Wound-Induced Trypsin Inhibitor in Alfalfa Leaves: Identity as a Member of the Bowman–Birk Inhibitor Family†

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ABSTRACT: The primary structure of the wound-inducible trypsin inhibitor from alfalfa (ATI) establishes it as a member of the Bowman–Birk proteinase inhibitor family. The time course of induction of ATI in alfalfa following wounding is similar to the induction of the nonhomologous proteinase inhibitors I and II in tomato and potato leaves, and, like inhibitors I and II, ATI is induced to accumulate in excised leaves supplied with the proteinase inhibitor inducing factor from tomato leaves. The similarity of the wound induction of ATI to that of inhibitors I and II indicates that wound-regulated systems are present in Solanaceae and Leguminosae plant families that possess a common fundamental recognition system regulating synthesis of proteinase inhibitors in response to pest attacks. ATI is the first Bowman–Birk inhibitor that has been found in leaves and is the only member of this family known to be regulated by wounding.

Wounding of leaves of solanaceous plants by chewing insects or by other forms of mechanical injury releases a systemic factor that travels rapidly throughout the plants where it induces the synthesis and accumulation of proteins that are powerful inhibitors of trypsin, chymotrypsin, elastase, and carboxypeptidases A and B (Ryan, 1983). This response is regarded as a natural defense system that reduces the digestibility of the leaf proteins toward attacking pests (Ryan, 1983).

A trypsin inhibitor was recently shown to be wound induced in leaves of alfalfa, a legume (Brown & Ryan, 1984). The inhibitor, called the alfalfa trypsin inhibitor (ATI)[1] with a molecular weight of about 7000, was specific for trypsin and did not inhibit chymotrypsin or the plant endopeptidase papain (Brown & Ryan, 1984). Its relationship to any known family of inhibitors was not established although the high cysteine content resembled members of either the potato inhibitor II (Bryant et al., 1976) or the Bowman–Birk (Norioka & Ikenaka, 1983) families of inhibitors. In this paper, we report the primary structure of wound-induced ATI which shows that it is unambiguously a member of the Bowman–Birk family of proteinase inhibitors. The Bowman–Birk family, found only in legumes, is one of the most extensively studied plant inhibitor families and heretofore has been shown to be present only in seeds, regulated during development. The accumulation of ATI in alfalfa leaves in response to wounding appears to be regulated in a similar way as inhibitors I and II in leaves of solanaceous plants.

Materials and Methods

Trypsin was purchased from Sigma and was 46% active as assayed by the method of Chase & Shaw (1967). LBI, TAME, and STI were also from Sigma. Goat anti-rabbit IgG horseradish peroxidase conjugate and 4-chloro-1-naphthol were from Bio-Rad. BBI was a gift of Dr. Irvin Liener of the University of Minnesota.

Proteinase inhibitor inducing factor (PIIF) was prepared as described previously (Bishop et al., 1984). Protein was determined by the method of Bradford (1976). Trypsin activity was assayed by the method of Hummel (1959) using TAME as the substrate. Proteinase inhibitor activity was assayed as previously described (Brown & Ryan, 1984).

Plants. Alfalfa (*Medicago sativa* L.), grown in the field in Pullman, WA, were 1–2 ft in height when harvested for use. Alfalfa plants grown in a growth chamber (17-h day, with 250 μEinstein m^{-2} s at a 30 °C daytime temperature and 20 °C nights) were 8–10 in. in height and 10–15 weeks old when used for experiments.

Preparation of Alfalfa Leaf Extracts. Unless indicated, all steps were performed at 4 °C. Approximately 100 mg of alfalfa leaves, consisting of only the upper two to three leaves, was ground by using a Dounce homogenizer in 300 μL of buffer I (10 mM Tris and 10 mM sodium metabisulfite, pH 6.0) and centrifuged at 10000*g* for 5 min. The supernatants were collected and recentrifuged at 105000*g* for 90 min. The clear supernatants were either stored at 4 °C or assayed immediately for protein and trypsin inhibitor activity.

Induction of ATI. Trypsin inhibitor activity was induced in intact alfalfa plants by mechanically wounding each of three lower leaflets per leaf once with a hemostat. The upper, unwounded leaflets were collected and leaf extracts prepared. Detached leaves were also induced to accumulate trypsin inhibitor activity by supplying the leaves with solutions of PIIF for 1 h through the cut petioles (Bishop et al., 1981). The leaves were then incubated in H_2O under light of 260 μEinstein m^{-2} s for 48 h and leaf extracts prepared. For each experiment

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[1] Abbreviations: kDa, kilodalton(s); ATI, alfalfa trypsin inhibitor; TAME, tosyl-L-arginine methyl ester; LBI, lima bean inhibitor; STI, soybean trypsin inhibitor; BBI, soybean Bowman–Birk inhibitor; PIIF, proteinase inhibitor inducing factor; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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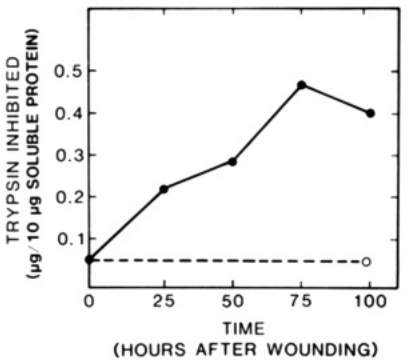

FIGURE 1: Wound-induced accumulation of trypsin inhibitor activity in alfalfa leaves. The bottom two leaves of young alfalfa plants were wounded with a hemostat at zero time and again at 8 h. The three or four upper (nonwounded) leaves were collected at the time indicated, and extracts were prepared and assayed for trypsin inhibitor activity as described in the text. Trypsin inhibitor activity was determined at several concentrations of each extract as described under Materials and Methods. The micrograms of trypsin inhibited by 10 μg of extract was calculated and plotted. Leaves from unwounded plants (○); unwounded leaves from wounded plants (●).

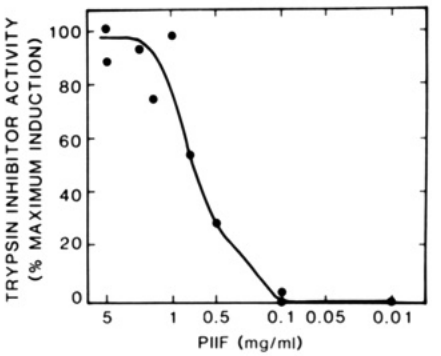

FIGURE 2: Effect of PIIF concentration on trypsin inhibitor activity in excised alfalfa leaves. The upper two to three leaves of alfalfa plants were detached and incubated with the indicated concentrations of PIIF in water for 1 h at room temperature. Control plants were excised and incubated in H_2O. Following incubation, the stems were rinsed and supplied with water in the growth chamber for 48 h. Extracts were prepared, and trypsin inhibitor activity was determined as described under Materials and Methods. Inhibitory activity of plants initially incubated at 5 mg/mL PIIF was arbitrarily assigned as 100% induction, and plants initially incubated in H_2O were assigned 0% induction. Data represent relative trypsin inhibitor activity in 15 μg of extract at each level of PIIF induction.

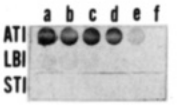

FIGURE 3: Immunoblots of ATI, LBI, and STI using rabbit anti-ATI serum and goat anti-rabbit peroxidase conjugate as described under Materials and Methods. Inhibitors were adsorbed to a nitrocellulose filter at the following amounts: (a) 14 μg; (b) 7 μg; (c) 1.4 μg; (d) 0.14 μg; (e) 0.014 μg; (f) 0 μg. Visualization was achieved with the peroxidase chromophoric substrate 4-chloro-1-naphthol.

reported, the leaves were from similar size plants planted on the same day.

Immunological Analysis. Antibodies to ATI were prepared as described previously (Brown & Ryan, 1984). 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). Enzyme-labeled immunoassays (ELISA) were performed by a modification (Brown & Ryan, 1984) of the method of Voller et al. (1978).

Immunological comparisons of ATI with other inhibitors were carried out by the method of dot blotting on nitrocellulose paper (Hawkes et al., 1982) followed by visualization by Immun-blot (Bio-Rad Corp. assay kit) using specific anti-ATI rabbit serum and anti-rabbit IgG coupled to horseradish peroxidase. The chromophoric substrate was 4-chloro-1-naphthol.

Sequence Analysis. ATI, purified as previously described (Brown & Ryan, 1984) (peak IV from HPLC), was employed for sequence analysis. The protein was reduced and carboxymethylated previous to analysis (Koide et al., 1978). For cysteine analysis, iodo[^{14}C]acetic acid (New England Nuclear) was used for the carboxymethylation reaction, and radioactivity was determined in fractions from HPLC. For digestion of the carboxymethylated protein, TPCK–trypsin and α-chymotrypsin (both from Worthington) were used. Peptides were purified by HPLC (Mahoney et al., 1980).

Amino acid analyses were performed with a Dionex D500 amino acid analyzer or with a Waters Pico-Tag system.

Automated Edman degradations were performed with an Applied Biosystems 470A protein sequencer using a program adapted from Hunkapiller et al. (1983). The products were identified by HPLC (Hunkapiller & Hood, 1983; Ericsson et al., 1977).

Results and Discussion

Wound Induction of Trypsin Inhibitor Activity in Alfalfa Leaves. Young alfalfa plants, wounded on their lower leaves, at zero time and again at 8 h, accumulate a trypsin inhibitor in their upper, unwounded leaves at a constant rate of about 0.45 μg h^{-1} (mg of leaf protein)$^{-1}$ for nearly 75 h (Figure 1) whereas leaves from nonwounded plants exhibited low levels of trypsin inhibitory activity throughout the experiments. The rate and amount of ATI induced by wounding are similar to the wound induction of inhibitors I and II previously quantified in young tomato plants (Ryan, 1983).

A small pectic fragment termed PIIF, isolated from tomato leaves, induces the accumulation of inhibitors I and II when supplied to young tomato plants through their cut petioles (Bishop et al., 1984). ATI accumulated in leaves of detached alfalfa plants when tomato PIIF was supplied through the cut petioles. PIIF induced ATI in a dose-dependent manner up to ~1 mg/mL (Figure 2). The range of concentrations of PIIF that induces ATI is identical with the range that induces inhibitors I and II in tomato plants (Bishop et al., 1984). Another inducer of inhibitors I and II in excised tomato plants is the β(1→4)-glucosamine polymer chitosan (Walker-Simmons & Ryan, 1984). Chitosan also induces ATI in excised alfalfa leaves. The concentrations that induce ATI in alfalfa leaves are ~0.1 mg/mL.

Identity of ATI as a Member of the Bowman–Birk Family. ATI had previously been shown to have a molecular weight of about 7000 and was rich in cysteine residues (Brown & Ryan, 1984). These properties are similar to those of the inhibitor II from tomato (Plunkett et al., 1982) and potato (Bryant et al., 1976) and to those of the Bowman–Birk family of proteinase inhibitors from legume seeds (Norioka & Ikenaka, 1983). Immunodiffusion experiments with inhibitor II, LBI, and BBI did not reveal any cross-reactivity with ATI antiserum (Brown & Ryan, 1984). However, in the more sensitive immunoblot assays, ATI did cross-react weakly with LBI, a member of the Bowman–Birk family (Figure 3). Neither BBI nor STI cross-reacted with anti-ATI serum, and antibodies to inhibitor II did not cross-react with ATI using immunoblot assays (data not shown). The cross-reactivity of ATI antibodies with LBI and the absence of reactivity with inhibitor II or BBI were confirmed by using the sensitive

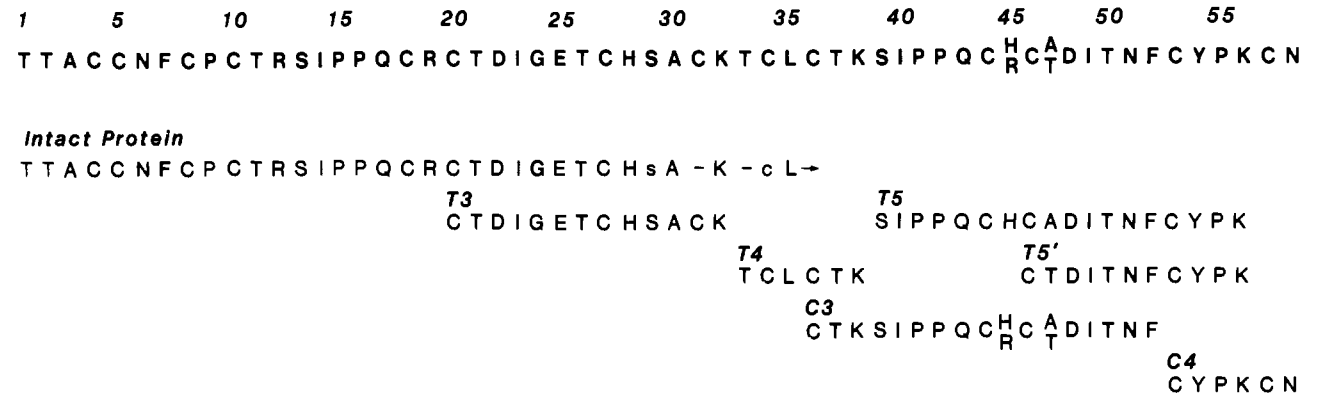

FIGURE 4: Summary proof of the sequence of ATI. The one-letter codes designate amino acid residues identified by Edman degradation unambiguously (capital letters) or tentatively (lower case letters). Those not identified in each peptide are shown by dashes. Prefixes *T* and *C* indicate that peptides were generated by trypsin and chymotrypsin, respectively. The final sequence derived is shown at the top in boldface letters. An arrow indicates that the C-terminal residues were not analyzed by Edman degradation.

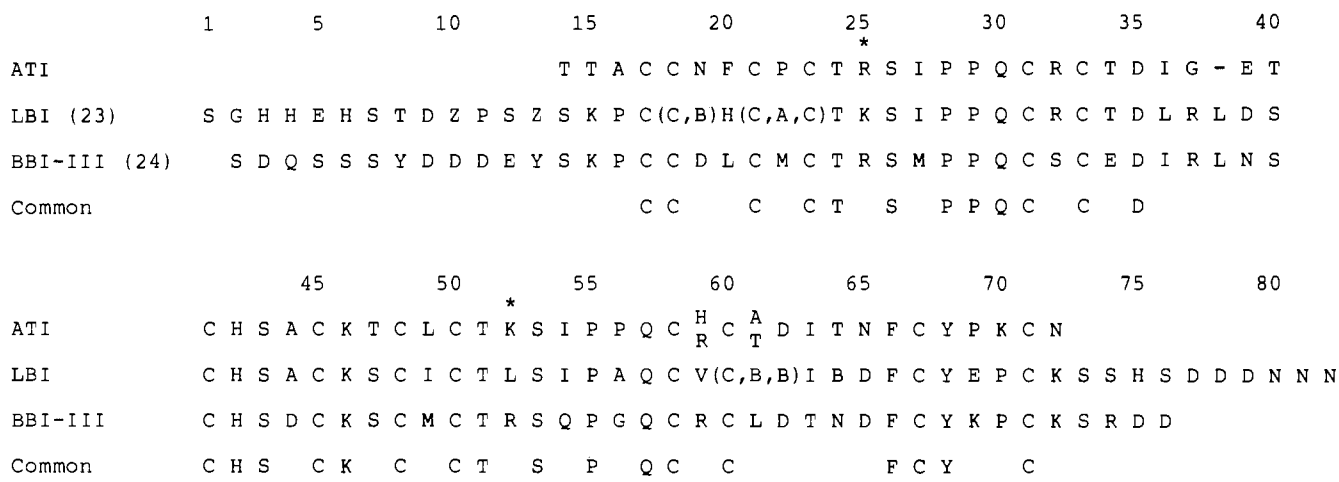

FIGURE 5: Comparison of the sequence of ATI with the Bowman–Birk III and lima bean of proteinase inhibitors. Numbering system for LBI is used. Asterisks indicate putative P_1 sites.

ELISA method of detection (data not shown).

The identity of ATI as a member of the Bowman–Birk family was established unambiguously from its amino acid sequence. The sequence was determined as summarized in Figure 4. This sequence was 58 residues in length with a molecular weight estimated to be 6387, making ATI the smallest member of the Bowman–Birk family yet reported. ATI exhibited microheterogeneity at residues 45 and 47. The inhibitor had previously been shown to be a mixture of isoinhibitors, and peak IV from HPLC purification is apparently a mixture of at least two isoinhibitor species.

The complete sequence of ATI is compared in Figure 5 with the sequences of LBI (Stevens et al., 1974) and BBI III (Odani & Ikenaka, 1977). Although ATI is shorter, a total of 29 residues are in identical loci in each inhibitor and the half-cystine residues are 100% conserved. At least 37 of the 58 residues of ATI are identical with one or the other of the two Bowman–Birk family members. The weak cross-reactivity of ATI antibodies with LBI (Figure 2), and the apparent absence of cross-reactivity with BBI, was somewhat surprising considering the high percentage of identity among them.

As observed with other Bowman–Birk inhibitors, the ATI sequence shows evidence of tandem gene duplication (residues 8–33 and 34–58, Figure 4). These regions have 56% identity between them and an alignment score of 9.4 standard deviation units according to the ALIGN program and the mutation data matrix (Dayhoff et al., 1983). The sequence also corroborates the specificity of ATI for trypsin since the duplicated P_1 sites contain Arg and Lys at residues 12 and 38, respectively (Figure 4).

The demonstration that ATI can be systemically regulated by wounding and can also be induced to accumulate in excised leaves in response to chitosan or tomato PIIF indicates that similar wound-regulated systems are present in both Leguminosae (alfalfa) and Solanaceae (tomato and potato) families. Thus, common fundamental recognition systems that respond to environmental signals to induce proteinase inhibitors are apparently present in at least two plant families. Although the inhibitors that are induced to accumulate are not homologous, the recognition systems may share a common ancestor and may have been conserved during evolution due to their functional roles in regulating active defense systems triggered by pest attacks. It is possible that similar gene-regulating systems are present in many plant families that activate genes coding for a variety of defense proteins. Isolation and characterization of the wound-induced ATI gene and its comparison to wound-induced tomato and potato inhibitor I and II genes should provide clues to help elucidate the processes that regulate the wound-induced synthesis of defense proteins in plants.

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Articles

3-Phenylpropenes as Mechanism-Based Inhibitors of Dopamine β-Hydroxylase: Evidence for a Radical Mechanism†

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ABSTRACT: A series of ring-substituted 3-phenylpropenes has been examined as mechanism-based inhibitors for the copper protein dopamine β-hydroxylase. *p*-HO-, *p*-CH_3O-, *m*-HO-, *m*-CH_3O-, *p*-Br-, and *p*-CN-substituted phenylpropenes all inactivate the enzyme under turnover conditions, requiring ascorbate and oxygen. Replacement of the benzylic hydrogens in 3-(*p*-hydroxyphenyl)propene with deuterium results in a kinetic isotope effect of 2.0 on k_{inact}/K_{O_2} but in no effect on the partition ratio, V_{max}/k_{inact}, consistent with a stepwise mechanism for hydrogen abstraction and oxygen insertion. The partition ratio is unchanged in the pH range from 4.5 to 7.1. Determination of the kinetics of inactivation and the partition ratios for each of these ring-substituted phenylpropenes has allowed determination of the respective V/K_{O_2} values. A linear free energy plot of these values as a function of σ^+ gives a ρ value of −1.2, while the partition ratios show only a slight decrease upon going from electron-donating to electron-withdrawing groups. The results are consistent with a mechanism for dopamine β-hydroxylase in which a hydrogen atom is abstracted to form a benzylic radical, which then partitions between hydroxylation and enzyme inactivation.

The physiological reaction catalyzed by the copper protein dopamine β-hydroxylase is the hydroxylation of dopamine to form norepinephrine, the final step in the biosynthesis of this important neurotransmitter. In vitro, however, the enzyme shows a broad substrate specificity (Creveling et al., 1962; Rosenberg & Lovenberg, 1980). It will hydroxylate benzyl cyanides, phenyl thioethers, phenethylamines, and phenylpropenes (Baldoni & Villafranca, 1980; May & Phillips, 1980; Klinman & Kreuger, 1982; Rajashekhar et al., 1984). This has been taken advantage of to probe the mechanism of C–H bond activation during catalysis, especially in the development of mechanism-based inhibitors or suicide substrates. Benzyl cyanides were the first class of compounds shown to inactivate

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