

The Plant Invertase Inhibitor Shares Structural Properties and Disulfide Bridges Arrangement with the Pectin Methylesterase Inhibitor

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Attempts to purify the inhibitor of pectin methylesterase (PMEI) from the soluble extract of ripe apricot (*Prunus armeniaca*) fruit led to isolation of a protein (Pa-INH) similar to PMEI, but having invertase inhibitory activity against vacuolar invertase from tomato. The molecular charge, the native and SDS-PAGE molecular weights were similar to those of PMEI. Partial amino acid sequence indicated a high level of identity with invertase inhibitors and a significant identity with PMEI. Circular dichroism analysis showed a mainly α -helix secondary structure for both the inhibitors and a higher thermostability of Pa-INH. Four Cys residues forming disulfide bridges in PMEI were conserved in Pa-INH. Similarly to PMEI, these residues were linked by disulfide bridges (first to second and third to fourth). The free Cys139 of PMEI is substituted by Ala in Pa-INH. The results reported in this study suggest a common structural arrangement of the two inhibitors.

KEY WORDS: Amino acid sequence; apricot; disulfide bridge; invertase inhibitor; pectin methylesterase inhibitor.

INTRODUCTION

Many carbohydrate-modifying enzymes in plants are modulated by inhibitory proteins. Pectin methylesterase (PME)⁴ is one of the first enzymes acting on pectin, and is involved in many physiological processes requiring remodelling of the plant cell wall. A proteinaceous inhibitor of pectin methylesterase (PMEI) was isolated from ripe kiwi (*Actinidia chinensis*) fruit and purified to homogeneity (Balestrieri *et al.*, 1990; Giovane *et al.*, 1995). It is active against plant PME, irrespective of the plant source assayed, whereas it is inactive against microbial PMEs. The inhibitor binds tomato PME with high affinity, forming a 1:1 non-covalent complex. The specificity toward plant PME suggests a physiological role of the inhibitor in the regulation of enzyme activity,

probably related to the fruit ripening process. However, the protein has been detected until now only in kiwi fruit.

The amino acid sequence of kiwi fruit PMEI reveals homology with different inhibitory proteins, namely inhibitors of invertase (both apoplasmic and vacuolar; Camardella *et al.*, 2000; Greiner *et al.*, 1998, 1999, 2000). Cell wall and vacuolar invertases play central roles in the sucrose metabolism of higher plants (Sturm, 1999, and literature cited therein). Sucrose and the resulting hexoses, glucose and fructose, released by invertase activity, are not only important carbohydrate metabolites but may also serve as metabolic signals affecting gene expression (Smeekens, 1998). Therefore, regulation of cell wall and/or vacuolar invertase activity is crucial for several developmental processes in plants, including defense against pathogens. Whereas induction of invertase activity is

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⁴ Abbreviations: CD, circular dichroism; HPLC, high-performance liquid chromatography; INH, invertase inhibitor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis; PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor.

usually mediated via increased transcription of the corresponding invertase gene, silencing of invertase activity appears to operate by post-translational regulation via inhibitory proteins (Pressey, 1966; Schwimmer *et al.*, 1961). Proteinaceous inhibitors of invertase (INHs) were found in different species (Isla *et al.*, 1991; Jaynes and Nelson, 1971; Matsushita and Uritani, 1976; Pressey, 1968; Weil *et al.*, 1994) and were purified from potato tubers (Bracho and Whitaker, 1990; Ovalle *et al.*, 1995), from tomato fruit (Pressey, 1994), and from tobacco cultured cells (Weil *et al.*, 1994). However, only recently the first *inh* cDNAs were cloned (Greiner *et al.*, 1998, 1999). Initially, the sequence analysis of the *Nicotiana tabacum* apoplasmic isoform *Nt-inh1* and the vacuolar isoform *Nt-inhh* revealed no homology to any other proteins of known function. Only after elucidation of the PMEI primary structure the relationship between the two different inhibitors was detected.

PMEI and INHs are monomeric proteins of similar size (the number of amino acid residues of mature proteins ranges between 143 and 152). The coding sequences of INHs were derived from the cDNAs, and were shown to contain signal peptides, which are cleaved off in the secretory pathway. The amino acid sequence of PMEI was obtained by direct sequencing of the mature protein and showed a ragged N-terminal end, with a partially removed N-terminal Ala residue (Camardella *et al.*, 2000). The identity level between PMEI and the *Nt-INH1* and *Nt-INHH* proteins are 30% and 26%, respectively. The most interesting observation is the conservation of four Cys residues, which in PMEI were shown to be connected by two disulfide bridges (first to second and third to fourth). Likewise, the presence of intramolecular disulfide bridges in INH was suggested by the inactivation of recombinant *Nt-INH1* protein after dithiothreitol treatment (Greiner *et al.*, 2000).

Here we report the purification of an INH protein from ripe apricot (*Prunus armeniaca*) fruit (Pa-INH) and its structural characterization comprising the determination of the disulfide bridges connectivity in the native protein. Pa-INH is active against tomato vacuolar invertase, and does not show any inhibitory activity toward PME.

MATERIALS AND METHODS

Reagents

Apricot and tomato fruit were purchased from local markets. Q-Sepharose was from Amersham-Pharmacia. SDS-PAGE reagents were from BioRad. HPLC grade acetonitrile was from Baker, trypsin from Boehringer, and dithiothreitol, Tris and 4-vinylpyridine from Sigma.

Sequencer grade reagents were from Applied Biosystems. All other reagents were of the highest commercially available quality.

Purification of Pa-INH Protein

Ripe apricot fruit were homogenized in water (1:1, mass/vol) and centrifuged at $13000 \times g$ for 20 min. The pH of the supernatant (3.8) was brought to 8.0 by adding NaOH. After addition of ammonium sulfate at 80% saturation and centrifugation at $13000 \times g$, the proteins were recovered in the pellet, redissolved in 10 mM Tris-Cl, pH 7.5 and dialyzed against the same buffer. The solution was then loaded on a Q-Sepharose column equilibrated in the same buffer. Elution was carried out by a linear gradient from 0 to 0.5 M NaCl in the equilibration buffer, following the absorbance at 280 nm. Aliquots of fractions collected at NaCl concentration around 0.25 M were analyzed by SDS-PAGE. Fractions containing invertase inhibitory activity were further purified by reverse-phase HPLC on a Vydac C₄ column (4.6×250 mm), using a System Gold apparatus (Beckman Instruments). Elution was carried out by a linear gradient of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. Collected fractions were diluted with water and concentrated under vacuum without complete drying, to remove acetonitrile and trifluoroacetic acid. The fraction containing INH activity was pure upon amino acid sequencing. Protein concentration was determined by the BIO-RAD protein assay.

Invertase and Invertase Inhibitor Activity

Invertase activity was assayed by measuring the amount of glucose produced by sucrose hydrolysis. 50 μ L aliquots of crude tomato extract (obtained by homogenization of ripe tomatoes and centrifugation of the cell wall insoluble fraction), used as source of invertase activity, were added to 100 μ L of 0.2 M sodium acetate buffer, pH 5.0. Reaction was started by the addition of 100 μ L of 0.5 M sucrose and incubation at 37°C. After 30 min, 30 μ L of incubation mixture were added to 100 μ L of 0.5 M potassium phosphate buffer, pH 7.0, to slow down the reaction. The amount of glucose produced was measured by the glucose oxidase-peroxidase method (Glu-cinet, Sclavo diagnostics, Kite Italia S.p.A.). To assay inhibition of invertase activity, the invertase sample was preincubated, before the addition of sucrose, with 20–50 μ L aliquots of fractions containing the invertase inhibitor, at 37°C for 15 min. A unit of

inhibitor is defined as that amount inhibiting one unit of invertase to 50% at pH 5.0 (Pressey, 1994).

Circular Dichroism Measurements

CD spectroscopy was performed on a J-710 spectropolarimeter (Jasco), equipped with the Neslab RTE-110 temperature controlled liquid system (Neslab Instruments) and calibrated with a standard solution of (+)-10-camphorsulfonic acid. Photo-multiplier high voltage did not exceed 600 V in the spectral region measured. Each spectrum was averaged five times and smoothed with Spectropolarimeter System Software version 1.00 (Jasco). All measurements were performed under nitrogen flow. The cell path was 1 cm. The protein concentration was approximately 10 $\mu\text{g/ml}$.

Reverse-Phase HPLC of Native and Denatured Invertase Inhibitor

The protein was subjected to reverse-phase HPLC on a Vydac C₄ column (4.6 $\times$ 250 mm), using a System Gold apparatus (Beckman Instruments) in native conditions and after denaturation and treatment with 4-vinylpyridine with or without previous reduction. Elution was carried out by a linear gradient of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm.

Amino Acid Sequencing

Denaturation and alkylation of the sulfhydryl groups with 4-vinylpyridine was carried out as described (Camardella *et al.*, 1995). Two 50 μg protein aliquots (one native and a second one denatured, reduced, and alkylated) were subjected to tryptic cleavage. Both aliquots were dissolved in 0.1 M Tris-Cl, pH 8.5, 10% acetonitrile and incubated at 37°C for 2 hr after addition of trypsin (1:20, w/w). The resulting peptides were separated by reverse-phase HPLC on a Vydac C₄ column (4.6 $\times$ 250 mm), using a Beckman System Gold apparatus. Elution was accomplished by a multistep linear gradient of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The separated fragments were manually collected and sequenced. Automated repetitive Edman degradation was performed on a ProciseTM Protein Sequencer model 492 (Applied Biosystems Division).

Mass Spectrometry

The molecular mass of peptides was measured by MALDI-TOF mass spectrometry on a Voyager-DE Biospectrometry Workstation (PerSeptive Biosystems). Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration 5 pmol/ μl) in 4 volumes of matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid) in 60% acetonitrile containing 0.3% trifluoroacetic acid.

RESULTS AND DISCUSSION

Purification and Functional Identification of Pa-INH, an Invertase Inhibitor from Apricot Fruit

Initially, fruits from several plant species were analyzed to detect the presence of PME inhibitors. The PME inhibition assay performed on the tissue soluble extracts gave negative results except in apricot fruit, where it yielded ambiguous results. Therefore, we decided to proceed with protein purification, following the same procedure used for the purification of PMEI from kiwi fruit. The proteins contained in the aqueous extract of ripe fruit were precipitated by ammonium sulfate and the resulting pellet was resuspended in 10 mM Tris-Cl buffer at pH 7.5 and subjected to anion exchange chromatography on a Q-Sepharose column. Aliquots of the fractions eluting at the same ionic strength as kiwi fruit PMEI (around 0.25 M NaCl) were analyzed by SDS-PAGE (Fig. 1), showing the presence of a band with molecular mass

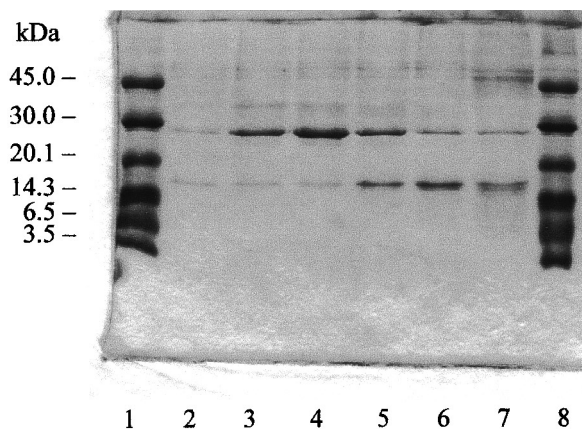


Fig. 1. SDS-PAGE (15% acrylamide) of fractions eluted from Q-Sepharose chromatography of the soluble extract from apricot fruit. Lanes 1 and 8 are molecular weight markers. Lanes 2-7 are fractions sequentially eluted by the NaCl gradient at a concentration around 0.25 M. Aliquots were precipitated with 10% TCA, and the pellets were dissolved in Laemmli sample buffer. The electrophoresis run was performed at constant 25 mAmps, at room temperature.

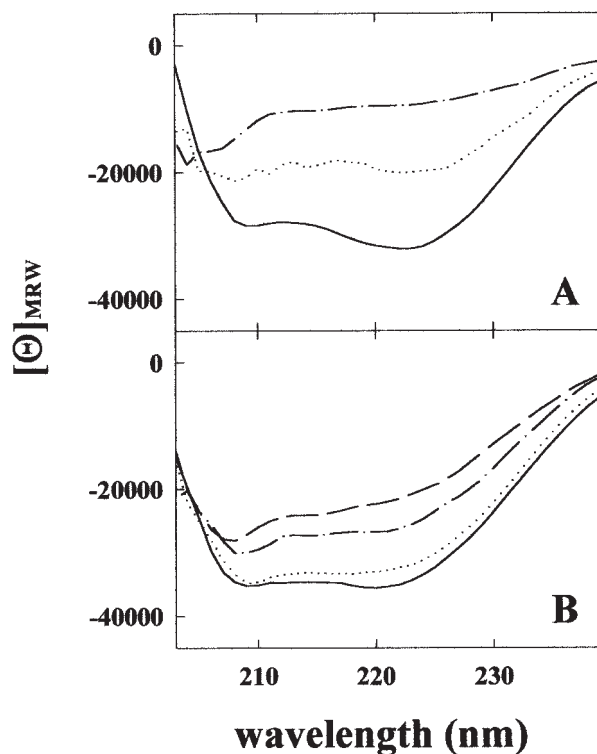


Fig. 2. Circular dichroism spectra of PMEI (A) and Pa-INH (B). Continuous line: 25°, 35°, and 45°C; dotted line: 55°C; dashed-dotted line: 65°C; dashed line: 75°C. Each spectrum was averaged five times and smoothed with Spectropolarimeter System Software version 1.00 (Jasco). All measurements were performed under nitrogen flow. The cell path was 1 cm. The protein concentration was approximately 10 $\mu\text{g/ml}$, in 10 mM Tris-Cl pH 7.5. $[\Theta]_{\text{MRW}}$, mean residue ellipticity ($\text{degree} \times \text{cm}^2 \times \text{dmol}^{-1}$).

close to 16,000 Da, similar to that of kiwi fruit PMEI. The band blotted from the gel was subjected to amino acid sequence analysis. The Edman degradation proceeded for 23 cycles, and no residues were detected at position 9 and 18. The protein was further purified by reverse-phase HPLC of the fractions from Q-Sepharose chromatography, reduced and alkylated and subjected again to a run of cyclic Edman degradation. Two Cys residues were detected at the previous blank positions (see Fig. 6). The N-terminal sequence revealed similarity with both kiwi fruit PMEI and tobacco INHs. The percent identity in this region was 35% with kiwi fruit PMEI, and 43%–61% with tobacco INHs. Furthermore, assays of enzyme inhibition performed on the fractions containing the 16,000 Da protein indicated the presence of invertase inhibitory activity, and absence of PME inhibitory activity. Thus, the search for a new PMEI led to the purification of an INH from apricot fruit with no PMEI activity. The exclusive target specificity of PMEI and INH proteins has been independently confirmed with

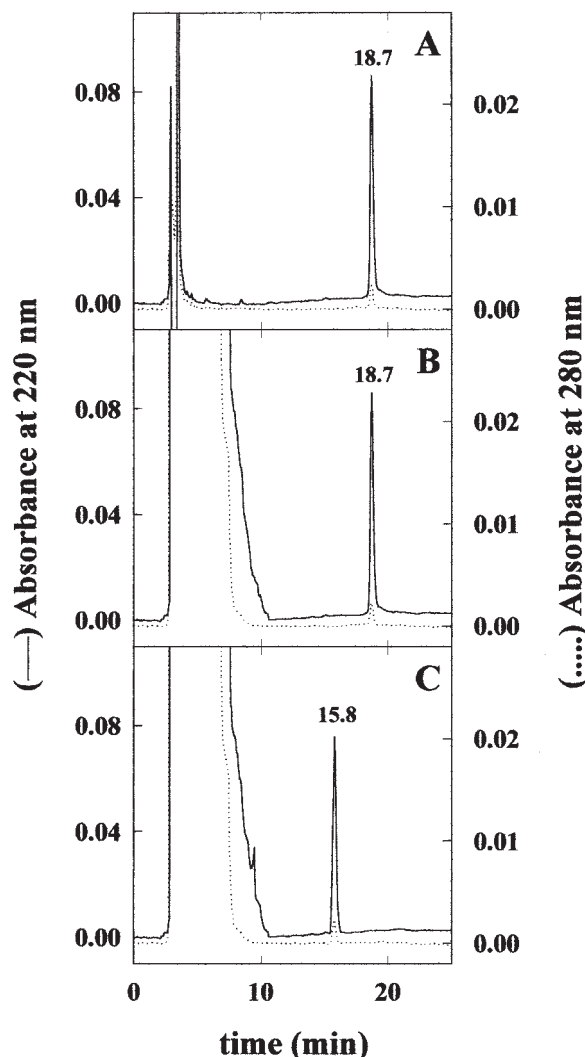


Fig. 3. HPLC on Vydac C₄ column (4.6 $\times$ 250 mm) of Pa-INH. A, native protein; B, protein denatured and treated with 4-vinylpyridine without previous reduction; C, protein treated with 4-vinylpyridine after denaturation and reduction. Elution was carried out by a linear gradient from 40% to 60% of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid) in 40 min, at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm.

tobacco INH and kiwi fruit PMEI, using different PME and invertase preparations (S. Grsic-Rausch and L. Camardella, unpublished).

The purified protein maintained the inhibitory activity, in spite of the HPLC conditions used, indicating that it is resistant to strong acidic environment and high organic solvent concentration. The protein had a specific activity of 20 U/mg when assayed toward soluble (vacuolar) invertase from tomato fruit. The molecular weight calculated by SDS-PAGE was 16,500 Da. The

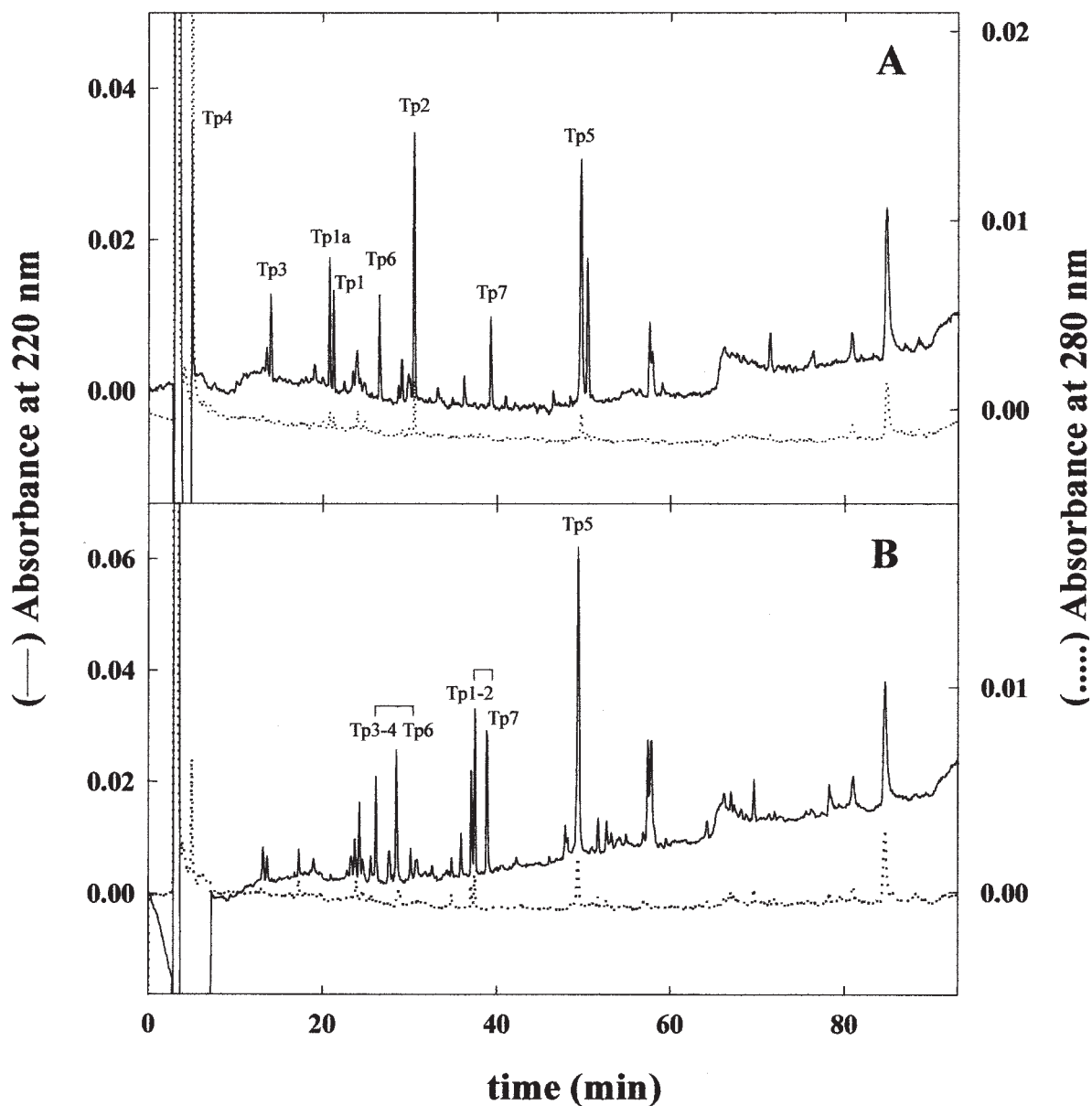


Fig. 4. HPLC on Vydac C₄ column (4.6 × 250 mm) of tryptic peptides of Pa-INH digested after denaturation, reduction and S-pyridylethylation (A) or in the native state (B). Elution was carried out by a multistep gradient, from 5% to 15% in 10 min, from 15% to 50% in 70 min, and from 50% to 60% in 10 min, of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid). Flow rate was 1 ml/min. The eluate was monitored at 220 and 280 nm.

apparent molecular weight of the native protein measured by gel filtration on a Superdex 75 column (Amersham-Pharmacia) was close to 20,000 Da, similar to that of PME1 from kiwi fruit.

Pa-INH was isolated from the aqueous extract of fruit tissue, therefore it is presumably a vacuolar isoform, and in fact is active against tomato fruit invertase, which was also found in the soluble fraction of the fruit. However,

this assumption requires independent confirmation, as the apoplasmic tobacco invertase inhibitor also inhibits vacuolar invertase from tomato fruit (Greiner *et al.*, 1998).

Circular Dichroism Spectra and Thermostability

Far UV circular dichroism spectra of both PME1 and Pa-INH showed a typical shape with two minima at 208

Tp1	DANLIDQTCK
Tp1a	ANLIDQTCK
TP2	QTPNYALCVSSLK
Tp1-2	DANLIDQTCKQTPNYALCVSSLK
Tp3	QRPGNQALK
Tp4	SCAR
Tp3-4	QRPGNQALKSCAR
Tp5	FYDTVVEALVPEAQGGFSLGXPX
Tp6	FAEQGMNDAAGEAETCK
Tp7	AVHDIAAVAAAIAR

Fig. 5. Sequenced tryptic peptides of native and S-pyridylethylated Pa-INH.

and 222 nm, indicating that they both have a mainly α -helical secondary structure (Fig. 2). Spectra recorded at increasing temperatures showed that both Pa-INH and kiwi fruit PMEI completely maintained their secondary structure up to 45°C (Fig. 2). Above this temperature, both proteins began to lose secondary structure, and kiwi fruit PMEI appeared completely unfolded at 65°C, whereas Pa-INH still retained some structure at 75°C, showing a

higher stability. Both proteins thus appeared quite thermostable, as their structure is stabilized by disulfide bridges.

Partial Amino Acid Sequence of Pa-INH and Identification of Disulfide Bridges

N-terminal amino acid sequence of pure protein yielded a unique sequence, however approximately 30% of the protein showed an additional Asp residue at the N-terminus. This finding may indicate that the protein is synthesized as a precursor, which after proteolytic processing maintains a partially removed N-terminal amino acid. The presence of signal peptides in INHs has been shown by analysis of the corresponding cDNAs (Greiner *et al.*, 1998, 1999). The PMEI from kiwi fruit also shows a ragged N-terminus due to the presence of a protein fraction containing an additional Ala residue.

Reverse phase HPLC analysis of the native protein and of the protein treated with 4-vinylpyridine without previous reduction showed that in both cases the protein was eluted at the same retention time, suggesting the absence of free Cys residues (Fig. 3A, B). Conversely, the elution time of the protein treated with 4-vinylpyridine after reduction in denaturing conditions was anticipated of approximately

	1	25
PMEI	-----ENH--LISEI	CPKTRNPSICLQAL
Nt-INH1	MKNLIFLTMTFLT-----ILLQTNANN--LVETTC	KNTPNYQLCLKTL
Le-INH	MKILIFLIMFLA-----MLLVTSGNN--LVETTC	KNTPNYLCVKTSLDKRSEK--AGDITT
Ib-INH	MKSLFTAMMLISSVLNGGKIGNTYSSDDDGSSNGGLINTAC	NNTPNYALCVSVLASDPR
Nt-INHH	MRNLFPIFMLIT-----NLAFNDDNNNSNN--IINTTC	RATTNYPCLCLTLHSDPRTSEAGADLTT
Pa-INH	DAN--LIDQTCKQTPNYALCVSSLK	
	50	75
PMEI	LGQFSIDIAQASAKQTSKIIASLTNQATDPKLGKRYET	CSENYADAIDS-LGQAKQFLTSGDYN
Nt-INH1	LALIMVDAIKAKANQAAVTISKLRHSNPPAAWKGPLKNC	AFSYKVILTASLPEAIEALTKGDPKFAEDGMVGSSG
Le-INH	LALIMVDAIKSKANQAANTISKLRHSNPPQAWKDPLKNC	AFSYKVILPASMPEALEALTKGDPKFAEDGMVGSSG
Ib-INH	LGLVMVDAVKAKAEEMIETIRELEKSKPV-EWRLPLSQ	YIYYAVVHADVPAAALKRGVVPKFAEDGMADA
Nt-INHH	LGLVMVDAVKLKSIEIMKSIKKLEKSNP--ELRLPLSQ	YIYYAVLHADVTVAEALKRGVVPKFAENGMDVAV
Pa-INH	QRPGNQALKSCARFYDTVVEALVPEAQGGFSLGXPX	FAEQGMNDAAG
	125	150
PMEI	GAGTCEDSFEGPP-----NIPTQLHQADLKLEDLC	DIVLVISNLLPGS
Nt-INH1	DAQECEEFKGS-----KSPFSALNIAVHELSDVGR	AIVRNLL--
Le-INH	DAQECEEFKATT-----IKYSPLSKLNIDVHELSDVGR	AIVRNLL--
Ib-INH	EAECEAAFKLQNEIDIIDYSGSAIDETNKDVIQLSA	VATSIKMLL--
Nt-INHH	EAECEFSFKYNG-----LVSPVSDMNKEI	IELSSVAKSIIRMLL--
Pa-INH	EAEETCK	AVHDIAAVAAAIAR

Fig. 6. Alignment of sequenced peptides of Pa-INH with kiwi fruit PMEI and INHs from *Nicotiana tabacum* (the apoplasmic Nt-INH1 and the vacuolar Nt-INHH isoforms), from *Lycopersicon esculentum* (Le-INH) and *Ipomoea batatas* (Ib-INH). The sequence accession numbers were P83326, O49908, AAN60076, O82001, and Q8LJU6, respectively. Alignment was performed with BLAST at ExPASy Molecular Biology Server. Conserved Cys residues are black-shadowed. Residues conserved in sequenced peptides of Pa-INH, in kiwi fruit PMEI and INH sequences are grey-shadowed. Black diamond indicates the free Cys residue found only in PMEI.

3 min, thus indicating that S-pyridylethylation occurred at Cys residues, which in these conditions became available to the alkylating agent (Fig. 3C).

Peptides obtained after trypsin digestion of the denatured, reduced, and alkylated protein (Fig. 4A) and of the native protein (Fig. 4B) were separated by reverse-phase HPLC. Sequenced peptides are reported in Fig. 5 and the corresponding peaks are indicated in Fig. 4A and B. In the denatured protein Tp3, Tp4, and Tp6 were eluted at different retention times (Fig. 4A). Cys residues were identified at positions 2 of Tp4 and 16 of Tp6. In the native protein a single peak was shown to contain peptide Tp3-4 (due to failure of cleavage by trypsin at Lys of Tp3) and Tp6 (Fig. 4B). A double sequence was obtained by amino acid sequence analysis, with no signal at positions corresponding to Cys residues, as identified in peptides derived from digestion of reduced and alkylated protein. Moreover, a single mass value (3,196 Da) was detected, corresponding to the sum of Tp3-4 and Tp6. Therefore, a disulfide bridge connecting the Cys residues of peptides Tp4 and Tp6 could be assigned. As shown in the alignment of Fig. 6, these Cys residues correspond to those conserved among kiwi fruit PMEI and INHs and that in PMEI are in sequence positions number 74 and 114.

In the denatured protein, peptides Tp1 and Tp2 (belonging to the N-terminal region) were eluted in different positions, and Cys residues at positions 9 and 18 (numbering from the N-terminal Asp residue) were detected. In the native protein trypsin failed to cleave at Lys10, thus generating peptide Tp1-2. Amino acid sequence of this fragment yielded a single sequence, which proceeded up to residue 23, and blank cycles were detected at positions where Cys residues were previously identified. A single mass value of 2,522 Da was detected for this peptide. Attempts to redigest more extensively the peptide with trypsin were unsuccessful. The native protein was then treated with 4-vinylpyridine without previous reduction (to selectively modify only free Cys residues) and analyzed by N-terminal sequencing. Again, blank cycles were observed at positions 9 and 18, thus indicating that these Cys residues are not available to the alkylating agent because they are linked by a disulfide bridge in the native protein. The observed lack of cleavage by trypsin at Lys10 in the native protein could be ascribed to the presence of the contiguous disulfide bridge, which may cause steric hindrance to trypsin action.

Peptide Tp7 was assigned to the C-terminal region by alignment with kiwi fruit PMEI and various INHs from tobacco, tomato, and sweet potato (Fig. 6). Interestingly, this region does not contain the Cys residue present at position 139 in PMEI, similarly to other INHs. A Ser residue is present in most INHs, as shown in

Fig. 6, whereas the protein described here shows Ala residue. The functional importance of this sequence position will be object of future investigation.

In conclusion, the reported data show that PMEI and INHs, although having different function and distinct target enzymes, not only have similarity of primary and secondary structure, but also share the same pattern of disulfide bridges. This finding is strongly indicative of a common structural arrangement of the members of this protein family, which will be finally elucidated by crystallographic studies, which are currently under way. The recent completion of the *Arabidopsis thaliana* genome sequence has revealed a larger gene family with only moderate protein sequence conservation, some members being related more closely with tobacco INH proteins while others group with the kiwi fruit PMEI (S. Greiner, personal communication). The subtle structural differences responsible of the target enzyme specificity remain to be elucidated.

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