

Review

Pectin methylesterase inhibitor

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Abstract

Pectin methylesterase (PME) is the first enzyme acting on pectin, a major component of plant cell wall. PME action produces pectin with different structural and functional properties, having an important role in plant physiology. Regulation of plant PME activity is obtained by the differential expression of several isoforms in different tissues and developmental stages and by subtle modifications of cell wall local pH. Inhibitory activities from various plant sources have also been reported. A proteinaceous inhibitor of PME (PMEI) has been purified from kiwi fruit. The kiwi PMEI is active against plant PMEs, forming a 1:1 non-covalent complex. The polypeptide chain comprises 152 amino acid residues and contains five Cys residues, four of which are connected by disulfide bridges, first to second and third to fourth. The sequence shows significant similarity with the N-terminal pro-peptides of plant PME, and with plant invertase inhibitors. In particular, the four Cys residues involved in disulfide bridges are conserved. On the basis of amino acid sequence similarity and Cys residues conservation, a large protein family including PMEI, invertase inhibitors and related proteins of unknown function has been identified. The presence of at least two sequences in the *Arabidopsis* genome having high similarity with kiwi PMEI suggests the ubiquitous presence of this inhibitor. PMEI has an interest in food industry as inhibitor of endogenous PME, responsible for phase separation and cloud loss in fruit juice manufacturing. Affinity chromatography on resin-bound PMEI can also be used to concentrate and detect residual PME activity in fruit and vegetable products.

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1. Pectin and pectin methylesterase (PME)

Pectin represents about 30% of polysaccharides constituting the cell wall in dicotyledonous plants. The main pectin component is homogalacturonan, consisting of α -D-galacturonic acid residues linked by a α -1,4 glycosidic bond. The carboxyl groups of the galacturonic acid residues can be methylesterified and the methylesterification degree changes significantly depending on the degree of differentiation of the cell and in the presence of some pathological conditions, such as infection by phytopathogenic fungi. Not only the number but also the distribution of free and unesterified galacturonate carboxyl groups along the homogalacturonan chain greatly influences the pectin properties

and the cell wall firmness [1]. Both the number and distribution of free carboxyl groups along the pectin molecule are regulated by the activity of PME (EC 3.1.1.11). PME catalyses the demethylesterification of galacturonic acid units of pectin, generating free carboxyl groups and releasing protons. Demethylesterified pectin may undergo depolymerisation by glycosidases. The enzyme was identified in carrots more than a century ago: in a study of the chemical processes occurring during fruit ripening, it was found that pectin modification occurred in the presence of “cette matière animale que l’on rencontre dans tous les fruits, et que l’on appelle albumine végétale” (“that animal matter found in all fruits, which is named plant albumin”) [2]. In particular, it was observed that the protein (the “albumine végétale”) present in the carrot root juice transformed the pectin in pectic acid, which underwent gelation. The enzyme is recently receiving renewed attention and its importance in plant physiology has been recognised (for a review see Ref. [3]). PME has been found in all higher plant

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examined, and is also produced by phytopathogenic fungi and bacteria.

1.1. Plant PME

Most of the purified plant PMEs have neutral or alkaline isoelectric points, and are bound to the cell wall via electrostatic interactions. However, the presence of neutral or acidic PME in the soluble fraction of plant tissues has also been detected [4–7]. Until few years ago, it was generally assumed that plant PMEs could act blockwise on pectin galacturonic acid residues, thus producing clusters of free carboxyl groups, whereas fungal PME could have a random demethylation activity. Recently it has been shown that some plant PME isoforms may exhibit both mechanisms depending on pH [8–10]. PME action on the components of plant cell wall may produce two diametrically opposite effects. On one hand, it contributes to the stiffening of the cell wall by producing blocks of unesterified carboxyl groups that can interact with calcium ions forming a pectate gel. On the other hand, the proton release may stimulate the activity of cell wall hydrolases [11,12], contributing to cell wall loosening. Manipulation of PME expression has been shown to influence physiological processes such as stem elongation and tuber yield [13], root development [14] and fruit softening [15]. Evidence has been provided that PME plays a role in nodule development on the tropical legume *Sesbania rostrata*, following interaction with its microsymbiont [16]. A role of PME in plant–pathogen interaction has also been proposed. In fact, a cell wall-associated PME of *Nicotiana tabacum* acts as a host-receptor for the tobacco mosaic virus (TMV) movement protein and this interaction is required for cell-to-cell translocation of the virus [17,18].

Several PME isoforms differing in molecular weight, pI and biochemical activity were detected in dicotyledonous plants [5,19–21]. PME isoforms are encoded by a family of genes, some of which are constitutively expressed throughout the plant [22], whereas others are differentially expressed in specific tissues and developmental stages [23]. Recently, the systematic sequencing of the *Arabidopsis* genome has led to the identification of the 67 PME-related genes in this species. PMEs are mostly encoded as large pre-pro-proteins. The pre-region or signal peptide is required for protein targeting to the endoplasmic reticulum. The pro-region consists of a large peptide (about 250 amino acid residues). Only the mature part of the PME (without the pro-region) is found in the cell wall. Therefore, the pro-peptide is cleaved off at some point of the secretory pathway. PME is thought to be secreted to the apoplast via the Golgi network together with highly methylated pectin. Although the role of the pro-region is not known, it has been hypothesised that it may act as an intramolecular chaperone, which could either prevent the correct folding of PME or directly inhibit the enzyme activity, in order to prevent premature demethylesterification of pectins before their insertion in the cell wall [3]. The similarity of the pro-

peptide of PME with the proteinaceous inhibitor identified in kiwi fruit supports this hypothesis.

1.2. Structure of PME

The first PME primary structure was determined by direct protein sequencing of the main PME isoform from tomato fruit [24,25]. Later on, DNA sequencing led to the observation that genomes contain several PME genes, coding for different isoforms, and that PMEs are synthesised as higher molecular weight precursors [22,26]. The sequencing of the entire genome of *Arabidopsis* revealed the presence of two types of PME genes: type I, having a long N-terminal pro-region and type II, having a short or absent pro-region. The N-terminal pro-peptides are variable in size and sequence and show a low level of amino acid identity. A higher conservation is instead observed in the C-terminal catalytic region, which constitutes the mature enzyme.

The three-dimensional structure of PME has been recently elucidated by X-ray diffraction studies. Crystal structures of PME from the phytopathogenic bacterium *Erwinia*

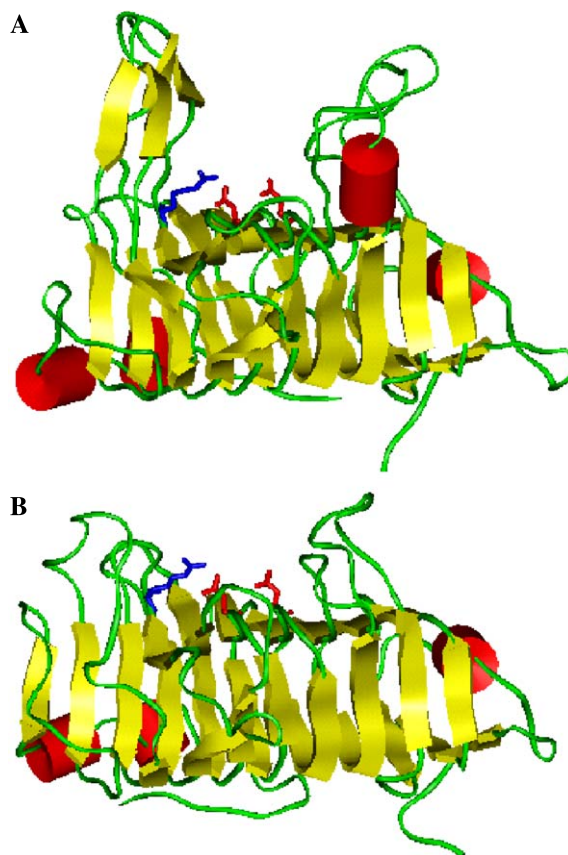


Fig. 1. Secondary render of *E. chrysanthemi* PME (A) and carrot PME (B) crystal structure. Yellow arrows indicate beta strands, red cylinders represent α -helices. The residues proposed to be part of the catalytic site (two Asp and one Arg residues) are shown. Secondary render representations are created by using Kabsch and Sander classification in InsightII program from Accelrys.

chrysanthemi, agent of the soft rot of plants [27], and of PME from carrot root [28] reveal that the enzyme folds into a right-handed parallel β -helix, as seen for the first time in pectate lyase [29]. It is proposed that the active site comprises two aspartic acid and one arginine residues, located on the surface of the parallel β -helix in a cleft formed by protruding loops, suitable for pectin binding. A remarkable difference between the plant and bacterial PME structures is in the shape of the active site cleft, which in the plant PME is shallower, with less pronounced walls (Fig. 1).

2. Regulation of PME activity

Regulation of PME activity is obtained in part by the differential expression of isoforms, and in part by posttranslational mechanisms. It was postulated that PME activity in the cell wall is regulated by H^+ concentration in a cyclic manner during cell growth [30]. The enzyme optimal activity occurs at pH close to neutrality and is reduced by the local decrease of pH generated by protons released as a consequence of the reaction. In turn, the pH decrease activates glycosidases and glycosyl-transferases, involved in cell wall extension and building up. The consequent dilution of negative charges and increase of local pH result in reactivation of PME, starting a new cycle.

Furthermore, the reported presence of PME inhibitors (PMEIs) from various plant sources led to the hypothesis that other mechanisms are involved in PME regulation.

Purification of a PME isozyme from potato tuber led to the concomitant isolation of a PMEI [31]. The inhibitor was present in the sap extract of potato tuber, and was purified by ion-exchange and gel-filtration chromatography. It was extremely thermostable (surviving incubation at 120 °C for 10 min), and its molecular weight estimated by gel-filtration chromatography was in the range 158–232 kDa. The purified inhibitor contained no detectable protein, whereas the presence of uronic acid and neutral sugars was detected by carbohydrates analysis. The inhibitory molecule was suggested to have a pectic configuration since it was deactivated by the pectic enzymes polygalacturonase (PG) and pectate lyase. The inhibitor was active against PME from potato and from different plant species, with an uncompetitive mode of action, whereas it did not affect bacterial and fungal PMEs.

An inhibitory activity was also found in banana fruit (*Musa sapientum* L.) [32], especially in the rubbery fruit. The inhibitory molecule was active against its own banana PME and the pea pod (*Pisum sativum*) PME, and was extremely thermostable (retaining 80% activity after 3 h in boiling water). The chemical nature of this inhibitor was not elucidated.

A highly thermostable inhibitory activity was also found in intact jelly fig (*Ficus awkeotsang*) achenes. This inhibitor, fractionated by gel filtration and concanavalin A-Sepharose chromatography, was found to consist of poly-

peptides with molecular weights ranging from 3.5 to 4.5 kDa [33].

2.1. The proteinaceous inhibitor of PME

During a study aimed at the characterisation of PME from kiwi fruit, a proteinaceous inhibitor of PME (PMEI) was discovered [34]. The protein is present in the aqueous phase of fully ripe fruit, and is probably synthesised as a larger precursor, undergoing proteolytic cleavage during the fruit ripening process [35]. PMEI is active against several plant PMEs (PMEs from kiwi, orange, apple, tomato, apricot, carrot, potato and banana were assayed), whereas it does not inhibit fungal PMEs [34]. We have recently shown that PMEI does not inhibit the bacterial PME from *E. chrysanthemi*, either. Modelling and fluorescence studies indicated that PMEI interacts with tomato PME at the level of the active site and we suggested that the more open active site cleft of plant PME, with respect to that of *E. chrysanthemi*, allows the interaction with the inhibitor, whereas the shape of the bacterial active site cleft, which is deeper, with pronounced walls, prevents the interaction [36]. The inhibition occurs through the formation of a reversible 1:1 complex. The stability of the complex is strongly influenced by pH, indicating that PME activity can be modulated by pH either directly or by modulation of the affinity between the enzyme and its inhibitor. The binding kinetics between tomato fruit PME and PMEI was investigated through Surface Plasmon Resonance technique. A dissociation constant value of 53 nM was calculated at pH 7; the value decreases 10 times (5 nM) at pH 6 [37]. Therefore, the affinity of the two proteins is higher at a pH close to that of the apoplasmic environment. The high affinity of the inhibitor for plant PME allowed the development of a purification procedure based on affinity chromatography on PME-bound Sepharose [35]. On the other hand, kiwi PMEI has been coupled to CNBr-activated Sepharose, yielding a powerful affinity chromatography support for purification of plant PME [38–40].

2.2. Primary structure of PMEI

The complete amino acid sequence of the natural PMEI purified from kiwi fruit was obtained by direct protein chemistry methods [41]. It consists of a single polypeptide chain, with a ragged N-terminus. In fact, part of the polypeptide chain has an additional N-terminal Ala residue, which could be a remnant of a partially cleaved signal peptide. The total sequence comprises 152 amino acid residues (not including the additional N-terminal Ala), yielding a molecular mass of 16,277 Da. It contains five Cys residues, four of which are connected by two disulfide bridges (first to second and third to fourth). The fifth Cys residue (position 139) has a free thiol group. Protein micro-heterogeneities were detected at five positions of the amino

acid sequence (Ala/Ser56, Tyr/Phe78, Ser/Asn117, Asn/Asp123, and Val/Ile142), indicating that several isoforms of the protein are present in the fruit [41]. Indeed, two entire cDNAs and one fragment coding for PME1 isoforms from *Actinidia deliciosa* have recently appeared in the EMBL database (accession numbers: [AB091088](#), [AB091089](#), [AB091090](#)), confirming the already determined amino acid sequence, including some of the microheterogeneities, with the exception of an additional Lys residue at the C-terminal end. The complete coding sequences of the entire cDNAs show a N-terminal extension of 32 amino acid residues, due to the presence of a signal peptide required for the extra-cellular targeting of the protein. The three-dimensional structure of PME1 has not yet been determined. However, secondary structure predictions and circular dichroism analysis indicate a predominance of α -helix structure and, although not having structural homologs in Protein Data Bank, threading techniques suggest a four-helix bundle

arrangement. The presence of the disulfide bridges seems essential for the maintenance of the secondary structure, since the treatment of the native protein with reducing agents yielded a dramatic loss of the α -helix structure. Furthermore, experiments of limited proteolysis revealed that Lys11 and Arg13 were susceptible to trypsin cleavage in the native protein, indicating that the stretch of amino acid residues spanning between Cys9 and Cys18, involved in the first disulfide bridge, is exposed on the surface of the protein.

In order to obtain PME1 in amounts suitable for structural characterisation, a synthetic PME1 gene has been constructed on the basis of the determined amino acid sequence and expressed in a heterologous system. Purification of the synthetic protein will hopefully overcome the difficulties encountered in crystallisation experiments, probably arising from the presence of different isoforms in the natural PME1 purified from kiwi fruit.

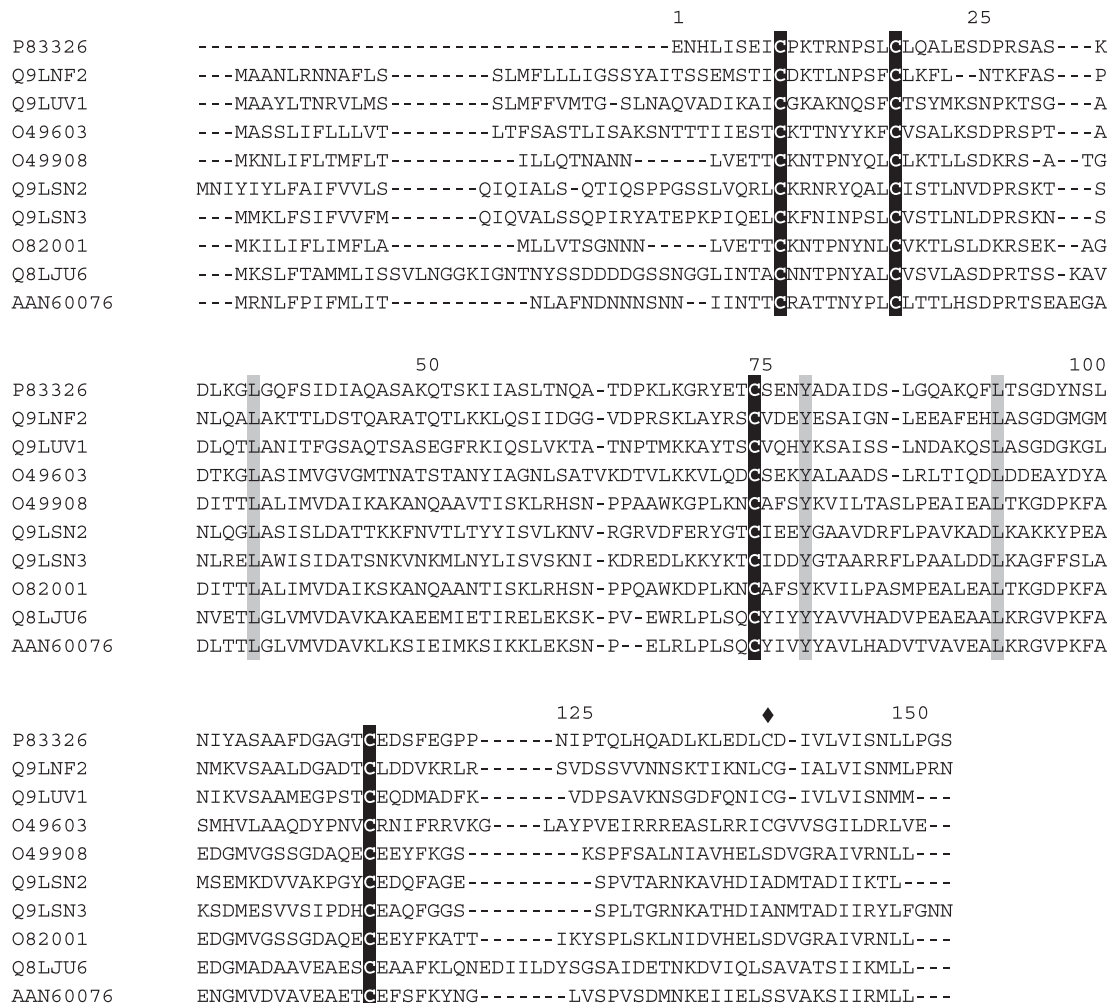


Fig. 2. Alignment of kiwi fruit PME1 (P83326) with the apoplasmic (O49908) and vacuolar (AAN60076) INH isoforms from *N. tabacum*, with INH from *Lycopersicon esculentum* (O82001), with INH-like protein from *Ipomoea batatas* (Q8LJU6), and with INH-like proteins (Q9LSN2, Q9LSN3), INH homolog (O49603) and other sequence-related proteins (Q9LNF2, Q9LUV1) from *A. thaliana*. Alignment was performed with BLAST at ExPASy Molecular Biology Server. Conserved Cys residues are black-shadowed. Other conserved residues are grey-shadowed. The black diamond indicates the free Cys residue found in PME1 and conserved in some of the reported sequences.

2.3. PME1 homology search

Search for homology in databank revealed similarity of PME1 with a huge number of sequences. The identity level is not very high, however, the position of the four Cys residues engaged in disulfide bridges is strictly conserved, leading to the recognition of a structural motif, which most probably confers a typical fold to the proteins of this family. We can distinguish two groups of related sequences, the first comprising cDNAs encoding proteins having about the same size as PME1, the second comprising large size cDNAs encoding precursors of PME. The first group includes cDNAs encoding for inhibitors of invertase (INH), and a number of sequences mostly coming from *Arabidopsis* genome, coding for hypothetical proteins of unknown function (a representative alignment is shown in Fig. 2). Sequences O49908 and AAN60076 from *N. tabacum* were proved to correspond to authentic INHs [42,43]. INHs are vacuolar or apoplasmic proteins that have been isolated from plant tissues, and specifically inhibit invertase of plant origin (for a review, see Rausch and Greiner, this issue). Sequences Q9LNF2 and Q9LUV1 from *Arabidopsis* have the highest identity level with PME1 (37%). They also share with PME1 the presence of the fifth Cys residue, not

involved in disulfide bridge, and absent in all INH sequences identified so far [44]. The sequence O49603 from *Arabidopsis* has 30% identity with PME1 and also shows the presence of the fifth Cys residue. The involvement of the free Cys residue in determining the PME1 enzyme specificity is under investigation. The second group contains the N-terminal pro-peptides, which are present in PME precursors and are removed in mature PMEs (a representative alignment is shown in Fig. 3). These sequences are quite variable; however, they maintain the conservation of the four Cys residues. Secondary structure predictions on the basis of the deduced amino acid sequence indicate in the pro-peptide region a prevalence of α -helix structure, in contrast with the mature protein, which is rich in β -sheets [22]. Several PMEs subjected to direct N-terminal sequencing showed an N-terminus resistant to Edman degradation. A cyclised glutamine was found in the most alkaline form of carrot PME [45], whereas an acetylated serine residue was found in PME from orange fruit peels [46]. Acetyl-threonine is present at the N-terminus of kiwi fruit PME. The cleavage, yielding mature PME and releasing the N-terminal pro-peptide, occurs close to the sequence R(R/K)L(L/M), conserved in plant PMEs. The fate of the released pro-peptides is not known. However, the amino acid sequence

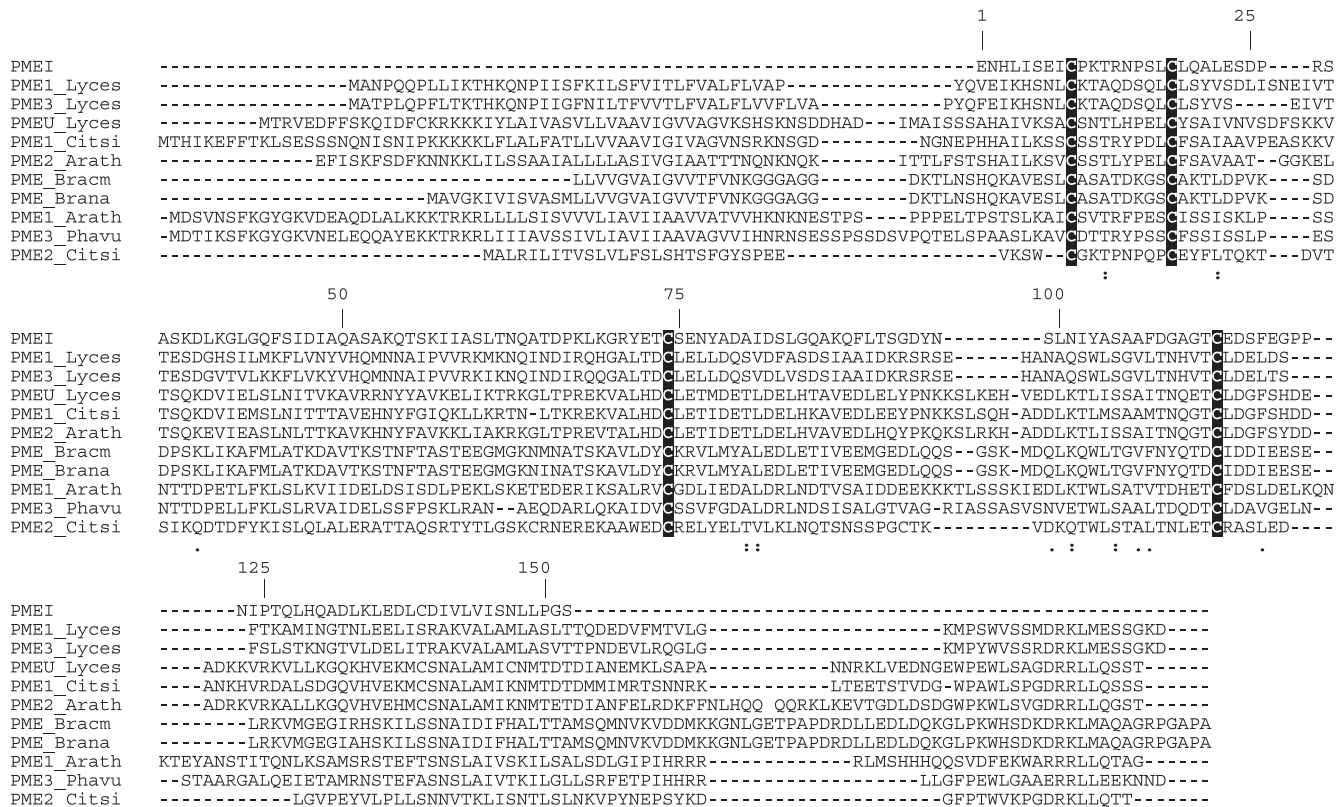


Fig. 3. Sequence alignment of PME1 with PME pro-peptides. The sequences are aligned with CLUSTALW. SwissProt accession numbers are: PME1 (P83326); PME1_Lyces (P14280); PME3_Lyces (Q96576); PME1_Lyces (Q43143), PME1_Citsi (O04886); PME2_Arath (Q42534); PME_Bracm (Q42608); PME_Brana (P41510); PME1_Arath (Q43867); PME3_Phavu (Q43111); PME2_Citsi (O04887). Sequence numbering refers to PME1. Four Cys residues conserved in all sequences are black-shadowed. Strongly similar residues (:) and weakly similar residues (.) are indicated. The conserved R(R/K)L(L/M) sequence, which is close to the cleavage site of PME precursor, is underlined.

and the predicted structure similarity with the kiwi PME are in favour of the hypothesis that the pro-peptide has an inhibitory function towards its own PME. It is likely that a genome rearrangement that occurred during evolution has produced the physical separation of the inhibitory domain initially linked to the catalytic domain, leading to a distinct protein with PME inhibitory activity.

3. Biotechnological applications of PME

Most of the vegetable transformation processes that are routinely employed in the food industry aim at the maintenance of firmness and texture of fruit and vegetable derived products [47–50]. The technologies employed are largely reliable, however, some problems concerning the quality of the product are not yet solved. Two problems in particular have been identified: the lack of firmness in canned vegetable during the storage and the cloud-loss of vegetable juices [51,52]. Firmness and texture changes of fruit and vegetable products during technological process and subsequent storage depend on the alterations of cell wall components, in particular the pectic substances [53]. Modification of pectin during processing is due to the action of endogenous pectin degrading enzymes, such as PME and PG. The synergic action of the two enzymes, both bound to the plant cell wall, yields short demethylated pectin chains, leading to softening of fruit and vegetable products and decrease of viscosity [54,55].

The fruit and vegetable juices can be considered biphasic systems, made up by a dispersing phase, called serum, in which the hydrosoluble components are dissolved, and a dispersed phase, called cloud, consisting of particles. The cloud phase, originating from plant tissue mechanically crushed in puree preparation, is constituted by solid particles with different size and shape [56]. These particles, essentially made of polysaccharides and, to a lesser extent, of proteins and cell wall-bound enzymes, are maintained in suspension by the interaction with soluble pectins. During the juice extraction step, PME de-esterifies the pectin. The low methylesterified pectin may interact with calcium ions forming insoluble pectates, which co-precipitate with the pulp-particles, thus causing cloud loss [53,57]. The degree of pectin methylation critical for this event is not yet well defined, due to the high heterogeneity of the pectic fraction in a complex system such as a vegetable juice. The juice stability also depends on the charge and hydration of pulp-particles in the suspension, both affected by the action of PME [58,59]. Therefore, PME is responsible for particle precipitation and cloud-loss of fruit juice. In order to prevent this phenomenon, PME must be inactivated. This is usually achieved by thermal treatments, such as pasteurisation or High Temperature Short Time (HTST) treatment. It is widely accepted that these treatments are sufficient to inactivate PME, although the assay of the residual PME activity in the thermically treated juices is generally per-

formed with the pH-stat method, which is not sensitive enough to detect weak enzymatic activity. In fact, cloud loss of thermically treated juices often still occurs and its control is a serious problem in the food industry. Much attention has been dedicated to the chemical and physical factors affecting the phenomenon, while the biochemical aspects of the problem have been investigated to lesser extent. PME in plant tissues exhibits different isoforms [20,55,60,61] with different thermal stabilities [21]. Only few data exist on the relation between the cloud loss of juices and PME thermostability [55,62]. The finding of residual PME activity in the commercial juices of some fruits [38,52] suggests the existence of extremely thermostable PME isoforms which survive the thermal treatment and indicates that some production process may be further improved.

The PME purified from kiwi fruit has a potential utilisation both in the detection of residual PME activity in fruit products and in PME inactivation.

A method using PME purified from kiwi fruit for the detection of low levels of PME activity in juices has been developed. The method is based on the affinity chromatography technique, employing a resin-bound PME, which selectively binds the native PME. The enzyme is eluted from the resin at pH 9.5 in a small volume and then assayed by the pH-stat method [63]. By this method, the presence of residual PME activity has been revealed in industrial tomato products showing cloud loss [52].

The most interesting biotechnological application of PME can be its use as adjuvant in the manufacturing of vegetable derived products. In fact, Castaldo et al. [64] found that the addition of PME to non-pasteurised orange juice prevented cloud loss after 9 months of storage at 5 °C. The PME-treated juices showed the same aspect as the pasteurised juices throughout the observation period. The analysis of the binding kinetics between PME and tomato PME indicates that the affinity between PME and PME increases as pH decreases, in the range 7.5–6.0 [36,37]. It is conceivable that the stability of the PME/PME complex is higher at acidic pH usually found in fruit juices. The PME inactivation by PME could allow a thermal treatment of juices at temperatures lower than those needed for inactivation of highly thermostable PMEs. Such milder thermal treatment could result in better flavour quality and higher vitamin content, thus producing high quality products.

4. Concluding remarks

PME has been found so far only in kiwi fruit. However, the specificity towards plant PME suggests a physiological role of this inhibitor. PME could be expressed in other plant or tissues only transiently, or in minute amounts or be tightly bound to its own PME, thus escaping biochemical detection. The observed similarity of the N-terminal non-catalytic extension of plant PMEs with PME supports the suggested inhibitory role of the PME pro-peptide region.

PMEI may be derived from a rearrangement of plant genome leading to a distinct protein with PME inhibitory activity. The common predicted structural arrangement shared by PMEI, INHs and PME pro-peptides might constitute a target required to direct the proteins to their cellular localisation.

A hint about the role of PMEI may also come from the observation that PME acts in the cell wall as a receptor of the tobacco mosaic virus movement protein, necessary for the translocation of the virus particles along plasmodesmata. Thus, PMEI could be involved in a defence mechanism against virus propagation through the formation of PME–PMEI complex. Future investigation will be focused on the characterisation of PMEI in other plant species, and the identification of amino acid residues or protein regions involved in the interaction between PMEI and PME, determining the specific recognition of the target enzyme.

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