Molecular basis of the activity of the phytopathogen pectin methylesterase

Markus Fries¹, Jessica Ihrig¹, Keith Brocklehurst¹, Vladimir E Shevchik² and Richard W Pickersgill^{1,*}

¹School of Biological and Chemical Sciences, Queen Mary, University of London, London, UK and ²CNRS, Composante INSA de l'Unité de Microbiologie et de Genetique, UMR 5122 CNRS-INSA-UCB, Bat. Andre Lwoff, Villeurbanne, France

We provide a mechanism for the activity of pectin methyl-esterase (PME), the enzyme that catalyses the essential first step in bacterial invasion of plant tissues. The complexes formed in the crystal using specifically methylated pectins, together with kinetic measurements of directed mutants, provide clear insights at atomic resolution into the specificity and the processive action of the *Erwinia chrysanthemi* enzyme. Product complexes provide additional snapshots along the reaction coordinate. We previously revealed that PME is a novel aspartic-esterase possessing parallel β -helix architecture and now show that the two conserved aspartates are the nucleophile and general acid-base in the mechanism, respectively. Other conserved residues at the catalytic centre are shown to be essential for substrate binding or transition state stabilisation. The preferential binding of methylated sugar residues upstream of the catalytic site, and demethylated residues downstream, drives the enzyme along the pectin molecule and accounts for the sequential pattern of demethylation produced by both bacterial and plant PMEs.

The EMBO Journal (2007) 26, 3879–3887. doi:10.1038/sj.emboj.7601816; Published online 23 August 2007

Subject Categories: microbiology & pathogens; structural biology

Keywords: mechanism; pectin methylesterase; processivity; specificity

Introduction

Pectin methylesterase (PME) (EC 3.1.1.11) from *Erwinia chrysanthemi* catalyses the important first step in the bacterial invasion of plant tissues, the deesterification of O6 methyl-esterified D-galacturonate (GalA) residues in pectic polysaccharides of the plant cell wall. The action of PME produces regions of polygalacturonic acid within the complex pectin polymer, which can then be depolymerised by the enzymes pectate lyase and polygalacturonase. This degradation of the plant cell wall allows the bacterial pathogen to invade the plant tissue and further spread disease. PME is not only

exploited by bacterial pathogens, but it is also essential for the spread of plant viruses, such as Tobacco mosaic virus, which uses endogenous plant PME as a vehicle for cell-to-cell movement (Dorokhov *et al*, 1999; Chen *et al*, 2000).

In contrast to PME secreted by *E. chrysanthemi* which is involved in bacterial invasion, endogenous plant PMEs are essential for remodelling the plant cell wall during growth and development. The *Arabidopsis thaliana* genome has more than 420 genes encoding enzymes involved in the synthesis and modification of the cell wall and over 150 genes for remodelling pectin (Arabidopsis Initiative, 2000). The crucial role of PME in plant development is emphasised by both the 79 isoforms found in *Arabidopsis* (Arabidopsis Initiative, 2000) and their involvement in a wide range of physiological processes including microsporogenesis, pollen tube growth, seed germination, root development, polarity of leaf growth, stem elongation, fruit ripening and loss of tissue integrity (Wen *et al*, 1999; Micheli *et al*, 2000; Pilling *et al*, 2000, 2004; Micheli, 2001). PMEs also act in defence mechanisms of plants against pathogens by releasing pectin fragments that function as messengers (Collmer and Keen, 1986) and by enhancing RNA silencing (Dorokhov *et al* 2006).

The common catalytic constellation of carbohydrate esterases is the Asp-His-Ser catalytic triad as seen in lipases and serine proteases. This catalytic triad is however not present at the active site of PME, which instead has two conserved aspartates and a conserved arginine as putative catalytic residues (Jenkins *et al*, 2001; Johansson *et al*, 2002). In addition, PME belongs, not to the typical α/β -hydrolase fold family of esterases, but to the family of parallel β -helix proteins (for a review, see Jenkins and Pickersgill, 2001).

Three patterns of action are generally proposed for the enzymatic conversion of polysaccharides: (i) a single-chain mechanism where the enzyme converts all substrate sites on the polymeric chain, (ii) a multiple-chain mechanism where the enzyme catalyses only one reaction and then dissociates from the substrate and, (iii) a multiple-attack mechanism where the enzyme catalyses a number of reaction cycles before the enzyme-polysaccharide complex dissociates. Plant and bacterial PMEs produce products with contiguous regions of GalA and both a single-chain and multiple-attack mechanism have been proposed (Dongowski and Bock, 1984; Kohn *et al*, 1985; Grasdalen *et al*, 1996; Christensen *et al*, 2001). In contrast, fungal PMEs attack more randomly and a multiple-chain mechanism has been proposed for those enzymes (Limberg *et al*, 2000; van Alebeek *et al*, 2003; Duvetter *et al*, 2006).

Here, we report the structures of four Michaelis complexes, each comprising an undistorted but catalytically inactive PME mutant and a specifically methylated hexameric substrate; these results reveal the molecular basis of the processive action of PME. Three product complexes, two formed using active enzyme and substrate, provide further crystallographic snapshots of the PME mechanism. These results, augmented with kinetic measurements of wild-type and

*Corresponding author. School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK. Tel.: +44 207 882 6360; Fax: +44 208 983 0973; E-mail: r.w.pickersgill@qmul.ac.uk

Received: 28 March 2007; accepted: 11 July 2007; published online: 23 August 2007

directed mutants, elucidate the mechanism, mode of action and specificity of PME.

Results and discussion

Choice of mutations and structure determination of mutant PMEs

The crystal structure of PME from *E. chrysanthemi* B374 revealed that the catalytic site comprises two conserved aspartates, Asp 178 and Asp 199, and a conserved arginine, Arg 267, which we presumed to be important for catalysis (Figure 1). To elucidate the individual roles of these three catalytic site residues, they were sequentially replaced by alanine in the present work. No activity was detected in any of the three mutants and to establish if the loss of activity was due to structural perturbation the structures of the three mutants were determined. Whereas the D178A mutant showed no difference in structure from that of wild-type PME, the D199A PME mutant had undergone significant

changes of the active centre architecture. Tyr 230 moved into the space once occupied by Asp 199 and Arg 267 changed its position and stacked on top of Tyr 230 (see the Supplementary data for more details). Surprisingly, the mutation of Arg 267 to alanine had no effect on the structural integrity of the active centre; despite this residue providing two hydrogen bonds to Asp 199 in the wild-type structure, the position of Asp 199 was not altered. However, as it was expected that the Arg 267 might be involved in binding the substrate, it was decided to use the other unperturbed PME mutant, D178A, for preparation of the Michaelis complexes.

Substrate specificity

The inactive D178A mutant was used to obtain complexes of PME with specifically methylated hexagalacturonates. Four Michaelis complexes were solved using the following substrates: Substrate I—MMCCCM; Substrate II—CCMMMC; Substrate III—MCCCCM; Substrate IV—CMMMMC (the oligosaccharide sequences are given from nonreducing to

Figure 1 Substrate binding to PME. (A) Stereoview of the cartoon representation of the three dimensional structure of PME with bound hexasaccharide (compound II) shown in 'stick' representation. Residues D199, R267 and D178A are also shown. The mutation D178A mutant was used to trap the Michaelis complex. (B) Surface representation of PME together with stick representation of hexasaccharide II showing the shape and position of the substrate-binding cleft, the reducing end of the hexasaccharide is to the right of this Figure and the substrate would move through the binding site from right to left. (C) The four specifically methylated hexasaccharides used in this work. Compound I: R1 = R2 = Me, R3 = H; compound II: R1 = R2 = H, R3 = Me; compound III: R1 = Me, R2 = R3 = H; compound IV: R1 = H, R2 = R3 = Me. Pymol (DeLano, 2002) was used to produce this figure and also Figures 2 and 3.

Table I Data collection and refinement statistics

Compound	I	II	III	IV	Product	pH 6.5	pH 3.2
Resolution (Å)	1.7	1.7	1.7	1.9	1.8	1.7	1.8
R_{merge} (%) ^{a,b}	7.5 (15.1)	8.5 (24.3)	6.6 (27.3)	10.3 (33.9)	6.3 (24.2)	6.5 (28.2)	11.0 (38.4)
Completeness (%)	99.8 (99.6)	96.3 (90.7)	95.2 (73.5)	90.1 (93.4)	99.9 (99.9)	100 (100)	100 (100)
Multiplicity	3.7 (3.6)	3.4 (3.2)	3.5 (2.6)	2.2 (2.2)	3.7 (3.7)	3.8 (3.8)	7.3 (7.4)
Mean I/σ (I)	16.3 (7.6)	12.8 (4.2)	14.4 (3.2)	9.1 (3.5)	16.8 (5.5)	15.0 (4.6)	16.2 (5.3)
R -factor (R -free) ^c	19.1 (20.7)	20.1 (23.2)	19.1 (21.3)	18.6 (20.5)	20.5 (24.5)	18.6 (21.9)	18.0 (21.2)
r.m.s.d. bonds ^d (Å)	0.007	0.007	0.007	0.008	0.008	0.007	0.008
r.m.s.d. angles ^d (deg)	1.0	1.0	1.1	1.1	1.1	1.1	1.1

^aValues in parentheses are for the final shell, which was in each case 0.1 Å wide.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$, where I_i is the intensity of the i th observation, $\langle I \rangle$ is the mean intensity of the reflection, and the summations extend over all unique reflections (hkl) and all equivalents (i), respectively.

^cThe R -factor = $\sum_{hkl} |F_o - F_c| / \sum_{hkl} |F_o|$, where F_o and F_c represent the observed and calculated structure factors, respectively. R -factor is calculated using 95% of the data included in refinement and R -free the 5% excluded.

^dr.m.s.d. is root mean square deviation and the target values were 0.02 Å and 2.0° for bonds and angles respectively.

reducing end where M and C denote a methylated GalA and an unmethylated GalA, respectively; also see Figure 1C). The structures were refined between 1.7 and 1.9 Å and the crystallographic statistics are summarised in Table I. The crystals have two PME molecules in the asymmetric unit, which were refined with tight noncrystallographic restraints.

For substrates I and III, there was electron density for three GalAs of the hexamer at a contour level of 1.5 σ in σ_A -weighted $2F_{\text{obs}} - F_{\text{calc}}$ syntheses. Additional electron density for the fourth, fifth and sixth GalA of substrates I and III was observed at a contour level of 0.5 σ . However, for substrate III, this electron density was less well defined. The GalAs of the bound pectin hexamers are all in the ⁴C₁ chair conformation. The subsites occupied by the hexasaccharides are summarised in Table II. Substrates I and III each bound PME in an identical way, with the nonreducing end at subsite −5 and the reducing end at subsite +1 (the active site is designated subsite +1). The binding mode of substrates II and IV was different from that of substrates I and III. Substrate II bound to subsites −2 to +4, whereas substrate IV occupied subsites −1 to +5. Well-ordered electron density was visible for five GalAs of the two hexamers (Figure 2). Electron density corresponding to the sixth GalA was observed at a lower contour level.

A systematic analysis of the ligand–protein interactions in the crystal suggests that the subsites involved in substrate binding are: −2, −1, +1, +2 and +3 (Figure 2 and Table II), and the most important subsites are −1 and +1. Subsites −1 and −2 were always occupied by nonmethylated GalAs, implying a strong preference for a carboxyl group. In contrast, in all Michaelis complexes, a methylated GalA was found at the catalytic site, indicating that the catalytic site has a strong preference for a methylster. At subsite +2 the C6 substituent faced the solvent as revealed in the Michaelis complexes II and IV. Hence, either a methylated or unmethylated GalA can be accommodated at subsite +2. The hydrophobic pocket found at subsite +3 explains why a methylster is favoured at that subsite. Substrates II and IV additionally occupy subsites +4 and +5, and substrates I and II additionally occupy −4 and −5, although hydrogen bonding at these sites is minimal. In conclusion, the complexes suggest the optimal substrate, binding to subsites −2 through +3, is CCMXM (Table II).

The software ConSurf 3.0 (Landau *et al*, 2005) was employed to identify the degree of conservation of residues

Table II Binding of pectin molecules to PME

	Subsites									
	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5
Preference				C	C	M	X	M		
Substrate I	<i>M</i>	<i>M</i>	<i>C</i>	C	C	M				
Substrate II				C	C	M	M	M	<i>C</i>	
Substrate III	<i>M</i>	<i>C</i>	<i>C</i>	C	C	M				
Substrate IV					C	M	M	M	M	<i>C</i>
Product				C	C	C	C	<i>C</i>		
PH 6.5			<i>M</i>	C	C	C	C	<i>X</i>		
PH 3.2		<i>X</i>	<i>M</i>	C	C	C	C			

GalA residues in bold and italics were visible at 1.5 and 0.5 σ , respectively in σ_A -weighted $2F_{\text{obs}} - F_{\text{calc}}$ syntheses. C indicates GalA, M indicates methylated GalA and X indicates the residue could be GalA or methylated GalA. The row labelled ‘preference’ gives the subsite preferences deduced from the structures of PME in complex with substrates I–IV.

involved in ligand binding amongst PME homologues. Conserved residues that bound to the various substrates were Thr 109, Gln 153, Gln 177, Val 198, Asp 199, Phe 202, Lys 223, Val 227, Ser 228, Gly 229, Arg 267 and Trp 269. Nonconserved amino-acid residues also contributed to ligand binding, namely, Ala 110, Arg 219, Asn 226, Tyr 230, Thr 272, Arg 279 and Met 306. Of particular interest are residues involved in binding the methylster and carboxylate groups of pectin, as they are obvious candidates for determining substrate specificity for specifically methylated pectin molecules. The lack of any protein contact with the pectin molecules at subsites −4 and −5 indicates that either a methylster or a carboxylate is tolerated to the nonreducing side of subsite −3. On the other hand, Arg 279 at subsite −3 bound to one of the oxygens of the carboxyl groups of substrate I (A chain only) and substrate III (B chain only). The proximity of this positively charged residue to the carboxyl group suggests a preference for a carboxylate rather than a methylster. At subsite −2 the main chain amide of Ala 110 and in some complexes also Thr 109 provide hydrogen bonds to both oxygens of the carboxylate, correlating with the preference for carboxylate at subsite −2. Likewise, the hydrogen bonds involving Trp 269 and Thr 272 (main chain amide and side chain hydroxyl) at subsite −1 explain the preference of a carboxylate group here. The specific binding of a methylster at the active site can be attributed to the

Figure 2 Enzyme-substrate interactions in detail. (A) Stereo-view of the stick model of the Michaelis complex formed using compound II overlaid with maximum likelihood/ σ_A weighted $2F_o - F_c$ syntheses contoured at 1.5σ and (B) schematic diagram showing the interactions involved. (B) was produced using LIGPLOT (Wallace *et al*, 1995).

hydrophobic environment created by Phe 202, Trp 269 and Met 306. At subsite +2 the methyl group of the ester faces the solvent and thus subsite +2 is not involved in determining substrate specificity. Val 198, Val 227 and Tyr 230 form a hydrophobic pocket for binding of the methyl function of the ester at subsite +3. The C6 substituents of GalA at subsites +4 and +5 do not interact with the protein, and thus subsites +4 and +5 are not expected to contribute to specificity for particular pattern of methylation of pectin substrate.

In conclusion, subsite -1 appears to be the key determinant of binding to PME, as in all complexes formed it is occupied by GalA (Table II). The importance of subsite -1 is highlighted by the number of interactions with bound GalA, involving at least six hydrogen bonds compared with between two and four for the other subsites. Pectins lacking a nonmethylated GalA, to bind to the -1 subsite, appear to bind very poorly as evidenced by unsuccessful attempts to prepare complexes with fully methylated tetra-GalA and hexa-GalA, respectively, despite the presence of the fully methylated

pectins at 100 mM concentration. The importance of subsite -1 is further emphasised by our observation that *E. chrysanthemi* PME is active against digalacturonate and trigalacturonate methylated at the reducing end, but not the corresponding oligogalacturonates methylated at the nonreducing end. The most important subsites are -1 and +1, which bind nonmethylated and methylated GalAs, respectively. Subsites -2 and +3 are of less relative importance, but reinforce the pattern of binding nonmethylated GalAs downstream and methylated GalAs upstream of the catalytic centre. Subsites -5, -4, -3, +2, +4 and +5 do not appear to significantly contribute to substrate specificity.

Product inhibition

Completely deesterified pectin, polygalacturonate, is a competitive inhibitor of PME with a reported K_i of 16 mM (Pitkänen *et al*, 1992). The lower value of K_m for pectin (2.6 mM) (Pitkänen *et al*, 1992) compared to the reported K_i for polygalacturonate may be attributed to the contribution of the methyl groups to substrate binding. To investigate the mechanism of product inhibition and to reveal any changes in the active centre after demethylation of a substrate, GalA6 was soaked into native PME crystals. The structure of the PME-product complex was determined at 1.8 Å resolution. There was electron density for four GalA residues at 1.5 σ binding to subsites -2 to +2 with refined B-factors of 17–22 Å². At 0.75 σ , two additional GalA residues were also visible binding subsites -3 and +3 (refined B-factors of 40 Å²). The product has a clear preference for subsites -2 to +2, and although mixed binding modes occupying the central subsites -2 to +2 is possible, a single binding mode with highly mobile terminal sugars would also account for the observed density. The same residues are involved in binding product as described and shown for substrate and there is no difference in the conformation of enzyme or ligand. Subsites that contribute to the stronger binding of pectin, compared to polygalacturonate, are +1 where Phe 202, Trp 269 and Met 306 contribute to binding the methyl group and +3 where Val 198, Val 227 and Tyr 230 contribute to the preference for a methyl group.

Time-dependent studies

To gain insight into the catalytic action of PME, wild-type PME crystals were incubated in cryobuffer with fully methylated GalA6 at pH 3.2 and at pH 6.5 for various times, and subsequently flash-frozen in liquid nitrogen. Complexes of structures with bound hexasaccharide were determined between 1.7 and 1.8 Å, and the clearest electron density was seen after 20 min; before 20 min, the electron density was weaker with no clear evidence of bound oligosaccharide. After 20 min, the product of the time course had bound in a similar way to hexagalacturonate. The enzyme has produced a predominantly demethylated substrate with evidence for a methylester group at the nonreducing end of the hexasaccharide (subsite -3).

A plausible explanation for these results is that, because fully methylated hexagalacturonate is a very poor substrate, most of the first 20 min was needed for removal of the first methyl group, after which the oligosaccharide becomes a substantially better substrate and is quickly turned into the final product with a single methyl group remaining at the nonreducing end. This result highlights the importance of

the GalA binding to subsite -1 for catalysis. The final product has a methylated GalA at its nonreducing end, because there is no residue available to bind to subsite -1 if the terminal sugar residue occupies subsite +1.

Molecular basis of the processive action of pectin methylesterase

The Michaelis complexes reveal a clear preference for the binding of methylated GalA residues upstream and demethylated residues downstream of the catalytic site (Figure 2 and Table II). The catalytic site itself has a preference for methylated GalA. The molecular details of the methyl-ester binding subsites +1 and +3 have been highlighted above (see substrate specificity) as have the details of the -1 and -2 subsites, which preferentially bind GalA. These preferences explain immediately why PME produces contiguous regions of GalA in its products, because it preferentially deesterifies a methylated sugar to the reducing side of a nonesterified sugar residue. The key structural features are the hydrophobic pocket at the +1 subsite and the hydrogen bonding at the -1 subsite. The former, methyl-ester binding site, is created by Phe 202, Trp 269 and Met 306 and the latter, carboxylate-binding site, by hydrogen bonding groups from Trp 269 and Thr 272. Thus, there is a direction of demethylation irrespective of whether the oligosaccharide diffuses out of the substrate-binding cleft between methyl-ester hydrolysis reactions or not. It is plausible that because of the entropic penalty involved in solvating the sugar and enzyme surfaces, diffusion out of the substrate-binding cleft is unlikely. Rather, entropic forces will tend to keep the oligosaccharide close to the enzyme, excluding water, whereas the hydrogen bonds break and reform as the oligosaccharide slides through the substrate-binding cleft. Our analysis also agrees with the direction of movement inferred by others (Grasdalen *et al*, 1996; Kester *et al*, 2000; van Alebeek *et al*, 2003), but the pentasaccharide used by van Alebeek *et al* (2003) is suggested to proceed through the substrate-binding cleft in the reverse direction.

Reaction mechanism

The structures of the Michaelis complexes of PME from *E. chrysanthemi* with various substrates, as well as the PME-product complex suggest the following residues are involved in the reaction mechanism: Asp 199, Asp 178 and Gln 177 (Figure 3). Arg 267, conserved amongst all PMEs, is not a direct participant in catalysis. It is reasonable to assume that Asp 178 is mainly in the protonated state due to its buried position in a hydrophobic environment. By contrast, Asp 199 is solvent accessible and is therefore likely to be substantially deprotonated at neutral pH. A hydrophobic patch created by Arg 267, Ala 233, Tyr 230 and Val 198 favours localisation of the negative charge of Asp 199 towards the solvent and incoming substrate. The hydrogen bonds between Arg 267 and the Asp 199 oxygens may help to maintain Asp 199 in a deprotonated state. Residues corresponding to Asp 199 and Asp 178 were proposed to be the nucleophile and the general acid, respectively. Gln 153 and Gln 177 were proposed to contribute to the oxyanion hole (Johansson *et al*, 2002).

The enzyme-substrate complexes clearly show that Asp 199 acts as a nucleophile that attacks the carbonyl carbon of the C6 ester. Attack on a trigonal (sp^2 -hybridised) carbon such as this always occurs in a plane orthogonal to the plane

Figure 3 Stereo-view of the active site residues in the Michaelis complex formed using compound II and the D178A mutant of PME. Asp 178 (the general acid-base) is built back into this structure in the conformation seen in the wild-type enzyme, the conformation of the other residues and substrate are as seen in the electron density map of the complex.

of the double bond and the orientation of Asp 199 is appropriate for such a nucleophilic attack. By contrast, Asp 178 is in approximately the same plane as the carbonyl group and, therefore, cannot be the nucleophile. Asp 178 is well positioned to act as a general acid-base catalyst in the reaction mechanism. Asp 178 forms a strong hydrogen bond with the carbonyl oxygen of the methylester (in the PME-product complex and the structures from the time courses). The PME-pectin complexes also reveal the function of the highly conserved Gln 177. It forms a strong hydrogen bond with the carbonyl group of the ester, suggesting that it is involved in stabilising the negative charge of the carbonyl oxygen that develops after the nucleophilic attack. The kinetic results (Table III) support the involvement of glutamine 177 in transition state stabilisation; the Q177A mutation results in a large decrease in k_{cat} and only a small increase in K_{m} . In contrast, the Q153A mutant shows a small decrease in k_{cat} and a large increase in K_{m} , revealing that the contribution of Gln 153 is to binding rather than transition state stabilisation. The values of $k_{\text{cat}}/K_{\text{m}}$ are very similar for these two mutants and significantly diminished. The critical importance of Arg 267 and Trp 269 is demonstrated by the complete absence of activity upon mutation of these residues to alanine. This may result from the loss of productive binding of substrate and, moreover, perturbation of the electrochemical environment of the catalytically essential aspartate residues. Trp 269 forms part of the hydrophobic pocket at the +1 site and provides a hydrogen bond and a hydrophobic contact to the GalA binding at subsite -1, as well as influencing the environment of Asp 178. Arg 267 makes a number of hydrogen bonds with Asp 199 and substrate. The effect of mutating Met 306, which forms part of the hydrophobic pocket at the catalytic site, is a decrease of k_{cat} and an increase of K_{m} , resulting in a much less efficient enzyme as indicated by the reduced $k_{\text{cat}}/K_{\text{m}}$ value. Mutation of Val 198, which forms part of the hydrophobic pocket at subsite +3 and the hydrophobic patch next to Asp 199 chiefly decreases k_{cat} . The decrease of k_{cat} might result from a direct influence on the catalytic residues, as well as decreased processivity for the polymeric substrate, which is assumed to slide through the active site cleft. The T272A mutation (subsite -1) mainly increased the K_{m} value presumably as two hydrogen bonds to the substrate are lost.

In conclusion, we propose the following reaction mechanism (Figure 4): Asp 178 activates the ester for nucleophilic

Table III Steady-state kinetic analyses of mutant PMEs

	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \text{mM}^{-1}$)
PME3937 wild-type	450 ± 12	0.13 ± 0.02	3462
Q153A	232 ± 19	2.2 ± 0.4	106
Q177A	40 ± 1	0.22 ± 0.01	181
D178A	—	—	—
V198A	68 ± 3	0.06 ± 0.01	1133
D199A	—	—	—
R267A	—	—	—
W269A	—	—	—
T272A	425 ± 8	0.77 ± 0.04	552
M306A	59 ± 1	0.53 ± 0.03	111

The calculation of K_{m} in mM assumes that all methylated GalAs are substrates and the degree of methylation of the pectin used (Sigma P9135) is 9%.

attack by hydrogen bonding to the oxygen of the carbonyl group. The negatively charged carboxylate of Asp 199 then attacks the carbonyl carbon. A tetrahedral intermediate is formed with a negative charge on the carbonyl oxygen. This negative charge is stabilised by interactions with Gln 177 and Asp 178. Methanol is then released involving protonation of the leaving group by Asp 178 to provide an anhydride intermediate. Nucleophilic catalysis by a carboxylate group in low M_{r} systems is limited to esters with good leaving groups (pK_{a} of the conjugate acid ≤ 7). Thus, the role of Asp 178 as a general acid catalyst of leaving group departure is clearly essential in the PME-catalysed hydrolysis of methyl esters. A water molecule (seen in the pH 3.2 structure) replaces the departing methanol molecule. The subsequent hydrolysis of the anhydride by this water molecule is base catalysed by Asp 178 and provides the final product, the demethylated GalA and the recycled enzyme.

The crystal structures provide clear insights into the reaction coordinate of PME at atomic resolution. Asp 199 is the nucleophile directly attacking the carbonyl carbon of the methyl ester and Asp 178 is the general acid-base in the reaction. There is no room for a water molecule to be accommodated between Asp 199 and substrate, and so the alternative mechanism where a water molecule is activated is ruled out. Simple activation of the methyl ester by Asp 178 without nucleophilic assistance would not explain the conservation of Asp 199 across the PME family and the stereochemistry observed at the catalytic site. Arg 267 and Trp 269

Figure 4 The proposed mechanism of PME based on the crystal structures and kinetic analyses of directed mutants.

are not directly involved in catalysis, but are involved in substrate binding. A chemically plausible hydrolytic water molecule, one that hydrolyses the acyl-enzyme, is seen in the low pH time course complex adjacent to the general base (Asp 178). The structures suggest that Gln 177 forms the oxyanion hole and its contribution to transition state stabilisation is confirmed by kinetic analysis; adjacent Gln 153 contributes mainly to substrate binding.

Conclusion

Knockout mutations confirm that PME from the plant pathogen *E. chrysanthemi* is essential for bacterial invasion of plant tissue and the resulting soft rot disease (Shevchik and Hugouvieux-Cotte-Pattat, 1997). We have successfully elucidated the molecular basis of the processivity, specificity and mechanism of PME, the knowledge of which may facilitate the design of inhibitors of this plant pathogen. The processive action of PME is driven by a preference for the binding of substrate methylesters upstream (reducing side) and carboxylates downstream (nonreducing side) of the active centre. Unsurprisingly, the active centre itself preferentially binds a methylester. Most importantly, there is a strong preference for binding an unmethylated GalA at subsite -1. Thus, both the molecular basis of the processivity of the enzyme and

the preference of PME for partially demethylated pectins (Grasdalen *et al*, 1996; van Alebeek *et al*, 2003) are explained. We envisage that, when there is at least one nonmethylated GalA downstream and methylated GalAs upstream, PME slides along the pectin polymer rather than dissociating and rebinding due to the entropic cost of dissociation, a result of binding water to both enzyme and substrate. The cost of PME's processivity is product inhibition, but this will not limit PME activity *in vivo*, because pectate lyase rapidly cleaves the demethylated pectins.

Materials and methods

Materials

Pectin from citrus peel (P9135) was purchased from Sigma. Selectively methylated hexa-GalAs were kindly provided by Clausen and Madsen (Clausen *et al*, 2001; Clausen and Madsen, 2003, 2004). Fully methylated hexa-GalA and completely unmethylated hexa-GalA were a kind gift from MacDoughall (Norwich).

Expression, purification and enzyme assay

E. coli strain NM522 (Stratagene) was employed to express wild-type and mutant PMEs from *E. chrysanthemi* 3937 encoded on plasmid pBCKSpemA. Mutants of PME from *E. chrysanthemi* 3937 were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The harvested cells from the expression cultures were

resuspended in ice-cold 30 mM Tris-HCl pH 8.0, 1 mM EDTA and the periplasmic protein was extracted from the periplasm by addition of the same volume of ice-cold 40% w/v sucrose and incubation on ice for 10 min. Subsequently, the extracted protein solution was dialysed against 20 mM MES (pH 6.0) and purified on a HiTrap S-Sepharose column (Amersham). Activity of the mutant PME was determined using a pH stat TitraLab 854 (Hach-Lange), following the release of protons during the deesterification of pectin from citrus fruit. The standard assay was with 0.7% pectin w/v, 100 mM NaCl at pH 7 at 30°C. The assay volume was 2 ml and 1 µg of PME was used. Kinetic parameters were determined by varying pectin concentrations from 1 to 0.002% (w/v). Data were analysed and fitted to a Michaelis-Menten curve using the Sigma Plot 8.0 software.

Crystallisation and structure determination

The purified PME was concentrated to 3 mg/ml by means of Millipore Centricons or Vivaspin concentrators and crystallised using 2 µl of protein and 2 µl of reservoir. The best crystals were obtained from 0.1 M MES (pH 6.5), 10% Dioxane and 1.6 M ammonium sulphate from Hampton Research Crystal screen 2 and dilutions of that crystallisation buffer with H₂O. Slightly smaller crystals were yielded from 0.1 M MES (pH 6.5) and 12% w/v PEG 20 000. The largest crystals grew as small rectangular plates with dimensions of $0.1 \times 0.17 \times 0.01$ mm³. The same cryoprotectant (0.1 M MES pH 6.5, 12% w/v PEG 20 000, 35% w/v glycerol) was used for crystals from both conditions. For the production of Michaelis complexes, crystals were soaked in 100 mM solutions of various oligo-GalAs.

Synchrotron X-ray data were collected at 100 K at Daresbury SRS stations 10.1 and 14.1 and at Grenoble ESRF at stations ID 14-1, 14-3 and 29. MOSFLM was utilised for indexing and integrating

(Leslie, 1992). The crystals were space group P2₁ with $a = 51.1$ Å, $b = 86.1$ Å, $c = 97.3$ Å and $\beta = 93.8^\circ$. The structures were solved by difference Fourier syntheses using the structure of PME from *E. chrysanthemi* B374 as the phasing model. SCALA (CCP4 Initiative, 1994) and REFMAC5 (Murshudov *et al*, 1997) of the CCP4 5.0 program suite were used for data reduction and refinement. A test set composed of 5% of the total reflections was excluded to allow calculation of the free *R* factor. Ligands were modelled into the electron density map using COOT (Emsley and Cowtan, 2004). Manual model building was followed by refinement with REFMAC5 and automated water addition using ARP/wARP (Perrakis *et al*, 1999).

Data deposition

The atomic coordinates and structure factor amplitudes for the seven structures described have been deposited in the Protein Data Bank, www.pdb.org (PDB codes: 2NSP, 2NST, 2NT6, 2NT9, 2NTB, 2NTP, 2NTQ).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research, and the Higher Education Funding Council for England. We thank Robert Madsen and Mads Clausen for compounds I to IV and acknowledge ESRF Grenoble and SRS Daresbury for the provision of synchrotron radiation and X-ray data collection facilities.

References

- Arabidopsis Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815
- CCP4 Initiative (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D* **50**: 760–763
- Chen MH, Sheng J, Hind G, Handa AK, Citovsky V (2000) Interaction between the tobacco mosaic virus movement protein and host cell pectin methyltransferases is required for viral cell-to-cell movement. *EMBO J* **19**: 913–920
- Christensen TMIE, Brunstedt J, Buchholt HC, Mikkelsen JD (2001) *Pectins and Pectinases*. The Netherlands: Rotterdam
- Clausen M, Madsen R (2004) Synthesis of oligogalacturonates conjugated to BSA. *Carbohydr Res* **339**: 2159–2169
- Clausen MH, Jorgensen MR, Thorsen J, Madsen R (2001) A strategy for chemical synthesis of selectively methyl-esterified oligomers of galacturonic acid. *J Chem Soc-Perkin Trans 1*: 543–551
- Clausen MH, Madsen R (2003) Synthesis of hexasaccharide fragments of pectin. *Chemistry* **9**: 3821–3832
- Collmer A, Keen NT (1986) The role of pectic enzymes in plant pathogenesis. *An Rev Phytopathol* **24**: 383–409
- DeLano WL (2002) *The PyMOL Molecular Graphics System*. San Carlos, CA, USA: DeLano Scientific
- Dongowski G, Bock W (1984) On the effects of molecular-parameters of pectin-substrates on the activities of pectin-esterases from *Aspergillus niger* and from higher plants. *Nahrung-Food* **28**: 507–516
- Dorokhov YL, Makinen JG, Frolova OY, Merits A, Saarinen J, Kalkinen N, Atabekov JG, Saarna M (1999) A novel function for a ubiquitous plant enzyme pectin methyltransferase: the host-cell receptor for the tobacco mosaic virus movement protein. *FEBS Lett* **461**: 223–228
- Dorokhov YL, Frolova OY, Skurat EV, Ivanov PA, Gasanova TV, Sheveleva AA, Ravin NV, Makinen KM, Klimyuk VI, Skryabin KG, Gleba YY, Atabekov JG (2006) A novel function for a ubiquitous plant enzyme pectin methyltransferase: the enhancer of RNA silencing. *FEBS Lett* **580**: 3872–3878
- Duvetter T, Fraeye I, Sila DN, Verlent I, Smout C, Hendrickx M, Van Loey A (2006) Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. *J Agric Food Chem* **54**: 7825–7831
- Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D* **60**: 2126–2132
- Grasdalen H, Andersen AK, Larsen B (1996) NMR spectroscopy studies of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism. *Carbohydr Res* **289**: 105–114
- Jenkins J, Mayans O, Smith D, Worboys K, Pickersgill RW (2001) Three-dimensional structure of *Erwinia chrysanthemi* pectin methyltransferase reveals a novel esterase active site. *J Mol Biol* **305**: 951–960
- Jenkins J, Pickersgill R (2001) The architecture of parallel beta-helices and related folds. *Prog Biophys Mol Biol* **77**: 111–175
- Johansson K, El-Ahmad M, Friemann R, Jorvall H, Markovic O, Eklund H (2002) Crystal structure of plant pectin methyltransferase. *FEBS Lett* **514**: 243–249
- Kester HC, Benen JA, Visser J, Warren ME, Orlando R, Bergmann C, Magaud D, Anker D, Doutheau A (2000) Tandem mass spectrometric analysis of *Aspergillus niger* pectin methyltransferase: mode of action on fully methyl-esterified oligogalacturonates. *Biochem J* **346** (Part 2): 469–474
- Kohn R, Dongowski G, Bock W (1985) The distribution of free and esterified carboxyl groups within the pectin molecule after the influence of pectin esterase from *Aspergillus niger* and oranges. *Nahrung-Food* **29**: 75–85
- Landau M, Mayrose I, Rosenberg Y, Glaser F, Martz E, Pupko T, Ben-Tal N (2005) ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures. *Nucleic Acids Res* **33**: W299–W302
- Leslie AGW (1992) Recent changes to the MOSFLM package for processing film and image plate data. *Joint CCP4+ESF-EAMCB Newsletter on Protein Crystallography* **26**
- Limberg G, Korner R, Buchholt HC, Christensen T, Roepstorff P, Mikkelsen JD (2000) Analysis of pectin structure part 1—analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A-niger*. *Carbohydr Res* **327**: 293–307
- Micheli F, Sundberg B, Goldberg R, Richard L (2000) Radial distribution pattern of pectin methyltransferases across the cambial region of hybrid aspen at activity and dormancy. *Plant Physiol* **124**: 191–199

- Micheli F (2001) Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends Plant Sci* **6**: 414–419
- Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* **53**: 240–255
- Perrakis A, Morris R, Lamzin VS (1999) Automated protein model building combined with iterative structure refinement. *Nat Struct Biol* **6**: 458–463
- Pilling J, Willmitzer L, Bucking H, Fisahn J (2004) Inhibition of a ubiquitously expressed pectin methyl esterase in *Solanum tuberosum* L. affects plant growth, leaf growth polarity, and ion partitioning. *Planta* **219**: 32–40
- Pilling J, Willmitzer L, Fisahn J (2000) Expression of a *Petunia inflata* pectin methyl esterase in *Solanum tuberosum* L. enhances stem elongation and modifies cation distribution. *Planta* **210**: 391–399
- Pitkänen K, Heikinheimo R, Pakkanen R (1992) Purification and characterization of *Erwinia chrysanthemi* B374 pectin methylesterase produced by *Bacillus subtilis*. *Enzyme Microb Technol* **14**: 832–836
- Shevchik VE, Hugouvieux-Cotte-Pattat N (1997) Identification of a bacterial pectin acetyl esterase in *Erwinia chrysanthemi* 3937. *Mol Microbiol* **24**: 1285–1301
- van Alebeek GJ, van Scherpenzeel K, Beldman G, Schols HA, Voragen AG (2003) Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*. *Biochem J* **372**: 211–218
- Wallace AC, Laskowski RA, Thornton JM (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng* **8**: 127–134
- Wen F, Zhu Y, Hawes MC (1999) Effect of pectin methylesterase gene expression on pea root development. *Plant Cell* **11**: 1129–1140