Isolation and Characterization of a Wound-Induced Trypsin Inhibitor from Alfalfa Leaves†

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ABSTRACT: A trypsin inhibitor from leaves of field-grown alfalfa plants has been purified and shown to be the same trypsin inhibitor that is wound induced in leaves of young growth chamber grown plants. This inhibitor accounts for the major trypsin inhibitory activity found in both field-grown and wound-induced plants. The inhibitor exhibits a molecular weight of about 7500 and is specific for trypsin with a K_i of 1×10^{-10} M. Analysis of the purified inhibitor by cation-exchange high-performance liquid chromatography revealed the presence of four isoinhibitor species that have identical immunological and inhibitory properties. The amino acid analysis of the four species indicates small but significant differences. Immunological double diffusion comparisons of the alfalfa inhibitor with the Bowman-Birk and Kunitz soybean inhibitors did not reveal any cross-reactivity although the amino acid content of the alfalfa inhibitor resembles those of Bowman-Birk family members.

Field-grown alfalfa plants contain a proteinaceous trypsin inhibitor that is a significant component of the soluble proteins of their leaves (Chein & Mitchell, 1970; Chang et al., 1978; Sukhinin et al., 1981). However, growth chamber grown alfalfa plants have only very low levels of trypsin inhibitor activity in leaves, but when detached and supplied with solutions containing the proteinase inhibitor inducing factor of tomato leaves (Walker-Simmons & Ryan, 1977), they accumulate, over the next 3 days, a 5-fold increase in trypsin inhibitory activity. We have recently determined that wounding of growth chamber grown alfalfa plants on the lower leaves also induces a striking increase in trypsin inhibitory activity in the upper leaves. This wound induction in alfalfa leaves is similar, in both the rate of induction and quantity of inhibitor accumulated, to the wound-induced phenomena described in potato and tomato plants (Ryan, 1983), which is considered to be an inducible defense response of those plants against attacking pests. No information was available to explain why leaves of field-grown plants contain significant levels of trypsin inhibitory activity while leaves of young growth chamber grown plants have very little activity, although the possibility of its induction by insects or microorganisms is being investigated. Furthermore, it was not known whether the trypsin inhibitor(s) induced in young growth chamber grown alfalfa plants is (are) identical with the inhibitor(s) present in plants grown in the field or if it (they) is (are) related to the wound-inducible potato or tomato inhibitors.

In this paper we report a new isolation procedure for the purification of trypsin inhibitor from field-grown alfalfa plants and extensively characterize the properties of the inhibitor. We demonstrate that it is immunologically identical with the trypsin inhibitor induced to accumulate in young growth chamber grown alfalfa plants. This inhibitor is present in alfalfa leaves as a family of isoinhibitors that does not cross-react immunologically with the wound-induced inhibitor I and II families of isoinhibitors found in potato and tomato plants nor to the Bowman-Birk or Kunitz families of legume protease inhibitors (Laskowski & Kato, 1980).

Materials and Methods

Trypsin was purchased from Sigma and was determined to be 46% active, as tested by the method of Chase & Shaw (1967). Kallikrein, TAME,[1] BTEE, and BSA were also from Sigma. Sepharose 4B and chromatofocusing matrix were from Pharmacia, and DE-52 resin was from Whatman. Bio-Gel P-10 was from Bio-Rad.

Protein was determined by the method of Bradford (1976). Trypsin and chymotrypsin activity was assayed by the method of Hummel (1959) using either BTEE (chymotrypsin) or TAME (trypsin) as substrates. Proteinase inhibitor activity was assayed as previously described (Gustafson & Ryan, 1976). One unit of trypsin inhibitor activity is that amount which gives 50% inhibition of 1.8 μg of active trypsin. Electrophoresis was performed in 15% polyacrylamide gels in 0.1% SDS and 7 M urea as described by Swank & Munkres (1971). Gels were stained with Coomassie blue. The molecular weight of alfalfa trypsin inhibitor (ATI) was determined by chromatography on either Sephadex G-50 or Sephadex G-75 columns equilibrated with 10 mM Tris-300 mM KCl, pH 7.0. The elution volume of ATI was estimated from the trypsin inhibitor activity. Standards were ovalbumin, myoglobin, cytochrome *c*, PTI, and potato carboxypeptidase inhibitor.

Amino acid analysis of ATI was determined by the method of Moore & Stein (1963). Samples were hydrolyzed in vacuo for 24 h at 110 °C in 6 N HCl containing norleucine as a standard. The samples were dried and dissolved in 100 μL of 200 mM citrate, pH 2.2. Portions were analyzed on a Dionex D-500 amino acid analyzer. Separate samples were analyzed for cysteine after periodate oxidation to cysteic acid (Edelhoch, 1967).

Radial diffusion assays were performed as described by Ryan (1967) and the data analyzed by the method of Trautman et al. (1971). Double diffusion experiments were performed by the method of Ouchterlony (1949) using 2% Noble agar plates made up of 0.01 M sodium barbital and 0.9% sodium chloride, pH 8.0. ELISA were performed by the

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[1] Abbreviations: TAME, *p*-toluenesulfonyl-L-arginine methyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; BSA, bovine serum albumin; ATI, alfalfa trypsin inhibitor; PTI, pancreatic trypsin inhibitor; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assays; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride.

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method of Voller et al. (1978) using goat antirabbit IgG–horseradish peroxidase conjugate (Bio-Rad) and 5-aminosalicylic acid.

Antibodies against ATI were prepared by injecting rabbits with 2 mg of pure inhibitor cross-linked to BSA, in incomplete Freund's adjuvant. ATI was cross-linked to BSA by incubating 4 mg of BSA/mg of ATI in 250 μL of 100 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide–10 mM PO_4, pH 7.5, at room temperature for 1 h, followed by extensive dialysis against water and lyophilized. Three injections over a 14-day period were made each month for 3 months.

Trypsin was cross-linked to Sepharose 4B by the method of Feinstein (1970). The capacity of the trypsin–Sepharose matrix to bind soybean trypsin inhibitor was approximately 170 μg/mL of gel. Columns containing 50 mL of gel were routinely employed.

HPLC was performed with a Beckman instrument, and samples were applied to a 0.41 × 25 cm column of CM-300 (Synchrom), equilibrated with 10 mM potassium phosphate buffer, pH 7.2. ATI was eluted with a linear gradient of 0–0.5 M sodium chloride in the equilibration buffer.

Field-grown alfalfa (*Medigo sativa* L.) plants, 1–3 ft in height, were grown near Pullman, WA. Plants grown in growth chambers were approximately 1 ft in height, grown under 800 ft-c of light for 18-h days at 28 °C.

Several hundred grams of field-grown alfalfa leaves was harvested, frozen at −20 °C, lyophilized, pulverized with an electric mill, and stored at −20 °C. In a typical experiment approximately 1500 g of the lyophilized tissue was extracted twice with acetone, and the dry powder was extracted by the method of Chang et al. (1978). The powder was dispersed in 10 mM Tris–10 mM $Na_2S_2O_3$, pH 6.5 (buffer I), that was adjusted to 200 mM NaCl and incubated at room temperature with stirring for 2 h. The slurry was filtered through Whatman No. 1 filter paper, and the filter cake was reextracted as above. The extracts were combined, and the protein was precipitated by adding solid ammonium sulfate to 65% saturation and centrifuged at 16000*g* for 20 min. The pellet was suspended in a minimal amount of buffer I and applied to a 5 × 70 cm column of Bio-Gel P-10 equilibrated with the same buffer. Fractions containing the trypsin inhibitor activity were pooled and designated the P-10 fraction. The P-10 fraction was immediately applied to 4 × 14 cm column containing DE-52 equilibrated with 10 mM Tris, pH 7.5. The flow-through material was collected and designated the DE-52 fraction. Solid NaCl was added to the DE-52 fraction to a final concentration of 1.0 M and applied to a 2 × 13 cm column of trypsin–Sepharose 4B equilibrated with 20 mM NH_4HCO_3–1 M NaCl, pH 8.0. The column was washed, successively, with 20 mM NH_4HCO_3–1 M NaCl, pH 8.0, 20 mM NH_4HCO_3, pH 8.0, and then 20 mM $HCOONH_4$, pH 1.5. All of the trypsin inhibitory activity eluted at the lower pH. The active fractions were pooled, the pH was immediately adjusted to 7.0 with ammonium bicarbonate, and the fractions were lyophilized. The lyophilized material was dissolved in a minimal amount of water and designated the trypsin–Sepharose fraction. The trypsin–Sepharose fraction was applied to a 1.5 × 80 cm column of Sephadex G-75 equilibrated with 50 mM NH_4HCO_3, pH 8.0. The elution profile is shown in Figure 1. Fractions containing the trypsin inhibitor activity were pooled, lyophilized, and resuspended in a minimal amount of 20 mM NH_4HCO_3, pH 8.0.

Results and Discussion

A summary of the purification of ATI is presented in Table I. A 330-fold purification was achieved with a 57% recovery of activity starting with the first gel filtration on Bio-Gel P-10. Prior to the gel filtration step, quantification of the inhibitory activity was not possible due to absorbing pigments in the extracts that interfered with the trypsin inhibitor assay. The final yield of pure inhibitor from lyophilized alfalfa leaves was 0.06 mg/g of leaf tissue, which represented about 0.2% of the protein calculated to be present in leaves, based on later immunological quantification when antibodies were prepared from the pure inhibitor (see below).

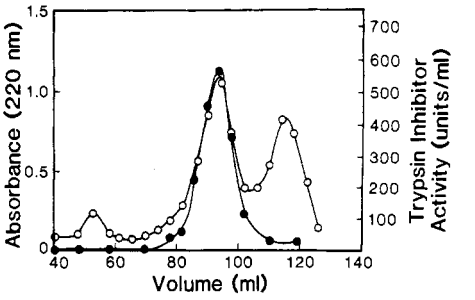

FIGURE 1: Gel filtration of affinity-purified ATI on Sephadex G-75. Details of the conditions are given under Materials and Methods. Absorbance at 220 nm (○); trypsin inhibitor activity (●).

Table I: Summary of Purification of ATI[a]

fraction	total mL	total mg	total units	% recovery protein	% recovery units
P-10	520	1210[b]	7900	100	100
DE-52	660	60[b]	6200	5	78
affinity chromatography	1.8	3.0[c]	5000	0.25	63
G-75 chromatography	2.0	2.1[c]	4500	0.17	57

[a] Details of purification procedures and assays are described under Materials and Methods. [b] Protein determined by the method of Bradford (1976). [c] Protein determined by absorbency at 220 nm.

Our procedure departed from the method of purification previously reported for the trypsin inhibitor from field-grown alfalfa (Chang et al., 1978; Sukhevin et al., 1980). Chang et al. (1978) reported recovery of a trypsin inhibitor in pure form by affinity chromatography of the crude extract. We could not obtain a pure inhibitor from crude extracts by that method. In our hands, affinity chromatography, even after our DE-52 chromatography step, still resulted in a somewhat impure preparation as judged by gel electrophoresis. Only after an additional gel filtration step on Sephadex G-75 (Figure 1) did a pure inhibitor result.

The electrophoresis of the pure inhibitor in 0.1% SDS and 7 M urea is shown in Figure 2. The band appeared to be somewhat diffuse and is probably in part due to its presence as multiple forms. In the following text we demonstrate that the inhibitor, like a number of other plant proteinase inhibitors, is composed of a family of isoinhibitors.

Molecular Weight Estimates. The molecular weight of ATI was estimated to be 6900 ± 300 when chromatographed on Sephadex G-50 with appropriate standards. This value is near the 7400 estimated by amino acid analysis and the value of 7600 ± 1400 estimated by titration with trypsin, calculated at 50% inhibition (see below). Comparisons of the mobility of electrophoretic band (Figure 2) with a series of known standards gave a value of 8000 ± 1000.

Proteinase Inhibitory Activity. ATI is a potent inhibitor of trypsin, exhibiting a K_i of 1×10^{-10} M, determined by the method of Green & Work (1953). This value is in good agreement with that for other trypsin inhibitors from the Leguminosae family (Kassell, 1970). The inhibitor has no measurable inhibitory activity toward the animal pancreatic serine proteinases chymotrypsin and elastase nor against the

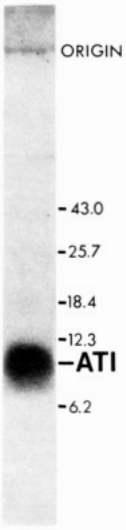

FIGURE 2: Electrophoretic analysis of purified ATI. Electrophoresis was carried out on slab gels in the presence of 0.1% SDS and 7 M urea as described under Materials and Methods. Twenty micrograms of ATI was applied to the gel and was visualized by staining with Coomassie blue. Molecular weights of known standards, $\times 10^{-3}$, are shown at the right.

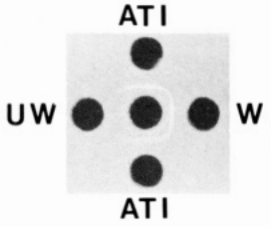

FIGURE 3: Immunological comparison of pure ATI from field-grown alfalfa plants with extracts from leaves of wounded and unwounded growth chamber grown plants. The center well is undiluted rabbit anti-ATI serum. In the outer wells are pure inhibitor (ATI) (1 μg), extracts from leaves of plants wounded 72 h previous to assays (W), and extracts from leaves of unwounded controls (UW).

plant sulfhydryl proteases papain and bromelain (data not shown). The inhibitor also did not inhibit kallikrein (data not shown), a trypsin-like enzyme from porcine pancreas (Fiedler, 1979).

Immunochemical Analyses. ATI, when injected into rabbits, did not readily elicit precipitating antibodies. However, when ATI was cross-linked to BSA, the protein produced good titers of precipitating antibodies. In Figure 3, a double diffusion experiment is shown in which ATI, purified from field-grown plants, is compared with an extract from leaves of growth chamber grown plants that had been wounded to induce trypsin inhibitor activity. Both pure ATI and the extract from wounded plants formed a single precipitin band with the rabbit anti-ATI serum, indicating complete identity (no spurs). No precipitin lines were detected when the serum was challenged with extracts from unwounded plants (Figure 3).

Antibodies prepared against the wound-inducible inhibitors I and II from tomato and potato did not cross-react with ATI (data not shown). In addition, antibodies against ATI did not cross-react with any plant protease inhibitor tested. When the sensitive ELISA technique was used, anti-ATI did not react with tomato inhibitors I and II or with a wound-inducible carboxypeptidase inhibitor from potato (Ryan, 1983). There was also no immunoprecipitation reaction with the Kunitz or Bowman–Birk soybean inhibitors, trypsin inhibitors that are found exclusively in the Leguminosae family (data not shown). However, the overall amino acid content of ATI is similar to

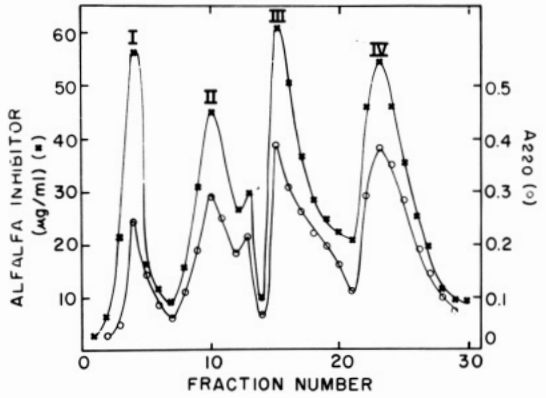

FIGURE 4: HPLC chromatography of pure ATI on a CM-300 column. ATI (200 μg) was applied to the column, and 0.4-mL fractions were collected and assayed. Conditions of separation and assay are in the text. (O) A_{220}; (X) ATI, assayed immunologically.

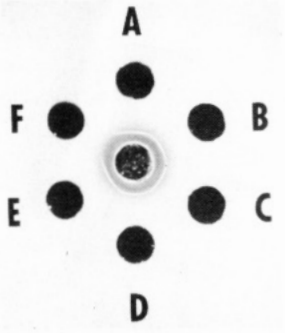

FIGURE 5: Immunological comparisons of isoinhibitors of ATI separated by HPLC. The solutions, all containing a total of 1 μg of proteins, were challenged with undiluted rabbit anti-ATI serum, prepared from the composite of isoinhibitors present in native ATI (center well). In the outer wells are pooled fractions as numbered in Figure 4: A, composite ATI; B, peak I; C, peak II; D, peak III; E, peak IV; F, lima bean inhibitors.

Table II: Specific Trypsin Inhibitory Activities of the Four Major Isoinhibitors of ATI

isoinhibitor peak[a] numeral	specific activity (units/μg)
I	4.46
II	4.32
III	4.57
IV	4.38

[a] From Figure 3.

those of several Bowman–Birk family members isolated and characterized from various legume seeds (Norioka & Ikenaka, 1983).

Isoinhibitor Isolation and Characterization. HPLC analysis of ATI on a CM-300 column revealed the presence of four major peaks (Figure 4). We investigated the possibility that the four peaks represented multiple forms of ATI. Individual fractions from the CM-300 assayed by spectral means (data not shown), by immunological means with radial diffusion techniques, and for protein (A_{220}) gave similar profiles (Figure 4). In addition, the specific activities of the individual peaks were nearly identical (Table II), and all cross-reacted completely (no spurs) by double diffusion analysis (Figure 5). Thus, the four species separated by HPLC are similar in molecular weight, immunological characteristics, and inhibitory properties and are considered isoinhibitors.

In order to qualitatively examine whether the multiple forms of ATI may have resulted from degradation during its isolation, the purification procedure was modified to minimize proteolysis. Fresh leaves were ground in ice-cold buffer I (see

Table III: Amino Acid Composition of ATI[a] and Its Isoinhibitors

amino acid	native ATI residue/mol	native ATI nearest integer	residues/mol ATI isoinhibitors I	II	III	IV	ATI[b]
Asp	6.17	6	4	5	5	6	6
Thr	8.32	8	5	6	6–7	8	7–8
Ser	4.64	5	3	5	4	6	3
Glu	3.82	4	3	3–4	3	3–4	4
Pro	7.77	8	4	5–6	5–6	7	7–8
Gly	1.92	2	3	3	2	1–2	1–2
Ala	3.00	3	3	3	3	3	3
$^1/_2$-Cys	11.79	12	ND[c]	ND	ND	ND	16
Val	0.22	0	0	0	0	0	1
Met	0	0	0	0	0	0	0
Ile	4.42	4–5	3	3–4	3–4	4–5	4–5
Leu	1.16	1	1	1	1	1	1
Tyr	1.22	1	0	0	1	1	1
Phe	2.75	3	1–2	2	2	2	2
His	1.81	2	2	3	3	3	1
Lys	3.05	3	1	1–2	1–2	1–2	3
Arg	3.79	4	2	3	3	5	1
Trp	0	0	0	0	0	0	1
totals		67–68					62–66
M_r		7370					

[a] Details of the analysis are described under Materials and Methods. [b] From Chang et al. (1978). [c] ND = not determined.

Materials and Methods) containing 1 mM PMSF, 4 mM EDTA, and leupeptin (1 μg/mL). After filtration through Whatman No. 1 paper, the solution was immersed in boiling water for 10 min [ATI is stable at 100 °C for at least 10 min (data not shown)]. The denatured protein was removed by centrifugation, and to the supernatant was added ammonium sulfate to 65% saturation. At this point ATI was purified as described under Materials and Methods except that all of columns were at 4 °C instead of room temperature. The HPLC profile of ATI purified in this manner (data not shown) was identical with that observed previously (Figure 4).

The amino acid content of the four isoinhibitor fractions was determined and compared in Table III with an analysis of the total mixture. The analysis indicates that overall the inhibitors are all similar, having no valine or methionine, a high cysteine content, and identical integers among several residues. Significant differences were noted, particularly between isoinhibitors from peaks I and IV, among a number of residues (Table III).

The analysis of the unfractionated ATI is also very similar to the analysis previously reported by Chang et al. (1978) for the alfalfa inhibitor. However, their sample was reported to contain 1 arginine and 16 cysteine residues, whereas the composite ATI reported here has 4 arginines and 12 cysteines. Other than the cysteine and arginine values, those of other residues correlate well with that reported by Chang et al. (1978) (Table III).

The amino acid analysis of isoinhibitor I indicates that it may be somewhat smaller than the other isoinhibitors. The electrophoretic analysis of the purified ATI also indicated that a component was present that was smaller than the others, and it is likely isoinhibitor I (cf. Figure 2). It is not yet known if the isoinhibitors are products of posttranslational degradation of a single translated species or if the isoinhibitors are discrete gene products. The data do not favor either possibility.

The low tyrosine and lack of tryptophan in ATI are reflected by its very low absorbance at 280 nm, and therefore the protein is more conveniently monitored at 220 nm. A 1 mg/mL solution of the purified mixture of isoinhibitors of ATI had an absorbance at this wavelength of 12.5. Like tomato and potato inhibitors I and II, the protein is quite basic and all of the subunits exhibit isoelectric points >9, as determined by chromatofocusing (data not shown).

Table IV: Quantification of ATI and Trypsin Inhibitory Activity in Alfalfa Plants Grown under Various Conditions

conditions of growth	units/g fresh wt[a]	μg/g fresh wt[b]	ATI units/μg	ATI % total protein
growth chamber[c]				
unwounded	60	15	4.0	0.02
wounded[c]	1470	385	3.8	0.5
field grown	470	123	3.8	0.2

[a] Assayed spectrophotometrically (Hummel, 1959). [b] Assayed immunologically (Ryan, 1967). [c] For conditions, see Methods and Materials.

Relationship of ATI to the Wound-Induced Trypsin Inhibitor. Since the trypsin inhibitor isolated from field-grown plants cross-reacted immunologically with the inhibitor isolated from wound-induced plants from the growth chamber (cf. Figures 3 and 4), the trypsin inhibitors from both field-grown and growth chamber grown plants are assumed to be identical. If this assumption is correct, then some environmental or developmental condition must have caused the expression of the inhibitors in field-grown plants. The upper, nonwounded leaves of young growth chamber grown plants wounded 72 h earlier contained 385 μg of ATI/g of leaf tissue (Table IV). In leaves from unwounded control plants the levels were about 15 μg/g of tissue. The levels of ATI in alfalfa leaves collected from three locations near Pullman, WA, varied between 60 and 140 μg/g of tissue. In field-grown plants the average level of ATI represents about 0.2% of the soluble proteins of the leaves (Table IV).

The trypsin inhibitory activities in extracts from field-grown alfalfa and of wounded and unwounded growth chamber grown alfalfa plants were also compared. In these experiments, also shown in Table IV, field-grown alfalfa contained 470 units/g fresh weight of tissue. The unwounded growth chamber grown plants had only 60 units/g of tissue, but within 72 h following wounding the plants possessed 1470 units/g of tissue, over a 10-fold increase due to wounding. The units of trypsin inhibitory activity per microgram of ATI, measured spectrally, correlated well with the micrograms of ATI, measured immunologically, among all three tissues (Table IV) and with the purified inhibitor (Table II). This strongly suggests that ATI is the major trypsin inhibitor present in alfalfa leaves.

This striking increase in a proteinase inhibitor due to wounding is similar to that found in leaves of two members of the Solanaceae family, i.e., tomato and potato (Ryan, 1983). It is clear from the immunological data that ATI is unrelated to the tomato and potato inhibitors I and II, even though wound regulated in much the same manner as the latter inhibitors (W. Brown and C. A. Ryan, unpublished results). The high specificity toward trypsin indicates that it probably possesses a lysine or arginine at its P_1 site, a characteristic of all trypsin inhibitors (Laskowski & Sealock, 1970). Alfalfa provides an opportunity to study the regulatory regions of a wound-induced trypsin inhibitor gene(s) in legumes to complement our similar studies with proteinase inhibitor genes from the Solanaceae family (J. Graham, G. Pearce, J. Merryweather, K. Titani, and C. A. Ryan, unpublished results). A similar wound-induced communication system appears to regulate expression of both alfalfa proteinase inhibitor genes and potato and tomato proteinase inhibitor genes (W. Brown and C. A. Ryan, unpublished results). These responses are thought to be part of the induced natural protection system of the plants in which wounding causes synthesis and accu-

mulation of proteins that severely reduce the digestibility and nutritional quality of the plant leaves to help ward off attacking pests (Ryan, 1983). The isolation and characterization of ATI and the acquisition of specific ATI antibodies are first steps in our efforts to isolate an ATI gene for further studies of wound-regulated expression in alfalfa leaves.

Acknowledgments

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Registry No. Trypsin inhibitor, 9035-81-8; trypsin, 9002-07-7.

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Metal Substitution of *Neurospora* Copper Metallothionein†

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ABSTRACT: The binding of diamagnetic Zn(II), Cd(II), and Hg(II) and paramagnetic Co(II) and Ni(II) ions to the apo form of *Neurospora* metallothionein (MT) was investigated by various spectroscopic techniques. In contrast to native copper MT, which was shown to bind 6 mol of Cu(I)/mol of protein (Lerch, 1980), all substituted forms reveal an overall metal to protein stoichiometry of 3. The charge-transfer (CT) transitions of the complexes containing diamagnetic metal ions as well as the d–d transitions of those with paramagnetic metal ions are indicative of a distorted T_d coordination. Electron paramagnetic resonance and absorption measurements of the Co(II) derivative are in agreement with the presence of a metal–thiolate cluster in this protein. Metal titration studies of the apoprotein reveal characteristic spectral features for the derivatives containing two metal equivalents as compared to those with a full complement of three metal ions. The former features are indicative of an exclusive T_d type of metal–sulfur coordination whereas the latter suggest that the third metal ion is coordinated in a different fashion. This finding is in agreement with the presence of only seven cysteine residues in *Neurospora* MT as opposed to nine cysteine residues in the three-metal cluster of the mammalian MT's [Winge, D. R., & Miklossy, K.-A. (1982) *J. Biol. Chem. 257*, 3471].

Metallothioneins (MT's)[1] are a class of low molecular weight, cysteine-rich proteins binding metal ions like Cd, Zn, and/or Cu (Nordberg & Kojima, 1979). After the first isolation and characterization of a Zn- and Cd-containing MT from equine kidney (Kägi & Vallee, 1961), most of the attention was focused on the proteins isolated from mammalian species. These proteins are characterized by a single polypeptide chain containing 20 cysteines out of a total of 61 amino acids and lacking aromatic residues and histidine. Several physicochemical investigations (Kägi et al., 1974; Otvos & Armitage, 1980; Vašák et al., 1981a,b; Vašák & Kägi, 1981) have shown that the metal binding occurs in the form of a metal–thiolate complex, the metal ions being organized in two clusters with distorted tetrahedral coordination. Metal coordination in the form of a metal–thiolate cluster has been proposed recently also in the case of the Cu-MT isolated from *Neurospora crassa* (Beltramini & Lerch, 1983). *Neurospora* MT consists of only 25 amino acids and binds six Cu(I) ions to seven cysteinyl residues (Lerch, 1980). Although this protein is the smallest MT isolated so far, it shows a striking

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[1] Abbreviations: CD, circular dichroism; CT, charge transfer; EPR, electron paramagnetic resonance; LMCT, ligand to metal charge transfer; MT, metallothionein; apoMT, apometallothionein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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