Purification and Characterization from Tobacco (Nicotiana tabacum) Leaves of Six Small, Wound-Inducible, Proteinase Isoinhibitors of the Potato Inhibitor II Family

Gregory Pearce, Scott Johnson, and Clarence A. Ryan*

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

Six small molecular mass, wound-inducible trypsin and chymotrypsin inhibitor proteins from tobacco (Nicotiana tabacum) leaves were isolated to homogeneity. The iso-inhibitors, cumulatively called tobacco trypsin inhibitor (TTI), have molecular masses of approximately 5500 to 5800 Da, calculated from gel filtration analysis and amino acid content. The amino acid sequence of the entire 53 residues of one iso-inhibitor, TTI-1, and the sequence of 36 amino acid residues from the N terminus of a second iso-inhibitor, TTI-2, were determined. The two iso-inhibitors differ only at residue 11, which is threonine in TTI-1 and lysine in TTI-2. The iso-inhibitors are members of the potato inhibitor II family and show considerable identity with the small molecular mass members of this family, which include the eggplant inhibitor, two small molecular mass trypsin and chymotrypsin inhibitors from potatoes, and an inhibitor from pistils of the ornamental plant Nicotiana alata. Antibodies produced against the iso-inhibitors in rabbits were used in radial immunoassays to quantify both the systemic wound inducibility of TTI in tobacco leaves and its constitutive levels in flowers.

Proteinase inhibitor proteins are among the defensive chemicals of plants directed against insects and pathogens (Ryan, 1990). At least eight families of Ser proteinase inhibitors in plants have been identified either in storage organs or in vegetative cells of virtually all plant families in which they have been sought (Garcia-Olmedo et al., 1987; Ryan, 1990). Members of four of the Ser proteinase inhibitor families are known to be systemically induced in various plants. These families include tomato and potato inhibitors I and II in solanaceous plants (Melville and Ryan, 1972; Bryant et al., 1976; Plunkett et al., 1982), Bowman-Birk inhibitors in alfalfa (Brown and Ryan, 1984), and a Kunitz inhibitor in poplar trees (Bradshaw et al., 1989).

As part of our program to understand the signal transduction pathways that regulate the localized and systemic wound-inducible expression of proteinase inhibitor proteins in different plant genera in response to pest attacks (Ryan, 1992), we have isolated and characterized a wound-inducible proteinase inhibitor from tobacco (Nicotiana tabacum) leaves. In a previous study we had measured an oligagalacturonide-

inducible trypsin inhibitor activity in tobacco leaves (Walker-Simmons and Ryan, 1977) but could not identify the inhibitor as a potato inhibitor I or II family member with the use of antibodies specific for these proteins (G. Pearce and C.A. Ryan, unpublished data). Recently, we found that the newly discovered polypeptide signal from tomato leaves, called systemin (Pearce et al., 1991), which activates inhibitor I and II genes in both tomato and potato leaves, did not activate the synthesis of the trypsin inhibitor activity in tobacco leaves. Therefore, we have isolated and characterized the wound-inducible trypsin inhibitor from tobacco leaves to further understand its structure, to obtain antibodies to quantify its wound inducibility in tobacco leaves, and to investigate the systemic signals that regulate its synthesis. We report the isolation and characterization of six iso-inhibitor forms of TTI. The complete amino acid sequence of one iso-inhibitor and partial sequence of another reveal that the systemically wound-inducible TTI iso-inhibitors are members of the potato inhibitor II family.

MATERIALS AND METHODS

G-75 Sephadex was purchased from Pharmacia (Piscataway, NJ), and dialysis membranes were purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA), SepPac C18 cartridges were purchased from Waters (Milford, MA). Tributylphosphate and 4-vinylpyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI), and BCA and TFA were purchased from Pierce Chemical Co. (Rockford, IL). Bovine trypsin (2X crystallized), bovine chymotrypsin (3X crystallized), carboxypeptidase P, p-tosyl-l-Arg methyl ester, N-benzoyl-l-Tyr ethyl ester, RSA, BSA, and Cyt c were purchased from Sigma Chemical Co. Kunitz trypsin inhibitor was purchased from Calbiochem (San Diego, CA), and V8 protease was from Boehringer Mannheim (Indianapolis, IN). Carboxypeptidase inhibitor and inhibitor II were purified from potato tubers as previously described (Bryant et al., 1976; Pearce and Ryan, 1983).

HPLC was performed on a Beckman system consisting of two model 112 pumps with a model 420 gradient controller. Eluted peaks were detected with a model 165 variable wave-

Abbreviations: BCA, bicinchoninic acid; PCL, polypeptide chymotrypsin inhibitor; PTI, polypeptide trypsin inhibitor; RP-HPLC, reverse-phase HPLC; RSA, rabbit serum albumin; SCX-HPLC, strong cation-exchange HPLC; TTI, tobacco trypsin inhibitor.
The amino acid protease (20 min. incubated at 37°C for 24 h. Reactants were separated on RP-
containing no peptide was also prepared, and both vials were
TTI-1 (200 μg) was added, and the sample was incubated
at 22°C for 4 h. The digested peptide fragments were sepa-
duced in volume by vacuum centrifugation to 200 μL, and 1
ammonium acetate was added dropwise to pH 4. V8
protease (20 μg) was added, and the sample was incubated
at 22°C for 4 h. The digested peptide fragments were sepa-
ated on RP-HPLC. Two fragments were identified, with
retention times of 28.99 and 33.05 (Fig. 1). The amino acid
sequence of the intact alkylated TTI-1 through residue 35
and the two proteolytic fragments were determined using
Edman chemistry on an Applied Biosystems model 475 se-
quence with pulse-liquid update using the manufacturer’s
protocol. To identify the carboxy-terminal residue of TTI-1,
the native protein (100 μg in 150 μL of water) was incubated
with carboxypeptidase P (7.5 μg) in 15 μL of 100 mM sodium
citrate buffer (pH 4.3) at 37°C. Aliquots (55 μL) were removed
at 10, 30, and 120 min and diluted with 250 μL of 0.1% TFA
in water and applied to a Sep-Pac C18 cartridge equilibrated
with 10% acetonitrile in 0.1% TFA/water. Free amino acids
released by carboxypeptidase P were eluted from the car-
tridge using 1 mL of 10% acetonitrile in 0.1% TFA/water.
The volume was reduced to 0.1 mL for amino acid analysis.

Antibodies were produced by cross-linking the major pep-
tide fraction from RP-HPLC to RSA (Doolittle, 1977). Briefly,
1 mg of RSA was dissolved in 40 μL of 0.4 m sodium
phosphate (pH 7.5). TTI (5 mg) in 100 μL of water was added
to the RSA solution. Glutaraldehyde (20 mM, 55 μL) was added with stirring for 5 min, and the sample was allowed
to stir for 30 min at room temperature. The remaining cross-
linking sites were blocked by the addition of 14 μL of 1 M Gly, followed by stirring for 30 min. The cross-linked proteins
were recovered in the void peak from a G-75 gel filtration
column equilibrated in 50 mM ammonium bicarbonate. The
void peak contents were analyzed by SDS-PAGE to ensure
that cross-linking had occurred. The void fraction contents
were injected into rabbits to elicit antibody production. The
antibodies were used to quantify TTI by immunoradial dif-
fusion analyses as described previously (Ryan, 1967; Traut-
man et al., 1971) using RP-HPLC-purified TTI as a standard.

RESULTS AND DISCUSSION

Oligouronide elicitors were previously shown to induce
trypsin inhibitor activity in leaves of young, excised tobacco
plants (Walker-Simmons and Ryan, 1977). Members of sev-
eral families of proteinase inhibitors are systemically induced
in plant leaves in response to wounding. This includes the
potato inhibitor I and II families in tomato and potato leaves
(Green and Ryan, 1972), a member of the Bowman-Birk
inhibitor family in alfalfa (Brown and Ryan, 1984), and a
Kunitz inhibitor family member in poplar trees (Bradshaw et
al., 1989). The identity of the tobacco trypsin inhibitor that
was induced by oligouronides (Walker-Simmons and Ryan,
1977) was of interest, because antibodies raised against potato
inhibitor I and II proteins, which strongly cross-reacted with
the homologous inhibitors from tomato plants, did not cross-
react with the extracts containing the oligouronide-inducible
inhibitors from tobacco (C. Ryan, unpublished data), indicat-
ing that it might be from a different family of inhibitors.

The oligouronide-inducible inhibitory activity was subse-
quently found to be systemically wound inducible (G. Pearce
and C.A. Ryan, unpublished data; cf. Fig. 7). To isolate the
wound-inducible tobacco inhibitor, mature tobacco plants
were grown to the flowering stage, each leaf was severely
wounded along the edges using a hemostat, and the wounded
leaves were harvested 48 h later for the isolation of TTI. The
leaves (630 g) were homogenized for 3 min in 500 mL of
cold buffer (0.81 mM sodium citrate, 1 mM NaCl [pH 4.3] with
3.5 g of sodium hydrosulfite) and squeezed through cheese-
cloth. The extracted juice was centrifuged at 10,000g for 20 min at 4°C. Ammonium sulfate was added to the supernatant to 80% saturation and stirred at 4°C for 2 h, and the precipitate was pelleted at 10,000g for 10 min. The resulting pellets were taken up in 200 mL of distilled water, stirred for 30 min, and then heated to 80°C on a steam bath. This solution was cooled to room temperature in an ice bath and then centrifuged at 10,000g for 15 min. The supernatants were pooled and dialyzed against four changes of 0.01 M Tris, 0.1 M KCl buffer (pH 8.1), using dialysis tubing with a molecular mass exclusion limit of 3500 D.

The retained solution was applied to a trypsin-Sepharose CL-4B affinity column equilibrated with the same buffer used for dialysis. The adsorbed inhibitor protein was eluted with 8 M urea adjusted to pH 3 with HCl, dialyzed against 50 mM ammonium bicarbonate, and lyophilized. The lyophilized powder (18 mg) was applied to a G-75 gel filtration column (1.3 × 106 cm) equilibrated with 50 mM ammonium bicarbonate, and the eluate were collected in 2-mL fractions. The A_{280} of fractions was determined, and inhibitory activities against trypsin were assayed. Tubes 53 to 68 (Fig. 2) were pooled and lyophilized. The elution profile of the TTI from Sephadex was compared with those of proteins of known mass (data not shown), and its molecular mass was estimated to be approximately 5500 D. This molecular mass is comparable to those of small proteinase inhibitors from potatoes (Pearce et al., 1982) and eggplant (Richardson, 1979), suggesting that it may be a member of the potato inhibitor II family. The yield of TTI was 7.2 mg.

The trypsin inhibitor fraction (200 µg) was dissolved in 0.1% TFA and applied to a semipreparative C_{18} RP-HPLC column. A linear gradient to 70% acetonitrile in 0.1% TFA was applied for 70 min, and 2-mL fractions were collected. One major and several minor peaks of protein eluted from the column (Fig. 3), and all exhibited inhibitory activity against trypsin. The major fraction was pooled and lyophilized. This fraction was further purified using SCX-HPLC.

Six major peaks exhibited strong inhibitory activity against trypsin and were assigned numbers of 1 through 6 (Fig. 4A). Peaks 4 through 6 were desalted and lyophilized. Peaks 1 through 3 were further purified using RP-HPLC with a shallow gradient (Fig. 4, B and C). The protein content of the six pure peptides was quantified using the BCA reagent with purified potato inhibitor II as a standard. Each inhibitor (5 µg) was analyzed by SDS-PAGE and stained with Coomassie blue. Each inhibitor migrated as a single component (Fig. 5) with slight differences apparent in their migrations, indicating that small differences in molecular mass may exist among them. The apparent mass of the inhibitors, estimated from those of standard proteins, was less than 8 kD, but in this size range we have found that estimates of proteinase inhibitor molecular masses by SDS-PAGE analyses are often not accurate.

The six purified peptides were assayed for their abilities to inhibit trypsin and chymotrypsin (Fig. 6). All of the polypeptides except number 3 were found to be strong inhibitors of trypsin. All six peptides were found to be strong inhibitors of chymotrypsin.

Rabbit antibodies were raised against the RP-HPLC fraction (Fig. 3) that contained the six isoformers of TTI by cross-linking the proteins to RSA using glutaraldehyde. The antibodies were found to cross-react strongly with all six of the isolated inhibitors (data not shown). In double-diffusion assays in agar gels, no spurs were found between any of the precipitin lines among the six proteins. This indicated that a high degree of structural similarity was present among the

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**Figure 2.** Gel filtration of tobacco trypsin inhibitory activity on Sephadex G-75. Affinity-purified tobacco trypsin inhibitor (18 mg in 1 mL of water) was applied to a 1.3- × 106-cm column of Sephadex G-75 equilibrated with 50 mM ammonium bicarbonate. Fractions (2 mL) were collected. Fractions containing trypsin inhibitory activity are indicated by the bar.

**Figure 3.** Separation of TTI on C_{18} RP-HPLC. An aliquot from the combined inhibitor fractions from Figure 2 containing 200 µg of protein was applied to the column and eluted with a gradient of 0 to 70% acetonitrile in 0.1% TFA for 70 min. The major absorbance peak (at 225 nm) containing the trypsin inhibitory activity was collected as marked above the elution profile.
Figure 4. A, SCX-HPLC separation of the trypsin inhibitory components of the major fraction from Figure 2. Protein from the Figure 3 fraction (120 μg) was applied to an SCX-HPLC column, equilibrated with 5 mM potassium phosphate (pH 3), containing 25% acetonitrile. The proteins were eluted for 60 min with a gradient of 500 mM KCl in the same buffer as above. Fractions (1 ml) were collected at a flow rate of 1 ml min⁻¹. B, Elution profile of the RP-HPLC of peptide number 1 from A. The conditions were as in Figure 3 except a shallower gradient of 0 to 40% acetonitrile was used for an 80-min period. Peak number 1 was recovered. C, RP-HPLC of combined peaks numbers 2 and 3 from A. Conditions were as described in B.

antigenic determinants of the proteins and confirmed the identity of all six proteins as iso inhibitor species.

To demonstrate that TTI was the wound-inducible inhibitor, the antibodies were used to quantify the induction of TTI in leaves of wounded tobacco plants. In Figure 7 is shown the induction of TTI in leaves of young tobacco plants that had been wounded on their two lower leaves; the levels of TTI were quantified in all four leaves. The induction of TTI in the young, upper, unwounded leaves by wounding demonstrates the strong, systemic response of tobacco plants to wounding. The upper leaves were induced to accumulate about 40 μg g⁻¹ of leaf tissue, whereas the inhibitor could not be detected in leaves of unwounded control plants. Lower wounded leaves were induced to accumulate about half of these levels of TTI. Mature tobacco plants in which all leaves were severely wounded accumulated about 80 μg of TTI g⁻¹ of tissue in the youngest leaves. As in young plants, the lower leaves did not respond as well, but leaves from the middle of the plants accumulated about 25 μg g⁻¹ of tissue. Leaves of unwounded plants did not exhibit the presence of TTI except for low levels in the uppermost leaves. The pattern of systemic induction in tobacco is similar to that found in tomato and potato plants, i.e. the younger, upper leaves respond more strongly to wounding than lower leaves.

The amino acid sequence of the iso inhibitor TTI-1 was determined using the reduced and alkylated protein (Ruegg and Rudinger, 1977), repurified using RP-HPLC (see "Materials and Methods"). The peptide was digested with V8 protease in ammonium acetate at pH 4, which specifically cleaves proteins with an internal Glu residue at the P1 position. Two
fragments of TTI were recovered (cf. Fig. 1) and subjected to sequence analysis by automated Edman degradation. The carboxy-terminal residue was determined to be a Ser by analyzing the release of amino acids by carboxypeptidase P. Only Ser was released, confirming that the C-terminal residue was Ser, as found by Edman degradation.

The complete amino acid sequence of TTI-1 is presented in Figure 8. TTI-1 consists of 53 amino acid residues with a calculated molecular mass of 5868 D. The N-terminal sequence of 36 residues of isoinhibitor TTI-5 was obtained by Edman degradation. TTI-1 and TTI-5 (sequence not shown) exhibit nearly complete sequence identity with each other through the 36 residues available for comparison. The two isoinhibitors differ only at residue 11, where TTI-5 exhibits a Lys residue and TTI-1 a Thr residue. This is consistent with the longer retention time of TTI-5 on SCX-HPLC (Fig. 4A). The sequence data revealed that TTI isoinhibitors are members of the potato inhibitor I family. When the two isoinhibitors with known inhibitor II family members were compared (Fig. 8), the high percentage of sequence identity between this new member of the potato inhibitor II family and other members is clearly evident. TTI-1 appears to be most closely related to the eggplant inhibitor rather than to the small potato PCI. TTI-1 exhibits 83% identity with the eggplant inhibitor compared to 69% with PCI and PTI from potato tubers, 68% for potato inhibitor II, and 60% homology with tomato inhibitor II (data not shown, Graham et al., 1985).

The reactive site of TTI responsible for the proteinase inhibitor activity is assumed to be at the same residues as in the other members of the inhibitor II family. The P1-P1' residues at the reactive site would, therefore, be Arg-Asn. These are the identical residues found at the reactive sites of both the eggplant inhibitor and the PTI.

In many plant species, the presence of defensive proteins in flowers has been reported. Recently, a small molecular mass proteinase inhibitor of the inhibitor II family was reported to be constitutively present in Nicotiana alata flowers in very high concentrations (Atkinson et al., 1993). Using anti-TTI serum to assay for the presence of TTI in the various flower parts, we have found that the flowers of N. tabacum also possess considerable quantities of TTI. Flowers were collected and dissected into various parts, and the tissues were crushed with a mortar and pestle to recover the expressed juice. The juice was centrifuged and assayed for soluble protein, for TTI protein levels, and for the presence of trypsin and chymotrypsin inhibitors. All flower parts exhibited high levels of TTI (Table I), with the sepals containing the highest levels. The mature fruit did not exhibit the presence of inhibitor. Modern tomato fruits do not contain proteinase inhibitor I or II, but some wild species accumulate the two inhibitors to extraordinary levels of 1 to 2 mg g⁻¹ of tissue (Pearce et al., 1988). Stigmas from N. alata are reported to contain about 20 to 30% of their soluble proteins as TTI (Atkinson et al., 1993).

Table I. Quantification of TTI and trypsin and chymotrypsin inhibition in various flower tissues

<table>
<thead>
<tr>
<th>Flower Part</th>
<th>Protein a</th>
<th>Inhibitor b</th>
<th>Enzyme Inhibition c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg mL⁻¹</td>
<td>µg mg⁻¹ of protein</td>
<td>µg of enzyme inhibited/mg of protein</td>
</tr>
<tr>
<td>Ovary</td>
<td>12.8</td>
<td>16.2</td>
<td>65.6</td>
</tr>
<tr>
<td>Sepal</td>
<td>6.6</td>
<td>28.6</td>
<td>79.5</td>
</tr>
<tr>
<td>Stamen</td>
<td>7.2</td>
<td>10.8</td>
<td>29.2</td>
</tr>
<tr>
<td>Petal</td>
<td>6.3</td>
<td>8.2</td>
<td>14.8</td>
</tr>
<tr>
<td>Mature fruit</td>
<td>35.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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a Protein was assayed using potato inhibitor II as a standard. b Inhibitor was assayed by immunoradiodiffusion using RP-HPLC-pure TTI as a standard. c Determined by spectrophotometer analysis (see "Materials and Methods").
cum that appeared to be the same proteins as the wound-inducible proteins in leaves. Atkinson et al. (1993) reported that the N. alata stigma trypsin inhibitor is a member of the potato inhibitor II family. The gene for this protein was much larger than that for the small inhibitor found in the stigma, indicating that the inhibitor was proteolytically processed from the large precursor.

Potato inhibitor II was the first member of this family to be isolated (Bryant et al., 1976) and is the modern product of an ancestral inhibitor gene that was duplicated and elongated to produce an inhibitor of 12,300 kD with two similar domains, each possessing a reactive site (Garcia-Olmedo et al., 1987). The sequences of two small, related inhibitors from potato tubers, called PCI and PTI (Hass et al., 1982), are nearly identical with internal sequences of potato and tomato inhibitor II, suggesting that posttranslational trimming of the inhibitor takes place at both the C terminus and the N terminus. PCI and PTI are composed of the last half of the N-terminal domain and the first half of the C-terminal domain (Hass et al., 1982), and their three-dimensional structure has been deduced by x-ray crystallography (Greenblatt et al., 1989). TTI has a sequence identical to the deduced sequence of the N. alata trypsin inhibitor (Atkinson et al., 1993) and is, therefore, likely to have been produced by a larger gene, as was found in N. alata.

Our interest in TTI is to characterize the signal transduction in tobacco that regulates the systemic induction of this inhibitor in leaves in response to wounding. This induction of TTI in tobacco leaves is similar in many ways to the wound induction of inhibitor I and II proteins previously found in leaves of tomato and potato plants. In these latter species, a polypeptide signal, called systemin, appears to be an integral part of the signal transduction system (Pearce et al., 1987). The sequences of two small, related inhibitors from potato tubers, called PCI and PTI (Hass et al., 1982), are nearly identical with internal sequences of potato and tomato inhibitor II, suggesting that posttranslational trimming of the inhibitor takes place at both the C terminus and the N terminus. PCI and PTI are composed of the last half of the N-terminal domain and the first half of the C-terminal domain (Hass et al., 1982), and their three-dimensional structure has been deduced by x-ray crystallography (Greenblatt et al., 1989). TTI has a sequence identical to the deduced sequence of the N. alata trypsin inhibitor (Atkinson et al., 1993) and is, therefore, likely to have been produced by a larger gene, as was found in N. alata.

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LITERATURE CITED


Richardson M (1979) The complete amino acid sequence and the trypsin reactive (inhibitory) site of the major proteinase inhibitor from the fruits of the aubergine (Solanum melongena L.). FEBS Lett 104: 322–326


