

Kiwi protein inhibitor of pectin methylesterase

Amino-acid sequence and structural importance of two disulfide bridges

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A protein acting as a powerful inhibitor of plant pectin methylesterase was isolated from kiwi (*Actinidia chinensis*) fruit. The complete amino-acid sequence of the pectin methylesterase inhibitor (PMEI) was determined by direct protein analysis. The sequence comprises 152 amino-acid residues, accounting for a molecular mass of 16 277 Da. The far-UV CD spectrum indicated a predominant alpha-helix conformation in the secondary structure. The protein has five cysteine residues but neither tryptophan nor methionine. Analysis of fragments obtained after digestion of the protein alkylated without previous reduction identified two disulfide bridges connecting Cys9 with Cys18, and Cys74 with Cys114; Cys140 bears a free thiol group. A database search pointed out a similarity between PMEI and plant invertase inhibitors. In particular, the four Cys residues, which in PMEI are involved in the disulfide bridges, are conserved. This allows us to infer that also in the homologous proteins, whose primary structure was deduced only by cDNA sequencing, those cysteine residues are engaged in two disulfide bridges, and constitute a common structural motif. The comparison of the sequence of these inhibitors confirms the existence of a novel class of proteins with moderate but significant sequence conservation, comprising plant proteins acting as inhibitors of sugar metabolism enzymes, and probably involved in various steps of plant development.

Keywords: pectin methylesterase inhibitor; invertase inhibitor; disulfide bridges; primary structure; *Actinidia chinensis*.

The plant enzyme pectin methylesterase (PME) hydrolyzes the pectin methylester groups producing pectin with a lower methylation degree and methanol. The enzyme plays a central role in all processes requiring remodeling of plant cell wall being involved in the cell extension and growth [1–3] and in the fruit ripening process [4–6]. Recently it has been reported that PME regulates methanol and ethanol levels in ripening tomato fruit [7]. We discovered in ripe kiwi (*Actinidia chinensis*) fruit a protein acting as powerful inhibitor of PME of plant origin, which inhibits the enzyme through the formation of a noncovalent 1 : 1 complex [8,9]. Pectin methylesterase inhibitor (PMEI), which has been detected only in kiwi fruit, is probably synthesized as a larger protein that is successively processed to a smaller one [9]. Unlike PME, PMEI is not tightly bound to cell walls and can be extracted easily from the ripe fruits at low ionic strength, allowing a simple separation from the endogenous PME, which can be extracted only by more concentrated salt solutions. A role for PMEI in the fruit defense mechanism against pathogens can be hypothesized, since polysaccharide degrading enzymes are produced by many microorganisms, and it is well known in plants the presence of proteins inhibiting the microbial polygalacturonase [10]. However, at present the inhibitor seems to be active only

when the PME is of plant origin; therefore a physiological role, possibly in the regulation of the fruit ripening process, can be hypothesized.

The kiwi protein inhibitor was proven to be specific for plant PME independently of the plant source assayed [8]. This raises a potential interest of the inhibitor in the technology of fruit products. Actually, PME is responsible for many commercially deleterious effects, such as loss of consistency in solid products and diminution of viscosity and turbidity in fruit juices, ultimately resulting in the phase separation (cloud-loss) of the juice, due to the precipitation of PME-hydrolyzed pectins as calcium pectate. Therefore, in order to inactivate PME, many plant products are subjected to thermal treatments that are harsher than those needed for pasteurization, with consequent loss of flavors and nutrients. We have recently demonstrated in some commercial fruit juices, showing the phenomenon of cloud-loss, the presence of PME activity, which evidently had survived the pasteurization treatment [11–13]. On the other hand, we have also shown that the addition of PMEI to fresh, nonpasteurized orange juices preserved the cloud stability [14]. This makes PMEI very attractive as a technological adjuvant in the fruit juice stabilization.

Therefore, in order to undertake overexpression of recombinant protein by molecular cloning techniques, we determined the protein primary structure. A search for homology has identified a relationship with plant invertase inhibitors, proteins which are likely to play a regulatory role in plant development [15]. In addition, a structural study of PMEI has identified the presence of two disulfide bridges which stabilize the protein structure, in which the α -helix secondary structure is prevalent.

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Abbreviations: PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; INH, invertase inhibitor.

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EXPERIMENTAL PROCEDURES

PMEI purification

The inhibitor was purified from fully ripened kiwi fruit as described [9]. The activity at pH 7.5 was determined as the capacity to block PME activity in a continuous spectrophotometric assay according to Hagerman and Austin [16]. The concentration of purified inhibitor was estimated spectrophotometrically by utilizing a molar extinction coefficient at 275.5 nm of $5960 \text{ M}^{-1}\cdot\text{cm}^{-1}$, based on tyrosyl residue content of four moles per mole of protein, and assuming for each tyrosyl residue the molar extinction coefficient of *N*-acetyl-L-tyrosinamide in water, i.e. $1490 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 275.5 nm [17]. Inhibitory activity on tomato invertase was assayed according to Pressey [18].

Protein alkylation

The purified protein was alkylated with iodoacetamide under denaturing conditions, with and without previous reduction. The protein (50 μg) was dissolved in 250 μL of 0.1 M Tris/HCl pH 8.0, 10 mM EDTA, 8 M guanidine hydrochloride. Dithiothreitol was added at a final concentration of 0.05 M and incubation was carried out at 37 °C for 30 min under nitrogen (this step was omitted in the sample alkylated without previous reduction). Iodoacetamide was added at a final concentration of 0.12 M and the reaction proceeded at room temperature for 15 min. The reaction was stopped by addition of 0.2 M 2-mercaptoethanol, and the sample was desalted by gel filtration on a prepacked PD-10 column (Pharmacia, Uppsala, Sweden). For amino-acid analysis, vapor phase hydrolysis was carried out with 6 M HCl containing 1% 2-mercaptoethanol and 1% phenol at 150 °C for 1 h. The analyses were performed as reported [19].

Primary structure determination

The lyophilized protein was resuspended in denaturing buffer containing 0.5 M Tris/HCl pH 7.8, 2 mM EDTA, 6 M guanidine hydrochloride. Dithiothreitol was added at a fivefold molar excess over cysteine residues and the solution was incubated at 30 °C under nitrogen for 3 h (this step was omitted in the sample alkylated without previous reduction). 4-Vinylpyridine (100-fold molar excess over cysteine residues) was added and the reaction proceeded at room temperature in the dark under nitrogen for 45 min. The protein was immediately desalted by gel filtration on a prepacked PD-10 column equilibrated with 0.1% trifluoroacetic acid.

Cleavage of the Asp-Pro bonds was performed on a protein aliquot (10 μg) in 70% formic acid at 42 °C for 20 h. The sample was applied to the sequencer: a first cycle with *o*-phthalaldehyde abolished all sequences starting with a primary amino group, which meant that only peptides starting with proline were sequenced [20]. An aliquot (0.2 mg) of the alkylated protein was digested with trypsin at 37 °C for 4 h in 1% ammonium bicarbonate (trypsin/protein ratio 1 : 50, w/w). A second aliquot was digested at 37 °C for 6 h with 2 μg of Asp-N endoproteinase in 250 μL of 50 mM sodium phosphate buffer pH 8.0, containing 10% acetonitrile. A third aliquot was digested at 37 °C for 6 h with 5 μg of endoproteinase Lys-C in 250 μL of 25 mM Tris/HCl buffer pH 8.5, 1 mM EDTA, 10% acetonitrile.

Peptides were purified by reverse phase HPLC on a Vydac C4 column ($4.6 \times 250 \text{ mm}$, 5 μm), using a Beckman system Gold apparatus. Elution was accomplished by a linear gradient of solvent B (0.08% trifluoroacetic acid in acetonitrile) in solvent A (0.1% trifluoroacetic acid) at a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. The eluate was monitored at 220 and 280 nm; the peaks were collected and sequenced.

Automated repetitive Edman degradation was performed on a Procise Protein Sequencer model 492, equipped with a 140C Microgradient System (PerkinElmer, Applied Biosystems Division). The total amino-acid sequence was reconstructed by peptide overlapping. Alignment of amino-acid sequences was obtained using the PILEUP and PRETTYPLLOT programs of the Extended GCG Wisconsin package.

Circular dichroism measurements

CD spectroscopy was performed at 20 °C on a J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with the Neslab RTE-110 temperature controlled liquid system (Neslab Instruments, Portsmouth, NH, USA) and calibrated with a standard solution of (+)-10-camphorsulfonic acid. Photomultiplier 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 percentages of secondary structures were estimated according to the Yang's method [21].

RESULTS

Amino-acid sequence

The complete amino-acid sequence of PME1 was obtained by combining direct N-terminal sequencing of the entire protein, sequencing of peptides derived from acid cleavage of the two Asp-Pro bonds, and sets of peptides derived from trypsin, Asp-N and Lys-C endoproteinase digestions (Fig. 1).

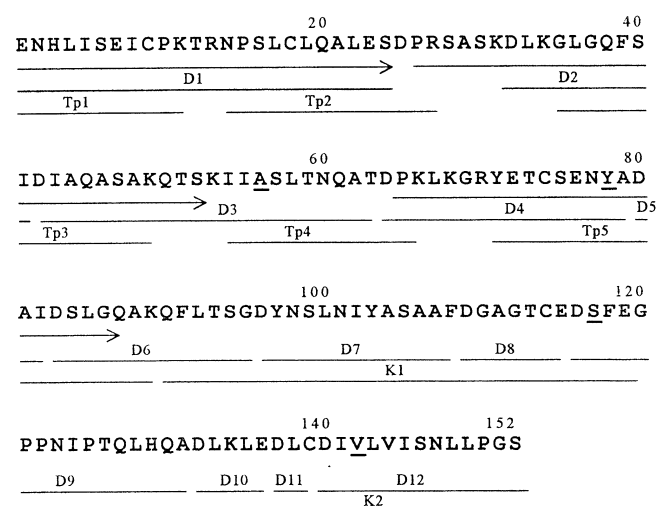


Fig. 1. Amino-acid sequence of PME1. The arrows indicate N-terminal sequence and sequences elucidated after acid cleavage of Asp-Pro bonds. The peptides necessary to reconstruct the sequence are indicated as Tp (trypsin), D (Asp-N), K (Lys-C) and numbered according to their order in the sequence. At the underlined positions 56, 78, 117 and 142, minor amounts of Ser, Phe, Asn and Ile were detected in addition to the principal amino-acid residues.

at the positions of Cys74 and Cys114. Therefore it was concluded that the two disulfide bonds were formed between Cys9 and Cys18, and between Cys74 and Cys114.

Sequence alignment

A comprehensive database search revealed homology of PMEI with invertase inhibitors (INH) from *Nicotiana tabacum* [15] and *Lycopersicon esculentum* [25], and with the related sequence deduced from the full length cDNA clone of *Arabidopsis thaliana* (accession no. Y12807), previously called invertase inhibitor homolog [15]. The alignment is shown in Fig. 3. The sequences of INHs and INH homolog are derived from full length cDNA clones, and show putative signal peptides at the N-terminal ends. The predicted N-terminus of the tobacco INH is identical to that of the mature protein isolated from tobacco cultured cells [15] and corresponds to that determined for INH purified from tomato fruit [18]. The identity level among the sequences is in the range 22–30%, except between the sequences of the proteins from tomato and tobacco (81%), which were identified as two authentic invertase inhibitors. It is noteworthy that the four Cys residues conserved in all sequences correspond to those involved in the two disulfide bridges in PMEI. Instead, the free Cys residue of the kiwi inhibitor is replaced by Ser in the two invertase inhibitors, whereas the sequence of the putative protein from *A. thaliana*, whose actual function is not known, presents a Cys residue. PMEI was tested for inhibitory activity on tomato invertase both at pH 4.5 and pH 6.0, and no inhibition was observed.

DISCUSSION

This paper reports the complete primary structure of PME protein inhibitor isolated from kiwi fruit. It is known that plants have evolved systems to modulate, in a tissue-specific and/or temporal manner, the activity of enzymes involved in carbohydrate metabolism. In addition to the well-known inhibitor of α -amylase from cereals, proteinaceous inhibitors of polygalacturonase, invertase, and xylanase have been reported from plants [10,15,26]. By contrast, the inhibitor of plant PME has been detected only in kiwi fruit. The possibility cannot be excluded that PMEI is transiently expressed in other tissues and plants also, and that it plays a more general physiological role as regulator of PME activity. Furthermore, a novel function has been recently attributed to plant PME: it has been shown that the enzyme acts as host-cell receptor for the tobacco mosaic virus movement protein, and that this interaction is required for viral cell-to-cell movement [27,28]. This finding suggests a possible role for PMEI in the plant defense mechanisms against plant pathogens.

The N-terminal PMEI sequence was previously determined [29], and utilized to design oligonucleotides, but any attempt to isolate the corresponding gene by analysis of a kiwi fruit cDNA library has been unsuccessful. The reason for this could reside in the well-known difficulty of obtaining RNA in good yield from fruit tissues, due to the abundance of carbohydrates and other compounds which may interfere with RNA purification. The availability of a source expressing a suitable amount of inhibitor, and a rapid procedure for the protein purification, have allowed determination of the primary structure by classical protein chemistry methods. In addition, it has been possible, by elucidating some structural aspects of the protein, i.e. the disulfide connectivities, to infer the same organization in homologous proteins whose sequence has only been deduced from DNA analysis.

The amino-acid sequence of PMEI presents some heterogeneity. The detection of different amino-acid residues at well-defined positions along the polypeptide chain can be attributed to the presence of multiple copies of the fruit gene. On the other hand, the presence of two forms, having or lacking the initial Ala residue, can be attributed to an incomplete processing of the protein. This finding points toward the probable existence of a signal sequence, which is cleaved by a proteolytic agent during the maturation of the protein, and is in line with the observation that PMEI is synthesized as an inactive precursor of higher molecular mass, subsequently transformed into the active protein during the fruit ripening process [9]. Considering that PMEI interacts with pectin methylesterase, which is localized in the cell wall, it is probable that a signal sequence is required to direct the protein toward its final cellular localization. In fact, preliminary experiments of tissue immunological staining indicate that PMEI is concentrated in a layer close to the cell membrane. The amino-acid sequence alignment identifies homology with members of a family of proteins, comprising invertase inhibitors and a putative protein deduced from a cDNA sequence of *A. thaliana*, named invertase inhibitor homolog [15]. At present, only the proteins encoded by the cDNAs of *N. tabacum* and *L. esculentum* have been characterized as true invertase inhibitors, whereas the function of the other member of the family is yet unknown. The overall amino-acid residue identity is high when comparing the sequences of the tobacco and tomato proteins, whereas it is low when comparing both invertase inhibitors with the homolog from *A. thaliana* and PMEI from kiwi. In this case the identity is restricted to few positions but, most interestingly, it includes the four Cys residues. The striking conservation of these Cys residues leads us to infer that also in those proteins, whose primary structures have been deduced from cDNA sequence, they are involved in disulfide bridges, as hypothesized by Greiner *et al.* [15]. We have now demonstrated by direct amino-acid sequence that the four conserved Cys residues present in PMEI are actually involved in two disulfide bonds and play a considerable role in the maintenance of the protein structure. PMEI polypeptide chain appears to have mainly α -helix secondary structure, as shown by CD spectral analysis. Secondary structure predictions, on the basis of the amino-acid sequence, indicate a similar structure for the homologous proteins. The lack of knowledge of three-dimensional structure for any homologous protein prevented the construction of a molecular model by homology modeling. Cloning of the gene and expression of recombinant protein will hopefully allow determination of the three-dimensional structure by X-ray crystallography or NMR, and study of the molecular interaction with the target enzymes.

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