was purified from isolate 86-124, sequenced by gas-phase amino acid microsequencing, and characterized by circular dichroism (CD) spectroscopy and isoelectric focusing. The purified protein had a similar amino acid composition and molecular weight as previously reported. Analysis of the CD spectrum from 178 to 250 nm indicated a protein consisting of 13% α -helix, 36% antiparallel β -sheet, 25% turns,

and 25% other structures. The Ptr necrosis toxin from isolate 86-124 has an isoelectric point near pH 10. Using overlapping proteolytic fragments obtained from the toxin, a sequence of 101 continuous amino acids was obtained, but the amino terminus was blocked and 9 to 16 amino acids could not be sequenced. Secondary structure prediction based on the amino acid sequence indicated a β -sheet protein with little α -helix, which is in agreement with the structure determined by CD spectroscopy. Sequence analysis indicated the presence of a possible membrane adhesion site and several possible phosphorylation sites that may be involved in phytotoxicity.

Additional keywords: protein isolation, *Triticum aestivum*, wheat.

Tan spot or yellow spot of wheat (*Triticum* spp.), caused by *Pyrenophora tritici-repentis* (Died.) Drechs., is an important foliar disease worldwide. Major areas of disease occurrence are the intensive wheat production areas of Australia, South America, and North America. Yield losses of 3 to 50% have been reported in these locations (21,33,37). Tan spot was ranked recently as the most serious of the economically important foliar wheat diseases in North Dakota (32). The name of the disease comes from characteristic light brown necrotic lesions often surrounded by chlorotic halos.

Tan spot is currently managed by crop rotation, plowing under wheat debris, and fungicides, because many of the widely grown wheat cultivars are susceptible (21,32,34). Genetic resistance, however, is the most environmentally safe and economical means of managing tan spot. Reports of inheritance of tan spot resistance in *Triticum* species have included both qualitative (29,30,45) and quantitative gene action (5,15,35). A single recessive gene conferred resistance to the necrotic reaction caused by a proteinaceous toxin produced by many pathogenic isolates of *P. tritici-repentis* (28,29). The single-gene model for reaction to the necrosis-inducing protein was independently confirmed (14), and flanking DNA markers have been located on chromosome 5BL (17). A spreading chlorosis symptom is caused by at least one other fungal metabolite and is under separate genetic control (16,27-29).

The necrosis-inducing protein was purified and partially characterized by two independent groups using essentially the same procedure (2,47). Recently, a similar toxin has been purified by another method (49). All groups isolated a water-soluble protein

with a molecular mass between 13,000 and 14,000 Da. Reduction of the protein by dithiothreitol resulted in loss of biological activity (2). Somewhat disparate results were observed by the different groups regarding thermal inactivation and the specific activity of the toxin (2,47,49). Tuori et al. (49) and Tomás et al. (47) claimed to have a thermally stable protein, still active after 10 min at 80 and 100°C, while the protein isolated by Ballance et al. (2) was only moderately stable, active after 15 min at 70°C, but inactive after 30 min at 70°C. Although the amino acid content and molecular mass have been determined by these research groups, there is no information available on the primary or secondary structure of the toxin or any of its other physical properties.

A method is described here for isolating necrosis toxin that is similar to that used by Tuori et al. (49) and briefly reported by our research group (31). This method allows the purification of large quantities of toxin for experimentation. We have also characterized the toxin by sodium dodecyl sulfate (SDS) gel electrophoresis, nonreducing isoelectric focusing, and circular dichroism (CD) spectroscopy. A major portion of the toxin was sequenced by gas-phase amino acid sequencing, and an extinction coefficient for both denatured and native toxin was determined. Based on the sequence data, a secondary structure for the toxin is predicted and is consistent with that determined by CD spectroscopy.

MATERIALS AND METHODS

Culture conditions. The isolate 86-124 of *P. tritici-repentis* was obtained from L. Lamari (University of Manitoba, Winnipeg, Canada) and subcultured on V8-potato dextrose agar (PDA) (150 ml of V8 juice, 10 g of PDA, 3 g of CaCO₃, 10 g of agar, and 850 ml of distilled water). This isolate was characterized as a producer of a necrosis toxin and nonproducer of chlorosis toxin (28). Cultures were stored at -80°C as dried mycelial plugs. These dried mycelial plugs were used to produce additional dried plugs that were stored at -20°C for the duration of the experiment.

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Inoculum was produced by placing two dried mycelial plugs 3 cm apart on petri plates containing V8-PDA medium. Plates were incubated for 5 to 6 days at 20°C under continuous darkness. Ten 0.5-cm-diameter plugs of mycelia were transferred to 50 ml of potato dextrose broth and cultured on a wrist-action shaker at room temperature for 4 to 6 days. Cultures were blended under sterile conditions and transferred with a syringe in 1-ml aliquots to 50 ml of liquid Fries medium (12). Cultures were held at 20 to 22°C in darkness for 28 days to produce necrosis toxin as previously described (28).

Extraction of necrosis toxin began with passage through a Whatman No. 1 filter (Whatman International, Ltd., Maidstone, England) under vacuum. Crude extract was checked for activity by infusion with about 25 µl of filtrate into the second or third leaf of ND495 (sensitive) and 'Erik' (insensitive). The effect of the filtrate was assayed using the scoring system proposed by Tomás and Bockus (46).

Ptr necrosis toxin purification. Crude extract, 200 to 2,000 ml, was centrifuged for 20 min at 35,000 × *g*. The supernatant was filtered through a 0.45-µm Whatman nylon filter (Whatman International, Ltd.), placed in dialysis tubing (3,500 molecular weight cut-off), and dialyzed overnight against 2 × 5 volumes of 20 mM sodium acetate buffer, pH 4.8. The dialyzed extract was loaded onto a fresh 10-ml S-Sepharose Fast Flow column (Pharmacia Biotechnology Inc., Uppsala, Sweden), 1.6 × 5 cm, previously equilibrated with 20 mM sodium acetate. After loading, the column was washed with four column volumes of buffer at a flow rate of 1 ml/min. The sample was eluted with a linear gradient from 0 to 300 mM NaCl over four column volumes, and the elution profile was measured spectrophotometrically at 280 nm. Samples from 80 to 230 mM NaCl were combined and concentrated approximately 10-fold by vacuum distillation at room temperature. The concentrated sample was chromatographed on a HiLoad 16/60 Superdex-30 gel filtration column (Pharmacia Biotechnology Inc.) at a flow rate of 0.5 ml/min. A peak centered near 14,000 Da was collected. In most preparations, the toxin is 80% pure at this step based on a comparison of peak areas observed in reversed phase high pressure liquid chromatography (HPLC). The sample was desalted by concentration to a volume of 2 ml using the Amicon Centriprep-3 ultra-filtration system (Amicon, Beverly, MA) and then diluted fivefold with 20 mM sodium acetate, pH 4.8. The desalted sample was loaded onto a HPLC sulfo-propyl ion exchange column (Beckman Spherogel TSK SP-5PW, 7.5 mm × 7.5 cm; Beckman Instruments, Inc., San Ramon, CA) and eluted at a flow rate of 1 ml/min with a linear gradient from 0 to 400 mM NaCl over 40 min.

Electrophoresis. Denaturing SDS-polyacrylamide gel electrophoresis was carried out in a Mini Protein II vertical gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA) using a 16%:3% gel (the first number denotes the total acrylamide concentration, i.e., both monomers, and the second number indicates the concentration of crosslinker) and the buffer system of Schägger and von Jagow (43). Samples of toxin and molecular weight standards, typically 5 to 10 µg of protein, were prepared by a fivefold dilution in sample buffer (5% 2-β-mecaptoethanol, 2% SDS, 63 mM Tris-HCl [pH 6.8], 10% glycerol, and 0.005% bromophenol blue) and heated for 5 min at 100°C. Molecular weight markers, MW-SDS-70L and MW-SDS-17S, were obtained from Sigma Chemical Co. (St. Louis). Samples were stained with either Coomassie brilliant blue (R-250) for 1 h and then destained or stained with the Bio-Rad Silver Stain Plus (Bio-Rad Laboratories).

Nondenaturing isoelectric focusing was performed with the Bio-Rad Mini Protein II system (Bio-Rad Laboratories). A 12-ml solution of 5% polyacrylamide (24.25% acrylamide:0.75% piperazine diacrylamide [Bio-Rad Laboratories]), 5% glycerol, and 2% ampholytes, Biolyte 3-10 (Pharmacia Biotechnology Inc.), was degassed and polymerization initiated by adding 20 µl each of 10% ammonium persulfate and *N,N,N',N'*-tetramethyleth-

ylenediamine (TEMED). After polymerization, the anode buffer, 0.02 M phosphoric acid, and cathode buffer, 0.04 M sodium hydroxide, were added, and the sample wells were filled with overlay buffer (0.87 ml of water, 0.1 ml of 50% glycerol, 15 µl of Biolyte 5-7 (Bio-Rad Laboratories), 5 µl of Biolyte 3-10 (Bio-Rad Laboratories), and 20 µl of 1% bromophenol blue). Samples and standards (isoelectric focusing standards [Bio-Rad Laboratories]) were dissolved in an equal volume of 50% glycerol and layered under the overlay buffer. Samples were electrophoresed by stepped increases in voltage (100 V for 15 min, 200 V for 30 min, 300 V for 45 min, and 400 V for 30 min). The outer buffer chamber was stirred with a magnetic stirrer throughout the electrophoresis to dissipate heat. After electrophoresis, the gels were carefully removed and fixed in a solution of 12% trichloroacetic acid, 4.5% sulfosalicylic acid, and 40% methanol for a period of 2 to 4 h with one change in the fixing solution. The gels were then washed for 2 to 8 h with water and stained with Coomassie brilliant blue R-250. The pH gradient was measured directly by slicing an unused portion of the gel in 3-mm sections and placing the sections in 1 ml of water under vacuum for 2 h.

In one experiment, an isoelectric focusing gel was run as described above with the toxin present in half the wells to check for biological activity. The gel was sliced in half vertically to separate the toxin-containing and toxin-free portions. These portions were then sliced horizontally in 3-mm strips and placed in water as described for measuring the pH gradient. The activity of each solution, sample and reference, was then bioassayed as described above.

Amino acid composition and sequencing. The amino acid composition was determined from two independent samples of 100 µg using a Beckman System Gold amino acid analyzer (Beckman Instruments, Inc.) after acid hydrolysis of the protein. For amino acid sequencing, the necrosis toxin was cleaved by several methods. In each case, 100 to 150 µg of protein was dialyzed against the appropriate buffer. Controls containing only the endoproteinase or chemical reagent were performed for each experiment. Endoproteinase aspartate-N (Boehringer Mannheim Corp., Indianapolis, IN) digests were performed with 1 µg of enzyme in 50 mM sodium phosphate buffer (pH 7.8) for 18 and 24 h. Endoproteinase glutamate-C digests (Boehringer Mannheim Biochemicals), 10 µg of proteinase, were performed in 75 mM ammonium bicarbonate buffer (pH 7.8) for 48 h. Trypsin digestion was performed using 20 µl of immobilized TPCK-trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin) (Pierce, Rockford, IL) in 200 mM ammonium bicarbonate buffer (pH 8.0) for 2 and 24 h. A third trypsin digest was performed in 100 mM potassium phosphate buffer (pH 7.8) for 24 min. The protein was digested for 18 h at room temperature with 3 µg of chymotrypsin in 100 mM Tris-HCl and 10 mM CaCl₂ at pH 7.8. The toxin was cleaved at the tryptophan residues by 2-(2'-nitrophenylsulfonyl)-3-methyl-3'-bromoindolenine (BNPS-skatole) according to the method described in Crimmins et al. (7), but with 75 µl of toxin (0.65 mg/ml) in 20 mM sodium acetate buffer added to an Eppendorf tube and dried. The protein was then resuspended in 10 µl of water, cleaved as described by Crimmins et al. (7), and the fragments separated as described below. A total of 10 different digests were performed.

After each cleavage, peptide fragments were separated by reversed phase HPLC using a Beckman 126 HPLC with a Beckman 210A injector (1-ml sample loop), and peptides were detected at 215 nm by a Beckman 166 UV detector (Beckman Instruments, Inc.). Samples were eluted from a C-18 column (Rainin Microsorb-MV, 4.6 mm × 25 cm; Rainin Instruments Co. Inc., Woburn, MA) with a linear gradient from 10 to 70% acetonitrile and 0.1% trifluoroacetic acid. In some cases, the gradient was modified to obtain better resolution near the uncleaved protein peak. Purified peptides were concentrated, spotted, and dried onto Porton peptide glass sample disks (Porton Instruments, Inc., Tarzana, CA). Peptides were sequenced by the Porton Instruments PI2090 gas-phase

automated protein sequencer (Porton Instruments, Inc.). In sequencing fragments from the endoproteinase digests, each portion of the protein was sequenced at least twice. Cysteine residues were determined by the method described by Brune (4).

Protein assay and secondary structure. The protein concentration was measured with the micro bicinchoninic acid (BCA) assay (Pierce) using bovine serum albumin as the standard or using the extinction coefficient derived below and a molecular mass of 13,200 Da (49).

To determine the secondary structure of a protein from CD spectroscopy, the concentration must be determined within 5% for most analysis methods, but protein concentrations determined by typical assay methods (i.e., Bradford, Lowry, or BCA), often have errors approaching 20% depending on the purity of the protein sample and the similarity of the protein standard to the sample (3,22). A more accurate method developed by Gill and von Hippel (20) is based on calculating the extinction coefficient from the

amino acid composition when the protein is denatured in 5 M guanidine hydrochloride. The extinction coefficient of the necrosis toxin, $19.9 \text{ mM}^{-1} \text{ cm}^{-1}$ at 280 nm (determined from 3-tryptophan and 2-tyrosine, obtained from our sequence, and 2-cysteine residues, Ballance et al. [2]) was calculated from the amino acid sequence according to Gill and von Hippel (20). The absorbance at 280 nm and this extinction coefficient was used to determine the toxin concentration in a stock solution. The same solution was then diluted into 50 mM sodium phosphate, 150 mM sodium chloride (pH 7.0), and the native extinction coefficient measured.

Fig. 1. Purification of the Ptr necrosis toxin from *Pyrenophora tritici-repentis* isolate 86-124. **A**, Chromatogram from low pressure S-Sepharose ion exchange chromatography showing the absorbance at 280 nm, the sodium chloride gradient, and the activity assayed as described in the text. **B**, Superdex HR-30 gel filtration chromatography of the concentrated active fractions from panel A. Shown are the absorbance at 280 nm and the activity assay. **C**, High pressure liquid chromatography sulfo-propyl cation exchange chromatography of the active samples from panel B. Shown are the absorbance at 280 nm and the sodium chloride gradient used to elute the protein.

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions obtained during purification of the necrosis toxin. Samples were taken of each step during the purification and electrophoresed. The following samples were separated: lane 1, cytochrome c, 12,500 Da; lane 2, Sigma molecular weight standards MW-SDS-70L (bovine serum albumin, 66,000 Da; egg albumin, 45,000 Da; glyceraldehyde-3-phosphate dehydrogenase, 36,000 Da; bovine erythrocyte carbonic anhydrase, 29,000 Da; soybean trypsin inhibitor, 20,100; and bovine milk α -lactalbumin, 14,200); lane 3, crude culture filtrate; lane 4, pooled active fractions from S-Sepharose column; lane 5, pooled active fractions from gel filtration column; lane 6, pure toxin obtained from high pressure liquid chromatography column; and lane 7, Sigma molecular weight standards MW-SDS-17S (myoglobin, 16,950 Da; myoglobin I + II, 14,440 Da; myoglobin I + III, 10,600 Da; myoglobin I, 8,160 Da; myoglobin II, 6,210 Da; glucagon, 3,460 Da; and myoglobin III, 2,510 Da [not visible]).

Fig. 3. The Ptr necrosis toxin and standards subjected to isoelectric focusing in a vertical slab gel system: lane 1, horse heart myoglobin, pI 6.8 and 7.2; lane 2, cytochrome c, pI 9.6; lane 3, Bio-Rad isoelectric focusing (IEF) standards; lane 4, empty; lane 5, necrosis toxin; lane 6, empty; lane 7, Bio-Rad IEF standards; and lane 8, lens culinaris lentil lectins, pI 8.8, 8.6, and 8.2.

The CD spectrum of the toxin was measured using the JASCO J-700 CD spectrometer (Japan Spectroscopic Co., Ltd., Tokyo). The purified toxin, 1.25 mg/ml (determined from the extinction coefficient and a molecular weight of 13,200), in 20 mM phosphate buffer (pH 6.8) was placed in a cylindrical quartz cuvette, 0.1-mm path length, and the CD spectrum measured from 178 to 260 nm. The CD spectrum was analyzed for secondary structure composition by the program SELCON (44).

RESULTS

Isolation and electrophoresis. The toxin eluted from the first ion exchange column in a volume of 15 ml (Fig. 1A), resulting in a 65-fold increase in concentration when starting with 1 liter of crude extract. Concentration of the active fractions to a volume of 2 ml and separation by gel filtration (Fig. 1B) resulted in an active fraction, from 50 to 60 ml, that was typically 50 to 80% pure based on relative areas in the HPLC separation (Fig. 1C). The final separation by ion exchange using HPLC resulted in one major active peak that eluted between 110 and 140 mM NaCl (Fig. 1C). The purification procedure, shown by an SDS polyacrylamide gel of the pooled active fractions from each step in the purification, resulted in the isolation of a single protein band (Fig. 2). Reversed phase HPLC analysis of the purified Ptr necrosis toxin exhibited a single asymmetric peak, indicating a single protein in the preparation (data not shown). The molecular mass determined from this gel is near 12.4 kDa, which is lower than previously reported. It should be noted that the molecular weight standard α -lactalbumin, lane 2 last band, has a molecular mass of 14.4 kDa and is coincident with cytochrome c, lane 1, which has a molecular mass of 12.5 kDa. A plot of both standard sets, lanes 2 and 7, indicated that α -lactalbumin exhibits anomalous behavior in this electrophoresis system (data not shown). Silver stain was used to increase the staining sensitivity in order to look for contaminating proteins. Although the toxin did not stain with the silver stain

Fig. 4. Ptr necrosis toxin was suspended in 20 mM sodium phosphate buffer, pH 6.8, at a concentration of 1.29 mg/ml, placed in a 0.1-mm path length circular cuvette and the circular dichroism spectrum measured. Shown is an average of 32 scans. Θ is in units of $M^{-1} cm^{-1}$.

TABLE 1. Secondary structure predicted from circular dichroism spectrum for the *Pyrenophora tritici-repentis* necrosis toxin

Relative protein concentration ^a	Secondary structure				Total
	α	β (antiparallel)	Turn	Other	
0.95	0.08	0.41	0.24	0.26	0.99
1.00	0.15	0.35	0.23	0.26	0.99
1.05	0.15	0.32	0.28	0.24	0.99

^a Allowing for a 5% margin of error in measuring total protein concentration.

used, no other proteins were detected even with excess amounts of toxin (data not shown).

A nondenaturing isoelectric focusing gel (Fig. 3) exhibited a single, very diffuse band with an isoelectric point near pH 10. No other band was distinguishable at other pH values, suggesting a pure protein. The broadness of the band may be due to microheterogeneity in the protein. Bioassay of extracts from horizontally sliced gel sections indicated activity only in the section containing observable protein. Thus, the protein isolated by this purification procedure was a necrosis-producing phytotoxin.

Protein concentration and secondary structure. To obtain an accurate measurement of the protein concentration for use in CD spectroscopy, we used the amino acid composition and sequence information. An extinction coefficient of $19.9 mM^{-1} cm^{-1}$ at 280 nm in the presence of 5 M guanidine hydrochloride was calculated based on 2-tyrosine, 3-tryptophan, and 2-cysteine residues (20). Using the protein concentration determined in the presence of guanidine hydrochloride, the extinction coefficient of the native protein in phosphate buffer (pH 7.0) was $18 mM^{-1} cm^{-1}$ at 280 nm.

The secondary structure of the toxin was determined using CD spectroscopy from 178 to 260 nm (Fig. 4). This spectrum is similar to that observed for α -chymotrypsin, which is mostly a β -sheet protein (48). Analysis of the CD spectrum using the program SELCON (44) for four components (α , β , turn, and other) indicated a mostly β -sheet protein with little α -helix (Table 1). Further analysis for five components (α , β -parallel, β -antiparallel, turn, and other) indicated that the β -sheet was completely antiparallel.

Amino acid composition and sequencing. The amino acid composition of our preparation can be compared with that obtained from other research groups (Table 2). Since we used the same isolate as Ballance et al. (2), we expected to obtain the same composition, and inspection of the table shows a close correlation between our results. Tuori et al. (49) and Tomás et al. (47) used closely related strains suggesting that the proteins would have a similar composition, but their results show differences in the mole percentages of serine and proline. The toxins produced by 86-124 and Pt-1C are similar in composition, but appear different in the mole percentages of proline, histidine, lysine, and arginine.

Figure 5 gives the sequence of a major portion of the toxin and indicates the overlapping fragments used to assemble the sequence. A total of 101 amino acids have been combined into a continuous sequence based on the sequence obtained from 50

TABLE 2. Amino acid composition (mole percentage) of isolated *Pyrenophora tritici-repentis* necrosis toxins

Amino acid	Pt-1C-BFP (ToxA) ^a	Pt-1C (Ptr toxin) ^b	86-124 (Ptr necrosis toxin) ^c	86-124 (Ptr necrosis toxin) ^d
Asp/Asn	17.7	18.7	17.2	18.5
Thr	7.7	7.0	7.9	7.3
Ser	6.3	10.7	7.3	6.0
Glu/Gln	7.9	9.2	8.1	8.8
Pro	5.3	8.4	3.0	3.7
Gly	11.0	10.5	11.2	11.5
Ala	3.2	4.3	3.1	3.4
Val	6.4	5.5	7.2	6.9
Met	1.6	0.7	1.3	1.4
Ile	9.8	4.3	10.3	10.5
Leu	6.9	4.9	7.0	7.0
Tyr	2.2	2.4	2.0	1.5
Phe	2.8	2.6	2.8	2.6
His	0.3	0.7	0.0	0.0
Lys	1.0	1.1	0.5	0.6
Arg	9.7	9.2	11.0	10.3

^a Data obtained from Tuori et al. (49).

^b Data obtained from Tomás et al. (46).

^c Data obtained from Ballance et al. (2).

^d Data reported here from two separate determinations.

Fig. 5. The partial Ptr necrosis toxin sequence was obtained by gas-phase amino acid sequencing of proteolytic fragments separated by reversed phase high pressure liquid chromatography. The following reagents were used: Trypsin (T), endoproteinase Glu-C (E), endoproteinase Asp-N (D), and 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine (BNPS-skatole) (W). The symbol X represents either an arginine or aspartate residue.

different fragments generated by the digests. Assuming a molecular mass between 12.5 and 13.2 kDa (49), a total of 9 to 16 amino acids are left to be sequenced. This should include a second cysteine residue based on the sensitivity of the protein to reduction by dithiothreitol (2). Since the N-terminal is blocked, it will be difficult to obtain these residues by direct amino acid sequencing. It appears that obtaining the complete amino acid sequence will require the cloning and sequencing of the cDNA.

Prediction of the secondary structure from the amino acid sequence by the methods of Chou and Fasman (6) as programmed by Deleage and Roux, and Deleage et al. (DR) (6,8-11) and the PHD algorithm (38-41) is shown in Figure 6. All three methods predicted a mostly β -sheet structure with an α -helix near the C-terminal end. In two of the three prediction methods, PHD and DR, a short helical region was also predicted near residue 67. These predictions were consistent with the CD analysis of the toxin, suggesting up to 40% β -sheet, but only 8 to 15% α -helix.

DISCUSSION

The purity of the toxin preparation obtained by our method was confirmed by SDS gel electrophoresis, reversed phase HPLC analysis, and isoelectric focusing. No proteins or biologically active components other than the native necrosis protein induced necrosis after isoelectric focusing. The toxin had an extremely basic pI in the native state and this result, and a small degree of heterogeneity in the protein structure, led to a diffuse band on the isoelectric focusing gel. The toxin was not stained by silver stain methods, although it was easily stained with Coomassie brilliant blue.

Purification of large quantities of toxin used for physical and biochemical analysis, genetic research, and wheat breeding requires an efficient method of concentrating the toxin from crude extract. Production of pure toxin by our method could easily be increased by increasing the column diameter for rapid loading of large quantities of crude extract. Our method has an advantage over that reported by Tuori et al. (49) in that colored material is removed early in the purification process. In addition, the elution from the first ion exchange column and the gel filtration separa-

tion may be performed within a single day, allowing the rapid production of partially purified toxin.

Comparison of the amino acid composition among the toxins from three fungal isolates indicates several amino acid differences (Table 2). Most differences occurred in the positively charged amino acids and may represent somewhat conservative amino acid replacements, because toxicity was not significantly affected. Variation in composition suggests that a pool of toxin variants exists in the fungal population, and these variants (or induced mutants) would be useful for determining the amino acid residues important for toxin function.

Although we did not obtain the complete amino acid sequence, our results demonstrate that, so far, there are no modified amino acids in the toxin. This is an important step in the characterization of the toxin and in determining the mechanism of the toxin. Unusual or modified amino acids have been found in some peptide toxins (23,25,26,50). Therefore, the results here eliminate, for the most part, unusual interactions between the toxin and its site of action. It should be relatively simple to use this amino acid sequence to confirm the toxin gene and, thus, obtain the complete amino acid and DNA sequences.

Analysis of CD spectra and primary sequence data indicated a mostly β -sheet protein. The number of β -strands predicted suggests that the toxin may form two overlapping β -sheets. Protein and DNA data bases were searched for proteins with similar sequences, but no significant matches were found, suggesting a unique biological function for this necrosis toxin. The comparison of the amino acid sequence with sequences of known function through PROSITE (1,18,19) resulted in two interesting observations. First, the threonine residues at positions 38, 46, 49, and 90 are in sequences typical for phosphorylation by phosphokinase C (51). In addition, the threonine residues at positions 55, 62, and 90 are also within sequences typical for casein kinase II phosphorylation (24,36). Although none of these amino acids were phosphorylated in the isolated protein, all of the possible phosphorylation sites except for threonine 91 are closely grouped and may be of importance to the mode of action if the protein is taken up by the host. Second, the tripeptide Arg-Gly-Asp at position 64 to 66, which was predicted as a turn region, has been found in a large number of proteins including collagens, fibrinogen, snake disintegrins, and slime mold discoidins (13,42). In each case, this tripeptide was involved in the adhesion of the protein to the plasma membrane, and it may play a similar role in the necrosis toxin. Further study of protein regulation, structure, specific activity, and mode of action will clarify the role of the Ptr necrosis toxin in the *Triticum-P. tritici-repentis* pathosystem.

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Fig. 6. The secondary structure of the *Pyrenophora tritici-repentis* Ptr necrosis toxin was predicted by the methods of Chou and Fasman (CF), Deleage and Roux (DR), and Rost and Sander (PHD). Unmarked regions indicate other structure. The following symbols indicate possible phosphorylation sites for protein kinase C (#), casein kinase II (!), and the possible cell adhesion tripeptide (+). The symbol X represents either an arginine or aspartate residue.

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