Partial Purification of Proteinase Inhibitors from Tomato Plants Infected with *Phytophthora infestans*

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ABSTRACT

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Chymotrypsin and trypsin inhibitors were partially purified from the leaves of tomato plants (*Lycopersicon esculentum*) by affinity chromatography and isoelectric focusing 48 hr after inoculation with *Phytophthora infestans*, tomato race O. Tomato selections 33 (TS 33), incompatible, and 19 (TS 19), compatible, to tomato race O of *P. infestans* were used in this study. The initial inhibitor activity was greater in uninoculated plants of TS 33 than in TS 19, and overall inhibitor activity declined in both tomato selections after inoculation. Measurements of individual inhibitor activity, after isoelectric focusing, showed that there was a decline in the levels of most inhibitors following inoculation. The same inhibitors were detected in inoculated and uninoculated tomato leaves. The results from this study failed to corroborate a previous report from this laboratory that an increase in proteinase inhibitor activity in crude extracts was associated with the incompatible response of TS 33 inoculated with race O of *P. infestans*.

Proteolytic enzymes are produced by several plant pathogenic fungi (16,17,22,23) and an increase in proteolytic activity has been measured in tissues of susceptible plants following infection by fungi (12,15,21). These results, coupled with the fact that proteins are present in plant cell walls, middle lamellae, and plasma membranes (1,6,11), have led to the suggestion that proteolytic enzymes are involved in the penetration and growth of fungal parasites in plant hosts.

Proteinase inhibitors are prevalent in the plant kingdom (18) and occur in high concentrations in members of several plant families (24). Green and Ryan (3,7,8) showed that proteinase inhibitors I and II accumulated throughout a tomato plant within a few hours after a single leaf was wounded by a feeding insect or mechanical abrasion, and speculated that proteinase inhibitors are protective agents against invading organisms. Results of other studies have strengthened their theory. Proteinase inhibitor accumulation occurred in excised tomato leaves treated with juices from several higher plants and three fleshy fungi (13). Proteinase inhibitors purified from potato tubers inhibited growth and proteolytic activity of several fungi and bacteria isolated from decaying tubers (20). In this laboratory, Peng and Black (14) observed an increase in proteinase inhibitor activity in crude extracts of tomato plants inoculated with an incompatible race (resistant reaction) of *Phytophthora infestans*, but not in plants inoculated with a compatible race (susceptible reaction).

The present investigation was initiated to explore further the previously reported (14) association of increased proteinase inhibitor activity with race-specific resistance to *P. infestans* in tomato plants. In subsequent experiments with the same host-parasite system, we were unable to verify the increased proteinase inhibitor activity in crude extracts. Therefore, we decided to isolate and identify specific proteinase inhibitors from infected tomato

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plants to determine if quantitative or qualitative changes in specific inhibitors are associated with compatible or incompatible host responses.

MATERIALS AND METHODS

Materials. Chemicals were reagent grade unless otherwise noted. Trypsin-agarose (172 units per milliliter) and chymotrypsin-agarose (156 units per milliliter) were obtained from U.S. Biochemicals, Cleveland, OH 44128. L-tyrosine ethyl ester hydrochloride (TEE), *p*-tosyl-arginine methyl ester hydrochloride (TAME), 3× crystallized α-chymotrypsin (7.5 units per milligram), 2× crystallized trypsin (40 units per milligram), and enzyme grade urea were obtained from Nutritional Biochemicals (ICN) (Cleveland, OH 44128). Ampholyte buffers and Sephadex G-75 superfine used in preparative flat-bed electrofocusing were obtained from LKB (Gaithersburg, MD 10877) and Pharmacia (Piscataway, NJ 08854), respectively.

Source of plants and fungal culture. Sibling tomato (*Lycopersicon esculentum* Mill.) selections TS 19 and TS 33 were used in this study and a previous study (14). *Phytophthora infestans* (Mont.) de Bary, tomato race O, isolate WV 75 was provided by M. E. Gallegly, West Virginia University, Morgantown. TS 19 is compatible (susceptible) with this race, while TS 33 is incompatible (resistant).

Preparation of inoculum. *P. infestans* was maintained on lima bean agar (5). Inoculum was prepared by washing sporangia from 10-day-old flood-seeded lima bean agar culture plates with 30 ml of water per 15 plates. Sporangia were pelleted by centrifugation at 12,000 *g* in an SS 34 Sorvall rotor and washed two times with lima bean agar extract obtained by blending the medium with equal volumes of water and filtering through cheesecloth. Suspensions containing 1.5×10^5 sporangia per milliliter were incubated at 12 C for 2 hr to induce zoospore liberation and then were used to

TABLE 1. Partial purification of chymotrypsin inhibitors by affinity chromatography of extracts from leaf tissue of TS 33 and TS 19 plants inoculated and uninoculated with *Phytophthora infestans*

Treatments	Total protein (mg)	CTIA[a]	Specific activity	Yield (%)	Purification[b]
Crude extract					
TS 33	2,006	128	0.06	100	1
TS 19	2,698	60	0.02	100	1
Race O, TS 33[c]	3,077	86	0.03	100	1
Race O, TS 19	2,788	0	0.00	100	1
Affinity chromatography					
10 mM HCl eluates, pH 2					
TS 33	5	75[d,e]	15	59	
TS 19	1	22[d,e]	22	37	
Race O, TS 33	4	24[e]	6	28	
Race O, TS 19	1	12[e]	12	...[f]	
6 M urea eluates, pH 7					
TS 33	7	35	5	27	
TS 19	1	21	21	35	
Race O, TS 33	6	34	6	40	
Race O, TS 19	2	15	8	...	
Total					
TS 33	12	110[d]	9	86	150
TS 19	2	43[d]	22	72	1,100
Race O, TS 33	10	58	6	67	200
Race O, TS 19	3	27	9	...	...

[a] Chymotrypsin inhibitor activity (measured in international inhibitor units). CTIA estimates from crude extracts and affinity column eluates are corrected for direct comparison with the results after isoelectric focusing (See Table 3).
[b] Degree of purification (onefold = crude extract, 150-fold = active material concentration increased ×150, etc).
[c] Race O = Tomato race O of *P. infestans*; Race O with TS 33 is an incompatible combination; Race O with TS 19 is a compatible combination.
[d] Single orthogonal comparisons of TS 33 vs. TS 19 showed statistical differences ($P = 0.05$). No statistical differences were shown in any of the Race O, TS 33 vs. Race O, TS 19 single comparisons.
[e] Pooled orthogonal comparisons of TS 33 and TS 19 vs. Race O, TS 33 and Race O, TS 19 showed a statistical difference.
[f] Unable to calculate since no activity was detected in crude extracts.

TABLE 2. Partial purification of trypsin inhibitors by affinity chromatography of extracts from leaf tissue of TS 33 and TS 19 plants inoculated and uninoculated with *Phytophthora infestans*

Treatments	Total protein (mg)	TIA[a]	Specific activity	Yield (%)	Apparent purification[b]
Crude extract					
TS 33	2,006	68[c]	0.34	100	1
TS 19	2,698	0	0.00	100	1
Race O, TS 33[d]	3,077	0	0.00	100	1
Race O, TS 19	2,788	0	0.00	100	1
Affinity chromatography					
10 mM HCl eluates, pH 2					
TS 33	5	149	30	...[e]	
TS 19	1	65	65	...	
Race O, TS 33	4	89	22	...	
Race O, TS 19	1	41	41	...	
6 M urea eluates, pH 7					
TS 33	7	41	6	...	
TS 19	1	0	0	...	
Race O, TS 33	6	34	6	...	
Race O, TS 19	2	4	2	...	
Total					
TS 33	12	190	16	...	47
TS 19	2	65	33	...	...
Race O, TS 33	10	123	12	...	...
Race O, TS 19	3	45	15	...	...

[a] Trypsin inhibitor activity (measured in international inhibitor units). TIA estimates from crude extracts and affinity column eluates are corrected for direct comparison with results after isoelectric focusing (See Table 3).
[b] This column is designated "Apparent" since results from crude extract trypsin inhibitor assays may be inaccurate.
[c] No statistical differences were shown in any of the comparisons. See Table 1 (footnotes d and e) for statistical analysis.
[d] See Table 1 (footnote c) for definition.
[e] Not calculated since crude extract measurements may be inaccurate.

TABLE 3. Chymotrypsin and trypsin inhibitor activity of isoelectrically focused inhibitor proteins in leaf tissue of *Phytophthora infestans*-inoculated and uninoculated TS 33 and TS 19 plants

Inhibitor peak	pI[b]	Inhibitor activity units[a]			
		Uninoculated		Inoculated	
		TS 33[c]	TS 19	TS 33	TS 19
Chymotrypsin					
A	9.5	15	4	6	6
B	8.4	29	8	11	9
C	7.9	21	14	9	9
D	7.5	17	12	9	11
Totals		82	38	35	35
Trypsin					
A	9.5	22	19	22	10
B	8.4	46	39	40	20
D	7.5	28	37	15	2
E	6.7	39	38	20	13
F	5.9	53	64	47	21
G	4.9	72	69	32	41
Totals		260	266	176	107

[a] Total inhibitory activity in the fractions under each peak.
[b] Isoelectric point.
[c] Inhibitors in the combined affinity column eluates of each treatment were isoelectrically focused. See experimental design in "Materials and Methods" and Fig. 2.

spray-inoculate the tomato plants.

Propagation and inoculation of plants. Plants were propagated from seed planted in a peat-vermiculite mix, Jiffy Mix Plus (Jiffy Products of America, West Chicago, IL 60185), in model 200 Todd Planter Flats (Speedling Inc., Sun City, FL 33586) with 5-cm cavities. Half-strength modified Hoagland's solution (10) was applied every 3 days between regular waterings. Plants were grown for 3–4 wk in an air-conditioned greenhouse at approximately 28 C and then transferred to an environmental growth chamber for 2 days with a day temperature of 24 C and a night temperature of 20 C. Daylength was 14 hr with 1.5×10^{-4} lx (1,400 ft-c) of fluorescent and incandescent light. Plants were then spray inoculated and kept in a dark humidity chamber for 24 hr at approximately 24 C before being returned to the growth chamber. After 24 hr in the growth chamber (48 hr after inoculation), all leaves were harvested, frozen with liquid nitrogen, and lyophilized. Uninoculated controls were treated similarly. Lyophilized leaf tissue was stored in vacuo at 4 C over $CaSO_4$ until used, at which time it was powdered in a Waring Blendor for 3 min. Proteinase inhibitors were extracted and partially purified from leaf powder as reported previously (4).

Extraction. Crude extracts were made by rapidly mixing 8.5 g of leaf powder in 170 ml of extraction buffer (0.1 M potassium phosphate at pH 6.5 containing 0.3 M KCl and 3 mM potassium metabisulfite) and stirring gently for 5 min at 4 C with a magnetic stirrer. Extracts were clarified by centrifugation at 33,000 *g* for 15 min in a Beckman Type 19 rotor. The supernatant was filtered through two layers of Whatman No. 1 filter paper and diluted 1:5 with the extraction buffer. The pH values determined for undiluted crude extracts ranged from 6.3 to 6.5.

Affinity chromatography. Affinity gel columns consisted of 7.5 ml of trypsin-agarose mixed with 22.5 ml of chymotrypsin-agarose in 1.5 × 20.0-cm columns. Diluted crude extracts (850 ml) at 6 C were allowed to flow from separatory funnels through affinity columns for 12–15 hr. Affinity gels were washed with extraction buffer until the absorbance of the eluate at 280 nm was near zero, then eluted with about 50 ml of 10 mM HCl (pH 2) containing 0.3 M KCl followed by about 30 ml of 6 M urea (pH 7) containing 0.1 M $CaCl_2$. The 10 mM HCl eluate fractions absorbing at 280 nm were pooled and dialyzed for 8 hr against 4 L of 1% glycine with two changes of dialysis buffer. The 6 M urea eluates were similarly pooled and dialyzed. Upon completion of the urea elution, urea was immediately washed from the affinity gels with 1 mM HCl containing 0.1 M $CaCl_2$ and the gels were stored in this solution at 4 C. Affinity gels were maintained by methods described previously (4). Loss of chymotrypsin activity was observed in affinity gels after elution with 6 M urea, but treatment with a chymotrypsin substrate (TEE) resulted in a restoration of chymotrypsin activity to near original levels. Trypsin remained fully active in the columns after

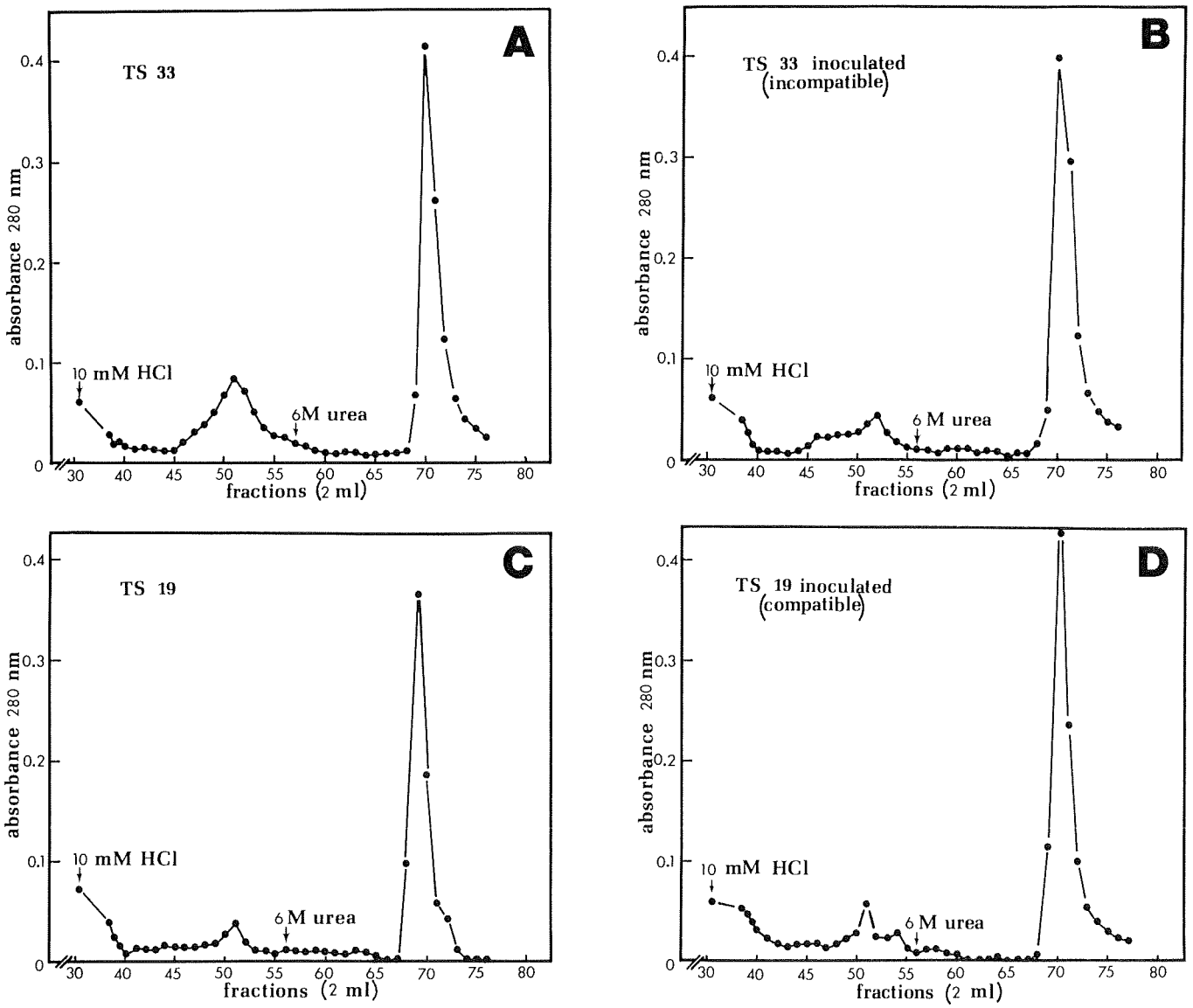

Fig. 1. Affinity chromatography of trypsin and chymotrypsin inhibitors from leaf tissue of TS 33 and TS 19 tomato plants inoculated and uninoculated with *Phytophthora infestans* (Race O).

urea treatment.

Preparative flat-bed electrofocusing. Proteins in the affinity gel eluates were separated by flat-bed isoelectric focusing. All dialyzed eluates from the affinity columns were pooled (about 80 ml total) and mixed with 5 ml of pH 3.5 to 10.0 and 1 ml of pH 9.0 to 11.0 carrier ampholytes to which 5 g of Sephadex G-75 superfine was added slowly while stirring. This combination of ampholytes resulted in slab gels with a pH gradient range, after electrofocusing, of approximately pH 4.0 to 9.5. The gels were poured to form slabs (10.5 × 22.5 cm) that were dried to 70 g under a gentle air stream from a small fan in a 10 C incubator. Electrode strips were soaked in 1 M NaOH for the cathode and 1 M H_3PO_3 for the anode. Initial settings on the power supply were 8 W and 1,500 V. Proteins were focused along the length of the slab gel for 12 hr and the gel was cut into 7.5-mm fractions. The fractions were eluted first with 1 ml of distilled H_2O, for pH measurements, and then with 3 ml of 0.5 M potassium phosphate buffer (pH 6.5). Control experiments with electrofocused ampholytes indicated that these buffers had no detectable inhibitory activity against chymotrypsin or trypsin. All steps in this procedure except drying the slab were at 4 C.

Proteinase inhibitor assays. At various stages during purification, the inhibitor preparations were assayed for level of inhibition of the esterase activities of chymotrypsin and trypsin. Chymotrypsin was assayed by the method of Schwert and Takenaka (19) with TEE as the substrate. Trypsin was assayed by the method of Hummel (9) with TAME as a substrate. Assay procedures for proteinase inhibitors were similar to those reported previously (4,14). They were conducted by preincubating 0.1 ml of chymotrypsin (200 $\mu g \cdot ml^{-1}$ in 1 mM HCl) or trypsin (50 $\mu g \cdot ml^{-1}$ in 1 mM HCl) with 0.1 ml of inhibitor preparation for 2 min at room temperature (~24 C). The assay for chymotrypsin inhibitors consisted of 2.5 ml of 1 mM TEE in 0.1 M potassium phosphate buffer at pH 7.0, plus 0.1 ml of the preincubated enzyme inhibitor solution in the reaction cuvette. The assay for trypsin inhibitors consisted of 2.5 ml of 1 mM TAME in 0.1 M tris-HCl buffer at pH 8.0, plus 0.1 ml of the preincubated enzyme inhibitor solution in the reaction cuvette. Inhibitor units were calculated by the method suggested by Vogel et al (24), ie, the quantity of inhibitor that reduces the rate of conversion of substrate by 1 μmole per minute under standard conditions is equal to one international inhibitor unit (IU). Protein content at various stages of purification was determined with the Biorad protein assay (2) by using bovine serum

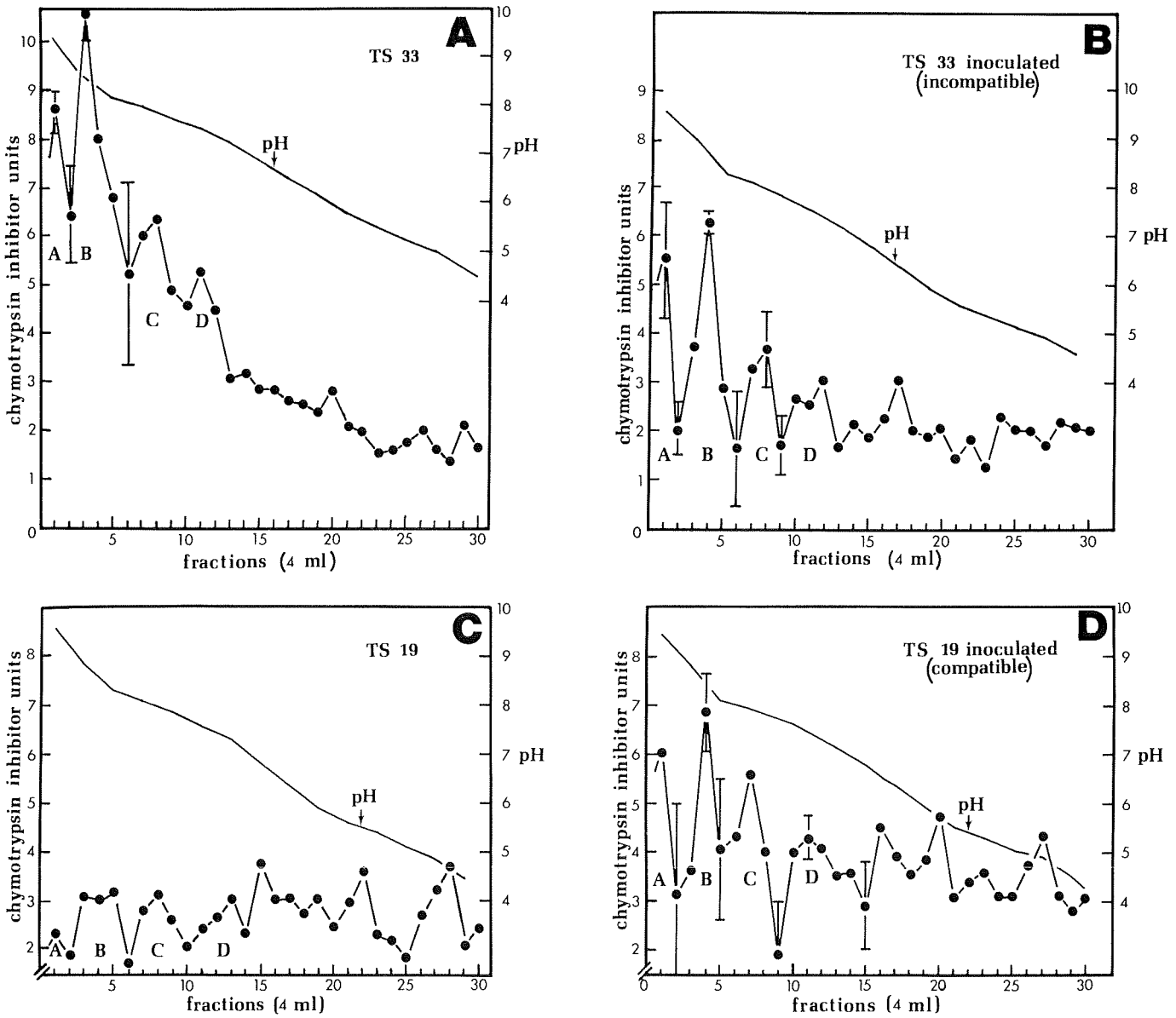

Fig. 2. Chymotrypsin inhibitor activity (CTIA) of isoelectrically focused inhibitor proteins in affinity column eluates, from TS 33 and TS 19 tomato plants, inoculated and uninoculated with *Phytophthora infestans* (Race O). Points on the curves represent the total quantity of CTIA, measured in international inhibitor units, in a single 4-ml fraction. Peaks of CTIA are labelled A to D.

albumin as a standard.

Experimental design and statistical analysis. Three separate crude extractions were made and passed individually through affinity chromatographic columns for each experimental treatment. The three replicated affinity eluates from each of the experimental treatments were combined for isoelectric focusing. This entire procedure was completed a minimum of two times. Independent orthogonal comparisons ($P = 0.05$) were used as a test for significance between treatments through the affinity chromatography step. Individual peaks of electrofocused inhibitor activity were subjected to statistical analysis. Peaks with statistically significant activity above background are indicated by a bar showing the confidence interval.

RESULTS

Inhibitor activity in crude extracts. Chymotrypsin inhibitor activity (CTIA) and trypsin inhibitor activity (TIA) in TS 33 and TS 19 plants (inoculated with *P. infestans* and uninoculated) were compared by assaying crude leaf extracts. There was an apparent decrease in levels of CTIA in both the TS 33 (incompatible) and TS 19 (compatible) plants following inoculation (Table 1). There was also an apparent decrease in TIA in the TS 33 plants following inoculation (Table 2). TIA and/or CTIA were not detected in some crude leaf extracts (Tables 1 and 2); however, both activities were detected in extracts from these plants after further purification. There were no significant differences in crude extract inhibitor levels between any of the treatments, but overall leaf extracts from the TS 33 plants nearly always contained more CTIA and TIA than extracts from the TS 19 leaves (Tables 1 and 2).

Affinity chromatography. Elution with about 50 ml of 10 mM HCl removed a peak of 280 nm absorbing material (Fig. 1A–D). Further elution with about 30 ml of 6 M urea removed a second peak. More inhibitor protein was specifically bound to the columns from the crude extracts of TS 33 tomato plants than extracts from TS 19 plants, as estimated by the absorbance at 280 nm of both HCl and urea eluates (Fig. 1A–D) and protein assays (Tables 1 and 2).

Inhibitor activity in affinity eluates. Levels of CTIA and TIA were measured in dialyzed HCl and urea eluates from affinity columns for each plant treatment. TIA levels were higher in HCl eluates than in urea eluates, whereas CTIA levels generally were similar in each of the eluates (Tables 1 and 2). The only exception was that CTIA levels were considerably higher in HCl eluates than in urea eluates from the uninoculated TS 33 plants.

Measurements of inhibitor activity in the affinity eluates of the various treatments suggested a decrease in total CTIA and TIA in both TS 33 and TS 19 plants following inoculation, but this decrease was not statistically significant (Tables 1 and 2). A

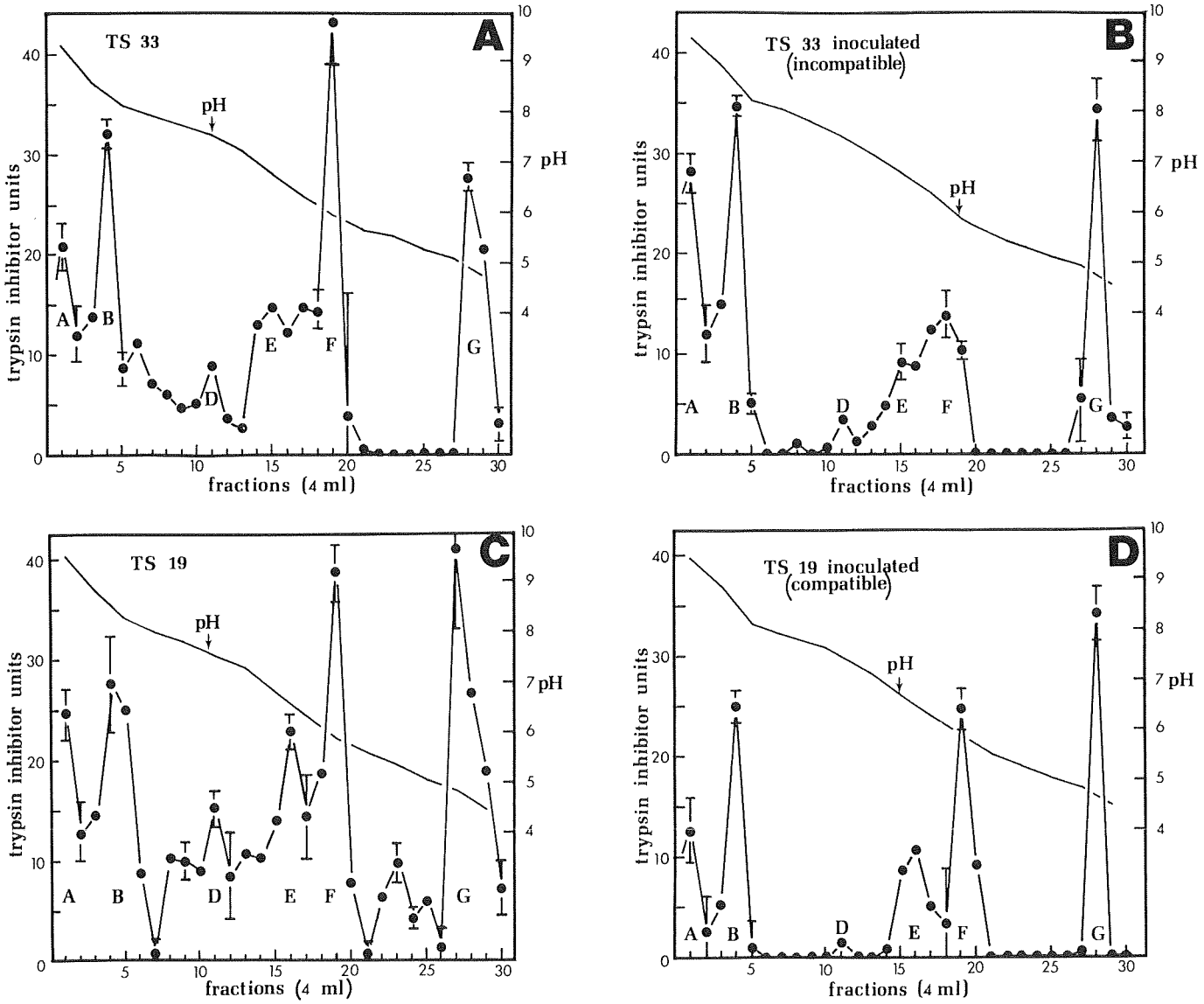

Fig. 3. Trypsin inhibitor activity (TIA) of isoelectrically focused inhibitor proteins from TS 33 and TS 19 tomato plants, inoculated and uninoculated with *Phytophthora infestans* Race O. Peaks of TIA are labelled A to G.

statistically significant difference was observed between individual treatments when levels of CTIA in the HCl eluates from the two uninoculated tomato selections were compared (Table 1). Levels of CTIA in HCl eluates and the total activity from both HCl and urea eluates indicated the presence of significantly more CTIA in the uninoculated TS 33 plants than in the uninoculated TS 19 plants. Additionally, a significant decrease in CTIA in HCl eluates was observed following inoculation when pooled data from TS 33 and TS 19 control treatments were compared with those from the inoculated treatments (Table 1). The same pattern was observed when TIA was compared in the TS 33 and TS 19 plants, but the differences were not statistically significant (Table 2).

Yields of CTIA from affinity chromatography were at least 67% in all experiments with at least a 150-fold purification (Table 1). The apparent degree of purification of TIA after affinity chromatography could only be calculated for the uninoculated TS 33 extracts, since it was the only treatment in which the TIA assay of crude extracts gave a positive reading. This activity estimate was considered too low to be accurate, and yields of TIA were not calculated. After affinity chromatography of the extracts, substantial levels of TIA were detected in all treatments (Table 2).

Isoelectric focusing. Seven main peaks (A through G) of CTIA and/or TIA were resolved from plant tissues of the various treatments by isoelectric focusing. These peaks were evident in Figs. 2 and 3, which show levels of CTIA and TIA, respectively, in 4-ml fractions eluted from gel slices in representative experiments. Not all inhibitor peaks were well resolved in all treatments. Only inhibitors that were consistently resolved into well-defined peaks are designated by letters. Peaks A through D were consistently detected using CTIA assays (Fig. 2). Inhibitors in peaks A, B, and D detected by CTIA assays (Fig. 2) are probably identical to those in peaks A, B, and D detected by TIA assays (Fig. 3), since they have identical pIs (Table 3). Peaks A, B, D, E, F, and G were consistently detected with TIA assays (Fig. 3).

Yields obtained from CTIA and TIA totals under peaks A through G (Table 3) demonstrated that little loss in activity occurred in any of the experiments following isoelectric focusing. In fact, higher levels of TIA were detected after focusing than in earlier stages of the purification procedure. Total levels of CTIA in peaks A, B, C, and D were considerably higher in the TS 33 plants than in TS 19 plants, which is in agreement with overall CTIA levels measured prior to isoelectric focusing (Table 1). Another observation, which is in agreement with results obtained prior to isoelectric focusing, was a general decline in CTIA and TIA in most individual electrofocused inhibitor peaks following infection of both TS 33 and TS 19 plants (Table 3).

DISCUSSION

The results obtained in this study failed to confirm those previously reported from this laboratory in which CTIA and TIA were shown to increase in crude extracts from tomato plants inoculated with an incompatible race of *P. infestans* (14). Using the same host-parasite system and similar inhibitor assay procedures as in the previous study, no increase in levels of CTIA or TIA could be measured in crude leaf extracts of tomato plants 48 hr after inoculation with an incompatible race of *P. infestans*. It was not determined why we were unable to repeat the results of Peng and Black (14). Perhaps it was due to some inadvertent difference in the experimental procedure. Regardless of the reason, it now appears that the inhibitor accumulation observed in the previous study was not responsible for the incompatible host-parasite reaction. This conclusion is drawn because in both studies, the incompatible reaction became apparent about 48 hr after inoculation by the development of necrotic flecks, but in the present study there was no detectable increase of inhibitor activity that accompanied the incompatible reaction. The present results showed that the endogenous levels of the chymotrypsin and trypsin inhibitors were higher in TS 33 than in TS 19, but the importance, if any, of this difference in relation to the response of the two tomato selections to inoculation with *P. infestans* is not known.

It is not known why CTIA and TIA sometimes were not detected in crude plant extracts (Tables 1 and 2). In those cases, the inhibitor may have been present in concentrations too low to detect in crude extracts, or there may have been substances in the extracts that masked CTIA or TIA. Another possibility is that inhibitors of CTIA and TIA were present in the crude extracts, which were removed during the purification procedure.

Seven main peaks of CTIA and/or TIA were detected by electrofocusing. Qualitatively, the isoelectrically focused inhibitor peak profiles for CTIA and TIA were essentially the same in extracts from inoculated and uninoculated TS 19 and TS 33 plants (Figs. 2 and 3). The lack of qualitative changes implies that within the detection limits of the techniques used, no new inhibitors were induced as a result of compatible or incompatible host-parasite combinations. Quantitatively, the isoelectrically focused inhibitor peak profiles showed a decrease in most of the peaks from extracts of TS 33 and TS 19 after inoculation with *P. infestans* (Figs. 2 and 3, Table 3). Apparently, this host response to the pathogen is general rather than specific, since levels of most of the inhibitors decline in both compatible and incompatible combinations.

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