

Pectin methyl esterase from *Aspergillus aculeatus*: expression cloning in yeast and characterization of the recombinant enzyme

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Seventeen full-length cDNAs encoding pectin methyl esterase I (PME I) have been isolated from the filamentous fungus *Aspergillus aculeatus* by expression cloning in yeast. Yeast colonies expressing functional PME I were identified on agar plates containing highly esterified pectin, and a cDNA encoding PME I was isolated. The deduced amino acid sequence of PME I is highly similar (74% identity) to the PME from *Aspergillus niger*. A full-length cDNA encoding PME I was cloned into an *Aspergillus* expression vector and transformed into *Aspergillus oryzae* for heterologous expression, purification and characterization of the recombinant enzyme. The recombinant PME I had a molecular mass of 36.2 kDa, an isoelectric point of pH 3.8, a pH optimum of 4.6 and a temperature optimum of 45 °C. The authentic PME I was purified from *A. aculeatus* culture super-

natant and subjected to amino acid sequencing. The peptide sequences covered 138 amino acid residues and were in complete agreement with the deduced PME I sequence. Both recombinant and authentic PME I were glycosylated, but the composition of the glycan moieties was different. PME I was able to remove 75–85% of the methyl groups in highly methylated pectin, and it did not remove acetyl groups from acetylated polysaccharides. When the enzyme was added together with polygalacturonases to pectin, a rapid depolymerization was observed. By comparison, polygalacturonases alone showed a very limited degradation of the methylated substrate. This demonstrates that PME I acts in synergy with polygalacturonases in the degradation of plant cell wall pectin.

INTRODUCTION

Dicotyledonous plants comprise some of the major crops cultured by man, such as beans, peas, beets, as well as most other fruits and vegetables. Pectic substances are major components of the primary cell wall and middle lamella of such plants. These complex polysaccharides are composed of smooth regions, containing mainly galacturonic acid, interrupted at regular intervals by so-called 'hairy regions', where multiple side-chains of neutral sugars are attached [1–3]. The smooth regions are linear polymers of up to 100 residues of α -(1,4)-linked D-galacturonic acid [4]. The galacturonic acid residues in the smooth regions can be methyl-esterified to a varying degree and the methylation is usually present in a non-random fashion, with blocks of polygalacturonic acid being completely methyl-esterified [5].

Pectin methyl esterases (PMEs; EC 3.1.1.11) hydrolyse the ester linkage between methanol and galacturonic acid in esterified pectin, and they are found both in plants and in some plant cell-wall-degrading micro-organisms [6–8]. Several plant PMEs have been subjected to molecular characterization [9–11], while the only fungal enzyme cloned and characterized to date is from the filamentous fungus *Aspergillus niger* [12,13].

PME activity is of major importance for complete degradation of pectin by polygalacturonases (PGs) and pectate lyases, since these enzymes are unable to recognize and cleave methyl-esterified pectin. Moreover, the physicochemical properties of the pectin polymer are controlled by the degree and distribution of methylation. Thus, PMEs play an important role in nature by modu-

lating the characteristics of pectin by altering the degree of esterification (DE), as well as the degradability of pectin polymers. In turn, this may be of importance in industrial processing of pectin and pectin-containing plant material.

Our aim was to clone and characterize the PME from the filamentous fungus *Aspergillus aculeatus*, which is known to produce a wide range of pectinolytic enzymes in culture [14–16]. Here, we describe the isolation of full-length cDNA clones encoding PME I by expression cloning in the yeast *Saccharomyces cerevisiae* [17–20]. The deduced PME amino acid sequence showed similarity to the PME from *A. niger* [12], as well as limited similarity to plant and bacterial PMEs. The *pme1* cDNA was expressed in *Aspergillus oryzae*, and the recombinant PME I was purified and characterized by a specific assay based on titration of free acid groups generated by the action of the enzyme. The biochemical properties of the enzyme were compared with authentic PME I purified from *A. aculeatus*.

MATERIALS AND METHODS

Materials

Highly esterified apple pectin was purchased from Herbstreich KG, Neunbürg, Germany (lot no. 710260) and purified by precipitation with 70% (v/v) 2-propanol/3.7% (w/v) HCl. The precipitate was washed with 75% (v/v) 2-propanol until all chloride was removed, and the pectin was dried at 20 °C. Citrus pectin (GENU) was obtained from Copenhagen Pectin, Lille Skensved, Denmark. Purified *A. niger* PG was purchased from

Abbreviations used; DE, degree of esterification; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); HPLC, high-performance size-exclusion chromatography; MTAB, mixed alkyltrimethylammonium bromide; PG, polygalacturonase; PME, pectin methyl esterase; *pme*, gene encoding PME; TFA, trifluoroacetic acid.

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Sigma, U.S.A. (cat. no. P3429). Reagents and solvents for amino acid sequencing were from Applied Biosystems (Foster City, U.S.A.). HCl for amino acid analysis was from BDH (Poole, Dorset, U.K.) and α -cyano-4-hydroxycinnamic acid was from Aldrich (Steinheim, Germany). All other chemicals were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Expression cloning of PME cDNAs

Construction of the cDNA library from *A. aculeatus* has been described previously [18,20]. Plasmid DNA from a cDNA library pool was transformed into *S. cerevisiae* W3124 [21] by electroporation [22], and the transformants were plated on to SC (7.5 g/l yeast nitrogen base, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids, 0.1 g/l tryptophan and 20 g/l agar)-plates [23] containing 2% (w/v) glucose. After incubation at 30 °C for 3 days, the colonies were replicated on to SC-plates containing 2% (w/v) galactose and incubated for 4 days at 30 °C. PME-expressing yeast colonies were identified by casting an overlay containing 1% of 75% methyl-esterified (DE 75%) apple pectin in 0.1 M sodium citrate/sodium phosphate buffer, pH 4.5, on the plates. After incubation for 24 h, the PME activity was visualized by precipitation with 1% mixed alkyltrimethylammonium bromide (MTAB). Total DNA from the PME-expressing yeast colonies was isolated according to Strathern and Higgins [24], and the plasmids were rescued by transformation of *Escherichia coli* MC1061 to ampicillin resistance.

The longest PME I cDNA clone (pC1PE5) was sequenced from both strands by the dideoxy chain-termination method [25] using purified plasmid DNA (Qiagen, U.S.A.), the Sequenase kit (US Biochemical Co., U.S.A.) and either pYES 2.0 polylinker primers or synthetic oligonucleotide primers. The sequence data were analysed according to Devereux et al. [26].

Construction of the *pme1* expression cassette and transformation of *A. oryzae*

Plasmid DNA from pC1PE5 was digested with *Bam*HI and *Xho*I, separated by electrophoresis in a 0.7% agarose gel, followed by purification of the 1.2 kb *pme1* cDNA insert using the GeneClean II kit according to the manufacturer's instructions (Bio 101 Inc., La Jolla, CA, U.S.A.) and ligation into *Bam*HI/*Xho*I-cleaved pHD464 vector [17]. Co-transformation of *A. oryzae* wild-type strain A 1560 was carried out as described by Christensen et al. [27]. Transformants were purified twice through conidial spores, and cultured in 5 ml of YP medium (20 g/l bacto-peptone, 10 g/l bacto-yeast extract and 20 g/l glucose) for evaluation of PME expression. The transformant producing the highest level of recombinant PME I was selected by SDS/PAGE and enzyme activity (overlay assay) for fermentation in a lab-scale fermenter.

Southern blot analysis

Genomic DNA from *A. aculeatus* was isolated [28] and digested to completion with *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III (10 μ g/sample), fractionated by electrophoresis in a 0.7% agarose gel, denatured and blotted on to a nylon filter (Hybond-N, Amersham) using 10 \times SSC as transfer buffer [28a] (1 \times SSC: 0.15 M sodium chloride/0.015 M sodium citrate). The 1.2 kb *pme I* cDNA was 32 P-labelled ($> 1 \times 10^9$ c.p.m./ μ g) by random priming and used as a probe in Southern analysis. The hybridization and washing conditions were as described [28a]. The filter was autoradiographed at -80 °C for 12 h.

Purification of PME I

Both recombinant and authentic PME I were purified by FPLC performed at 4 °C to minimize proteolytic degradation, but without addition of specific protease inhibitors to the buffers. All ultrafiltration was performed with a 10 kDa cut-off membrane (Amicon, U.S.A.).

The authentic enzyme was purified from a commercially available *A. aculeatus* supernatant (Pectinex Ultra SP, Novo Nordisk, Denmark). Initially, a 100 ml sample with a protein concentration of approx. 100 g/l was transferred into 0.02 M Tris/HCl, pH 6.5, by ultrafiltration and applied on a QMA 7.5 cm \times 20 cm anion-exchange column (Waters, U.S.A.). Bound proteins were eluted with a linear NaCl gradient and the PME-containing fractions were pooled, changed into 0.02 M Tris/HCl, pH 8.0, by ultrafiltration and re-chromatographed on the same column. $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 1 M to the PME-containing sample, followed by application on a 5 cm \times 24 cm Phenyl-Toyopearl 650 M column (TosoHaas, Japan). The pool of unbound proteins from the hydrophobic column contained the PME activity, and it was buffer-changed into 0.02 M sodium phosphate, pH 7.5, by ultrafiltration. The sample was loaded on to a 2.6 cm \times 30 cm Accell QMA column (Waters, U.S.A.) and the enzyme was eluted with a decreasing pH gradient from pH 7.5 to 5.0. PME I-containing fractions were pooled, concentrated and buffer-changed into PBS by ultrafiltration. This preparation was subjected to amino acid sequencing and characterization.

Recombinant PME I expressed in *A. oryzae* was purified from a culture supernatant harvested after 5 days of fermentation in a 1 litre fermenter. The fungal mycelium was removed by centrifugation, the supernatant was filter sterilized, and concentrated by ultrafiltration to approx. 20% dry matter. A 40 ml aliquot of the concentrated sample was diluted 10-fold in 0.02 M Tris/HCl, pH 8.0, applied to a XK16/20 Q-Sepharose fast flow column (Pharmacia, Sweden) at 1.5 ml/min, and eluted with a linear gradient from 0 to 0.5 M NaCl at approx. 0.4 M NaCl. The fractions containing PME activity were pooled, transferred into 0.02 M citrate, pH 4.0, by ultrafiltration and loaded on to an XK16/20 S-Sepharose fast flow column (Pharmacia, Sweden) at 1.5 ml/min. PME I was eluted with a linear NaCl gradient at approx. 0.2 M NaCl. The fractions containing PME activity were pooled, buffer-changed into PBS by ultrafiltration and used for characterization as described below.

Amino acid analysis and sequencing

Lyophilized samples of PME I were hydrolysed in evacuated sealed glass tubes with 100 μ l of 6 M HCl containing 0.1% phenol for 16 h at 110 °C. Amino acid analysis was carried out using an Applied Biosystems 420A amino acid analysis system.

Reduced and *S*-carboxymethylated PME I (≈ 1 mg) was digested with either trypsin or chymotrypsin in 0.02 M NH_4HCO_3 for 16 h at 37 °C with an enzyme-to-substrate ratio of 1:100. The resulting peptides were separated by reversed-phase HPLC using a Vydac C_{18} column (Vydac, U.S.A.), eluted with a linear gradient of 80% 2-propanol containing 0.08% trifluoroacetic acid (TFA) in 0.1% aqueous TFA (4–72% 2-propanol over 85 min). Isolated peptides were sequenced in an Applied Biosystems 473A protein sequencer according to the manufacturer's instructions.

Titration of cysteine and cystine content of authentic PME I

Two identical samples containing 3.6 nmol of PME I each were lyophilized. The sample for titration of free cysteine was dissolved

in 20 μl of water. The sample for determination of total cysteine content following reduction of disulphide bonds was dissolved in 20 μl of freshly prepared 2.5% NaBH_4 in water. The samples were incubated for 1 h at 50 °C before addition of 30 μl of 0.3 M HCl to inactivate the remaining NaBH_4 . Following incubation for 30 min at 25 °C, 200 μl of 0.11% 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.04 M sodium phosphate, pH 8.1, was added. The formation of TNB^{2-} , resulting from the reaction of DTNB with cysteine, was measured after 5 min as the absorbance at 412 nm. The amount of free cysteine and total cysteine was calculated by using the molar absorption coefficient $\epsilon = 13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for TNB^{2-} .

SDS/PAGE and isoelectric focusing

SDS/PAGE was performed essentially according to the method of Laemmli [29], with the modifications described [20]. Isoelectric focusing was carried out in precast Ampholine PAG plates pH 3.5–9.5 (Pharmacia, Sweden) on a Multiphor electrophoresis unit according to the manufacturer's instructions. Gels were stained with a 0.1% solution of Coomassie Brilliant Blue R250 in 40% (v/v) ethanol and 5% (v/v) acetic acid.

Electroblotting on to poly(vinylidene difluoride) membranes (Immobilon-P, Millipore, U.S.A.) following SDS/PAGE was done by semi-dry electroblotting. The membranes were probed with lectins as described [20].

Monosaccharide analysis of protein-bound glycans

Lyophilized samples were hydrolysed in evacuated sealed glass tubes with 100 μl of 2 M TFA for 1 h and 4 h at 100 °C. Monosaccharides were separated by high-performance anion-exchange chromatography using a Dionex CarboPac PA1 column (Dionex, Sunnyvale, U.S.A.), eluted with 0.016 M NaOH and detected by pulsed amperometric detection. Due to the different stability and release of monosaccharides in 2 M TFA, the amounts of glucosamine and mannose were determined after 4 h of hydrolysis, while the amount of galactose was determined after 1 h of hydrolysis.

MS analysis

MS was done using matrix-assisted laser desorption ionization time-of-flight MS in a VG Analytical ToFSpec (VG Analytical, Manchester, U.K.). A 2 μl sample was mixed with 2 μl of saturated matrix solution [α -cyano-4-hydroxycinnamic acid in 0.1% TFA/acetonitrile (70:30, v/v)] and 2 μl of the mixture was spotted on the target plate. The solvent was removed by evaporation and the target plate was inserted in the mass spectrometer. Samples were desorbed and ionized by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions were detected by a microchannel plate set at 1850 V. The spectra were calibrated externally with proteins of known masses.

Titration analysis of PME activity

The following equipment was used: titrator TTT80, Autoburette ABU80, Titrigraph module REA160, pH stat unit REA 270 (all manufactured by Radiometer, Copenhagen, Denmark).

The incubations were done in a 15 ml reactor cell connected to a thermostat and a magnetic stirrer. The reactor was filled with 15 ml of a 0.2% solution of DE 75% apple pectin in water. The pH was adjusted to 4.6 with NaOH or HCl (for determination of pH optimum, the pH was adjusted to other values as described below), and the temperature of the reaction cell was maintained at 30 °C (except for determination of temperature optimum, as

described below). The enzyme sample was injected, the reagents were stirred for 1 min, and the reaction was measured over an additional 1.5 min. 0.01 M NaOH was injected in the reactor cell to maintain constant pH, and the number of hydrolysed methyl ester linkages (hmel) was determined according to the following equation:

$$n(\text{hmel}) = n(\text{NaOH})\{1 + ([\text{H}^+]/K_a)\}$$

The pK_a of galacturonic acid is approx. 3.5 at 30 °C in aqueous solutions [30], and this value has been used for polygalacturonic acid.

The reaction speed, V_{max} , and K_m were determined for the enzyme by varying the substrate concentration from 0.01% to 0.4%, and plotting S/v as a function of S (substrate concentration) [31].

For determination of the pH optimum, pH in the reaction cell was adjusted to values varying from 2.5 to 6.0. A 1.5 μg sample of purified enzyme was added to the substrate solution and measurements were carried out as described. pH stability was determined by adding 1.5 μg of PME I to 10 ml of 0.1% substrate solution in the reactor cell, followed by adjustment of the pH to a value between 2.5 and 8. After 1 h of incubation at 30 °C, 5 ml of 0.4% substrate solution was added, the pH adjusted to 4.6, and activity measurements were carried out as described above.

The temperature optimum of PME I was determined by measuring the reaction at temperatures varying from 25 °C to 62 °C. A 1.5 μg sample of purified enzyme was added to the substrate solution and measurements carried out as described. For measurement of temperature stability, the enzyme samples were incubated without substrate at various temperatures between 30 °C and 80 °C for 0, 1 and 2 h and the activity was measured as described.

For determination of the influence of MgCl_2 , CaCl_2 and NaCl on PME I activity, the salts were added to the reaction mixture in concentrations of 0.01, 0.05, 0.075, 0.1, 0.15 and 0.2 M, and the enzyme activity was determined as described above. Influence of methanol and galacturonic acid on the enzyme activity was studied by adding various amounts of these compounds to the reaction mixture prior to measurement of the enzyme activity. Activity on acetylated polysaccharides was measured as previously described [28a].

Enzymic demethylation of pectin

A 2% solution of methyl-esterified citrus pectin (DE 72%; GENU pectin, Copenhagen Pectin, Denmark) was adjusted to pH 4.6 and aliquoted in 6.25 ml portions. PME I was added, the aliquots were incubated for 0, 10, 30, 60, 120 or 180 min and the enzyme was inactivated by boiling for 10 min. Free carboxyl groups were determined by titration with 0.2 M NaOH to pH 7.0.

High-performance size-exclusion chromatography (HPSEC) of polysaccharides

Enzyme incubations for HPSEC analyses were carried out by adding enzyme (1 μg of PME I and *A. niger* PG alone or in combination) to 1 ml of a 1% apple pectin (DE 75%) in 0.1 M acetate buffer, pH 4.0. After incubation at 30 °C for 0, 1, 2, 4 or 24 h the enzyme was heat-inactivated. A 25 μl aliquot of each sample was analysed for size distribution of the soluble oligomeric and polymeric degradation products as described [19,20].

RESULTS AND DISCUSSION

The PME assay

A. aculeatus was cultivated in a complex, soybean meal-containing medium, which has previously been shown to induce the expression of several enzymes acting on pectic hairy regions [19,20,28a]. A PME assay for use in agar plates was developed by incorporating 1% apple pectin with DE of 75% (DE 75%) into

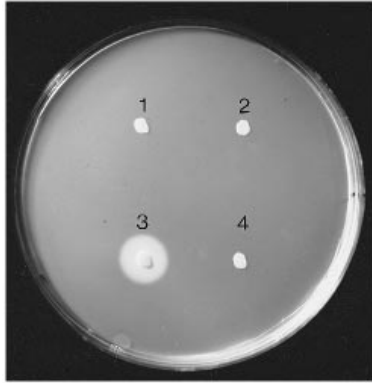


Figure 1 Expression of the *A. aculeatus* PME in *S. cerevisiae*

Four recombinant *S. cerevisiae* yeast strains harbouring the *A. aculeatus* PG I and II expression constructs pC1PG1 (1) and pC1PG2 (2), pC1PE5 (3) encoding the PME I, and the yeast expression plasmid pYES 2.0 (4), were grown on SC-agar containing 2% galactose for 3 days, overlaid with 1% apple pectin (DE 75%) and incubated for 24 h at 30 °C. The pectin was precipitated with the quaternary ammonium ion MTAB. The PME activity is seen as an opaque precipitate around the yeast clone harbouring the PME I expression vector (3).

the plates. Samples of the culture supernatant were applied into the plates, and they were incubated at 30 °C overnight. PME activity could then be visualized by incubating the plates with a solution containing quaternary ammonium ions (MTAB), resulting in a turbid pectate precipitate around the application wells, where the enzyme had demethylated the pectin (Figure 1 and results not shown). However, PME activity could not be directly quantified in *A. aculeatus* supernatants, because of high levels of PG and pectin lyase activity. These enzymes rapidly degraded the pectin, thus giving rise to a transparent zone around the sample well.

Expression cloning and characterization of cDNAs encoding PME I

Highest levels of pectinolytic activity were detected in 5-day-old cultures of *A. aculeatus*. Hence, poly(A)⁺ RNA isolated from fungal mycelia from day 5 was used for the construction of a cDNA library in the yeast expression vector pYES 2.0 [18–20,28a]. Aliquots of the library were transformed into *S. cerevisiae*, and the transformants were screened for PME activity by replica plating the yeast colonies on to agar plates and overlaying with 1% apple pectin (DE 75%). Yeast colonies producing functional PME were identified by precipitation with MTAB (Figure 1). The *S. cerevisiae* strain used in this study expresses no endogenous PME, pectin lyase or PG activity, which could interfere with the plate assay (Figure 1). Of the 30000 transformants screened from the yeast sub-library, 17 clones showed PME activity. All clones contained a 1.2 kb cDNA insert representing transcripts of the same gene (designated here as *pme1*).

The 1195 bp cDNA clone pC1PE5 contains a 993 bp open reading frame initiating with an ATG codon at nucleotide position 32, and terminating with a TAA stop codon at nucleotide

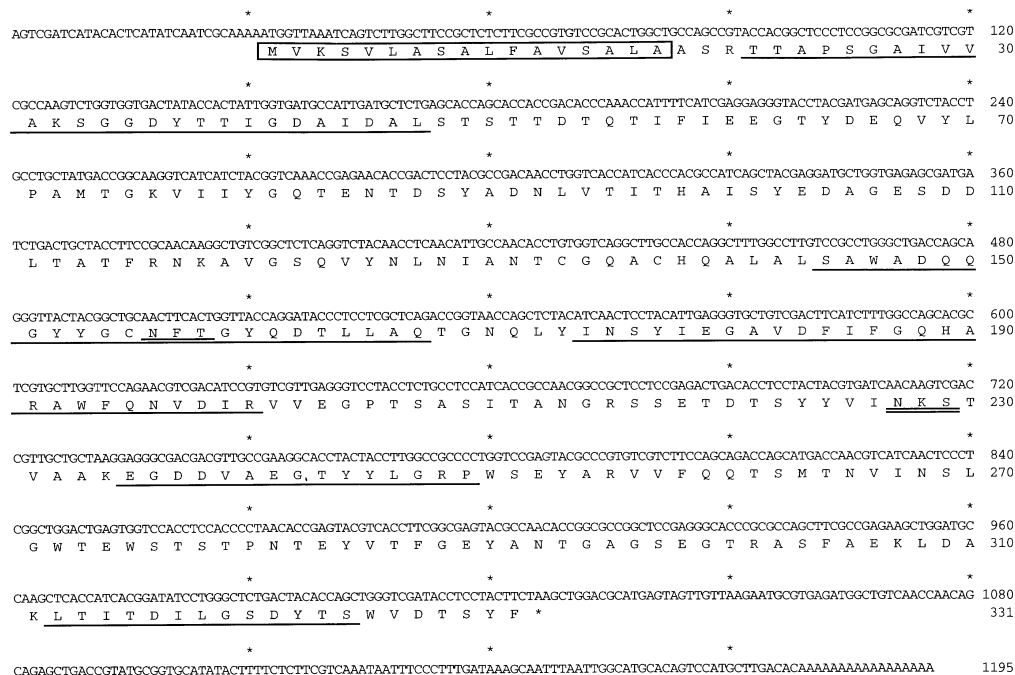


Figure 2 The nucleotide sequence of the *pme1* cDNA, and the deduced amino acid sequence of PME I from *A. aculeatus*

The signal peptide is boxed, and the N-terminal sequence as well as internal peptide sequences obtained from authentic PME are underlined and the two potential N-glycosylation sites are indicated by double underlines. The nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with accession number U49378.

<i>A. aculeatus</i>	1	MVKSLVLSALFLAVSALAASRTTAPSGAIVVAKSGGDYTTIGDA	43
<i>A. niger</i>	1	MVKSLVLSALFLAAATLAASRMATAPSGAIVVAKSGGDYTTISAA	43
<i>A. aculeatus</i>	44	IDALSTSTTDITOTFIEEGTYDEQVYLPALMIGKVIIVYGQTEIN	86
<i>A. niger</i>	44	IDALSTSTTEITQTIFIEEGSYDEQVYLPALMIGKVIIVYGQTEIN	86
<i>A. aculeatus</i>	87	DSYADNLVITITHASIEYDAGESDDLTATFRNKAVGSGVYVNLNI	129
<i>A. niger</i>	87	DTLITNINLVNITTHAALADVDNDDLTATFRNKAVGSGVYVNLNI	128
<i>A. aculeatus</i>	130	ANTGGQACHQALALSAWADQGGYVGNFTGYQDTLLAQFTGNQL	172
<i>A. niger</i>	129	ANTGGQACHQALALSAWADQGGYVGNFTGYQDTLLAQFTGNQL	171
<i>A. aculeatus</i>	173	YINSYIEGAVDFIFGQHARAWFQNVDIRVVEGPTTSASITANGR	215
<i>A. niger</i>	172	YIAGTYIEGAVDFIFGQHARAWFHECDIRVVEGPTTSASITANGR	214
<i>A. aculeatus</i>	216	SSEYDITSYVYVINKSTVAAKEGDDVAEGTYYLGRPWSQYARVVF	258
<i>A. niger</i>	215	SSEYDITSYVYVINKSTVAAADGNDVYSGTYYLGRPWSQYARVVF	257
<i>A. aculeatus</i>	259	QQTSMNTNINSLGWTEWSTSTPNTETVTFVGEYANTGAGSEGTFR	301
<i>A. niger</i>	258	QKTSMNTNINSLGWTEWSTSTPNTETVTFVGEYQNTGTGAGSEGTFR	300
<i>A. aculeatus</i>	302	ASFAEKDAKLITITDILGSDYTSWVDTSYF	331
<i>A. niger</i>	301	ANESSELLTEPILITISWLLGSDWEDWVDTSYIN	331

Figure 3 Alignment of the deduced amino acid sequence of PME I from *A. aculeatus* with that of the PME from *A. niger* [12]

Identical residues (boxed) are indicated around the corresponding amino acids.

position 1025, thus predicting a 331-residue polypeptide of 35679 Da (Figure 2). The open reading frame is preceded by a 31 bp 5' non-coding region, and followed by a 171 bp 3' non-coding region and a poly(A) tail. The deduced primary structure of PME I matches the N-terminal and internal peptide sequences determined from the purified authentic enzyme (Figure 2). The putative start of the mature protein is, however, not preceded by a typical signal peptidase cleavage site [33]. By contrast, the *pme1* cDNA encodes an apparent signal sequence of 17 amino acids with a typical signal cleavage site between Ala-17 and Ala-18, suggesting that the authentic PME I has been proteolytically modified during purification from *A. aculeatus*.

The copy number of the *pme1* gene in the *A. aculeatus* genome was determined by Southern blot hybridization (results not shown). Total DNA isolated from the fungal mycelium was digested to completion with *Bam*HI, *Bgl*II, *Eco*RI or *Hind*III and hybridized with the *pme1* cDNA. The *pme1* probe detected only single strongly hybridizing fragments in each digest, indicating that the *pme1* gene is present as a single copy in the *A. aculeatus* genome, and that no homologous or closely related genes are present. The fact that all PME-encoding cDNAs isolated by expression cloning from the *A. aculeatus* cDNA library represent transcripts from the same gene further indicates that only one *pme* gene is present in the *A. aculeatus* genome.

An alignment of the deduced amino acid sequence of PME I from *A. aculeatus* with that of PME from *A. niger* [12] revealed 83% similarity including 74% identity (Figure 3). In addition, limited homologies were found to PMEs I and II from tomato (approx. 28% identity) [7], from *Erwinia chrysanthemi* (25% identity) [9] and from *Pseudomonas solanacearum* [34]. The primary structure of the previously known PMEs have been compared [7], and four highly conserved segments were found. All four regions are also present in PME I from *A. aculeatus*: the N-terminal segment GXYXE from residue 62 to 66, XQDTL from residue 160 to 164, DFIFG from residue 183 to 187 and LGRXW from residue 246 to 250 (Figure 2). These structural elements have been proposed to play an important functional role in the PMEs and their conservation in PME I from *A. aculeatus* strongly suggests that it belongs to the same enzyme family.

Heterologous expression of PME I and purification of the enzyme

The full-length, 1195 bp *pme1* cDNA (Figure 2) was cloned into the fungal expression vector pHD464 [17] and transformed into *A. oryzae* wild-type strain A1560. This strain does not express

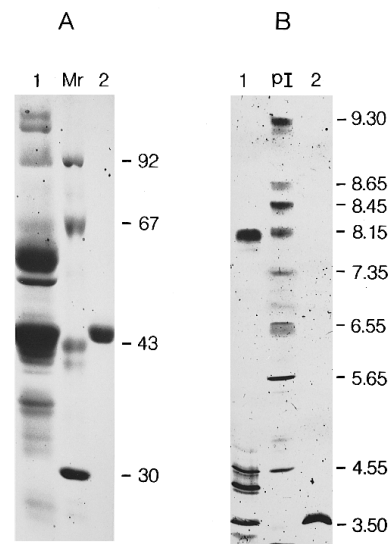


Figure 4 (A) SDS/PAGE gel stained with Coomassie Brilliant Blue showing an *A. aculeatus* culture supernatant as well as purified recombinant *A. aculeatus* PME I expressed in *A. oryzae* and (B) Coomassie Brilliant Blue-stained isoelectric focusing gel

(A) Lane 1, *A. aculeatus* culture supernatant (10 μ g); lane 2, purified recombinant PME I (1 μ g); lane Mr, molecular mass markers (rabbit muscle phosphorylase B, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa). (B) Lane 1, *A. aculeatus* culture supernatant; lane 2, purified recombinant PME I; lane pI, isoelectric point markers (pI indicated on the left).

endogenous PME or PG activity and thus, heterologously expressed PME I could readily be detected by the PME plate assay and SDS/PAGE. The transformant giving the highest production of the recombinant enzyme was grown in a 1-litre fermenter.

After 5 days culture in a medium with maltodextrin as the main carbon source, PME I comprised 20–30% of the total protein present in the culture supernatant. The recombinant enzyme could be purified from this culture supernatant in a two-step procedure involving anion exchange on a Q-Sepharose column followed by cation exchange on a S-Sepharose column. The resulting enzyme preparation was more than 95% pure as determined by SDS/PAGE (Figure 4A).

A more complex procedure had to be followed for purification of authentic PME I from *A. aculeatus*, because it comprises less than 1% of the total protein that the fungi secretes under the employed culture conditions. The authentic enzyme was purified by a four-step procedure involving anion-exchange and hydrophobic-interaction chromatography.

Comparison of authentic and recombinant PME I

Direct N-terminal amino acid sequencing of the purified authentic and recombinant PME I revealed different N-terminal sequences for the two enzymes. The authentic enzyme gave a sequence identical with the deduced amino acid sequence from residue 21 (TTAPSGAIVVAKSGGDYTTIGDAIDAL) (Figure 2). However, the recombinant enzyme was found to have an N-terminal sequence starting at the predicted signal peptidase cleavage site at residue 18 (ASRTTAP) (Figure 2) [33]. The reason for this discrepancy may be that the recombinant enzyme is processed differently compared with the authentic enzyme from *A. aculeatus*, or that the authentic enzyme has been proteolytically

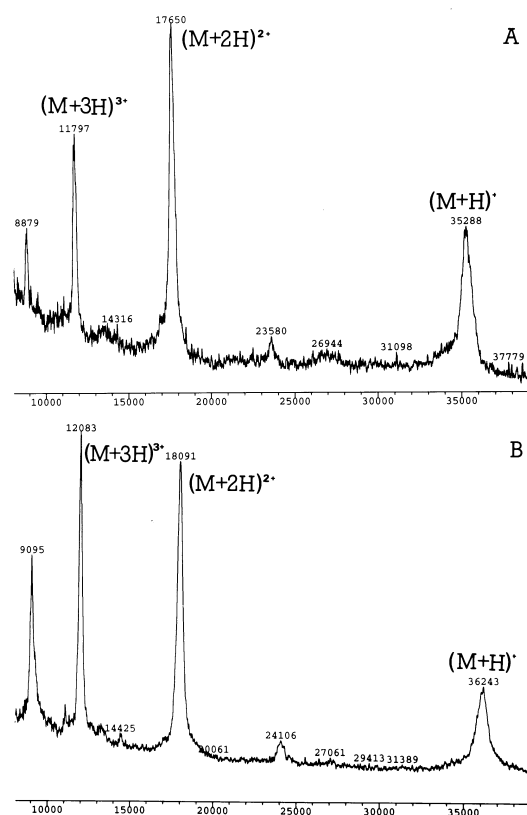


Figure 5 MS analysis of authentic and recombinant *A. aculeatus* PME I

Matrix-assisted laser desorption ionization time-of-flight MS of authentic (A) and recombinant (B) PME I. The molecular ions carrying 1, 2 and 3 positive charges are indicated as $(M+H)^+$, $(M+H)^{2+}$ and $(M+H)^{3+}$. The unit of the x-axis is m/z .

processed during purification. Observations which support the latter suggestion are that small amounts of PME I with the same N-terminus as the recombinant enzyme were found in preparations of the authentic enzyme (results not shown), as well as the better agreement with the consensus signal peptidase cleavage site between Ala-17 and Ala-18 [33]. Furthermore, the authentic enzyme was subjected to a longer purification procedure, increasing the probability for proteolytic degradation to occur.

Amino acid analysis was carried out to determine the amino acid composition of the authentic and recombinant PME I. The two forms of PME I had identical amino acid compositions, and the results were in good agreement with the theoretically determined values deduced from the primary PME I sequence (results not shown).

Purified authentic PME I was digested with proteases, and internal peptide fragments were isolated and subjected to amino acid sequencing. The sequences cover 138-amino-acid residues, and they are in complete agreement with the deduced amino acid sequence of the enzyme (Figure 2). Five of the residues in the isolated peptides were not identified, three of them correspond to the three cysteine residues in the enzyme (Cys-134, Cys-138 and Cys-155), one of them to an asparagine residue (Asn-156) and one to a threonine residue (Thr-244). Asn-156 is one of the two predicted attachment sites for N-linked glycosylation (Figure 2). Thus, the absence of a signal in amino acid sequencing at this position indicates that it may carry a glycan moiety. Determinations of the cysteine and cystine content of authentic PME

Table 1 Monosaccharide composition of authentic and recombinant PME I

The values given are the averages of four determinations.

Monosaccharide	Authentic PME (pmol/pmol of enzyme)	Recombinant PME (pmol/pmol of enzyme)
Glucosamine	2	3
Galactose	0	1
Mannose	6	10

I using titration with DTNB showed that PME I contains one free cysteine residue and one disulphide bond, as the total amount of cysteine residues before and after reduction was found to be one and three, respectively.

Biochemical characterization of PME I

PME I has an apparent molecular mass of 43 kDa as determined by SDS/PAGE (Figure 4A), and an isoelectric point of 3.8 (Figure 4B). Identical values were obtained for the purified authentic PME from *A. aculeatus* (results not shown). Whereas the isoelectric point is in accordance with the theoretical value of pH 4.04, the observed molecular mass is approx. 9 kDa higher than the calculated one of the mature protein (34.1 kDa). When purified recombinant PME I was analysed by MS, the average molecular mass was found to be 36.2 kDa with the molecular masses ranging from 35.0 to 37.0 kDa (Figure 5A). The authentic enzyme had an average molecular mass of 35.3 kDa, with molecular masses ranging from 34.7 to 36.2 kDa (Figure 5B). This is in better agreement with the calculated molecular mass, but the discrepancy of 1–3 kDa between the calculated and observed molecular masses strongly suggests that both forms of PME I are glycosylated. Furthermore, it appears that the recombinant enzyme is differently glycosylated compared with authentic PME I, and that both forms of the enzyme are heterogeneously glycosylated due to the broad peaks observed in MS (Figure 5).

Monosaccharide compositional analysis confirmed that authentic and recombinant PME I are differently glycosylated (Table 1). Differences are apparent in both the amount and the composition of the monosaccharides of the attached carbohydrates. The presence of glucosamine indicates that at least some of the carbohydrates are N-linked in accordance with the presence of two consensus sequences for N-linked glycosylation at amino acid positions 156 and 227 (Figure 2) [35]. Amino acid sequencing of a peptide from authentic PME I containing Asn-156 gave a blank cycle in this position, strongly indicating that this residue serves as an attachment point for a glycan moiety. The threonine residue at position 244 was also blank, suggesting that this residue may serve as an attachment point for an O-linked carbohydrate moiety. The residues around the other potential attachment site for N-linked glycosylation (Asn-227) were not determined.

The glycosylation of the enzyme was also investigated by studying the binding of lectins to the denatured enzyme electroblotted on to poly(vinylidene difluoride) membranes following SDS/PAGE. GNA lectin (from *Galanthus nivalis*), specific for high-mannose N-linked glycosylation with terminal mannose, PNA lectin (from peanut), which is specific for the Gal- β -1,3-GalNAc in some N-linked glycans, and DSA lectin (from *Datura stramonium*), specific for β -1,4-linked N-acetylglucosamine, and

Table 2 Enzymic characteristics of purified recombinant PME I

Characteristics	Value	Unit
Molecular mass	43 000	Da
pI	3.8	pH
pH optimum	4.5	pH
pH stability	> 5.5–6.0	pH
Temp. optimum	45	°C
Temp. stability	< 40	°C
K_m	2.7%	75% DE pectin
Specific activity	5500	$\mu\text{mol}/\text{min per mg}$

terminal *N*-acetylglucosamines were unable to bind to PME I, indicating that the structural elements recognized by these lectins are absent in PME I (results not shown).

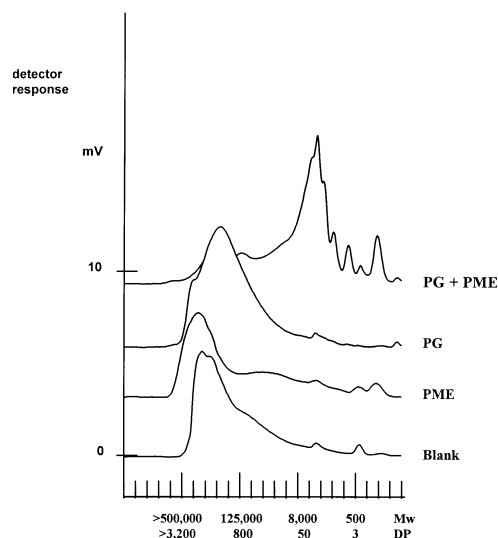
Characterization of the enzymic properties of PME I

The pH optimum of PME I was determined to be pH 4.6 and the temperature optimum was 45 °C, with 90% of the activity present in the interval from 35 ° to 50 °C (Table 2). These are similar to the values reported for a partially purified PME from *A. niger* (pH 5.0, 45 °C)[13], in accordance with the homology between the two enzymes (Figure 3). The activity of PME I declines sharply at pH values above 4.6 and temperatures above 50 °C, whereas the decrease in activity at lower temperatures and pH is less pronounced. PME I appears to be most stable at neutral pH (pH 6–8) (Table 2), but even at pH 2.5 more than 40% of the initial activity is preserved after 1 h of incubation with the substrate at 30 °C. After 1 h at 50 °C and pH 4.6, almost 80% of the activity was lost (Table 2) and at higher temperatures, the enzyme was rapidly inactivated.

The K_m was measured to 2.8% (w/v) apple pectin (DE 75%), a relatively high value, which indicates that the enzyme has a low affinity for the substrate used in the experiment (Table 2). The specific activity was determined to be 5.5 mmol/min per mg, suggesting that the enzyme has a very high catalytic capacity. However, in practice this is probably never fully utilized, due to the high K_m value.

Apart from pectin, very few naturally occurring polysaccharides with methyl ester linkages exist in plant cell walls. Acetyl esters are more common in various plant polysaccharides such as xylan, mannan and pectin [28a,36]. PME I was unable to hydrolyse such ester linkages, showing that the enzyme is limited to methyl esterase activity.

Presence of the bivalent cations Mg^{2+} and Ca^{2+} stimulated the activity of the enzyme. The largest effect was observed at a concentration of 0.05 M MgCl_2 (approximately a twofold stimulation of the activity), and 0.01 M CaCl_2 (approximately 150% of the activity without Ca^{2+}) (results not shown). Also NaCl stimulated the activity of PME I; at 0.075 M concentration the activity was 140% of the baseline activity without NaCl. At higher salt concentrations the activity decreased, but even at 0.2 M of either one of the three salts, the activity was still higher than the baseline activity without salts. The bivalent cations may affect the enzymic activity by forming non-covalent complexes with demethylated pectin, thus, removing the reaction products of the enzyme. Alternatively, the salts may exert their effect directly on the enzyme. The stimulatory effect of bivalent cations is in good accordance with results observed with other PME enzymes [37,38]. However, PME from *A. niger* was reported to be partially inhibited by Mg^{2+} and stimulated by Na^+ [13].

**Figure 6** Synergistic degradation of highly methylated apple pectin (DE 75%) by PME I and *A. niger* PG

Aliquots of a 1% solution of the apple pectin were incubated for 4 h without addition of enzymes (blank), with PME I or PG alone, or with PME I and PG in combination, followed by analysis of the samples by HPSEC on a DIONEX system. The y-axis shows the detector response in mV.

The activity of PME I was not inhibited by high concentrations of methanol (15%, v/v) or by the presence of polygalacturonic acid (up to 0.75%, w/v), demonstrating that the enzyme is not subjected to product inhibition (results not shown). This is in contrast to what is reported for PME from *A. niger* [13], where methanol was found to be a non-competitive inhibitor and polygalacturonic acid a competitive inhibitor. The discrepancies between PME I and PME from *A. niger* with regard to product inhibition and influence of bivalent cations is surprising, considering the high degree of sequence similarity between the two enzymes (Figure 3). Different assay conditions and substrates may have influenced the results. It appears that the results reported here for PME I from *A. aculeatus* are more consistent with the results obtained with other fungal PMEs [37,38].

When the enzyme was incubated with highly esterified pectin (citrus pectin DE 72%), a rapid de-esterification was observed although a significant portion of the methyl groups (15–25%) remained on the pectin even after prolonged incubation (4–6 h) with the enzyme (results not shown). This could be due to other modifications on the pectin (i.e. acetylation or attachment of xylose to the galacturonic acid), which may reduce the accessibility of the substrate to the enzyme. Alternatively, the enzyme may only work on highly methylated pectin and fails to recognize the substrate when the distribution of the remaining methyl groups reaches a certain random level [5].

The acidic pH optimum of the enzyme indicates that it may be of great value in fruit juice processing, where the pH frequently is below 5.0. Soluble pectin polymers are a common problem in juice-manufacturing, because they tend to precipitate during storage, thus giving rise to an unwanted haze in the juice. By adding PME during the juice processing this problem can be avoided, since demethylated pectin can be precipitated with calcium or degraded by PG into small, soluble oligomers (Figure 6). The enzyme may also be of use in the wine, feed and food industry, where pectin-containing plant material is processed. Furthermore, it may substitute for saponification in the manu-

facturing of demethylated pectin, which is used for gelation and thickening in food.

The enzyme acted in synergy with PG for pectin degradation. Figure 6 shows the degradation products from apple pectin (DE 75%), obtained by incubation for 4 h with an *A. niger* PG and PME I alone or in combination. Alone, PME I has no depolymerizing effect, but rather causes an increase in apparent molecular mass, probably by facilitating aggregation of pectin [39]. PG alone causes very limited depolymerization of the substrate (Figure 6) and incubation for 24 h did not increase the depolymerization. However, PME I significantly enhances the PG-catalysed pectin degradation (Figure 6), facilitating the complete degradation of the substrate into oligomers with a size of less than ten galacturonic acid residues. The enzymic properties of PME I and the synergistic action with PG described here, suggest that this enzyme is an important component in the inventory of pectinolytic enzymes from *A. aculeatus*, which the fungus uses for degradation and assimilation of nutrients from the environment.

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