

Influence of pH on the Structure and Function of Kiwi Pectin Methylesterase Inhibitor

Alessandro Bonavita,[†] Vitale Carratore,[†] Maria Antonietta Ciardiello,[†] Alfonso Giovane,[‡] Luigi Servillo,[‡] and Rossana D'Avino^{*,†}

[†]Institute of Biosciences and BioResources, C.N.R., Napoli, Italy

[‡]Department of Biochemistry, Biophysics and General Pathology, Second University of Napoli, Napoli, Italy

ABSTRACT: Pectin methylesterase is a pectin modifying enzyme that plays a key role in plant physiology. It is also an important quality-related enzyme in plant-based food products. The pectin methylesterase inhibitor (PMEI) from kiwifruit inhibits this enzyme activity and is widely used as an efficient tool for research purposes and also recommended in the context of fruit and vegetable processing. Using several methodologies of protein biochemistry, including circular dichroism and fluorescence spectroscopy, chemical modifications, direct protein-sequencing, enzyme activity, and bioinformatics analysis of the crystal structure, this study demonstrates that conformational changes occur in kiwi PMEI by the pH rising over 6.0 bringing about structure loosening, exposure, and cleavage of a natively buried disulfide bond, unfolding and aggregation, ultimately determining the loss of ability of kiwi PMEI to bind and inhibit PME. pH-induced structural changes are prevented when PMEI is already engaged in complex or is in a solution of high ionic strength.

KEYWORDS: *Actinidia deliciosa*, pectin methylesterase, kiwi pectin methylesterase inhibitor (PMEI), pH-induced conformational changes, pH-dependent protein stability, protein–inhibitor interaction, thiol–disulfide reshuffling, unfolding

INTRODUCTION

Pectin methylesterase (PME, EC.3.1.1.11) is a cell-wall associated pectin modifying enzyme present in higher plants and in phytopathogenic microorganisms that catalyzes the demethylation of the pectic homogalacturonans, markedly affecting the cell wall mechanical properties.^{1,2} Ubiquitously expressed in plants, PMEs play a key role in many fundamental vegetative and reproductive plant processes^{3,4} and in plant–pathogen interaction.^{5,6} PME activity is likely to be regulated in plants through several factors, including the interaction with its specific protein inhibitor.⁷ Plant PMEs are also relevant as a biotechnological tool in plant-derived food products processing. PME activity may have both a favorable or deleterious role on the quality of the pectin-containing food products. It may contribute to the softening of the cell wall determining texture or viscosity loss in fruits and vegetables thus increasing the extraction yields of juices or may have the opposite effect of cell wall stiffening and gel formation thus reducing unwanted thermal texture loss but also inducing cloud destabilization of juices and gelation of concentrates.⁸ Pectin-related properties of plant-derived food products may be controlled via PME activation or inactivation by classical or new implemented processing technologies, such as thermal or high hydrostatic pressure processing⁹ and ultrasound application,¹⁰ the goal of the food industry being the preservation of the product characteristics often adversely modified by the processing conditions. Otherwise, the control of undesired PME activity in food may be obtained through enzyme inhibition by the action of PME natural inhibitors.^{7,11,12} Studies were performed to assess the biotechnological potential of the inhibitor from kiwi in the regulation of PME activity in plant-derived food products.^{13,14}

The pectin methylesterase inhibitor (PMEI) was first discovered in 1990 in ripe green kiwi fruit (*Actinidia deliciosa*)¹⁵ and later detected in many plant species.^{16–21} Further investigations demonstrate the involvement of PMEIs in growth and developmental processes,^{19–23} such as pollen tube growth, enhanced cell expansion, cell growth acceleration, fruit development and ripening, and in plant resistance against pathogens.^{17,24–26} PMEI coexpression with PME in *A. thaliana*,^{23,27} in tomato fruit²⁰ and in kiwi fruit²⁸ and their interaction²⁰ in planta were demonstrated.

The high affinity of the inhibitor for plant PME¹⁵ allowed the development of a PMEI purification procedure based on affinity chromatography on PME bound sepharose²⁹ that is widely adopted for research purposes. Although it seems expressed in several plants, the natural form of PMEI has been so far found in significant concentration only in kiwifruit²⁹ from which it is purified in sufficient amount to be used for research purposes. For instance, kiwi PMEI is commonly used as a powerful affinity chromatography support for analytical and laboratory-scale PME purification and for PME/PMEI binding/inhibition studies. Kiwi PMEI natural protein was used to obtain a PMEI-based molecular probe for in situ detection of plant PME activity³⁰ and anti-PME monoclonal antibodies.²⁸ To promote its application in food processing, recombinant kiwi PMEI was expressed in *E. coli*³¹ and more recently in *Pichia pastoris*.³²

PMEI belongs to a large protein family (PFAM 04043) comprising also invertase inhibitors (INH), PMEI domains of PME precursors, and proteins of unknown function.⁷ Kiwi

Received: April 15, 2016

Revised: June 20, 2016

Accepted: June 23, 2016

Published: June 23, 2016

PMEI is an acidic protein of 152 amino acids, including five Cys residues, four of which are connected by disulfide bridges, whereas the fifth one bears a free thiol group.³³ The crystal structures of tobacco INH³⁴ and kiwi PME³⁵ show that the two proteins equally fold into an up and down four α -helix bundle bearing a N-terminal protruding helical hairpin. They also have the same disulfide bridge arrangement that probably confers the same fold to the proteins of this family.^{7,36}

Disulfide bonds are among the most common motifs enhancing the stability of proteins. They are particularly important in secreted proteins that function in a fluctuating cellular environment, exposed to changing conditions such as pH and temperature.³⁷ Ionic interactions may also affect the protein stability. In particular, subtle environmental pH changes may have an important effect on the stability of partially buried ionic interaction. In fact, the involved ionizable groups can have a greatly lowered or raised pK_a value depending on factors such as the vicinity to other charged residues and the involvement in H-bonds. Consequently, changes in ionization of these groups might occur at a pH very distant from their pK_a value.³⁸ For instance, the loss of interaction of between kiwi PME and tomato PME, occurring at pH 8.5,³⁹ is suggested to be ascribed to the presence at the binding interface of two proton linked acidic residues undergoing deprotonation, and subsequent repulsion, at unusually high pH.³⁵

Kiwi PME forms with plant PMEs a noncovalent 1:1 complex.²⁹ The crystal structure of the tomato PME–kiwi PME complex shows that the inhibitor covers the putative active site cleft of the enzyme making a large number of intermolecular contacts and hydrogen bonds.³⁵ This interaction, however, rapidly decreases with increasing pH, the dissociation constant value of the binding (K_d) being 70-fold higher at pH 7.5 than at pH 6.0. No complex formation between kiwi PME and tomato PME is observed at pH 8.0¹³ and 8.5.³⁹ The inhibitory capacity of kiwi PME to banana, carrot, and strawberry PME is also reported to invariantly decrease at pH above 7.0.³⁹ Generally, the interaction of kiwi PME with PME from other sources is abolished at slightly alkaline pH value.^{13,14,39–41} In contrast, the complex formed by PME and PME from the same source, namely, from kiwi fruit, shows the highest stability to pH variations, the complete dissociation occurring only at pH 10.0. The presence of additional salt link interactions at the binding interface may explain the higher pH stability of the complex formed by the two kiwi proteins.⁴²

The significant interest that kiwi PME is attracting, due to its established exploitation for research purposes and recommendation for biotechnological applications in food industry, prompted this study focused on the effects of pH on the structure and function of this protein, purified from the natural source. Different methodologies, including circular dichroism and fluorescence spectroscopy to monitor the protein folding, classical biochemical methods to analyze the disulfide bridges modification, enzyme inhibition assays, and chromatographic analysis to detect the PME–PME complex formation and bioinformatics analysis of the available crystal structure to interpret the observed structural changes have been used in this study. In addition, a possible molecular mechanism sustaining the observed pH-induced structural changes is proposed.

MATERIALS AND METHODS

Materials. Green kiwi (*Actinidia deliciosa*) and tomato (*Solanum lycopersicon*) fruits were purchased from the local markets. Dithiothreitol (DTT), citrus pectin (66% degree of methylation), and trifluoroacetic acid (TFA) were from Fluka. Iodoacetamide (IAM), iodoacetic acid (IAA), 4-vinylpyridine (4VP), Tris, and EDTA were from Sigma. The 4VP was vacuum distilled and kept under nitrogen at -20°C . Ethylenimine was from Chem-Service. HPLC-grade acetonitrile was from Lab-Scan Analytical. TPCK (L-1-tosylamide-2-phenylethylchloromethylketone)-treated trypsin was from Cooper Biomedical and DEAE-cellulose (DE52) was from Whatman; Mono S column (HR 5/S), Superdex75 column (HR10/30), SP-Sepharose, and CNBr activated Sepharose 4B resins were from Pharmacia. Dialysis membranes CelluSept T2 6000–8000 Da cutoff were from Orange Scientific. Amicon Ultra 3k centrifugal devices were from Millipore. Sequencer-grade reagents were from Applied Biosystems. All other reagents were of the highest purity commercially available.

Protein Purification. Tomato PME (PME1) was purified from ripe tomato fruit according to Giovane et al.²⁸ Kiwi PME was purified according to Ciardiello et al.⁴² PME was purified from mature kiwi fruits. Peeled fruits were homogenized in water at 1:1 (w/v) ratio and centrifuged at $15000g \times 30$ min at 4°C . Supernatant proteins were precipitated with ammonium sulfate (70% saturation) and separated on DE-52 (Whatman, 2.5×12.7 cm) by using a linear gradient of NaCl (0 to 0.5 M) in 10 mM Tris-HCl at pH 7.5. The fractions containing PME inhibitory activity were pooled and subjected to affinity chromatography on PME–CNBr–sepharose²⁹ equilibrated in 10 mM Tris-HCl at pH 7.0. After exhaustive washing with 0.5 M NaCl, PME was eluted with 10 mM Tris-HCl at pH 9.0 containing 1 M NaCl. Active fractions were pooled, adjusted to pH 7.0, desalted by dialysis against 10 mM sodium phosphate, at pH 6.0, concentrated on Amicon Ultra 3K and stored in aliquots at -20°C . Protein concentration was determined using the extinction coefficients 14.97, 17.84, and 3.29 (at 280 nm, 1%) for tomato PME, kiwi PME, and kiwi PME, respectively. SDS–PAGE was performed on 12.5% or 15% gels. To protect the disulfide bridges, a sample buffer devoid of reducing agent was used.

Enzyme Activity and Inhibition Assays. PME and PME activity was routinely tested by a PME activity qualitative assay, evidencing the decrease of pH upon demethylesterification of pectin using bromothymol blue as the indicator. The assay solution contained 0.1% citrus pectin adjusted to pH 7.5 with NaOH in 0.1 M NaCl. PME specific activity was determined on a Mettler Toledo SL 50 pH-stat equipped with an autoburet, at room temperature ($\sim 22^\circ\text{C}$), by the addition of 0.5 μg of PME to 5 mL of 0.1 M NaCl containing 0.1% citrus peel pectin equilibrated at pH 6.2. Titration of released protons was carried out with 10 mM NaOH for 5 min. PME inhibitory activity was determined by measuring the PME residual activity following PME (0.5 μg) addition to the reaction mixture containing the inhibitor at 1:1, 1:1.5, and 1:2 PME/PMEI molar ratio. The specific activity of tomato PME used for inhibition experiments was 560 U/mg.

Circular Dichroism Measurements. Far-UV CD measurements were performed at room temperature, on a spectropolarimeter (Jasco J-710) calibrated with a standard solution of (+)-10-camphorsulfonic acid, after diluting the same amount of PME in final 5 mM buffer at different pH values (sodium acetate pH 5.0 and 6.0, Tris-HCl pHs from 7.4 to 9.0 and glycine-NaOH buffer pH 10). Cells with 0.2 cm path length and protein concentrations of 4 μM were used. Photo multiplier high voltage did not exceed 600 V in the measured spectral region (240–190 nm). Each spectrum was averaged five times and baseline corrected by subtracting the buffer spectrum. The percentages of secondary structure were estimated according to Chen et al.⁴³

CD measurement was also performed after the reduction of native PME disulfide bridges. The protein in 10 mM sodium phosphate at pH 7.0 was treated with DTT (200-fold molar excess over the Cys residues) for 4 h at 37°C . DTT was removed by gel filtration on a Sephadex G25 fine column (1×15 cm) equilibrated in the same

buffer. CD spectra were immediately recorded, together with those of the protein treated ones in the same way without addition of DTT.

Fluorescence Experiments. Fluorescence measurements were performed on a PerkinElmer LS 55 fluorometer equipped with a PTP-1 Peltier control system. The excitation and emission band widths were set at 5 nm. When kinetically assessing the pH effect on the protein fluorescence, 50 μ L of the concentrated PME1 solution (1.2 mg/mL) was directly diluted in the cuvette containing 1.0 mL of buffer at the chosen pH. The buffers were 50 mM sodium acetate for pH 5.0 and pH 6.0, 50 mM HEPES for pH 7.0, and 50 mM Tris-HCl for pHs between 7.5 to 8.5. The salt effect was explored at pH 8.5 in 50 mM Tris-HCl containing 0.1, 0.5, and 1 M NaCl, respectively. The kinetic assays (excitation 275 nm; emission 315 nm) started soon after the protein addition. In all the measurements, the absorbance was always less than 0.05 at the excitation wavelength. It is worth noting that when an excitation wavelength of 275 nm is used, Raman scattering of light occurs at around 305 nm where the maximum of the tyrosine fluorescence emission also occurs.⁴⁴ Therefore, to avoid the interference arising from the Raman light scattering, the fluorescence emission measurements were performed with excitation at 275 nm and emission at 315 nm.

Denaturation and Alkylation. Aliquots of PME1 (400 μ L, 10 nmol) were subjected to alkylation with different alkylating agents in native and non-native conditions.

Alkylation with 4-VP and IAA after Denaturation in Reducing and Nonreducing Conditions. Aliquots of PME1, dried in a Savant speed-vac concentrator, were dissolved in 200 μ L of 100 mM Tris-HCl at pH 8.5, containing 6 M guanidine HCl, and 2 mM EDTA. DTT was added (5-fold molar excess over the thiol groups of the protein), and the solution was incubated at 30 °C for 3 h. PME1 aliquots were also denaturated in nonreducing conditions, that is, without DTT addition. 4-VP or IAA was added to the mixtures (150-fold and 30-fold molar excess over the total thiols, respectively). The incubation proceeded for 50 min (4-VP) and 30 min (IAA).

Alkylation with 4-VP and IAM in Native Conditions. Dried PME1 aliquots were dissolved in 100 mM Tris-HCl at pH 7.5 (300 μ L total volume) and subjected to alkylation with 4-VP (450-fold molar excess over the free Cys,³⁵ 50 min incubation) and with IAM (30-fold molar excess over the total thiols, 30 min incubation).

Alkylation with IAA and Ethylenimine in Native Conditions. A PME1 aliquot was subjected to alkylation in 100 mM Tris-HCl at pH 8.5 with IAA (30-fold molar excess over the free Cys, 30 min incubation) and with ethylenimine (5-fold molar excess over the free Cys, 30 min incubation).

All the alkylation reactions were performed at room temperature, in the dark, under nitrogen. The reagents were removed by gel filtration on a Sephadex G25 fine column (1 $\times$ 15 cm) equilibrated with 10 mM Tris-HCl at pH 7.0. The alkylated PME1 samples were lyophilized in an Edwards Modulyo apparatus, then digested with trypsin and separated by RP-HPLC.

Tryptic Digestion and Amino Acid Sequencing. Lyophilized alkylated PME1 samples were dissolved in 200 μ L of 100 mM Tris-HCl at pH 8.5 and 10% acetonitrile. Trypsin (0.5 mg/mL in 1 mM HCl) digestion was carried out at an enzyme/protein ratio of 1/25 (w/w) at 37 °C for 4 h. The obtained peptides were separated by RP-HPLC on a μ Bondapack C18 column (Waters, 0.39 $\times$ 30 cm) using 0.1% TFA (solvent A) and 0.08% TFA in acetonitrile (solvent B) as eluents, at a flow rate of 1 mL/min in a multistep linear gradient (0–17.5% B in 15 min, 17.5–34% B in 24 min, 34–65% B in 16 min, and 65–90% B in 5 min). The eluate was monitored at 220 and 280 nm. Peaks were manually collected and vacuum-dried. Before sequencing, samples were dissolved in 50% acetonitrile, 0.1% TFA. Amino acid sequencing was performed with an Applied Biosystems Procise 492 Sequencer connected with online detection of the phenylthiohydantoin amino acids.

Mass Spectrometry Analysis. MALDI-TOF mass spectrometry was carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager-DE Biospectrometry Workstation. Analyses of the dried tryptic peptides, suspended in 50% acetonitrile 0.1% TFA, were performed on premixed solutions prepared by diluting samples (final

concentration 5 pmol/ μ L) in 4 volumes of matrix, namely, 10 mg/mL α -cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% TFA.

Incubation of PME1 at Different pH Values. Aliquots of purified kiwi PME1 (30 nmol in 1.2 mL) were equilibrated to different pH values from 5.0 to 9.0 with appropriate buffers. After 30 min of incubation, the samples were adjusted to pH 5.0 by the addition of 100 mM sodium acetate at pH 4.0. Titrations were made in Eppendorf tubes, after gently mixing the sample following each addition, by using a PHM83 AUTOCAL pHmeter (RADIOMETER, Copenhagen) equipped with a SLIMTRODE electrode (Hamilton, Bonaduz, Switzerland) for small volumes.

Cation Exchange Chromatography. pH-treated PME1 samples (10 nmol) were incubated with tomato PME (at a molar ratio 1:1.3) for 15 min. PME1 samples alone and in complex with PME were submitted in duplicate to cation exchange chromatography on a Mono S (HR5/5) column by using an FPLC ÅKTA (Amersham Pharmacia Biotech). The samples were diluted to 1 mL final volume with 20 mM sodium acetate at pH 5.0, centrifuged for 10 min at 12000 rpm and loaded on the column equilibrated with the same buffer. Elution was performed by applying a linear gradient of NaCl from 0 to 0.5 M in the equilibration buffer, at a flow rate of 1 mL/min and collecting 1 mL fractions. FPLC-eluted peak area was calculated by the chromatography software UNICORN 4.10 (Amersham Pharmacia Biotech).

pH-untreated kiwi PME1 was mixed with kiwi PME at a molar ratio 1:1 in 20 mM sodium acetate at pH 5.0. An aliquot of the mixture was equilibrated to pH 9.0 for 30 min. Complex detection of pH-untreated and treated mixtures was performed as just described.

Turbidimetric Analysis. Kiwi PME1 was diluted at a concentration of 40 μ g/mL in 50 mM sodium phosphate buffer at pH 7.0 and in 50 mM glycine-NaOH buffer at pH 9.0 in the absence and in the presence of 1 M NaCl. Optical absorbance spectra were taken in the visible range between 300 and 420 nm, immediately and after 1 h of incubation, on a Varian Cary 50 Bio UV–vis spectrophotometer.

In Silico Analysis. Analysis of the crystal structure of kiwi PME1 (PDB code: 1XG2:B) elucidated in complex with tomato PME (PDB code: 1XG2:A) and of the homologous tobacco invertase inhibitor Nt-CIF (PDB code: 1RJ1), as well as the solvent accessibility measurements, was performed by using the InsightII molecular modeling system from Accelrys Inc. (San Diego, CA).

RESULTS

Kiwi PME1 Purification. The traditional procedure used to purify kiwi PME1 includes a chromatography on PME-CNBr-Sepharose affinity. A single sharp protein peak was eluted by high salt concentration (1 M NaCl) at pH 9.0 and, up to now, directly desalted by dialysis against a 10 mM buffer at pH 7.5 before storage. A necessary variation has been now introduced, that is, the pH lowering at least to 7.0 of the protein solution immediately after the elution from affinity chromatography and before desalting by dialysis against a buffer at pH 6.0. PME1 was then concentrated at 0.41 mg/mL, aliquoted, quickly frozen, and stored at –20 °C. The PME1 recovery was about 3 mg/kg of peeled kiwi-fruits. The PME1 purity was checked by N-terminal sequencing, size exclusion chromatography, and SDS–PAGE analysis. The inhibitor, showing a single N-terminal sequence and eluting from gel-filtration as a single peak, was separated in SDS–PAGE into two close bands of equal intensity, having an estimated molecular mass of 16.4 and 16.8 kDa. This difference is likely due to the microheterogeneities detected in kiwi PME1 amino acid sequence.³³ Purified PME1 fold was routinely controlled by far-UV CD analysis. Despite the correctness of the purification procedures, CD spectra often showed a decrease of the protein helical content when the protein eluted from the affinity was directly desalted at pH 7.5, skipping the pH lowering immediately after the elution from affinity chromatography.

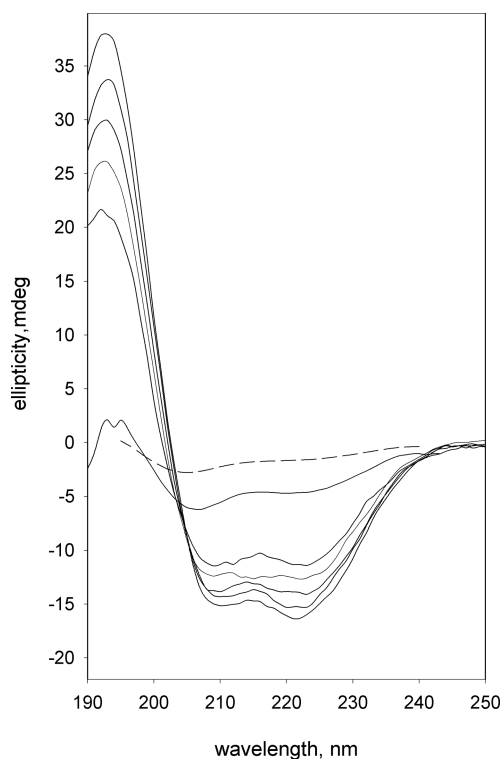


Figure 1. Far-UV CD spectra of native kiwi PMEI as a function of pH. Spectra acquired after diluting PMEI in final 5 mM buffers at different pH values. Starting from the bottom to the top: pH 7.5 (spectra at pH 5.0 and pH 6.0 were coincident), pH 8.1, pH 8.4, pH 8.6, pH 9.0, and pH 10 (solid lines). The spectrum of DTT-treated protein is also shown (dashed lines). Protein concentration was 4 μ M.

Detection of pH-Dependent Secondary Structure Changes by Far-UV Circular Dichroism (CD). Figure 1 shows the CD spectra of native PMEI registered in the pH range 5.0–10.0. An all α -helix fold with an helical content of 75% was estimated by the Yang algorithm⁴³ in the pH range 5.0–7.5. When the pH was increased from 7.5 to 9.0, the α -helical content progressively decreased from 75% to about 40%. A much higher unfolding was observed at pH 10.0, with an estimated α -helical content of 15%. Attempts to recover the native structure by lowering the pH of the sample from 9.0 to

7.0 and monitoring the spectra for 60 min were unsuccessful, thus suggesting that PMEI partial unfolding was irreversible.

CD analysis of the native PMEI, performed at pH 7.0 after treatment with DTT showed that the reduction of the disulfide bridges induced an almost complete loss of protein fold (Figure 1).

Analysis of the pH Influence on the Tertiary Structure by Fluorescence Studies. Kiwi PMEI contains four tyrosine residues and is devoid of tryptophan. The maximum of the emission spectrum of the protein excited at 275 nm is centered at about 305 nm, which is typical of fluorescence arising from a protein containing only tyrosyl residues as fluorophores.⁴⁴ The spectra obtained (Figure 2) suggest that the tertiary structure changes occurring in kiwi PMEI follow an uncommon fluorescence behavior, bringing about a decrease of the protein fluorescence emission. PMEI unfolding following guanidine-denaturation (data not shown) also led to a decrease of the intrinsic fluorescence rather than an increase as normally observed when tyrosine containing proteins unfold.

As shown in Figure 2 (left panel), the PMEI fluorescence emission keeps constant in the pH range 5.0–6.0, while a decrease over time is observed at pH values higher than 6.0, becoming faster between pH 7.0 and 7.5. At these pH values, no secondary structure changes were detected by CD, signifying that the observed fluorescence decrease is not attributable to the α -helix unfolding but to changes in the chemical environment around tyrosyl residues. Furthermore, the native PMEI fluorescence emission was recovered bringing back the pH of the sample from 7.5 to 6.0, indicating that the changes occurring in this pH range are reversible. No further remarkable fluorescence decrease was observed by increasing the pH to 8.0 and 8.5. The addition of 0.5 or 1 M NaCl protected the PMEI structure when the pH of the solution was raised to 8.5 (Figure 2, right panel), whereas a lower NaCl concentration (0.1 M) was not equally effective.

Detection of pH-Induced Thiol–Disulfide Reshuffling. To investigate whether PMEI disulfide connectivity could be affected by increasing pH, we analyzed the disulfide bridges at different pH values. For this purpose, the protein was alkylated in the absence of denaturing and reducing agents by using different sulphhydryl-reactive reagents requiring incubation buffers with different optimal pH (IAM at pH 7.0; 4-VP at pH 7.5; IAA and ethylenimine at pH 8.5). In addition, PMEI

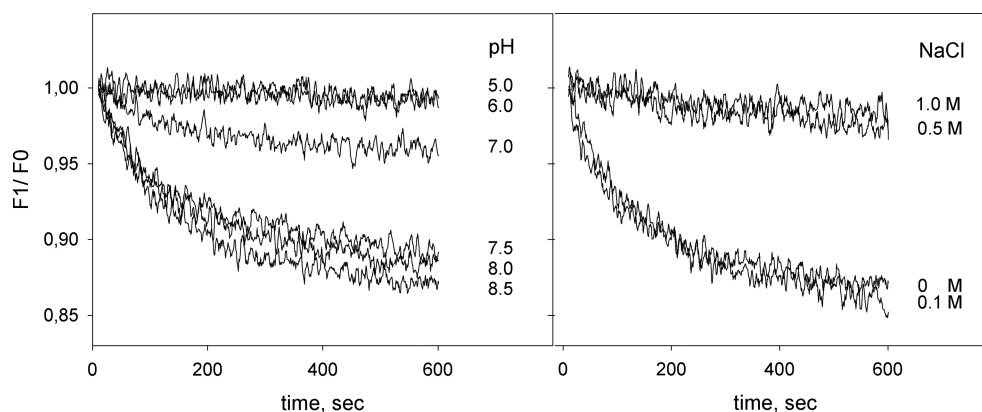


Figure 2. Time course of kiwi PMEI fluorescence emission as a function of pH. Left panel: ratio of the fluorescence emission over time measured at pHs ranging from 5.0 to 8.5. Right panel: ratio of the fluorescence emission over time at pH 8.5 in the absence (gray) and in the presence of different NaCl concentrations. The fluorescence emission was recorded soon after PMEI addition to the different solutions.

was alkylated in denaturing and reducing conditions with 4-VP and IAA, and in only denaturing conditions with IAA.

Kiwi PME1 (AcPME1, SwissProt accession number P83326) contains four Cys residues involved in disulfide bridges (disu 9–18 and 74–114) and one free Cys (Cys139) (see Table 1).

Table 1. Kiwi PME1 Amino Acid Sequence and Tryptic Peptides

20		40	
ENH	LISEICPK	TRNPSLCLQ	ALES
T1		T2	DRSASKDLKGLGQFS
60		80	
IDIAQ	ASAKQTSKII	IASLTNQ	ATDPK
T3		T4	KLKGRYETCSENYAD
100		120	
AIDSL	GQAKQFL	TSGDYN	SLNIYAS
T5		T6	AFDAGTCE
140		160	
PPNIPT	QLHQADIK	LEDLC	DIVLVIS
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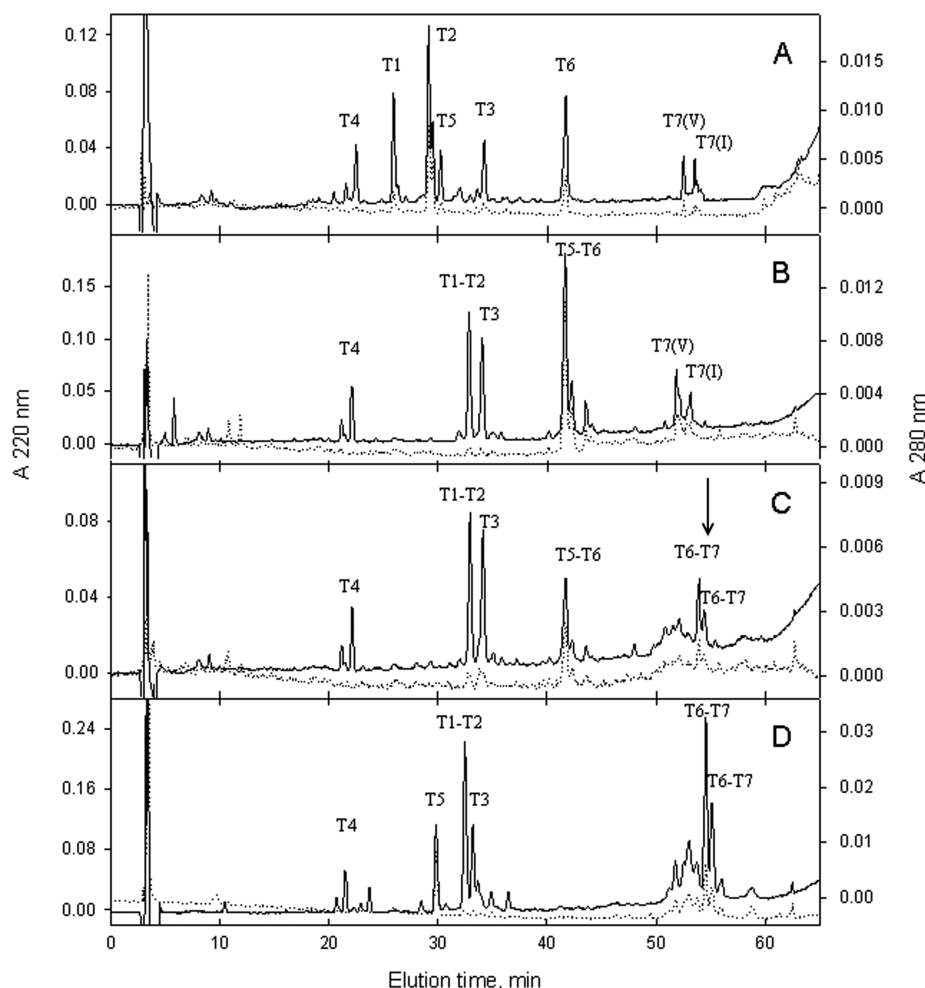


Figure 3. RP-HPLC profiles of kiwi PMEI after alkylation in different conditions and digestion with trypsin. Peptides are numbered from T1 to T7 according to their order in the amino acid sequence (see Table 1). Panel A: PMEI alkylated with 4-VP, at pH 7.5 in the presence of reducing and denaturing agents. The five tryptic peptides T1, T2, T5, T6, and T7 contain each an alkylated cysteine. T7, containing Cys139, is present as a double peak (T7_V and T7_I) due to the microheterogeneity Val/Ile in position 142. PMEI carboxymethylated (IAA) under the same conditions at pH 8.5 gave the same elution profile (not shown). Panel B: PMEI alkylated at pH 7.5 (with 4-VP) in the absence of reducing and denaturing agents. T1-T2 and T5-T6 are disulfide-bridged (disu 9–18 and 74–114, respectively). Panel C: PMEI alkylated at pH 8.5 (with IAA) in the absence of reducing and denaturing agents. Appearance of a new peptide, T6-T7, containing the non-native disulfide 114–139. The peptide containing the native disulfide 74–114 (T5-T6) is concomitantly reduced. Panel D: PMEI alkylated at pH 8.5 (with IAA) in denaturing and nonreducing conditions. The peptide containing the native disulfide 74–114 (T5-T6) completely disappears, while Cys 139 is completely involved in the non-native disulfide 114–139 (peptide T6-T7).

Table 2. Tomato PME Activity Following PME175 and PME185 Addition

molar ratio PME/PMEI	residual PME activity ^a	
	PME175	PME185
1:1	36%	56%
1:1.5	14%	43%
1:2	0	30%

^aTitrimetric assays were performed at pH 6.2.

eluted unbound (measured by the Unicorn program), which exactly accounts for the molar excess added over the inhibitor. PMEI exposed to pH higher than 7.5 formed progressively lower amounts of complex, while the amount of PME left unbound concomitantly increased (panels B and C), thus indicating that the amount of active inhibitor decreased by increasing the pH. We also expected to detect an increasing

amount of free inhibitor; nevertheless, no unbound PMEI was present in the elution profiles.

The comparative analysis of the elution profiles obtained by injection of the inhibitor alone is shown in Figure 4, right panel. PMEI exposed to pH 6.0, 7.0, and 7.5 (panel D) gave rise to identical elution profiles. The two PMEI peaks, separately collected and analyzed, showed the same full inhibitory activity and CD spectra overlapping that of the native PMEI before injection. The PMEI exposed to pH 8.5 and 9.0 (panels E and F) equally eluted into the two same peaks, containing a fully active and folded inhibitor, but their amount progressively decreased by increasing the pH. The two PMEI subpopulations were reduced by the same amount (as measured by the Unicorn program), thus indicating that, despite the microheterogeneity, they undergo the same structural modifications. No peaks containing inactive PMEI were obtained from the elution patterns. This observation suggested further experi-

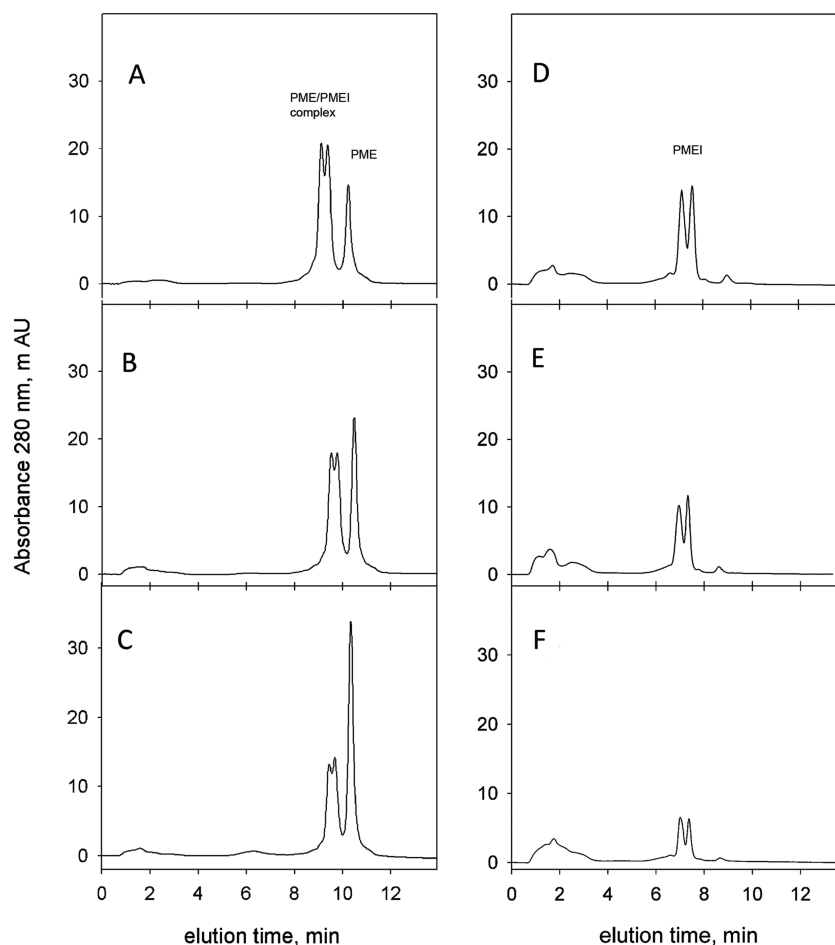


Figure 4. Cation exchange chromatography of the PME–PMEI mixture and PMEI “alone” following PMEI incubation at different pH values. Left panel: kiwi PMEI (1 nmoles) after incubation at pH 6.0, 7.0, 7.5 (A), 8.5 (B), and 9.0 (C) mixed with tomato PME (1.3 nmol). The absorbance peak of tomato PME (1.3 nmoles) was 64 mAU (not shown). Right panel: the same kiwi PMEI “alone” (5 nmoles) after incubation at pH 6.0, 7.0, 7.5 (D), 8.5 (E), and 9.0 (F). Elution is performed on a Mono S column by applying a NaCl gradient (0–0.5 M) in 20 mM Na acetate buffer at pH 5.0.

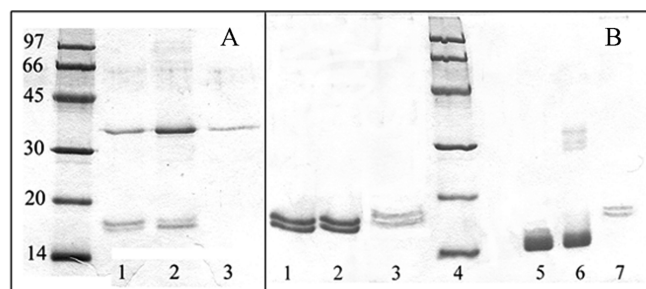


Figure 5. SDS–PAGE. Panel A: FPLC-eluted peaks of the PME–PMEI mixture. Samples are from the separation reported in Figure 4A: fractions 8 (lane 1), 9 (lane 2), and 10 (lane 3). Lane 1 and lane 2 contain the complex (PMEI, approximately 16.8 and 16.4 kDa and PME 35 kDa), and lane 3 contains PME. Molecular weight markers are shown on the left. Panel B: Native PMEI, supernatant, and pellet of PMEI after incubation at pH 9.0 (PMEI 90) are analyzed in the presence (lanes 1–3) and in the absence (lanes 5–7) of β -mercaptoethanol. Molecular weight markers are shown in lane 4.

ments to investigate the fate of inactive PMEI that was not eluted from the chromatographic column.

Detection of Aggregate Formation by SDS–PAGE. To detect if any PMEI remained at the bottom of the tubes after centrifugation, native PMEI was brought to pH 9.0 (PMEI 90) and centrifuged after 30 min of incubation. Native PMEI, PMEI

90 supernatant, and PMEI 90 pellet (not visible with the naked eye) were analyzed by SDS–PAGE in reducing and non-reducing conditions (Figure 5, panel B). Under reducing conditions, the protein classically splits into two bands with an apparent molecular mass of 16.4–16.8 kDa (lane 1), while under nonreducing conditions (lane 5) its mobility increases, as occurs when proteins have the Cys residues engaged in native disulfide bridges.⁴⁵ Similar to native PMEI, the PMEI 90 supernatant showed in reducing conditions (lane 2) a higher apparent molecular mass than under nonreducing conditions (lane 6). Furthermore, thin bands of higher molecular mass (around 30 kDa) were observed in the absence of reducing agents, suggesting the presence of a very small amount of disulfide-bridged dimers at this pH. The mobility of PMEI from the PMEI 90 pellet (lanes 3) was instead not affected by the absence of reducing agents (lane 7), indicating that the aggregated protein does not contain the native disulfide pattern.

Detection of Aggregate Formation by Turbidity Experiments. Turbidity measurements confirmed that the inactive PMEI molecules undergo pH-dependent aggregation (Figure 6). PMEI was diluted (40 $\mu\text{g}/\text{mL}$) in buffers at pH 7.0 and pH 9.0, with or without 1 M NaCl. The spectra showed that the absorbance of PMEI at pH 9.0 was constantly higher than that measured for PMEI at pH 7.0 (6 times higher at 420 nm and 2 times higher at 360 nm). The spectrum obtained at

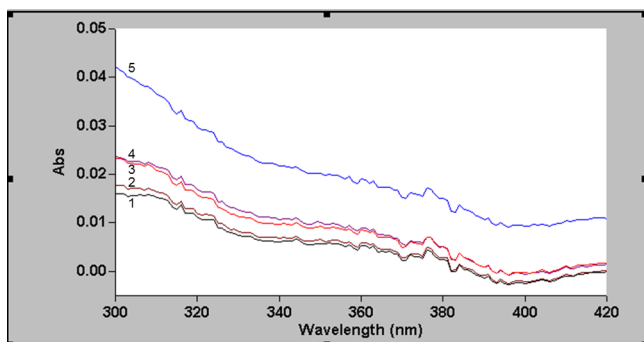


Figure 6. Turbidity measurements. PMEI spectra after protein incubation at pH 7.0 and pH 9.0 in the absence and in the presence of 1 M NaCl. Optical absorbance spectra (1–5) were acquired by using a Varian Cary 50 Bio UV–vis spectrophotometer. The figure was created with the Cary software, and the screen shot is shown. (1) Buffer pH 7.0 and pH 9.0 (overlapping spectra); (2) buffer pH 7.0 + 1 M NaCl and pH 9.0 + 1 M NaCl (overlapping spectra); (3) PMEI pH 7.0 with and without 1 M NaCl (overlapping spectra); (4) PMEI pH 9.0 + 1 M NaCl; and (5) PMEI pH 9.0.

pH 9.0 in the presence of 1 M NaCl was instead superimposable to that obtained for PMEI at pH 7.0, indicating that PMEI aggregation is prevented by increasing the ionic strength of the environment.

Prevention of Kiwi PMEI pH-Induced Conformational Changes by Kiwi PME Binding. We have analyzed the stability to the pH of the kiwi PME/PMEI complex by comparing the amount of complex present in solution at pH 5.0 and in the same sample brought to pH 9.0 for 30 min. Detection of complex formation, performed by cation-exchange chromatography, showed that the same amount of complex was eluted in both runs, indicating that the interaction with the kiwi PME protects the inhibitor from structural changes.

Analysis of Kiwi PMEI 3D Structure. Kiwi PMEI crystal structure (PDB, 1XG2:B), elucidated in complex with tomato PME at pH 6.0,³⁵ shows that the inhibitor folds as an up and down four α -helix bundle bearing at the N-terminus a protruding α -helical hairpin (Figure 7). Cys9 and Cys18 connect the small helices of the N-terminal helical hairpin, while Cys74 and Cys114 connect the second and third helix of the bundle that are the helices interacting with PME in the complex formation. Solvent accessibility measurements show that the free Cys139 and the two disulfides are completely buried. The free Cys139 lies on the fourth helix of the bundle, far from disulfide 74–114 (13.36 Å to Cys114 and 14.84 Å to Cys74), which at pH 8.5 likely brings a nucleophile attack producing the non-native disulfide bridge detected in the alkylation experiments described above. Therefore, a protein rearrangement has to occur by increasing pH to bring Cys139 and disulfide 74–114 at the distance required to interact. Alkylation experiments suggest that the protein in solution must have already at pH 7.0 a less compact packing than that observed in the crystal structure since Cys139 results in being accessible to the alkylating agents at this pH. Structure loosening at this pH is moreover confirmed by our fluorescence experiments.

A possible molecular mechanism promoting the destabilization of the PMEI native state is suggested. It is based on the identification in the interior of the four-helix bundle and in close proximity to the 74–114 disulfide bridge of an H-bond network of polar residues connecting the protein interior to the

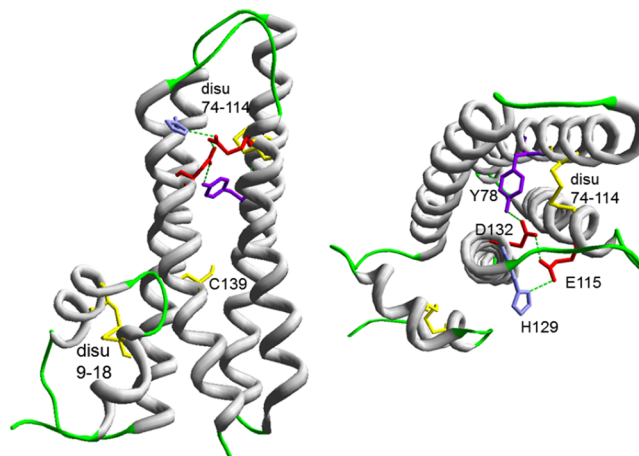


Figure 7. Kiwi PMEI fold. Left panel, protein front view; right panel, protein top view. The two disulfide bridges 9–18 (at the N-terminal hairpin) and 74–114 (buried in the four-helix bundle) and the free Cys 139 are shown and labeled. In close proximity of the disulfide 74–114, an H-bond network of polar residues connects the protein interior to the surface. The residues involved in the network, Tyr78, Asp132, Glu115 (contiguous to Cys114), and His129, are shown and labeled. The connecting H-bonds are colored in green (dotted lines). The distances between residues are Tyr78:O–Asp132:OD1, 2.61 Å; Asp132:OD2–Glu115:OE1, 2.59 Å; Glu115:OE2–His129:ND1, 2.93 Å.

surface (see Figure 7). The network comprises the buried Tyr78 (whose changes in exposure are likely monitored by fluorescence) and two proton-linked acidic residues, Asp132 and Glu115 (contiguous to Cys114), the former completely buried into the bundle and the latter partially exposed to the solvent (their solvent accessibility does not change when PMEI is in complex with tomato PME). With the pH increasing above neutrality, the deprotonation of these acidic residues, having an unusually high pK_a , conceivably generates a repulsion event causing (i) the disruption of the H-bond network, (ii) a local opening up of the bundle and structure loosening, and also (iii) the access to the solvent of the 74–114 disulfide, which will be then cleaved.

Our analysis further indicates that the internal H-bond network is equally present in the homologous inhibitor of invertase from tobacco (PDB ID: 1RJ1) that however shows a nonionizable Asn in place of Asp132.

DISCUSSION

This study highlights the strong sensitivity of kiwi PMEI to pH values generally considered to have no deleterious effects on protein structure and function. For instance, pH around neutrality and sometimes even values up to 9.0 are routinely used for research purposes. Therefore, taking into account that kiwi PMEI finds a large use for research purposes and also can be exploited for biotechnological applications, the results described in this article not only increase the knowledge on this protein but also are likely to have a significant impact on the procedure chosen to handle it.

We have investigated the effect of pH on fold and activity of kiwi PMEI discovering that it undergoes, when exposed to pH values above 6.0, conformational modifications that have a clear influence on its interaction with PME and that ultimately lead to protein misfolding and loss of function. Our data suggest that the destabilization of the PMEI fold is induced by two

different events mediated by changes in the state of groups ionizable at different pH values. Reversible tertiary structure changes, detected by fluorescence spectroscopy, start to occur at pH values above 6.0, the largest variations being observed in the pH range 7.0–7.5, whereas irreversible secondary structure changes, detected by CD, take place only at a pH above 7.5 and gradually increase with increasing pH, bringing about a progressive decrease of the α -helical content of the protein.

The tertiary structure changes initiate the process of protein destabilization apparently without greatly altering the protein fold, as suggested by their reversibility, the absence of concomitant secondary structure loss, and by the conserved ability of kiwi PMEI to bind and inhibit tomato PME when exposed to pH values up to 7.5. However, in the same pH range in which these changes are detected the strength of the interaction between kiwi PMEI and tomato PME rapidly decreases, the dissociation constant value of the binding being 70-fold higher at pH 7.5 than at pH 6.0.^{13,39} A similar trend is also observed for kiwi PMEI interaction with other plant PMEs, the inhibitory activity invariantly decreasing at pH above 7.0 independently of the source of the PME tested.^{13,40} Kiwi PMEI and tomato PME complex formation is then abolished at pH 8.0¹⁰ or 8.5,¹³ which is the pH at which PMEI partial helical unfolding is detected. Collectively, these data suggest that kiwi PMEI structural changes have a significant role in the loosening of the PMEI/PME interaction and also in determining the loss of PMEI activity at slightly alkaline pH values.

Further investigations shed more light on the behavior of PMEI misfolding and inactivation and highlight the essential role that the buried disulfide bridge 74–114, conserved in all of the PMEI-like proteins, has in the PMEI stability. In the course of alkylation experiments, we discovered that this disulfide, likely exposed to solvent by the effect of the tertiary structure changes, undergoes cleavage when the protein is exposed at pH above 7.5. The cleavage possibly occurs via thiol–disulfide reshuffling by the action of the ionized free Cys139, as suggested by the formation of the non-native disulfide 114–139. Chromatographic analyses performed by using PMEI previously treated at increasing pH values provide evidence that the pH increase acts on kiwi PMEI giving rise to two distinct populations of folded fully active and misfolded fully inactive protein. They also demonstrate that PMEI inactivation is directly related to the cleavage of the 74–114 disulfide. In fact by the pH increasing over 7.5, the first population, containing only the native disulfide, is increasingly converted into a fully inactive one containing the non-native disulfide species. The conversion becomes more prominent with increasing pH probably because the thiol–disulfide exchange reaction is accelerated by the concomitant increasing ionization of the free Cys.

The absence of formation of any other non-native disulfide species further suggests that a specific molecular mechanism leads to the cleavage of the 74–114 disulfide only. It is worth noting that, in agreement with our hypothesis indicating the pH-induced loss of Asp132–Glu115 proton-linked interaction as the starting event for PMEI structural changes, the homologous inhibitor of invertase from tobacco, showing a non ionizable Asn in place of Asp132, has a pH-insensitive structure.⁴⁶ In addition, it has been demonstrated that AtPMEI1 and AtPMEI2 from *Arabidopsis* exhibit a Ser residue instead of Asp132 and form a complex with a lower pH dependence with tomato PME.¹⁶ Our suggestions might help to engineer more tolerant variants for industrial needs.

In any case, breaking of the native disulfide bond induces helix instability and randomization, leaving normally buried hydrophobic residues accessible for intermolecular interactions, an event reported to lead to *in vitro* and *in vivo* protein aggregation.^{37,47,48} Indeed, the reported results indicate that the inactive misfolded inhibitor undergoes noncovalent aggregation.

Revisiting our previous data in light of the detected PMEI pH-dependence, the experiments showing that only extreme pH conditions (pH 10) can dissociate the kiwi PME/PMEI complex⁴² suggested that the pH increase does not affect the PMEI fold when the inhibitor is in complex with its natural partner. We actually found that PMEI changes are prevented if the pH is raised after complex formation with kiwi PME, confirming that the interaction with the enzyme protects the inhibitor from structural changes. The protective effect of this complex formation might be explained by the burial at the binding interface of the ionizable residues responsible for the destabilization of the PMEI fold. This finding is likely highly specific considering that the complexes formed by kiwi PMEI with other plant PMEs are all sensitive to pH changes and dissociate at much lower pH.^{10,13,40}

Fluorescence experiments further show that PMEI changes are prevented by the addition of salt, the protective effect being attributable to the shielding of specific charged residues changing their ionization state in the pH range 6.0–7.5. In fact, the increase of ionic strength stabilizes the PMEI structure directly counteracting the tertiary structure modifications, as indicated by the absence of any fluorescence decrease observed when the experiments are conducted after the addition of NaCl. The protective effect of the high ionic strength well explains the reason why, during the usual purification procedures (buffer at pH 9.0, containing 1 M NaCl), kiwi PMEI can be eluted from the affinity at high pH values without losing its inhibitory activity.

Furthermore, the detailed knowledge of the pH-dependent stability of kiwi PMEI is of practical interest since the kiwi inhibitor is widely used in experimental procedures dealing with plant PME and is an important tool in several biotechnological applications. It also allows a reinterpretation of the interaction/inhibition experiments conducted by using the inhibitor at pH values over the neutrality taking into account that the amount of interacting PMEI greatly depends on the pH of the solution used. Defining optimal buffer conditions for keeping PMEI in native conformation is thus a crucial point, and the data here described may contribute to the improvement of the experimental procedures and biotechnological exploitation of this molecule.

AUTHOR INFORMATION

Corresponding Author

*Institute of Biosciences and BioResources, C.N.R., Via P. Castellino 111, I 80131 Napoli, Italy. Phone: +39 081 6132551. Fax: +39 081 6132248. E-mail: rossana.davino@ibbr.cnr.it.

Funding

This work was supported by a dedicated grant from the Italian Ministry of Economy and Finance to the National Research Council for the project “Innovazione e Sviluppo del Mezzogiorno - Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare - Legge n. 191/2009”.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Laura Camardella (now retired), who promoted the research on PME1 in our group since 2000, for the helpful discussion of the manuscript.

ABBREVIATIONS USED

PME, pectin methylesterase; PME1, pectin methylesterase inhibitor; disulfide, disu; 4-VP, 4-vinylpyridine; IAA, iodoacetic acid; IAM, iodoacetamide; TFA, trifluoroacetic acid; SPR, surface plasmon resonance

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