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Purification and Immunological Characterization of Toxic Components from Cultures of *Pyrenophora tritici-repentis*

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To facilitate the genetic analysis of pathogenicity in the wheat–*Pyrenophora tritici-repentis* interaction, a host-selective toxic protein, designated ToxA, was purified from culture filtrates of this fungus. ToxA was shown to be a 13.2-kDa heat-stable protein which induced visible necrosis in sensitive wheat cultivars at an average minimum concentration of 60 nM. Polyclonal antibodies raised against ToxA were shown by Western analysis and indirect immunoprecipitation to be specific for this protein. Bioassays of immunoprecipitated protein and ToxA protein eluted from polyacrylamide gels indicated that ToxA protein is the toxic agent. Other less abundant necrosis-inducing components that are chromatographically and immunologically distinct from ToxA were also detected in culture filtrates of *P. tritici-repentis*. These components were found in cationic and anionic protein fractions and, like ToxA, induced cultivar-specific necrosis.

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoemaker) is the causal agent of tan spot of wheat (*Triticum aestivum* L.), an economically significant disease that has been reported worldwide (Hosford 1982). Certain isolates of *P. tritici-repentis* were shown to produce in culture a cultivar-specific toxic compound or compounds that induced typical tan spot necrosis upon infiltration into tissue of susceptible wheat cultivars (Tomas and Bockus 1987). Host sensitivity to the toxic compound or compounds was shown to correlate strongly with susceptibility to the disease (Tomas and Bockus 1987; Lamari and Bernier 1989), suggesting an involvement of the toxin in pathogenesis.

The toxic compound or compounds from *P. tritici-repentis* culture filtrates have been purified and partially characterized by two groups employing slightly different procedures. Both groups concluded that the toxin was a low-molecular-weight protein (Ballance *et al.* 1989; Tomas *et al.* 1990). Ballance *et al.* (1989) reported that the toxin was a heat-labile protein of M_r 13,900 (designated Ptr necrosis toxin) with an average minimum active concentration of 0.2 nM. Tomas *et al.* (1990) described the toxin as a heat-stable protein of M_r 14,700 (designated Ptr toxin) with an average minimum active concentration of 90 nM. Comparison of these proteins showed only minor differences in amino acid content. Differences in molecular weight, specific activity, and heat lability suggest that multiple toxins may be produced by *P. tritici-repentis*.

This work describes the purification and immunological characterization of the major toxin, which we have designated ToxA, from cultures of *P. tritici-repentis*. To determine whether multiple toxins are produced by *P. tritici-repentis*, we developed polyclonal antibodies against the ToxA protein and used them to perform an immunological analysis of toxic components from culture filtrates of *P. tritici-repentis*. Characterization of the ToxA protein and other toxins will facilitate the molecular genetic analysis of the corresponding toxin gene or genes.

RESULTS

ToxA purification.

Gel permeation chromatography separated toxic agents from the majority of colored material and larger proteins found in crude culture filtrate (CCF) of *P. tritici-repentis*. Activity was typically found within the range of fractions 35–60 and did not correspond to a distinct UV absorbing peak. Further purification by cation exchange Mono-S fast protein liquid chromatography (FPLC) (Fig. 1A) resulted in the consistent isolation of a major peak (ToxA), which corresponded to a protein band of approximately 14 kDa, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B, lane 2). Analysis of purified ToxA by mass spectroscopy indicated a size of 13.2 kDa. Nondenaturing gel electrophoresis (Fig. 1C) further demonstrated the purity of the ToxA protein. No protein other than the 13.2-kDa band was detected when as much as 20 μg of purified ToxA was run on SDS gels stained with Brilliant Blue G–Colloidal. The 13.2-kDa protein was established as the toxic agent by bioassay of protein bands eluted from polyacrylamide gel slices and by bioassay of indirect immunoprecipitation products (see below). The amino acid composition of ToxA was determined (Table 1) and found to be similar to both previously published results (Tomas *et al.* 1990; Ballance *et al.* 1989).

This purification procedure resulted in the recovery of approximately 6.7 μg of ToxA per milliliter of CCF. The average minimum concentration of ToxA required to induce a visible tan necrosis in susceptible wheat plants was determined to be 80 ng per 100-μl infiltration (60 nM). Minimum

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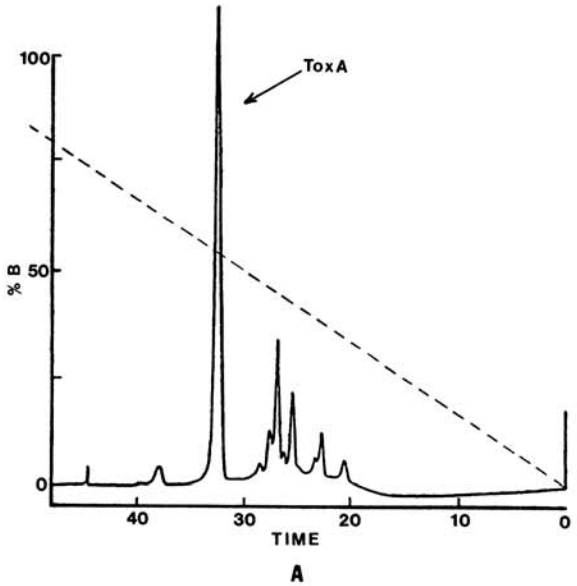
active concentrations ranged from 50 to 120 ng per 100-µl infiltration (38–91 nM).

Heat and Pronase treatment of ToxA.

Treatment of ToxA with Pronase E resulted in the loss of the 13.2-kDa band when the sample was analyzed by SDS-PAGE and loss of all necrosis-inducing activity when the sample was infiltrated into leaves of the susceptible wheat cultivar. Infiltrations of buffer or Pronase E alone did not result in damage to leaves, and ToxA incubated without Pronase retained activity. ToxA samples heated for 10 min at 80 or 100° C retained activity.



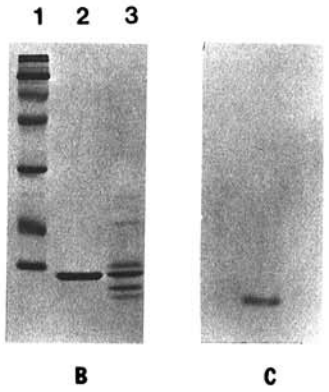


Fig. 1. A, Mono-S cation exchange chromatography. The dashed line indicates increasing salt gradient (from right to left) from 10 mM sodium acetate, pH 4.8, to 300 mM sodium chloride in 10 mM sodium acetate, pH 4.8 (buffer B), over 60 min. **B,** Electrophoresis on 14% sodium dodecyl sulfate–polyacrylamide gel stained with Brilliant Blue G–Colloidal: molecular weight markers of (from bottom to top) 14.3, 21.5, 30, 46, 69, 97.4, and 200 kDa (lane 1); purified ToxA (approximately 5 µg) from Mono-S cation exchange chromatography (lane 2); concentrated active fractions from Sephadex G-50 column chromatography (lane 3). **C,** Electrophoresis of 5 µg of purified ToxA on 10% native gel stained with Brilliant Blue G–Colloidal.

Immunological characterization.

Western analysis indicated that polyclonal antibodies raised in mice against ToxA reacted with and are specific to the 13.2-kDa protein associated with toxin activity (Fig. 2). No other protein bands were detected with the anti-ToxA polyclonal antibodies.

Indirect immunoprecipitations of crude toxin preparations confirmed the specificity of the antibodies for the 13.2-kDa ToxA band and indicated that the 13.2-kDa protein was the major toxic agent (Figs. 3 and 4). Electrophoretic analysis of immunoprecipitated products revealed a unique 13.2-kDa protein band that co-migrated with purified ToxA on polyacrylamide gels and was present only when anti-ToxA antisera were used for the reaction (Fig. 3). Bioassay of the immunoprecipitated 13.2-kDa band indicated specific necrosis-inducing activity upon infiltration into leaves of the susceptible wheat cultivar (Fig. 4). Infiltration with anti-ToxA antiserum (boiled and unboiled) did not elicit a response. The 13.2-kDa toxin band did not react with control serum from preinoculated mice, as determined by electrophoresis (Fig. 3) and bioassay (Fig. 4).

Other toxic components.

Necrosis-inducing activity, distinct from the 13.2-kDa ToxA band, was often detected when protein fractions from the Mono-S cation exchange column were assayed by leaf infiltration. These components eluted in two fractions during chromatography: 1) in the flow-through, i.e., material which did not bind to the cation exchange resin (anionic toxic component, or AI); and 2) immediately following elution of the ToxA protein (cationic toxic component, or CI). Both CI and AI were present in relatively small quantities in comparison to the amount of ToxA activity observed.

Table 1. Detectable amino acid composition of toxins (mol%)

Amino acid[a]	ToxA[b]	Ptr toxin[c]	Ptr necrosis toxin[d]
Asp/Asn	17.7	18.7	17.2
Thr	7.7	7.0	7.9
Ser	6.3	10.7	7.3
Glu/Gln	7.9	9.2	8.1
Pro	5.3	8.4	3.0
Gly	11.0	10.5	11.2
Ala	3.2	4.3	3.1
Val	6.4	5.5	7.2
Met	1.6	0.7	1.3
Ile	9.8	4.3	10.3
Leu	6.9	4.9	7.0
Tyr	2.2	2.4	2.0
Phe	2.8	2.6	2.8
His	0.3	0.7	0.0
Lys	1.0	1.1	0.5
Arg	9.7	9.2	11.0

[a] Values were calculated from amounts of each amino acid detected (in nanomoles) divided by the total amount of all detectable amino acids (in nanomoles). Although the tryptophan and cysteine content of proteins can be calculated by other methods, as was done for Ptr toxin and Ptr necrosis toxin, they are not detectable by standard amino acid analysis. These data were not determined for Tox-A, and thus these values were not included for Ptr toxin and Ptr necrosis toxin.

[b] Based on two independent determinations.

[c] Data obtained from Tomas *et al.* (1990).

[d] Data obtained from Ballance *et al.* (1989), converted to mole percent detectable amino acid.

The CI fractions eluted in the Mono-S gradient in two to six fractions after the elution of ToxA and, thus, are chromatographically distinct from ToxA fractions. However, because of the similar retention times of the CI and ToxA fractions, the CI fractions often contained detectable amounts of ToxA. Therefore, we determined whether the quantity of contaminating ToxA detectable by electrophoretic or immunological analysis could account for the biological activity of these fractions. Three representative CI fractions were analyzed biologically, electrophoretically, and immunologically (Fig. 5). The three fractions analyzed were obtained from two different isolates (CI-1 and CI-2 from Pt-1C-BFP, and CI-3 from SD-21) and, thus, indicate that the CI fractions were not restricted to a particular fungal isolate. Similar necrosis-inducing CI fractions were also obtained from several other isolates. It is not known if the necrosis-inducing activity of the different CI fractions is due to the same toxic component or to different components within these fractions. All CI fractions tested displayed cultivar-specific activity in the wheat cultivar TAM-105, and all were inactive when infiltrated into the cultivar Auburn. SDS-PAGE of CI fractions revealed a predominant band migrating near ToxA, often with smaller bands running immediately below. Multiple bands were clearly resolved by native PAGE (Fig. 5).

Three representative CI fractions and a range of ToxA standards were analyzed by leaf infiltration bioassay (Fig. 5A). The same quantity of CI-1 infiltrated (50 µl) was also run on native gels (Fig. 5B) and immunoassayed by slot blot analysis (Fig. 5C). This process was repeated exactly for CI-2 and CI-3. Fraction CI-1 induced necrosis equivalent to a minimum of 500 ng of ToxA (Fig. 5A), and yet no ToxA was detected by native gel electrophoresis (Fig. 5B) or slot blot analysis (Fig. 5C). Fraction CI-2 induced necrosis equivalent to a minimum of 1 µg of ToxA (ToxA in amounts of 1 µg or more induced complete necrosis of the infiltrated area), but far less than 500 ng was detected by electrophoretic analysis, and only approximately 100 ng was detected by slot blot analysis. Fraction CI-3 also induced necrosis equivalent to 1 µg or more of ToxA, and yet slot blot analysis detected levels of ToxA no higher than that of the buffer control. Electrophoretic analysis of fraction CI-3 revealed a protein migrating slightly slower than ToxA, as well as two faster-migrating protein bands. The slot blot immunoassay was repeated three times with similar results. The results shown in Figure 5C are all from the same slot blot.

When CI fractions were tested for heat stability by incubating for 10 min at either 80 or 100° C, most necrosis-inducing activity was lost, whereas ToxA activity was stable under these conditions.

Chromatography of the AI fractions on a Mono-Q anion exchange column yielded protein fractions which eluted during the gradient and induced cultivar-specific necrosis in the wheat cultivar Norkan. ToxA, an apparently cationic protein, does not bind to the Mono-Q column. AI fractions, such as the representative AI-1, consistently induced necrosis in Norkan (Fig. 5A) but not in the cultivars Auburn or Arkan (Fig. 5A). Inconsistent necrosis-inducing activity was observed when AI-1 was infiltrated into TAM-105. Fraction AI-1 could not be analyzed by the native gel system used for CI fractions, because it contained anionic proteins. Fraction AI-1 showed no detectable reaction with the anti-ToxA polyclonal antibody when analyzed by slot blot (Fig. 5C). The necrosis

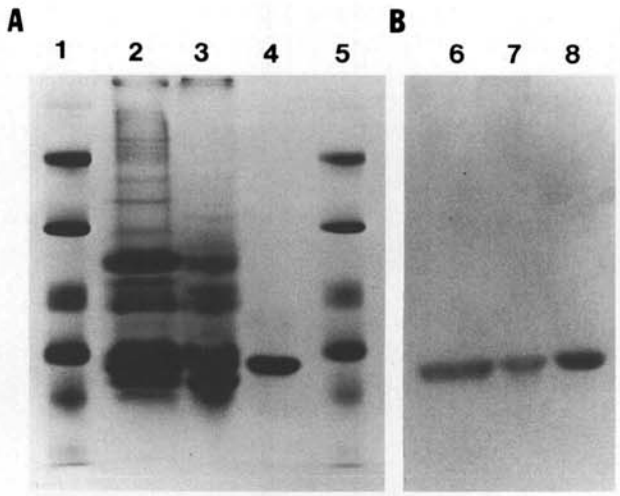


Fig. 2. Electrophoresis on 14% sodium dodecyl sulfate–polyacrylamide gel stained with Brilliant Blue G–Colloidal (**A**) and Western analysis (**B**): molecular weight markers of (from bottom to top) 6.5, 14.3, 21.5, 30, and 46 kDa (lanes 1 and 5); concentrated crude culture filtrate of *Pyrenophora tritici-repentis* (lanes 2 and 6); concentrated active fractions from Sephadex G-50 column chromatography (lanes 3 and 7); purified ToxA (approximately 10 µg) from Mono-S cation exchange chromatography (lanes 4 and 8). Western analysis (**B**) shows polyclonal antibodies specific for ToxA (lanes 6, 7, and 8).

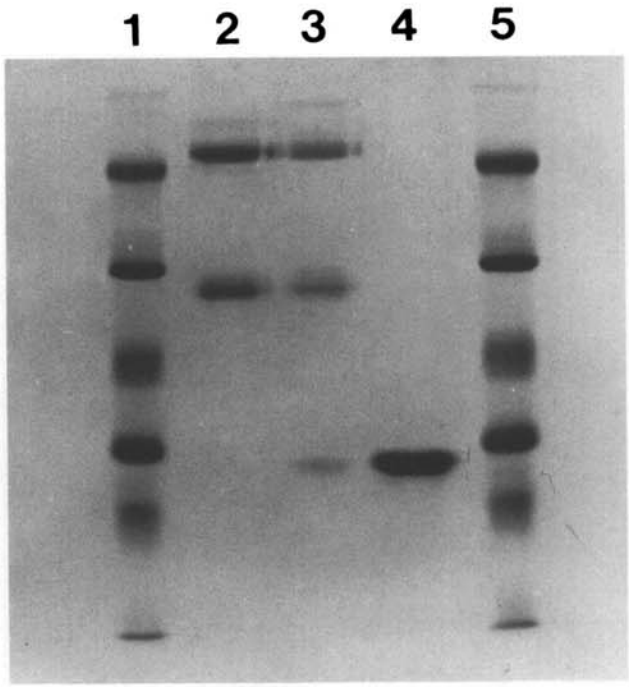


Fig. 3. Indirect immunoprecipitation products on 14% sodium dodecyl sulfate–polyacrylamide gel stained with Brilliant Blue G–Colloidal: molecular weight markers of (from bottom to top) 6.5, 14.3, 21.5, 30, and 46 kDa (lanes 1 and 5); indirect immunoprecipitations from crude toxic fractions (Sephadex G-50 column–enriched toxin preparations) (lanes 2 and 3); immunoprecipitation with normal (control, preimmunized) mouse serum (lane 2); immunoprecipitation of sample with polyclonal antibodies from mouse immunized with ToxA (lane 3); purified ToxA (approximately 10 µg) (lane 4).

induced by AI fractions showed a distinctive progression of symptoms. AI-induced lesions originally appeared chlorotic, whereas ToxA infiltrations initially produced a gray, collapsed appearance. Within 5–7 days, however, necrosis with pink to red margins was visible in most reactions.

DISCUSSION

The purification procedure reported here resulted in approximately 6.7 μg of purified ToxA protein per milliliter of CCF, with an average minimum active concentration of 0.8 μg/ml (60 nM). This concentration for activity is far higher (300-fold) than that obtained for Ptr necrosis toxin (Ballance *et al.* 1989), but it is comparable to that obtained for Ptr toxin (Tomas *et al.* 1990).

We were unable to obtain sufficient quantities of purified toxin by the procedure of Tomas *et al.* (1990) or Ballance *et al.* (1989). With the protocol of Ballance *et al.* (1989), toxic compounds eluted during the gradient as reported. However, in two of the four attempts, toxic activity was also detected in fractions that eluted prior to the salt gradient. This activity of compounds eluting prior to the gradient could be the same as that of the AIs described here. In all four instances, the gradient fractions with necrosis-inducing activity contained the ToxA band as well as other contaminating protein bands when analyzed by SDS-PAGE.

The heat stability of ToxA is similar to that determined for Ptr toxin (Tomas *et al.* 1988) but contrasts with the heat lability of Ptr necrosis toxin (Ballance *et al.* 1990), which lost most activity after treatment for 30 min at 70° C. This indicates that ToxA may be similar to or the same compound as Ptr toxin. The loss of most activity of CIs following a 10-min treatment at 80 or 100° C indicates that these fractions contain a toxin different from ToxA, and it suggests that the cationic activity is caused by the same compound as Ptr necrosis toxin.

Polyclonal antibody raised in mice against ToxA was useful in characterizing the toxic agents produced by *P. tritici-repentis* in several ways: 1) The anti-ToxA antibodies were specific for this protein among all extracellular proteins produced by *P. tritici-repentis,* as determined by Western blot analysis and indirect immunoprecipitations. Western blot analysis showed that, even in lanes overloaded with crude protein preparations, only the 13.2-kDa protein reacted with anti-ToxA antibodies (Fig. 2). When these crude protein preparations were used for indirect immunoprecipitations, again only the 13.2-kDa ToxA protein eluted from the polyclonal antibodies and was detected on SDS-polyacrylamide gels (Fig. 3). 2) Bioassay of the immunoprecipitated products (Fig. 4) demonstrated most convincingly the causal nature of the ToxA protein in necrosis. The immunoprecipitation results, demonstration of antibody specificity, results of the bioassay of protein eluted from slices of polyacrylamide gels, and the Pronase E sensitivity of ToxA provide strong evidence that the 13.2-kDa protein is a toxic agent. 3) Other toxic components were produced by *P. tritici-repentis* that are immunologically and chromatographically distinct from the ToxA protein.

Toxic components were isolated that either did not bind to the Mono-S column under the conditions used (AI) or bound to the column but eluted later in the gradient than ToxA (CI-1, CI-2, and CI-3). Although these fractions contained several proteins, crude preparations of both the CI and AI active fractions were obtained with little or no ToxA contamination. The CI fractions were the most difficult to resolve free of ToxA contamination. However, the necrosis induced by the three CI fractions clearly cannot be accounted for by the amount of ToxA detected electrophoretically and immunologically.

The proteins in AI fractions have distinctly different chromatographic and immunological properties from ToxA. Because of the initial chlorotic appearance of AI-induced lesions, the possibility that these fractions cause the chlorotic response described by Lamari and Bernier (1989) was investigated by infiltration of leaves of the wheat cultivars Norkan and Arkan with these fractions. The wheat response of extensive chlorosis in infection by *P. tritici-repentis* has been de-

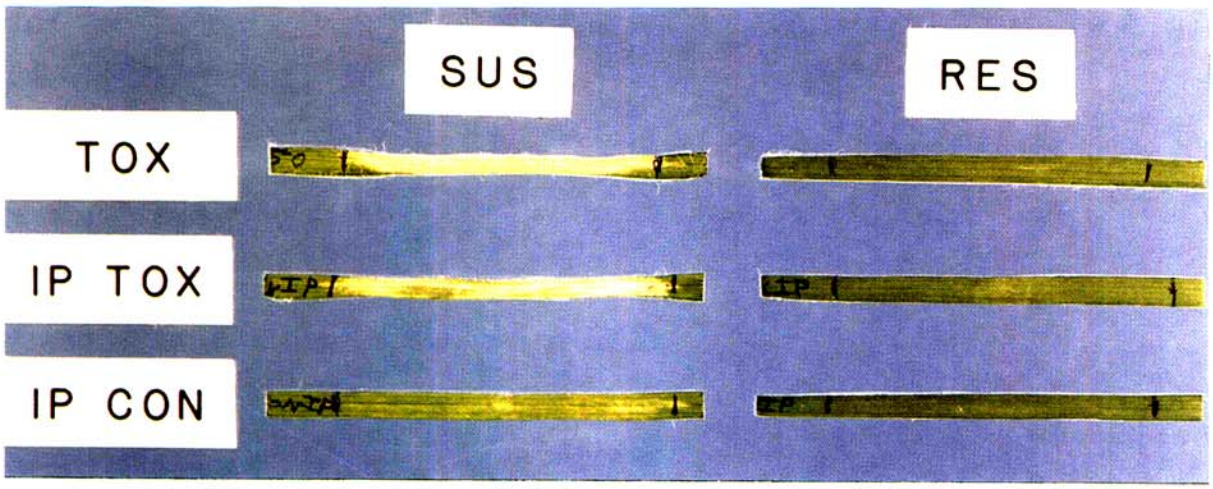


Fig. 4. Leaf infiltration bioassay of indirect immunoprecipitation products. The susceptible (SUS) and resistant (RES) wheat cultivars TAM-105 and Auburn, respectively, were infiltrated with purified ToxA (TOX), immunoprecipitated products obtained from anti-ToxA polyclonal antibodies (IP TOX), or immunoprecipitated products obtained from control mouse antisera (IP CON). Typical toxin-induced tan necrosis is shown on leaves of the susceptible wheat cultivar infiltrated with purified ToxA and with the protein recognized by the anti-ToxA polyclonal antibodies. No necrosis was present in the resistant cultivar or in either wheat genotype when immunoprecipitated products obtained from control mouse antisera were used for the infiltration bioassay.

termined to be genetically distinct from the development of necrosis (Lamari and Bernier 1991). Both Norkan (nec^+chl^+) and Arkan (nec^-chl^+) are susceptible to infection and react with extensive chlorosis to certain pathotypes of *P. triticirepentis*. Norkan also shows a necrosis response similar to that of TAM-105 (susceptible, nec^+chl^-). AI fractions obtained from all isolates consistently induced necrosis when infiltrated into leaves of Norkan and did not induce necrosis or chorosis in the cultivars Auburn or Arkan. The AI toxin, therefore, is not responsible for the chlorosis observed by Lamari and Bernier (1989). The AI fractions from some isolates, however, produced an inconsistent necrotic response in

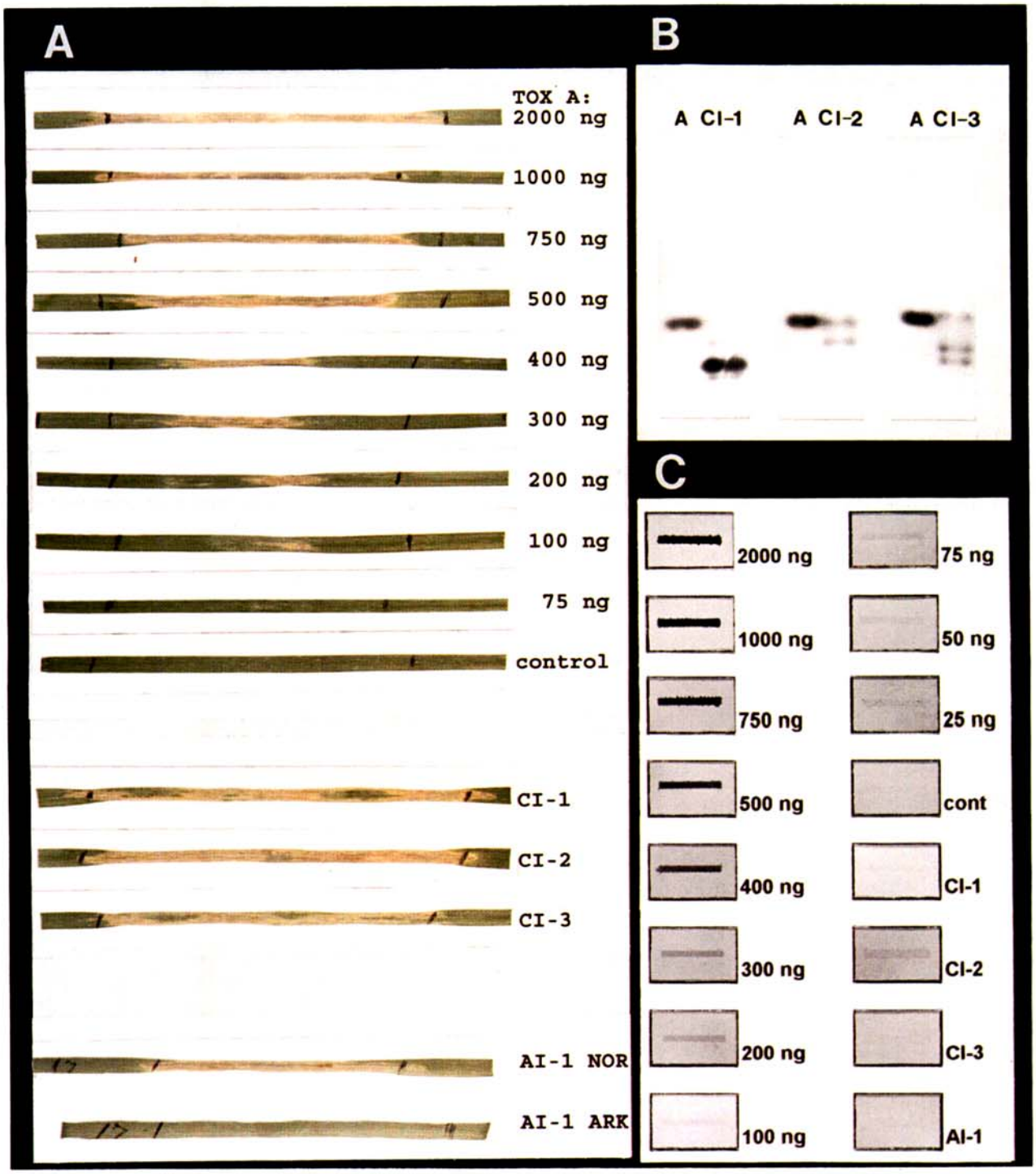


Fig. 5. Biological, electrophoretic, and immunological analyses of cationic toxic component (CI) and anionic toxic component (AI) fractions. **A,** Leaf infiltration bioassay. Leaves of the susceptible wheat cultivar TAM-105 infiltrated with a range of purified ToxA standards, from 75 to 2,000 ng and, for comparison of activity levels, with 50 µl each of three representative CI fractions (CI-1, CI-2, and CI-3). Wheat genotypes Norkan (NOR) and Arkan (ARK) infiltrated with 50 µl of a representative AI fraction (AI-1). All infiltrations were brought up to a final volume of 100 µl with distilled water. **B,** Silver-stained native gel with 500 ng of ToxA standard run next to 50 µl each of CI-1, CI-2, and CI-3. **C,** Slot blot immunoassay of a range of ToxA standards, from 50 to 2,000 ng, and 50 µl each of CI-1, CI-2, CI-3, and AI-1. The control (cont) is 10 mM sodium acetate buffer, pH 4.8.

TAM-105 plants. AI fractions obtained from isolate Pt-1C-BFP produced necrosis in TAM-105 in approximately 50% of the infiltrations, while fractions obtained from isolate SD-8 produced necrosis in TAM-105 in all infiltrations. Infiltration of TAM-105 with ToxA in amounts greater than the minimum active concentration of 60 nM always resulted in visible necrosis. These results suggest 1) there is more than one toxic component in the AI fractions; 2) the production of the different toxic components is isolate-dependent; 3) the different AI components interact specifically with different wheat genotypes that are present in TAM-105; and 4) there are additional genes in wheat that condition a necrotic response to toxic components from *P. tritici-repentis*. Clearly, the AI toxin or toxins are significantly different from both ToxA and the previously described toxins, Ptr toxin (Tomas *et al.* 1990) and Ptr necrosis toxin (Ballance *et al.* 1989). Thus, the AI toxin or toxins can be considered novel cultivar-specific toxins produced by this fungus.

It is possible that the ToxA protein described here is distinct from both Ptr toxin and Ptr necrosis toxin. However, Ptr toxin obtained as a gift from A. Tomas co-migrated on SDS-polyacrylamide gels and reacted with anti-ToxA polyclonal antibodies on a Western blot (data not shown). These data, along with the similar molar activities and heat stability, indicate that Ptr toxin and ToxA are likely the same protein. Ptr necrosis toxin and ToxA, however, are probably different proteins. ToxA appears distinct from Ptr necrosis toxin in molecular weight as determined by mass spectrometry, heat stability, and molar activity. The CI we observed was of low molecular weight and heat-labile. Thus, the CI fractions could contain Ptr necrosis toxin, which may be produced at low levels by our isolate.

MATERIALS AND METHODS

Fungal cultures and plant material.

Isolates of *P. tritici-repentis* were obtained from William Bockus, Kansas State University. Isolate Pt-1C exhibited high toxin production as determined by symptom expression in susceptible wheat cultivars. Susceptible host plants were inoculated with single conidia of this isolate, and mycelium from lesions was cultured in modified Fries medium as described by Tomas and Bockus (1987). Filtrates from these cultures were tested for toxic activity by leaf infiltration assays. A fast-growing subculture of Pt-1C with a distinctive pink coloration was named Pt-1C-BFP. Conidia of Pt-1C-BFP were isolated as described by Hunger and Brown (1987) and used as an inoculum source for liquid cultures and toxin purification. Standing cultures were produced in 500-ml flasks containing 100 ml of modified Fries medium, inoculated with 6 ml of a suspension of approximately 3×10^4 conidia per milliliter and incubated for 17–21 days at 25° C under constant fluorescent light. Several other isolates of *P. tritici-repentis,* which we designated isolates SD-1 through SD-21, were obtained from Gary Buchenau, South Dakota State University.

Wheat plants used for bioassay were grown at 25° C with a 16-hr photoperiod. Plants were grown to the four-leaf stage, and samples were infiltrated into the third or fourth leaves with a Hagborg device (Hagborg 1970). Toxin activity was assayed in the wheat cultivars TAM-105 (susceptible) and Auburn (resistant). Plants were maintained at the temperature and photoperiod previously described until necrosis developed (3–5 days). Necrosis-inducing toxic activity of anionic protein fractions was detected by infiltration into the wheat cultivars Norkan (obtained from Warren Kronstad, Oregon State University), Auburn and Arkan (obtained from Greg Shaner, Purdue University), as well as TAM-105 and (obtaianed from Dave Marshall, Texas A & M University).

Protein purification.

Toxin was purified by a modification of the procedures established by Ballance *et al.* (1989) and Tomas *et al.* (1990). CCF was filtered through four layers of cheesecloth, and proteins were precipitated from the filtrate with ammonium sulfate at 75% saturation at 4° C overnight. Proteins were pelleted by centrifugation for 20 min at 8,000 × *g*, and the supernatant was centrifuged again to further recover proteins. The pellets were combined, resuspended in water, and centrifuged at 10,000 × *g* for 20 min to remove debris, and the supernatant was applied to a 100 × 2.5 cm gel filtration column of Sephadex G-50 (Sigma). The column was equilibrated and eluted with a solution containing 10 mM sodium acetate and 100 mM sodium chloride, pH 4.8, at 0.5 ml/min, and 6-ml fractions were collected every 12 min over a 16-hr period. Fractions were assayed for toxic activity, and active fractions were combined and concentrated approximately 20-fold by rotoevaporation at 40° C. Concentrated material was desalted on a disposable PD-10 Sephadex G-25 desalting column (Pharmacia) equilibrated with 10 mM sodium acetate, pH 4.8 (buffer A).

Proteins were separated by FPLC on a Mono-S cation exchange column (Pharmacia) equilibrated in buffer A. The sample was applied to the column by pumping at 1.0 ml/min in buffer A. Proteins were eluted from the column at 0.5 ml/min with a 60-min linear gradient of 0–300 mM sodium chloride in 10 mM sodium acetate, pH 4.8. One-milliliter fractions were collected, assayed for toxic activity *in planta,* and analyzed by gel electrophoresis. The total yield of purified protein was determined by a modification of the Folin assay (Peterson 1977).

Anionic proteins (those which were not retained on the Mono-S column and eluted in the void volume, designated Mono-S flow-through) were separated on a Mono-Q anion exchange column (Pharmacia) equilibrated in 30 mM Tris, pH 9.0. The Mono-S flow-through was concentrated 10-fold by rotoevaporation at 40° C, dialyzed against 30 mM Tris, pH 9.0, in a 3,500 molecular weight cutoff membrane and applied to the Mono-Q column at 1.0 ml/min. Proteins were eluted from the column at a flow rate of 0.5 ml/min with a 30-min linear gradient of 0–1 M sodium chloride in 30 mM Tris, pH 9.0. One-milliliter fractions were collected and assayed for toxic activity *in planta.*

Gel electrophoresis.

SDS-PAGE was performed on a vertical minigel apparatus (Dual Vertical Minigel Unit, C.B.S. Scientific Co.). Proteins were resolved with a 17% resolving gel and 5% stacking gel in the buffer system described by Laemmli (1970). Gels were fixed for 1 hr in an aqueous solution containing 7% glacial acetic acid in 40% methanol (v/v) and stained with Brilliant Blue G–Colloidal Concentrate (Sigma). Nondenaturing gel

electrophoresis was performed with the low-pH discontinuous buffer system described by Hames (1981) with a 10% resolving gel and 4.6% stacking gel. Samples were loaded on the gel at the anode and electrophoresed toward the cathode for 3.5 hr at a constant current of 30 mA. Native gels were silver-stained as described by Heukshoren and Dernick (1985). ToxA protein was extracted from SDS gels following electrophoresis according to the protocol described by Tomas *et al.* (1990), and extracted protein was assayed by infiltration *in planta*.

Pronase treatment of ToxA.

Purified ToxA protein (20 µg) was treated with Pronase E (Sigma), 1 µg/µl, in a solution containing 10 mM Tris, 10 mM EDTA, and 0.5% SDS at 37° C for 18 hr. After the Pronase treatment, 5 µg was infiltrated into TAM-105 and Auburn leaves after being diluted sixfold in distilled water, so that the final SDS concentration was 0.08% (150 µl final volume). The other 10 µg was analyzed by SDS-PAGE.

Heat stability of toxic components.

The heat stability of ToxA was determined by incubating 1-µg samples diluted to 100 µl with distilled water for 10 min at 80 or 100° C. The heat stability of cationic toxic activity fractions was also assessed by incubating undiluted samples for 10 min at 80° or 100° C. The samples were cooled on ice and infiltrated into TAM-105 and Auburn leaves.

Western blotting.

Western blotting of polyacrylamide gels and immunological detection of proteins was performed by a modification of the protocol described by Wolpert and Macko (1991). SDS gels were immersed for 10 min in 25 mM Tris, 192 mM glycine, and 20% methanol (v/v) (transfer buffer), and proteins were transferred to a nitrocellulose membrane (Millipore) at 1.0 mA/cm^2 of membrane for 1 hr with an electroblotting apparatus (Transblot, Bio-Rad). After transfer, blots were equilibrated for 1 hr in a solution containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST) and 3% nonfat dried milk. Blots were washed three times for 5 min each in TBST to remove the nonfat dried milk and then were incubated for 1 hr with polyclonal antisera diluted 1:4,000 in TBST. Blots were washed three times for 5 min each in TBST and then incubated with alkaline phosphatase–conjugated anti-mouse immunoglobulin G (Sigma) at a 1:4,000 dilution in TBST. Blots were again washed, and the color was developed as described by Wolpert and Macko (1991).

Slot blots were prepared with a Bio-Rad Bio-Dot SF apparatus as described by the manufacturer, with Immobilon-P membranes (Millipore). Samples were adjusted to 20% methanol prior to loading in wells. Proteins were blotted and membranes were incubated in blocking solution and subsequently treated the same as Western blots.

Indirect immunoprecipitations.

Indirect immunoprecipitations were performed by mixing 400 µl of crude toxin preparation (concentrated and desalted active fractions from the Sephadex G-50 gel filtration column) containing approximately 200 µg of total protein with 1.6 ml of TBST and 12 µl of polyclonal antisera. Samples were vortexed and incubated for 16 hr at 4° C. The following day samples were centrifuged in a microcentrifuge for 5 min, and the supernatant was transferred to a fresh tube; 120 µl of a 1:1 suspension (w/v) of protein A–agarose (Sigma) was added to each sample, and the samples were incubated with shaking for 2 hr at room temperature. The protein A–agarose had previously been washed and resuspended in 190 mM NaCl, 60 mM Tris-HCl (pH 7.4), and 6 mM EDTA (wash buffer) with 2.5% Triton X-100. Protein A–agarose beads were pelleted in a microcentrifuge for 15 sec, and the supernatant was removed and discarded. Pellets were washed five times with wash buffer (no detergent). For SDS-PAGE analysis, the final protein A–agarose pellet was resuspended in 62.5 mM Tris, 5% β-mercaptoethanol (v/v), and 2.3% SDS (w/v), pH 6.8 (SDS loading buffer), boiled for 5 min, and centrifuged, and the supernatant was loaded onto a gel. For *in planta* bioassays, the final protein A–agarose pellet was resuspended in 200 µl of water, boiled for 5 min, and centrifuged, and the supernatant was infiltrated into TAM-105 and Auburn leaves. Control immunoprecipitations with antisera from preimmunized mice were conducted and analyzed identically.

Antibody production.

Polyclonal antibodies were raised against purified ToxA in mice at the Monoclonal Antibody Facility of the Center for Gene Research and Biotechnology at Oregon State University. Mice were injected with 20 µg of ToxA protein and given two booster injections of the same amount of protein at 1-week intervals.

Amino acid analysis.

The amino acid composition of purified ToxA was determined with a Beckman 126AA System Gold HPLC Amino Acid Analyzer at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Samples were hydrolyzed at 110°C for 20 hr in 6 N HCl containing 1% phenol. Ninhydrin-derivatized amino acids were detected after separation on a Beckman Spherogel ion exchange column.

Mass spectrometry.

The molecular weight of purified ToxA was determined by mass spectrometry at the Mass Spectrometry Lab at Oregon State University. A custom-built time-of-flight mass spectrometer equipped with a frequency-tripled (355 nm) Nd:YAG laser was used for positive-ion MALDI mass spectrometric analyses (Jensen *et al.* 1993). The time-of-flight spectra consisted of the summed data generated from 50 consecutive laser pulses. The data were analyzed with M over Z software provided by Ronald C. Beavis, Memorial University of Newfoundland. Samples were prepared by mixing 1 µl of protein solution in 2 µl of matrix solution (a 10-g/L solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/acetonitrile (2:1)). A 1-µl sample of this mixture was applied to the mass spectrometer probe and dried under a stream of air. The sample was then rinsed with cold (4° C) water, dried, and inserted into the mass spectrometer. Equine cytochrome *c* (molecular weight 12,360) was used as an internal mass calibrant.

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