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Isolation and Characterization of a Proteinaceous Inhibitor of Microbial Proteinases Induced During the Hypersensitive Reaction of Tobacco to Tobacco Mosaic Virus

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A proteinase inhibitor is strongly induced in tobacco leaves reacting hypersensitively to tobacco mosaic virus. The tobacco inhibitor is highly active against four different serine endoproteinases of fungal and bacterial origin (EC 3.4.21.14) but inhibits poorly two serine endoproteinases of animal origin, trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). The inhibitor has been purified to homogeneity by successive steps of conventional and high-performance liquid chromatography. When electrophoresed under denaturing conditions, it behaves as a small polypeptide with a molecular weight of about 6,000. From its amino acid composition and NH_2-terminal amino acid sequence analysis, it appears that the inhibitor belongs to the potato inhibitor I family. A polyclonal antiserum was raised against the purified tobacco inhibitor and was used in immunoblotting experiments to follow inhibitor accumulation during the hypersensitive reaction of tobacco to tobacco mosaic virus. The inhibitor is highly efficient and might represent a potent fungicide and/or bactericide to be used in plant biotechnology.

Additional keywords: antibodies, purification to homogeneity, specificity.

In the animal kingdom serine proteinases are present in virtually all living organisms and are known to be involved in a wide variety of processes such as digestion of food, the immune reaction to foreign cells, blood clotting, or fertilization of the ovum (Stroud 1974). Unwanted proteolysis can be prevented by specific proteinaceous inhibitors of proteinases that have been found in animals but also in plants and in microorganisms (Ryan 1973; Richardson 1977; Laskowski and Kato 1980; Barrett and Salvesen 1986).

Much of the work on proteinase inhibitors of plant origin has focused on the inhibitors of trypsin and chymotrypsin, two proteinases of the digestive tract of animals. These inhibitors have been extensively studied in the Leguminosae, Gramineae, and Solanaceae (Ryan 1973; Richardson 1977) and can be classified in different families on the basis of amino acid sequence homology (Laskowski and Kato 1980; Ryan 1989). For instance, inhibitors of the potato inhibitor I family have been found in potato tubers (Melville and Ryan 1972), in wounded leaves of tomato and potato (Plunkett *et al.* 1982; Graham *et al.* 1985a; Cleveland *et al.* 1987), in tobacco callus tissues (Wong *et al.* 1975), in etiolated tobacco leaves (Kuo *et al.* 1984), in grain of barley (Svendsen *et al.* 1982), in seeds of broad bean (Svendsen *et al.* 1984), and even in a lower animal, the leech (Seemüller *et al.* 1981). Microorganisms also produce serine proteinases (EC 3.4.21.14) resembling animal proteinases (Morihara 1974; Pladys *et al.* 1981), and plant proteinase inhibitors are consequently thought to be involved in plant defense against attacking pests (Ryan 1973; Richardson 1977). There is only one case, however, in which the production of the proteinase inhibitor has been shown to be induced by a pathogen: in melon plants infected by *Colletotrichum lagenarium* (Passerini) Ellis and Halsted, an increase of activity inhibiting a proteinase produced by the fungus has been demonstrated (Roby *et al.* 1987). Other plant-pathogen systems are known, however, in which no increase in proteinase inhibitory activity could be detected upon infection (Brown and Adikaram 1983; Cleveland and Black 1983).

Here we show that an inhibitory activity against microbial serine proteinases is strongly induced in tobacco reacting hypersensitively to tobacco mosaic virus (TMV). The proteinase inhibitor has been purified to homogeneity from tobacco leaves infected with TMV and shown to be a small polypeptide that very efficiently inhibits the proteolytic activity of the four microbial enzymes which have been tested. In contrast, trypsin and chymotrypsin were poorly inhibited. Amino acid analysis and NH_2-terminus sequence determinations have shown that the inhibitor belongs to the potato inhibitor I family. Antibodies raised in rabbit were used in immunoblotting experiments to study the kinetics of the accumulation of the inhibitor during the hypersensitive reaction of tobacco to TMV.

The strong induction of the tobacco inhibitor with its high level of specificity for microbial enzymes could represent a plant defense complementary to the potential lytic activity of chitinases and 1,3-β-glucanases that have also been shown to be induced by TMV infection (Legrand *et al.* 1987; Kauffmann *et al.* 1987).

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MATERIALS AND METHODS

Plant material. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown in a greenhouse under

controlled conditions. Three leaves of 3-month-old plants were inoculated with a suspension of purified TMV. The plants were then inoculated in a growth chamber with a 16-hr photoperiod. The leaves bearing ~ 200–300 lesions were harvested 6 days after inoculation, frozen in liquid nitrogen, and stored at −80° C.

Extraction and purification of the proteinase inhibitor. One hundred and twenty grams of leaves was ground in 150 ml of 0.5 M sodium acetate, pH 5.2, containing 15 mM 2-mercaptoethanol. After centrifugation at 15,000 × *g*, the supernatant was desalted on a Sephadex G-25 column (4.5 × 60 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.2. The protein fraction was kept at 4° C for a few hours and centrifuged at 20,000 × *g*, and the precipitate was discarded. The supernatant was loaded on an S-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column (2.2 × 8.5 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.2. Elution was performed with 0.5 L of a linear gradient from 0 to 0.4 M NaCl. Fractions containing inhibitory activity were pooled and adjusted to 1.2 M $(NH_4)_2SO_4$ with solid salt, and the solution was filtered through 0.22-μm filters. After injection onto a TSK-Phenyl 5PW column (LKB, Bromma, Sweden), elution was performed by a decreasing gradient of salt concentration obtained by diluting a 1.2 M $(NH_4)_2SO_4$ solution with water under the control of the programmer of a fast protein liquid chromatography apparatus. Inhibitory fractions were pooled, concentrated to 200 μl on Centricon 10 concentrators (Amicon Corporation, Danvers, MA), filtered, and injected onto a Superose 12 column (1 × 30 cm, Pharmacia). Elution with 0.1 M sodium phosphate (pH 6.9) containing 0.2 M NaCl yielded the purified proteinase inhibitor.

Polyacrylamide gel electrophoresis and staining. Electrophoresis was performed on slab gels under denaturing conditions by the method of Laemmli (1970) with a 5% stacking gel and a 16% separating gel that contained 6 M urea. Electrophoresis was conducted in a Midget unit (LKB) at a constant 110 V. Gels were fixed for 1 hr in 10% trichloroacetic acid and then stained overnight by the method of Neuhoff *et al.* (1988). At the final stage of the purification procedure, the homogeneity of the preparation was checked by an additional step of silver staining (Morrissey 1981).

Production of serum. The antigen was emulsified with either Freund's complete adjuvant for the first injection or incomplete adjuvant for all the following boost injections. Fifty micrograms of antigen was administered at each injection, and 10 days after each boost, serum was collected.

Immunoblotting. The basic procedure of Towbin *et al.* (1979) was used for transfer. The nitrocellulose sheet was incubated at 37° C according to Meyer *et al.* (1988): after soaking for 1 hr with a solution containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 3% gelatin, the membrane was washed four times in the same solution without gelatin and then incubated overnight with the serum diluted 1,000-fold with 20 mM Tris-HCl buffer, pH 7.5, containing 0.05% Tween 20 and 1% defatted milk powder. After four washes with the same solution without serum, the blot was allowed to react for 2 hr with a goat anti-rabbit antibody labeled with phosphatase, washed, and revealed as described in Legrand *et al.* (1987).

Assay of inhibitory activity against proteinases. Inhibitory activity was measured by preincubating 1 μg of a commercial proteinase (trypsin, chymotrypsin, Carlsberg subtilisin, proteinase K, and proteinases from *Aspergillus oryzae* (Ahlburg) Cohn [Type XXIII] or *Streptomyces griseus* (Krainsky) Waksman and Henrici [Type XXI], all from Sigma Chemical Co., St. Louis, MO) with the inhibitor solution for 3 min at 37° C and assaying the residual proteinase activity with Azocoll (Sigma) as substrate. Before use, Azocoll was washed three times with 50 mM Tris-HCl buffer, pH 8.0, at 37° C (about 2 L per gram) to decrease the blank value of the enzymatic test. The reaction mixture contained 3 mg of washed Azocoll, 10 μg of bovine serum albumin in 1 ml of 50 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$. After incubation for 15 min at 37° C on a test tube rotator, the mixture was centrifuged at 7,000 × *g* for 10 min. The absorbance of the supernatant at 520 nm is a measure of the residual proteinase activity. Controls included enzyme and substrate blanks. The inhibitory activity of a preparation is obtained by measuring the difference between the proteolytic activity in the absence and presence of the inhibitor. This value was used to calculate the amount of proteinase inhibited, and inhibitory activity was expressed in these terms.

Amino acid analysis and NH_2-terminus sequence determination. Amino acid analysis and sequence determinations were conducted in the laboratory of Y. Boulanger

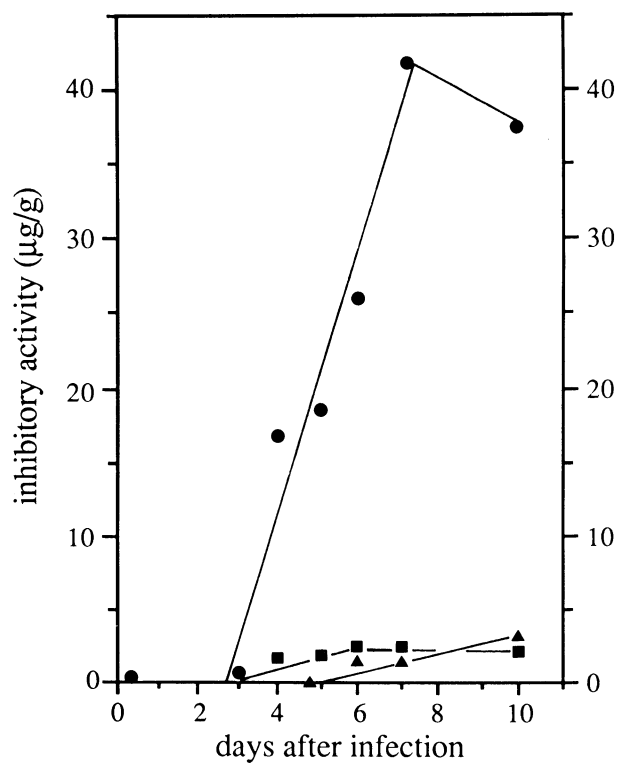

Fig. 1. Time course curve of proteinase inhibitory activity. Inhibitory activity was measured on the void volume fraction of the Sephadex G-25 column as described in the text and is expressed as micrograms of proteolytic enzyme inhibited per gram of fresh weight. Activity was tested in inoculated leaves against subtilisin (●—●) and trypsin (■—■). Extracts from upper uninoculated leaves were assayed for subtilisin inhibitory activity (▲—▲).

and J. Reinbolt (Institut de Biologie Moléculaire et Cellulaire [IBMC], Strasbourg). An HPLC-WISP 712 system (Waters Associates, Milford, MA) was used for amino acid analysis after performic acid oxidation and HCl hydrolysis of the purified protein. Determination of the NH_2-terminal amino acid sequence by Edman degradation was performed following the method of Hewick *et al.* (1981) and using an Applied Biosystems (Foster City, CA) 470A sequencer and its on-line phenylthiohydantoin amino acid analyzer.

RESULTS

Induction of inhibitory activity during the hypersensitive reaction of tobacco to TMV. Figure 1 presents time course curves of proteinase inhibitory activity in Samsun NN tobacco leaves infected with TMV. Inhibitory activity has been measured toward one microbial enzyme (subtilisin) and one digestive enzyme (trypsin). On inoculated leaves, lesions appeared about 36 hr after infection. The inhibitory activity measured toward subtilisin was undetectable until the third day of infection and then was strongly induced and reached a maximum 4 days later. An increase of inhibitory activity was also detected with trypsin as the proteolytic enzyme but was much less pronounced. A similar level of trypsin inhibitory activity has been reported in Xanthi-nc tobacco infected with TMV (Pierpoint *et al.* 1981). Since proteinase inhibitors are known to be induced in a systemic fashion throughout the plant (Graham *et al.* 1985a, 1985b; Sanchez-Serrano *et al.* 1986), subtilisin inhibitor activity was also evaluated in upper uninfected leaves. In this material, the inhibitory activity became detectable by the sixth day of infection and reached, by the tenth day, 5–10% of the activity of inoculated leaves (Fig. 1).

Purification and characterization of the subtilisin inhibitor. The protein extract from tobacco leaves infected with TMV was desalted by chromatography on Sephadex G-25 and clarified by centrifugation. The supernatant was fractionated by cation exchange chromatography. The elution profiles are shown in Figure 2. The inhibitory activity of the fractions was tested against subtilisin and trypsin. As expected from the results obtained with crude extracts (Fig. 1), the fractions inhibited subtilisin much more efficiently than trypsin. Moreover, the maximum of inhibitory activity directed against the two proteinases was found in different fractions, indicating that different inhibitors account for the inhibition of the two proteolytic enzymes. Fractions containing subtilisin inhibitor activity were pooled and further purified by hydrophobic-interaction chromatography (Fig. 3) and by molecular sieving (Fig. 4) under HPLC conditions. At each step of the purification procedure, the protein content of the preparation was analyzed by electrophoresis under denaturing conditions (Fig. 5). At the final stage of the purification, only a single band was detected on the gel, even after silver staining. The band was rather broad and had a relative mobility corresponding to a molecular weight of about 6,000. However, due to unreliable electrophoretic mobilities of small proteinase inhibitors (Seemüller *et al.*

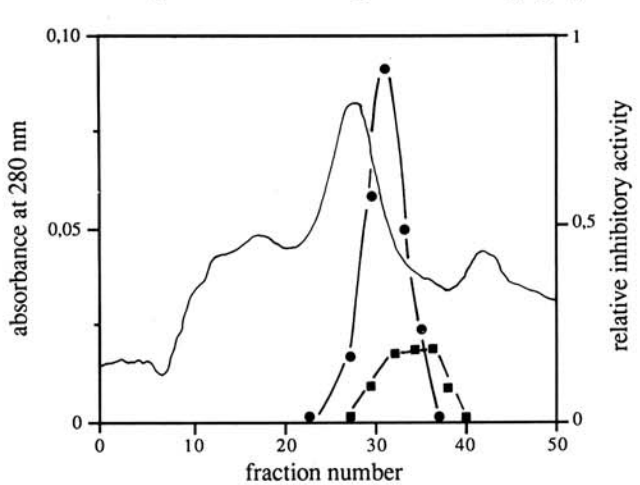

Fig. 2. Elution profiles after cation exchange chromatography. The column was equilibrated, loaded, and eluted as described in the text, and absorbance (—) was monitored at 280 nm. Twenty microliters of each fraction was assayed for inhibitory activity toward subtilisin (●—●). Aliquots of 40 μl were used for trypsin inhibitory assays (■—■).

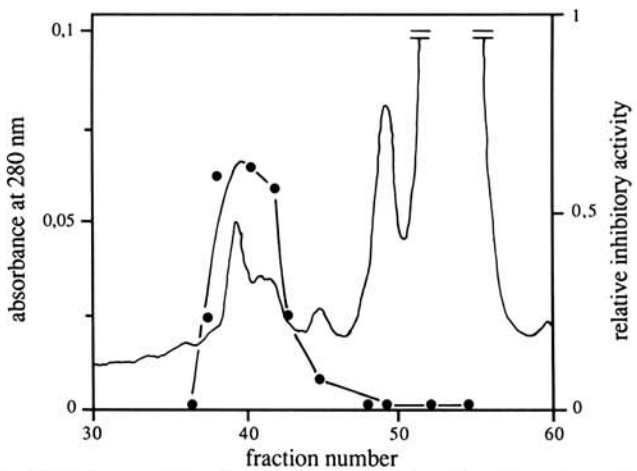

Fig. 3. Elution profiles after hydrophobic-interaction chromatography. Inhibitory fractions from the cation exchange column were adjusted to 1.2 M $(NH_4)_2SO_4$ and loaded on a TSK-Phenyl-5PW column. Elution was with a decreasing gradient of salt concentration, and absorbance was monitored at 280 nm (—). Subtilisin inhibitory activity (●—●) was measured on 10-μl aliquots.

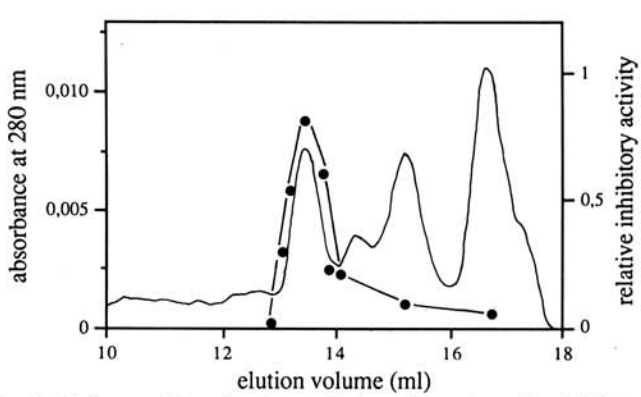

Fig. 4. Elution profiles after size exclusion chromatography. Inhibitory fractions from hydrophobic-interaction chromatography were pooled, concentrated, and loaded on a Superose 12 column. Ten microliters of each 110-μl fraction was tested for inhibitory activity toward subtilisin (●—●). Absorbance was monitored at 280 nm (—).

1981; Svendsen *et al.* 1982; Graham *et al.* 1985a), knowledge of the complete nucleotide sequence of the cDNA will be required to establish the precise molecular weight of the monomer and the molecular structure of the native inhibitor, which behaves as a multimer of 43,000 ± 2,000 upon molecular sieving under HPLC conditions (data not shown). The purified inhibitor was used for amino acid analysis, NH_2-terminal sequence determination, and raising polyclonal antibodies.

Amino acid composition and NH_2-terminus sequence determination. Amino acid analysis was performed as described in Materials and Methods. The results are presented in Table 1 and compared to data obtained from the amino acid sequences of tomato and potato inhibitors I and II deduced from the cDNA sequences (Graham *et al.* 1985a, 1985b; Sanchez-Serrano *et al.* 1986; Cleveland *et al.* 1987). The two types of inhibitors are distinguishable in their content in valine, tyrosine, and cysteine residues (Table 1). The contents of the tobacco inhibitor in these three residues resemble those of inhibitors I from potato and tomato. The purified inhibitor was sequenced from the NH_2-terminus through 14 cycles. No phenylthiohydantoin derivative was identified in the fifth cycle of Edman degradation, suggesting that the residue (X) at position 5 is either a cysteine or a modified amino acid residue. The NH_2-terminal sequence of the tobacco inhibitor is presented in Figure 6 and shows homology with NH_2-termini of inhibitors of the potato inhibitor I family: eight residues were found at the same position as in the potato or tomato inhibitors, and five of them (underlined in Fig. 6) are identical in the six members of the potato inhibitor I family (Svendsen *et al.* 1984; Graham *et al.* 1985a). These data, together with amino acid composition, indicate that the tobacco inhibitor belongs to the inhibitor I family.

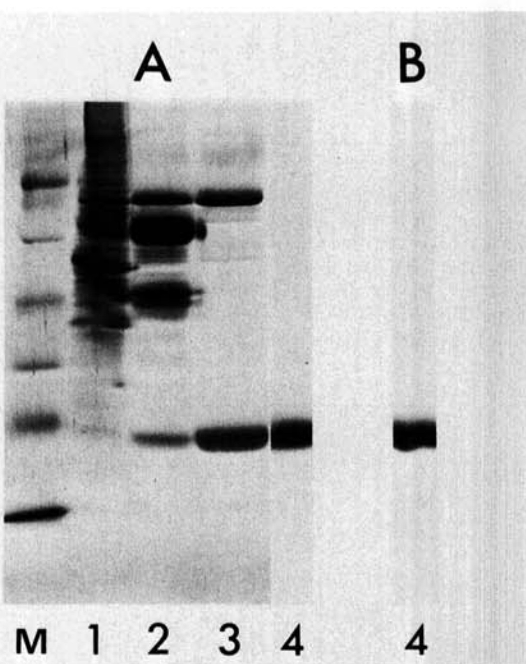

Fig. 5. Electrophoretic analysis of inhibitory fractions at different stages of purification. Fractions obtained after gel permeation through the Sephadex G-25 column (1), cation exchange chromatography (2), hydrophobic-interaction chromatography (3), and size exclusion chromatography (4) were analyzed by electrophoresis under denaturing conditions. **A**, Coomassie Brilliant Blue G 250 staining; **B**, Silver staining. M, molecular weight markers, which are from top to bottom: ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6,200), and insulin (3,000).

Specificity of tobacco inhibitor I. Proteinase inhibitors belonging to the potato inhibitor I family are known to be effective against serine endoproteinases (Ryan 1973; Laskowski and Kato 1980). We have tested the activity of the tobacco inhibitor toward commercial serine proteinases of different origins: two digestive animal enzymes (trypsin and chymotrypsin), two bacterial enzymes (the Carlsberg subtilisin from *Bacillus subtilis* (Ehrenberg) Cohn and a proteinase from *S. griseus*), and two fungal proteinases (proteinase K from *Tritirachium album* Limber and an alkaline proteinase from *A. oryzae*). The tobacco inhibitor was found to be a potent and a specific inhibitor of the four proteinases of microbial origin. As examples, Figure 7 shows the inhibition curves of trypsin, chymotrypsin, subtilisin, and proteinase K in the presence of increasing amounts of purified tobacco inhibitor. About 0.25 μg of inhibitor was sufficient to completely inhibit the activity of 1 μg of subtilisin or proteinase K, whereas the same amount of inhibitor reduced chymotrypsin or

Table 1. Amino acid content[a] of proteinase inhibitors from Solanaceae

		Inhibitor I		Inhibitor II	
Designation	**Tobacco**	**Tomato[b]**	**Potato[c]**	**Tomato[d]**	**Potato[e]**
Asx	9.7	9.0	12.9	11.0	9.7
Glx	15.0	10.1	12.9	7.6	8.9
Ser	2.6	3.4	2.2	5.1	4.7
Gly	4.3	3.7	3.6	6.3	7.0
Arg	8.8	4.1	9.8	6.9	4.8
Thr	7.4	5.3	5.1	4.5	4.6
Ala	2.2	0.93	1.8	2.1	4.3
Pro	7.7	7.6	4.9	7.2	7.4
Val[f]	10.3	7.7	8.7	1.5	0.76
Met	1.6	5.1	1.6	1.9	2.0
Ile	4.1	11.8	9.9	5.0	4.3
Leu	10.2	13.3	9.9	2.5	3.5
Phe	5.8	3.8	3.7	6.5	4.5
Lys	5.7	6.7	8.0	8.5	9.8
Cys[f]	1.8	2.7	2.6	12.2	12.6
Tyr[f]	0.0	2.1	0.0	10.8	10.0
Trp	2.7	2.4	2.3	0.0	0.0

[a] Expressed as percentage per mole.
[b,c,d,e] Data calculated from Graham *et al.* (1985a), Cleveland *et al.* (1987), Graham *et al.* (1985b), and Sanchez-Serrano *et al.* (1986), respectively.
[f] The contents in these amino acids differ clearly between the two families of inhibitors.

Tomato			$^{+}H_3N$	Leu	Met	Cys	Glu	Gly	Lys	Gln	Met	Trp	Pro	Glu	Leu
Tobacco	$^{+}H_3N$	Lys	Gln	Leu	Arg?	X	Asn?	Gly	Lys	Glu	Ser?	Trp	Pro	Glu	Leu
Potato	$^{+}H_3N$	Lys	Glu	Phe	Glu	Cys	Asn	Gly	Lys	Gln	Arg	Trp	Pro	Glu	Leu

Fig. 6. Comparison of the NH_2-terminal amino acid sequence of tobacco inhibitor and tomato and potato inhibitors I. One residue (X) cannot be identified. Some ambiguity in the identity of three other residues is shown by a question mark. Amino acids found at the same position in the tobacco inhibitor and one of the two others are boxed. Amino acids found at the same position in the four other inhibitors of the inhibitor I family are underlined.

trypsin activity by less than 30% (Fig. 7). Proteinase K is widely used for nucleic acid isolation because it is a highly active and rather unspecific proteinase. Nevertheless, proteinase K is the enzyme that was most efficiently inhibited by the tobacco inhibitor. The specificity of tobacco inhibitor I for the microbial serine proteinases (EC 3.4.21.14) is particularly striking since inhibitors I from tomato and potato, two other Solanaceae, were referred to as chymotrypsin inhibitor I due to their high inhibitory activity toward this animal enzyme (Gurusiddaiah *et al.* 1972; Melville and Ryan 1972).

Accumulation of inhibitor I during the hypersensitive reaction induced by TMV. Polyclonal antibodies were raised in rabbit against the purified inhibitor and used in immunoblotting experiments to study inhibitor accumulation during the hypersensitive reaction of tobacco to TMV. Extracts from tobacco leaves at different stages of infection were desalted on a Sephadex G-25 column, clarified by centrifugation at 20,000 × *g*, and electrophoresed under denaturing conditions. Proteins were either stained (Fig. 8A) or transferred onto nitrocellulose sheets and immunodetected (Fig. 8B). Among the numerous proteins stained with Coomassie Brilliant Blue G 250, several accumulated during infection and are, in fact, the well-known pathogenesis-related proteins of tobacco (Van Loon 1985; Legrand *et al.* 1987; Kauffmann *et al.* 1987). Inhibitor I was not present in sufficient amounts to be clearly visible after protein staining but was revealed by the antibodies by the fourth day of infection. The intensity of the immunological reaction estimated by densitometry (data not shown) increased with the infection time in parallel with the level of inhibitory activity (Fig. 1). This indicates that, in infected tobacco leaves, inhibitor I accounts for the major part if not for all of the inhibitory activity effective against microbial proteinases. No serological cross-reaction was detected with any other protein of the extracts and, in particular, with the inhibitor(s) of trypsin present in the crude extract (Fig. 1).

DISCUSSION

The inhibitor I family is the most widely distributed family of proteinase inhibitors in nature, being found in both the animal and plant kingdoms. Potato, tomato, barley, broad bean, and leech inhibitors have been shown to share homologous sequences (Laskowski and Kato 1980; Seemüller *et al.* 1981; Svendsen *et al.* 1982, 1984; Graham *et al.* 1985a). With the exception of the inhibitor from the seeds of broad bean, all of these proteins inhibit the activity of chymotrypsin and are referred to as chymotrypsin inhibitors (Melville and Ryan 1972; Gurusiddaiah *et al.* 1972; Svendsen *et al.* 1982). Surprisingly, the inhibitor that is induced in tobacco reacting hypersensitively to TMV is a poor inhibitor of chymotrypsin but a very potent inhibitor of microbial proteinases. However, NH_2-terminal sequence determination and amino acid analysis have shown that the tobacco inhibitor does indeed belong to the inhibitor I family, in agreement with the immunological relationships which had been found between inhibitors from different genera of Solanaceae (Gurusiddaiah *et al.* 1972). Inhibitors of the same family had previously been detected immunologically in tobacco tissues under abnormal or stress conditions (Wong *et al.* 1975, 1976). From tobacco leaves maintained in total darkness, an inhibitor of this family has been characterized (Kuo *et al.* 1984). This inhibitor has been shown to inhibit trypsin and chymotrypsin, but its activity against microbial enzymes was not investigated. Inhibitors from broad bean seeds, barley grains, and tomato leaves were found to be active on subtilisin, but these inhibitors clearly differ from the one described here and accumulate under completely different environmental conditions (Svendsen *et al.* 1982, 1984;

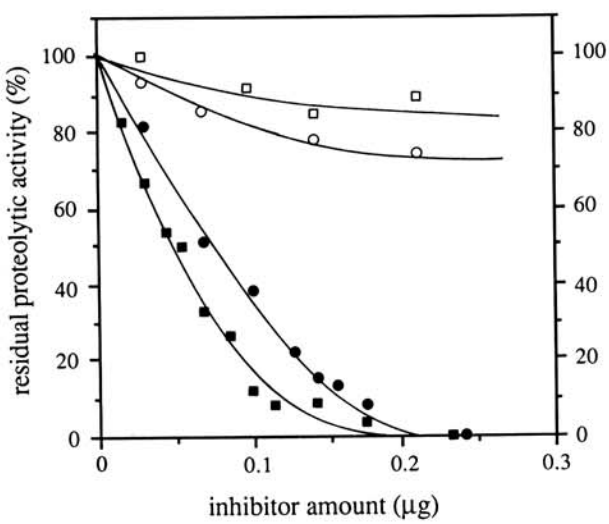

Fig. 7. Inhibition curves of tobacco inhibitor toward different proteolytic enzymes. Residual activity is expressed as percentage of activity of the proteinase in the absence of inhibitor (= 100%). Inhibition curves of trypsin (□—□), chymotrypsin (○—○), subtilisin (●—●), and proteinase K (■—■) are presented.

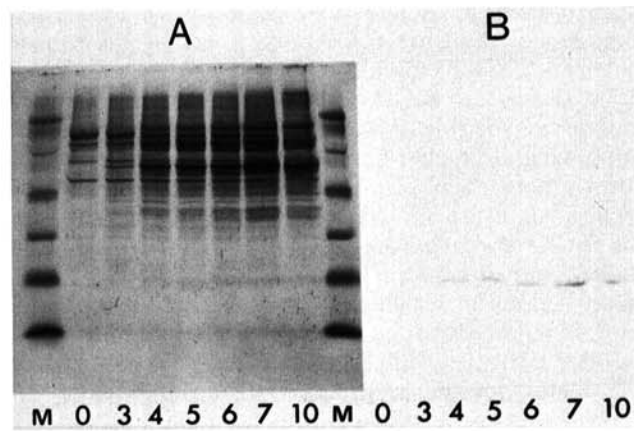

Fig. 8. Kinetics of accumulation of the inhibitor during the hypersensitive reaction induced by tobacco mosaic virus. Extracts from tobacco leaves inoculated with water (lane 0) or with purified tobacco mosaic virus for 3, 4, 5, 6, 7, and 10 days were electrophoresed on polyacrylamide gels under denaturing conditions. **A**, Protein staining. The gel was fixed and stained with Coomassie Brilliant Blue G 250. **B**, Immunoblotting. The proteins were transferred onto nitrocellulose at the end of electrophoresis and immunodetected with the antiserum raised against the tobacco inhibitor. Molecular weight markers (M) are as given in Figure 5.

Plunkett *et al.* 1982). In tomato, two different inhibitor I genes have been characterized. One of them is induced by wounding (Graham *et al.* 1985a), whereas the other is not, but it is expressed in response to an increased level of ethylene (Margossian *et al.* 1988). DNA sequence analysis suggests that this ethylene-responsive gene encodes an inhibitor of microbial proteinase, but the protein encoded by the gene has not been characterized.

In this study inhibitor I was purified to homogeneity from tobacco leaves infected with TMV. When electrophoresed under denaturing conditions, it behaved as a small polypeptide with a molecular weight of about 6,000 and was used to raise polyclonal antibodies. Using immunoblotting experiments, the inhibitor could be detected in tobacco leaves soon after the appearance of symptoms and has been shown to accumulate in tobacco leaves infected with TMV. The inhibitor showed no serological relationship with inhibitors of trypsin, which were detected by their activity in the same material.

The kinetics of induction of inhibitor I during the hypersensitive reaction of tobacco to TMV strikingly resemble the time course of induction of pathogenesis-related proteins and, in particular, of hydrolases such as chitinases and 1,3-β-glucanases (Legrand *et al.* 1987; Kauffmann *et al.* 1987). These hydrolytic enzymes are considered to be direct defense enzymes of plants against pathogens because they are capable of attacking cell walls of fungi and bacteria (Boller *et al.* 1983; Mauch *et al.* 1988). The accumulation of inhibitor I, which is a specific and a potent inhibitor of microbial proteinases, represents a defense reaction complementary to the lytic action of hydrolases. Thus it appears that, upon a pathogen attack, the plant acquires not only the ability to actively counterattack by producing defense enzymes but also to neutralize some of the invader's weapons. In fact, infection with TMV induces the production of an entire set of antimicrobial proteins whose role in antiviral defense is not clear.

The defense capability of a cowpea trypsin inhibitor toward insects has been recently shown by the constitutive expression of the foreign inhibitor in transgenic tobacco (Hilder *et al.* 1987). Inhibitor I and II genes isolated from tomato and potato are also being used to transform plants and to assess the role of these inhibitors in plant resistance to pathogens (Sanchez-Serrano *et al.* 1987; Ryan 1989). In this respect it is of primary importance to be aware that infection of transgenic tobacco can also induce tobacco inhibitor I and complicate the interpretation of the results obtained with these transgenic plants. Furthermore, since tobacco inhibitor I is highly efficient against microbial proteinases, the corresponding gene might be a much better candidate than the inhibitor I and II genes isolated from tomato and potato for use in transforming plants and assessing the role of the inhibitor in plant resistance to pathogens.

ACKNOWLEDGMENTS

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