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# Pectin acetylesterases from *Aspergillus* are able to deacetylate homogalacturonan as well as rhamnogalacturonan

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ABSTRACT

An acetylesterase was purified from *Aspergillus aculeatus* on the basis of its ability to deacetylate homogalacturonan. A series of well-characterized substrates was prepared and used to investigate its specificity, including pectin with various degrees of methylation and acetylation, pectic domains with various structures, and oligomers. It was then compared to a rhamnogalacturonan acetylesterase previously isolated from the same fungus. Both enzymes were active towards various acetylated pectins, and were able to release acetyl groups from acetylated homogalacturonan, oligogalacturonates as well as rhamnogalacturonan at different extent. It was thus concluded that while the two enzymes differed in their efficiency, they are both pectin acetylesterases, able to act in the "smooth" as well as in the "hairy" regions of pectin.


## 1. Introduction

Pectins are complex plant cell wall polysaccharides characterised by a high content of galacturonic acid (GalA). They are divided into structurally different regions, mainly the homogalacturonan (HG) or "smooth" region, and the rhamnogalacturonan I (RGI) or "hairy" region (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). HG consists of a (1 → 4)-α-linked D-GalA backbone with a degree of polymerisation (DP) of 100–200 residues (Bonnin, Dolo, Le Goff, & Thibault, 2002; Thibault, Renard, Axelos, Roger, & Crépeau, 1993; Zhan, Janssen, & Mort, 1998). In RGI backbone domains, (1 → 4)-α-linked D-GalA units are interrupted by (1 → 2)-linked rhamnopyranosyl residues (Rha). RGI may carry side chains, typically α (1 → 5)-linked arabinans or β (1 → 4)-linked galactans, primarily attached to the C-4 of the Rha residues (Albersheim, 1978). The carboxyl group of GalA residues may be methylesterified whereas the secondary alcoholic functions on C-2 or C-3 may be O-acetylated. The degree of methylation (DM) and degree of acetylation (DAc) are defined as the percentage of GalA esterified with methanol and acetic acid, respectively. Depending on species and tissues, native pectins are generally highly methylated and slightly acetylated (Voragen et al., 1995). The distribution of both methylation and acetylation between HG and RG is not clearly established. Acetyl groups were claimed by some authors to be highly present in the RGI, such as in bamboo shoot (Ishii, 1995), flax (Rihouey et al., 1995), potato, leek, apple or onion pectins (Schols & Voragen 1996). In contrast, in sugar beet pectin, it was recently shown that around 90% of the GalA and 75% of the acetyl groups belong to the HG backbone, the remaining GalA and acetyl belonging to the RGI regions (Ralet et al. 2005).

Pectin is used as a gelling agent in many industrial applications, due to its ability to form gels in the presence of $Ca^{2+}$ ions, which can cross-link their free carboxylic functions. *In planta*, the pectic cement holds cells together via calcium ions. The presence of acetyl groups may alter the physicochemical properties of pectin in its industrial uses as well as in planta. It hinders the association of pectins (Ralet, Crépeau, Buchholt, & Thibault, 2003) and therefore, acetylated pectins have poor gelling properties (Kertesz, 1951). Acetyl groups also obstruct pectin-depolymerising enzymes and therefore limit the industrial use of these enzymes (Benen, Kester, & Visser, 1999; Bonnin, Le Goff, van Alebeek, Voragen, & Thibault, 2003; Chen & Mort, 1996). In the cell wall, the pectic network could be disturbed by the presence of acetyl groups (Ridley, O'Neill & Mohnen, 2001), which could decrease cell adhesion (Liners, Gaspar, & Van Cutsem, 1994).

Enzymes are powerful tools to elucidate the fine chemical structure of pectins and may in addition modify their structure and functionality. To remove acetyl groups from pectin, several acetylesterases (EC 3.1.1.6, CE family 12 http://afmb.cnrs-mrs.fr/CAZY/) have been reported in some microorganisms and plants. Two different acetylesterases were purified from orange peel, one having a Mw of 29–30 kDa and a p*I* of 5.1 (Williamson, 1991), the other

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having a Mw of 42 kDa and a p*I* > 9 (Christensen, Nielsen, & Mikkelsen, 1996). Another acetylesterase purified from mung bean hypocotyl cell walls (Bordenave, Goldberg, Huet, & Pernollet, 1995) had physicochemical properties (Mw and p*I*) similar to the high molar mass acetylesterase from orange (Christensen et al., 1996). These plant acetylesterases were active on sugar beet or flax pectin and, thus, were called pectin acetylesterases (PAE). Both orange and mung bean PAEs showed a preference for beet pectin when compared to activity on apple pectin (Bordenave et al., 1995; Christensen et al., 1996), in which acetyl groups are claimed to be mainly bound to RG (Schols, Posthumus, & Voragen, 1990). However, both PAEs had the highest activity on synthetic substrates such as triacetin or *p*-nitrophenyl acetate.

Acetylesterases have also been reported in fungi as well as bacteria. Two different acetylesterases were described in *Aspergillus niger* (Searle-van Leeuwen, Vincken, Schipper, Voragen, & Beldman, 1996). One was more active on sugar beet pectin and was referred to as a PAE, while the other was mainly active on modified "hairy" region (MHR), corresponding to the RG fraction resistant to the degradation of fresh apple by liquefying enzymes (Schols et al., 1990). Due to this specificity, this enzyme was called rhamnogalacturonan acetylesterase (RGAE). A similar RGAE was found in *Aspergillus aculeatus* (Kauppinen et al., 1995; Searle-van Leeuwen, van der Broek, Schols, Beldman, & Voragen, 1992) but had no activity towards beet pectin (Searle-van Leeuwen et al., 1992).

Acetylesterases were also reported in some bacteria. PaeY and PaeX from *Erwinia chrysantemi* (Shevchik & Hugouvieux-Cotte-Pattat 1997, 2003) and YxiM from *Bacillus subtilis* (Bolvig, Pauly, Orfila, Scheller, & Schnorr, 2003) were highly active on acetylated oligogalacturonates but their best substrates were the synthetic ones. Combined action of PaeX and PaeY on acetylated pectic oligomers improved deacetylation in comparison with the action of each enzyme alone suggesting that the two *E. chrysanthemi* enzymes have different substrate specificity (Shevchik & Hugouvieux-Cotte-Pattat, 2003).

Taken together, well-characterised substrates would be highly essential to study the specificities of different acetylesterases. To this end we prepared a collection of substrates that varied with respect to their contents of HG, RG, acetyl and methyl groups and purified an acetylesterase from *A. aculeatus* (AspacAE) using acetylated HG to conduct the purification. We compared its activity towards the different substrates to the activity of the RGAE from *A. aculeatus* (Kauppinen et al., 1995).

## 2. Materials and methods

### 2.1. Substrates

Water extractable pectin (Pw) was isolated from sugar beet pulp after extrusion-cooking and was used to isolate acetylated homogalacturonan (AcHG) as previously described (Bonnin et al., 2002).

This procedure was further adapted for the preparation of acetylated rhamnogalacturonan (AcRG) by degrading Pw with a combination of fungal pectin methylesterase (f-PME, Swiss–Prot entry Q12535), galactanase (Swiss–Prot entry P48842), arabinanase (UniProt entry Q9HFS9) and endopolygalacturonase PGII (UniProt entry P26214). The reaction products were fractionated on a DEAE-Sepharose CL6B column (GE Healthcare, Orsay, France) and the most retained fraction having the highest ratio Rha/GalA was collected. This fraction was again degraded by the galactanase and the arabinanase in combination with a β-galactosidase (E.C. 3.2.1.23, *Aspergillus oryzae*, G5160 Sigma Chemicals, L'Isle d'Abeau, France) and a α-arabinofuranosidase (EC 3.2.1.99, *Aspergillus oryzae*) to completely remove the side chains. The reaction products were separated on a Sephacryl-S200 size-exclusion chromatography (GE Healthcare, Orsay, France) and the high molecular weight fraction corresponded to AcRG.

A mixture of oligogalacturonates was prepared by autohydrolysis of 5 g polygalacturonic acid (P3889, Sigma Chemicals, L'Isle d'Abeau, France) in 500 mL water. After 24 h autohydrolysis at 100 °C followed by a concentration to 150 mL, the soluble fraction was precipitated by ethanol at 64%. After centrifugation, the supernatant was recovered and dialysed against water for 2 h. The remaining fraction contained oligogalacturonic acids with various DP centred on 9. After mixing with tetrabutylammonium, the tetrabutylammonium oligogalacturonate was freeze-dried and acetylated in a DMSO medium and in the presence of pyridine and acetic anhydride (Renard & Jarvis, 1999). The reaction mixture was extensively dialysed against water and was referred to as O79.

The raw and modified pectins from sugar beet were provided by Danisco A/S (Copenhagen, Denmark) and their preparation is described in Buchholt, Christensen, Fallesen, Ralet, and Thibault (2004). They are referred to as a letter explaining the mode of treatment, followed by a first number for the DM and a second number for the DAc. The mother pectin having a DM = 62 and a DAc = 30 (SBP6230) was chemically overmethylated in methanolic medium leading to E7329 which was further used for controlled demethoxylation by enzymatic as well as chemical means. The B-series was obtained by base deesterification (B3124, B0915), the F-series was demethoxylated by a fungal PME (F4429, F2803) and the P-series was demethoxylated by a plant PME (P4628).

The monosaccharide composition of all the substrates was determined following hydrolysis and derivatisation in alditol acetates (Blakeney, Harris, Henry, & Stone, 1983). The GalA content was determined by the automated *m*-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). The DM and DAc were determined by hplc (Lévigne, Thomas, Ralet, Quémener, & Thibault, 2002). Borwin software (JMBS Developments, Grenoble, France) was used for hplc data acquisition and processing. Composition of the pectin and oligomers is shown in Table 1.

### 2.2. Enzymes

The AspacAE was purified from Ronozyme VP, a commercial preparation from *Aspergillus aculeatus* kindly given by Hoffman-La Roche (Basel, Switzerland). The RGAE from the same fungus was kindly provided by Dr. A. Mølgaard and Pr S. Larsen, University of Copenhagen, Denmark (Kauppinen et al., 1995).

The endopolygalacturonase PGII, galactanase, arabinanase, arabinofuranosidase and f-PME were provided by Novozymes (Bagsvaerd, Denmark) as monocomponent preparations.

### 2.3. Purification of AspacAE

All purification steps were carried out at 4 °C.

The DEAE FastFlow column (250 × 26 mm, GE Healthcare, Orsay, France) was eluted at 2 mL/min with 3 column volumes (CV) of 100 mM phosphate buffer pH 6.5, and a 0–0.35 M NaCl gradient in the same buffer over 8 CV. Fractions of 5 mL were recovered. The cross-linked alginate column (160 × 16 mm) was prepared as described by Rozie et al. (1988), equilibrated with 50 mM succinate buffer pH 4 and eluted at 0.2 mL/min. Fractions of 2 mL were collected. Two Superdex 75 columns (GE Healthcare, Orsay, France) were mounted in series and equilibrated with 50 mM succinate buffer pH 4.5. The elution was carried out at 0.3 mL/min and 1.8-mL fractions were collected. The column of PhenylSepharose (110 × 26 mm, GE Healthcare, Orsay, France) was eluted at 2 mL/min with 50 mM Tris–HCl pH 6.5 buffer containing 2 M ammonium sulphate, and a multi-step gradient of ammonium sulphate: 2–1 M in 1 CV, 1–0.5 M in 4 CV, and 0.5 M to 0 in 2 CV. The sample

**Table 1**
Composition of the substrates

| | GalA (mg/g of dry matter) | Rha (mg/g of dry matter) | Ara (mg/g of dry matter) | Xyl (mg/g of dry matter) | Gal (mg/g of dry matter) | Glc (mg/g of dry matter) | DM (%) | DAc (%) |
|---|---|---|---|---|---|---|---|---|
| *Extracted from sugar beet pulp* | | | | | | | | |
| SBP6230[1] | 548 | 53 | 123 | 3 | 104 | 5 | 61.6 | 29.8 |
| Pw | 421 | 21 | 177 | 0 | 46 | 14 | 59.0 | 45.0 |
| *Esterified pectin*[1] | | | | | | | | |
| E7329 | 581 | 54 | 82 | 2 | 100 | 4 | 73.2 | 28.7 |
| *Base-deesterified pectin*[1] | | | | | | | | |
| B3124 | 577 | 56 | 117 | 3 | 98 | 4 | 31.4 | 24.0 |
| B0915 | 569 | 48 | 102 | 2 | 93 | 3 | 9.0 | 14.9 |
| *Fungal PME-deesterified pectin*[1] | | | | | | | | |
| F4429 | 567 | 52 | 110 | 3 | 92 | 3 | 43.9 | 29.3 |
| *Plant PME-deesterified pectin*[1] | | | | | | | | |
| P4628 | 572 | 54 | 111 | 0 | 96 | 4 | 45.6 | 27.6 |
| *Pectin sub-units* | | | | | | | | |
| AcHG[2] | 586 | 35 | 53 | 2 | 53 | 0 | 24.0 | 45.0 |
| AcRG | 273 | 85 | 103 | 2 | 106 | 2 | 10.2 | 49.4 |
| O79 | 657 | 6 | 1.5 | 1.5 | 11 | 2 | 0.0 | 79.0 |

[1] Values from Buchholt et al. (2004).
[2] Values from Sakamoto, Bonnin and Thibault (2002).

was adjusted to 2 M ammonium sulphate prior to application. Fractions of 4 mL were recovered. The Superose 12 column (580 × 16 mm, GE Healthcare, Orsay, France) was eluted in 5 mM succinate buffer pH 4 at 0.5 mL/min and 2-mL fractions were recovered.

### 2.4. Enzymatic assays

Enzymatic activity was expressed in nkat, 1 nkat corresponding to the activity necessary to release 1 nmol of product per s.

For acetylesterase determination, the release of acetic acid from pectin was determined by hplc on a C18 column (Superspher 100 RP-18, 250 × 4 mm, Merck KGaA) eluted with 4 mM $H_2SO_4$ using isopropanol as the internal standard (Lévigne et al., 2002). When O79 was used as the substrate, the release of acetic acid was quantified by the enzymatic kit Enzytec (Scil, Viernheim, Germany). The two methods gave similar results on known samples.

To measure acetylesterase activities as well as to follow the substrate deacetylation, substrates were dissolved in 50 mM succinate buffer pH 4.5 at a constant concentration in acetyl groups of 150 μg/mL taking into account the DAc of each substrate. 700 μL of substrate solution were incubated with 30 μL of appropriately diluted enzyme at 40 °C. To study the synergy with PGII or fPME, 9 nkat PGII or 5 nkat fPME were added in the medium. Each assay was performed in triplicate. The average of the three results is presented and the deviation did not exceed 1.25% of it.

PG II activity was determined using polygalacturonic acid (Sigma Chemicals, L'Isle d'Abeau, France) dissolved in 50 mM succinate buffer pH 4.5. It was calculated from the increase in reducing ends (Nelson, 1944) carried out in microplates (Sturgeon, 1990), using GalA as a standard. Reaction mixtures contained 450 μL substrate (5 g/L) incubated with 50 μL appropriately diluted polygalacturonase at 40 °C.

Viscosimetry was chosen as the most sensitive method to check the absence of endopolygalacturonase activity in the purified fraction. The reaction mixture contained 3.5 mL pectin (5 mg/mL) or polygalacturonic acid (8 mg/mL) in 50 mM succinate buffer pH 4.5 and 0.15 mL enzyme. The viscosity was measured for 20 h using an automated viscosimeter (AVS 310, Schott Geräte, Germany) fitted with an Ubbelohde capillary tube (3.5 mL, diameter 0.4 mm). Relative viscosities were calculated as $t/t_0$ where $t$ is the flow time of reaction mixture, and $t_0$ is the flow time of the buffer (84.99 s). Specific viscosities were calculated as $\eta spe = (t_0 - t)/t_0$.

### 2.5. Protein determination and electrophoresis

Protein content was determined using Bio-Rad reagent and bovine serum albumin as standard (Bradford, 1976).

The molar mass of AspacAE was determined by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulphate in a MiniProtean II apparatus (Bio-Rad, Marnes la Coquette, France) using a continuous 10–20% polyacrylamide gel (Laemmli, 1970) calibrated using a Low Molecular Weight kit (GE Healthcare, Orsay, France).

## 3. Results

### 3.1. Preparation of pectin subunits

Pectin is composed of different blocks, HG and RG, in which the latter carries neutral sugar side chains (Schols & Voragen, 1996). These sub-units were obtained by enzymatic digestion from the highly acetylated pectin Pw and used to unambiguously determine the specifity of the enzymes. 200 mg Pw were degraded by a mixture of rhamnogalacturonase, f-PME, arabinanase and galactanase, and 111 mg AcHG were generated, in which GalA represents 77% mol and the DAc = 45 (Table 1) after fractionation of the hydrolysis products. The significant residual DM (DM = 24) suggests that acetyl groups may hinder the PME. The relevance of our enzymatic method for the preparation of AcHG was already shown in a previous study (Bonnin et al., 2002).

The enzymatic mixture was adapted to the isolation of AcRG by replacing the rhamnogalacturonase by PGII. After fractionation of the hydrolysis products by anion exchange and size exclusion chromatographies, the AcRG fraction represented 5% of initial Pw and had a DAc = 49, a DM = 10 and a ratio GalA/Rha = 2.67 (Table 1).

To complete our collection of acetylated substrates, we prepared acetylated oligogalacturonate by autohydrolysis of polygalacturonic acid. The reaction mixture was separated by ethanol precipitation, and the dialysis of the soluble fraction eliminated about 40% of the monomer, 33% of the dimer, 23% of the trimer and 20% of the tetramer. The dp of the final mixture was centred on 9. Next, we applied a chemical acetylation on tetrabutylammonium salt using acetic anhydride as previously published for the acetylation of HG (Renard & Jarvis, 1999). The final oligomer mixture had an average DAc of 79 and was referred to as O79 (Table 1).

Its content in GalA represents 96%mol while Rha content was only 1%mol. The method was shown not to affect the backbone dp and to lead to a homogeneous distribution of acetyl groups (Renard & Jarvis, 1999). However, from this previous work, it can be anticipated that part of the acetyl groups are carried on diacetylated derivatives.

### 3.2. Purification and biochemical characterization of AspacAE

Thirty commercial preparations from various fungal or bacterial strains were screened for acetylesterase and PG activities. Using Ronozyme VP, originating from *Aspergillus aculeatus*, the release of acetyl from Pw reached 46.8% of the initial content in acetyl groups, and PG activity was 2990 nkat/mg protein. Due to its efficiency to release acetyl groups from Pw and to its relatively low PG activity when compared to the other preparations screened (up to 10,000 nkat/mg for a close efficiency to release acetyl esters), Ronozyme VP was selected as the best source of acetylesterase that will be referred to as AspacAE.

After dialysis of Ronozyme VP against cold water, five chromatographic steps were performed. After each step, fractions were assayed for acetylesterase towards AcHG (HGAE) and PG activity using polygalacturonic acid as the substrate. As the first step, the dialysate was loaded on a DEAE Fast Flow column in pH 6.5 phosphate buffer (Fig. 1). The HGAE activity eluted together with the PG in 0.18 M NaCl. At this point, SDS–PAGE evidenced one major band around 30 kDa and two minor bands with higher molar masses (see lane 3, Fig. 3). The corresponding fractions were pooled and freeze-dried, and they were applied to a cross-linked alginate column. The conditions of chromatography were set up as follows: pH 4 buffer (2 column volumes), pH 6 buffer (2 column volumes), NaCl gradient up to 1 M in pH 6 buffer over 4 column volumes. In these conditions, HGAE eluted in the pH 6 buffer step together with a peak of PG. The HGAE active fractions were then loaded on

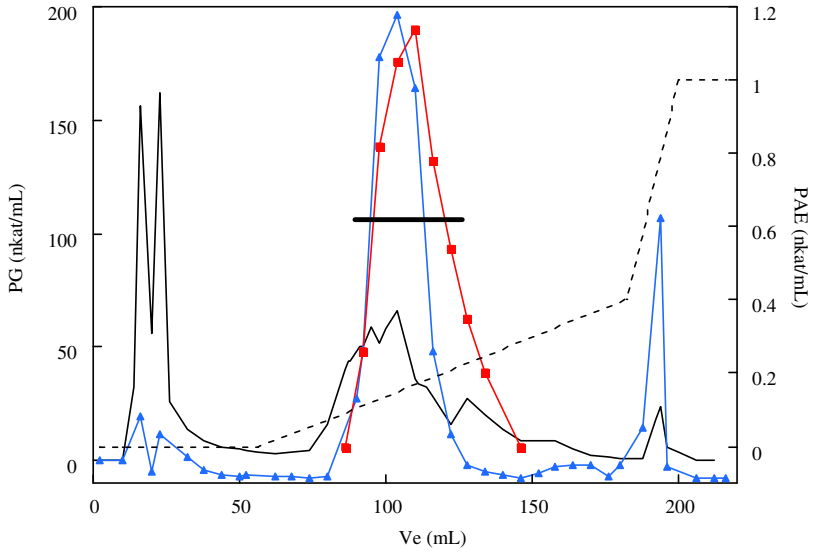


**Fig. 1.** Anion exchange chromatography (DEAE Fast Flow in 0.1 M phosphate buffer pH 6.5) of Ronozyme VP. $A_{280}$ (full line), AE activity (squares), PG activity (triangles), NaCl (dotted line).

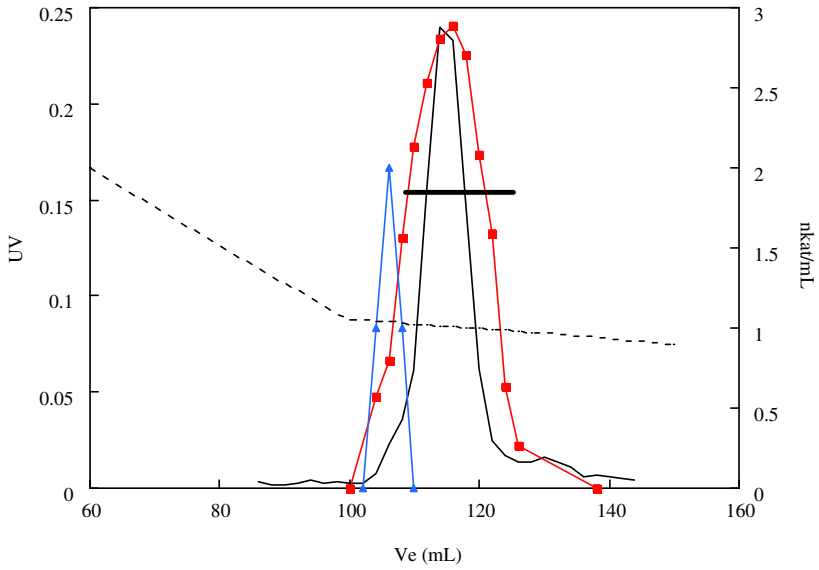


**Fig. 2.** Hydrophobic interaction chromatography (Phenyl Sepharose in 0.05 M Tris–HCl pH 6.5) of AspacAE-containing fractions from Superdex 75 conditioned in 2 M ammonium sulphate. $A_{280}$ (full line), AE activity (squares), PG activity (triangles), ammonium sulphate (dotted line).

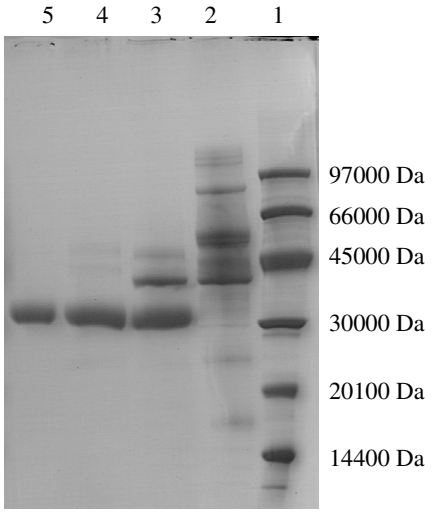


**Fig. 3.** PAGE–SDS of the different fractions along the purification: (lane 1) Mw markers; (lane 2) initial Ronozyme VP; (lane 3) after chromatography on DEAE Sepharose; (lane 4) after chromatography on Phenyl Sepharose; (lane 5) after chromatography on Superose 12.

Superdex 75 gel, allowing to eliminate part of the PG activity. The sample was conditioned in ammonium sulphate and fractionated on PhenylSepharose gel (Fig. 2). The first shoulder corresponded to another PG whereas the HGAE eluted in the main protein peak around 1 M ammonium sulphate. The fractions active on AcHG were pooled. As a trace of PG activity was still detectable by viscosimetry, the sample was loaded on a Superose 12 in 5 mM succinate buffer, the low ionic strength favouring the PG to bind the matrix. This last step yielded the purified AspacAE. Activity of AspacAE towards AcHG was 9.7 nkat/mL, the yield of purification was 1.5% and the purification factor 66 (Table 2). The viscosity of a reaction mixture containing either SBP6230 or polygalacturonic acid was stable over 20 h, demonstrating that AspacAE was free of depolymerising activity (data not shown). It was also free of PME as shown by the absence of methanol peak on the hplc chromatograms after a prolonged incubation with SBP6230.

AspacAE showed a unique band in SDS–PAGE (Fig. 3) and has a molar mass of 32.4 or 34.5 kDa from SDS–PAGE or gel filtration determination, respectively.

The activity of the purified enzyme was tested towards Pw in various conditions of pH or temperature (Fig. 4). The highest activity was found between pH 4.5 and 5 in 50 mM succinate buffer and to 5.7 to in 50 mM citrate-phosphate buffer. It was 5× less active at pH 3.5 than at pH 4.5 and decreased again 5× from pH 5.5–6.6. However, the maximum of activity in citrate-phosphate buffer was 40.5% of that in succinate buffer showing the effect of nature of the ions present in the medium. The activity increased rapidly between 30 and 45 °C, and was highest at 45 °C and beyond. In the optimal conditions of buffer, i.e. 50 mM succinate buffer, the addition of 5 mM $CaCl_2$ or NaCl had no significant effect on the activity.

For comparison, optimal conditions for RGAE activity were reported to be pH 5.5 and 40 °C when measured on MHR from apple (Searle-van Leeuwen et al., 1992).

### 3.3. Time course of hydrolysis of Pw by AspacAE and RGAE

Similar amounts of enzyme and optimum conditions of pH and temperature were used to follow the time course of deacetylation of Pw using AspacAE and RGAE (Fig. 5). The time courses were

**Table 2**
Purification of AspacAE from Ronozyme VP

| | Volume (mL) | Proteins (mg) | Protein yield (%) | HGAE activity (nkat) | Activity yield (%) | Specific activity (nkat/mg) | Purification factor |
|---|---|---|---|---|---|---|---|
| Ronozyme | 50 | 1547 | | 95500 | | 62 | |
| After DEAE | 850 | 550 | 35.6 | 84150 | 88.1 | 153 | 2.5 |
| After alginate | 45 | 10.5 | 0.7 | 14175 | 14.8 | 1350 | 21.8 |
| After superdex | 22 | 2.9 | 0.2 | 2310 | 2.4 | 797 | 12.8 |
| After phenyl | 56 | 1.2 | 0.08 | 3528 | 3.7 | 2940 | 31.3 |
| After superose | 152 | 0.36 | 0.02 | 1474 | 1.5 | 4094 | 66.0 |

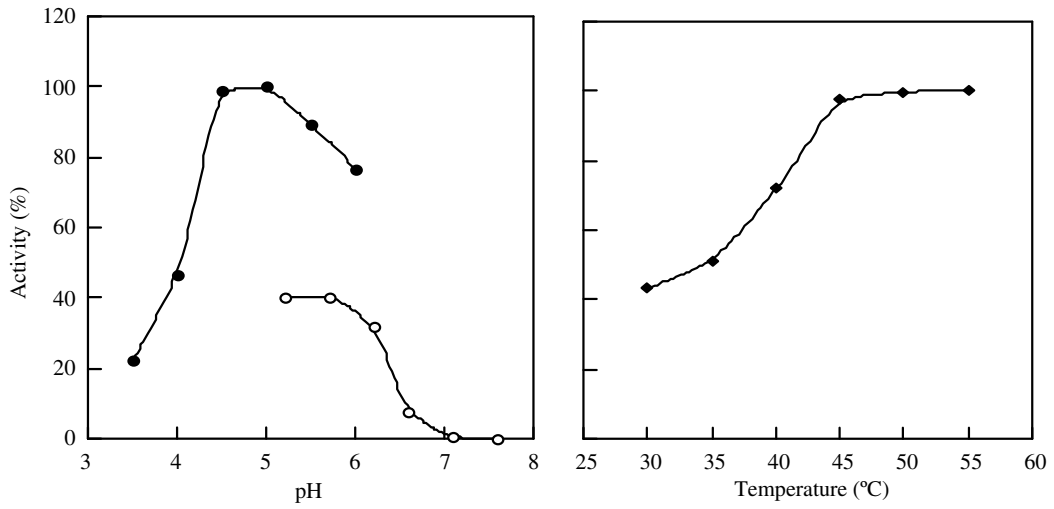


**Fig. 4.** Influence of pH and Temperature on the activity of AspacAE. Activity is expressed as a percentage of the maximum activity. For the effect of pH, the activity was measured in 5 mM succinate buffer from pH 3.5 to 6 (closed symbols) or in 50 mM citrate-phosphate buffer from 5.2 to 7.6 (open symbols).

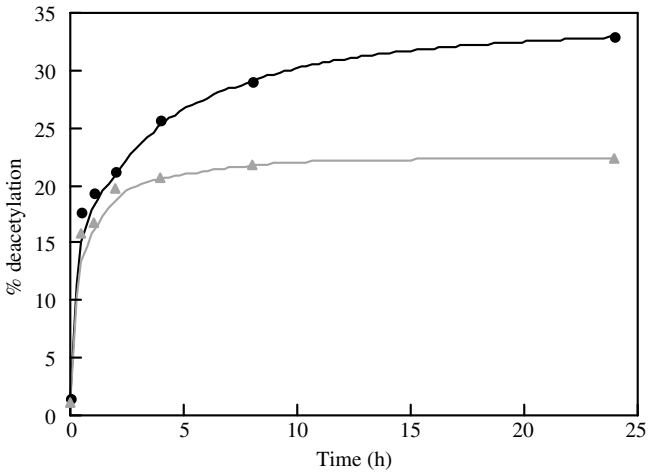


**Fig. 5.** Time course of hydrolysis of Pw with AspacAE (circles) and RGAE (gray triangles). The release of acetate is expressed as a percentage of the initial contents in acetate in Pw.

rather similar for both enzymes with a very high rate of hydrolysis during the first hour diminishing afterwards. With RGAE, the plateau was reached after 4 h hydrolysis, earlier than with AspacAE. After 24 h treatment, the deacetylation was 32.6% and 22.2% with AspacAE and RGAE, respectively. Fresh enzymes were added after 24 h but did not increase the acetyl release, showing that the final state of hydrolysis was already reached. This result shows that RGAE is able to deacetylate beet pectin, contrary to previously published data (Searle-van Leeuwen et al., 1992).

### 3.4. Specificity of acetylesterases towards pectin subunits

The specificity of the two enzymes was studied towards the pectin structural domains. In each case, the final percentage of deacetylation is given (Table 3).

AspacAE was active on AcHG, AcRG and O79, with 30.2%, 37.0% and 32.9% deacetylation, respectively. Therefore, it can be concluded that AspacAE can work in the HG as well as in the RG area.

Concerning RGAE, its best substrate is AcRG (36.4%) followed by O79 (31.9%) and AcHG (23.5%), showing that RGAE is more favoured by the Rha–GalA environment, but is also active on a pure GalA polymer or oligomer. Thus, our results demonstrate that RGAE is able to de-esterify HG as well as RG domains, similarly to AspacAE.

It is noteworthy that the deacetylation by both enzymes was a little higher on O79 than on AcHG. As the main dp of O79 is 9 and thus already long enough compared to the probable size of the active site, this difference is probably more ascribable to the higher initial DAc of O79 and to the residual DM of AcHG which may hinder the enzyme.

**Table 3**
Final percentage of deacetylation of the different substrates by the two enzymes

| | AspacAE | RGAE |
|---|---|---|
| SBP6230 | 34.7 | 20.5 |
| Pw5945 | 32.6 | 22.2 |
| E7329 | 15.9 | 7.5 |
| B3124 | 48.3 | 23.5 |
| B0915 | 57.3 | 21.1 |
| F4429 | 35.7 | 22.2 |
| P4628 | 33.7 | 21.5 |
| AcHG | 30.2 | 23.5 |
| AcRG | 37.0 | 36.4 |
| O79 | 32.9 | 31.9 |

The final percentages of deacetylation with the two enzymes were about similar for AcRG and O79 while AcHG was more efficiently deacetylated by AspacAE than by RGAE (30.2% *vs* 23.5%).

### 3.5. Effect of DAc on acetylesterases

To study the influence of DAc on the enzyme activities, different pectins with different DAc were tested (Table 3). The final percentage of deacetylation reached with RGAE was always in the range of 21–23.5% for DAc from 15 to 45. Only one exception at 7.5% deacetylation was observed for E7329. With the same substrates, the deacetylation by AspacAE varied from 32% to 57%, suggesting that AspacAE was more tolerant to variation in substrate structure. However, all these substrates vary not only in their DAC but also in their DM. Indeed, comparison of final deacetylation of E7329, F4429 and P4628 by the two enzymes clearly shows that the DM has a great impact on the final results and that it is necessary to study the effect of methyl-esterification on the acetylesterase activity.

### 3.6. Effect of methyl-esterification of the substrates on acetylesterases

AspacAE released about one third of acetyl groups on the native pectin SBP6230 and Pw5945 (Table 3). When increasing the DM of the substrate (E7329), AspacAE was twice less effective to deacetylate pectin. On the opposite, after alkaline de-esterification, the deacetylation with AspacAE was more effective (48.3% and 57.3% total deacetylation on B3124 and B0915, respectively). So, the highest is the DM, the lowest is the deacetylation. On the other hand, it is not clear whether or not the use of PME favours the AspacAE activity probably because the DM was not lowered enough to show an effect on the deacetylation. In the same way, the source of PME used for the demethylation step does not crucially modify the AspacAE efficiency (33.7% and 35.7% total deacetylation on P4828 and F4429, respectively).

The behaviour of RGAE was also studied on these substrates. Most often, it releases one fifth of acetyl groups, for DAc varying from 15 to 45 and DM from 9 to 62. The deacetylation is lowered when the DM is much higher (7.5% de-acetylation on E7329). Similarly to what was observed with AspacAE, the methyl distribution had no effect on the deacetylation (22.2% for F4429 *vs* 21.5% for P4628). As it is known that the two PMEs used to prepare the pectins have different modes of action leading to blockwise or random distribution of the residual methyl groups on P4828 and F4429, respectively, our results suggest that the acetylesterases have a multichain attack mode of action and that none of them glide on the backbone. Each enzyme-substrate encounter corresponds to one catalytic step.

By plotting the percentage of deacetylation of pectin as a function of substrate DM, it is clear that RGAE is less active than AspacAE and that the two enzymes have different susceptibility to methyl groups (Fig. 6). DM has a quite linear effect on final deacetylation by AspacAE in the range of 9–79 while RGAE deacetylation is rather constant when increasing DM up to 60 and decreases beyond.

### 3.7. Synergy of acetylesterases with other pectin-degrading enzymes

To investigate further the specificity of both enzymes, hydrolyses of Pw were carried out with the acetylesterases in combination with f-PME or PG II (Table 4). The combination with PGII does not increase the final deacetylation as the result is similar to that of the sum of each acetylesterase with PGII (21.7% and 32.6% for RGAE+PGII and AspacAE+PGII, respectively). Thus, neither one nor the other of the two enzymes is specific for the polymer or the oligomer.

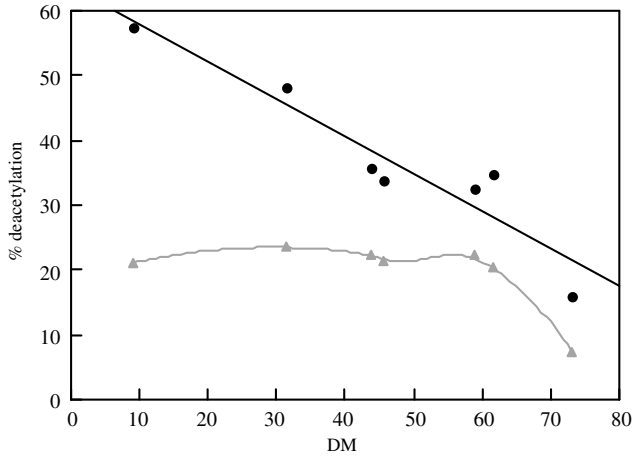


**Fig. 6.** Impact of pectin methyl-esterification on the efficiency of AspacAE (circles) and RGAE (gray triangles).

**Table 4**
Final deacetylation of Pw5945 by different combinations of enzymes

| | Deacetylation (%) |
|---|---|
| AspacAE+PGII | 32.6 |
| RGAE+PGII | 21.7 |
| AspacAE+fPME | 39.4 |
| RGAE+fPME | 21.6 |
| AspacAE+RGAE | 33.9 |
| AspacAE+RGAE+fPME | 40.3 |

When used together with f-PME, AspacAE was more efficient than alone (39.4% *vs* 32.6% deacetylation), confirming that it is hindered by methyl groups. Conversely, RGAE gave similar results when used alone or with f-PME (19.9% *vs* 23.9% deacetylation), confirming that RGAE is less sensitive to methyl groups.

When the two acetylesterases are used together, the final deacetylation reaches a value similar to that obtained with AspacAE alone (32.6%) showing that no synergy is observed between the two acetylesterases. By adding f-PME to the two acetylesterases, the final deacetylation is increased similarly to what was obtained with AspacAE+f-PME (40.3% *vs* 39.4% deacetylation). As O-2 as well as O-3 of GalA may be acetylated (Quémener, Cabrera-Pino, Ralet, Bonnin, & Thibault, 2003), this last series of experiments shows that the two enzymes releases the same acetyl groups and that none of them is specific for one or the other of the two positions.

## 4. Discussion

*Aspergillus aculeatus* is known to produce various pectin-degrading enzymes, such as PGs, PMEs, rhamnogalacturonase, etc. The RGAE was first purified from the commercial preparation Ultra-Sp-L from Novozymes (Searle-van Leeuwen et al., 1992). This enzyme was active on MHR from apple (DM 42, DAc 60), and thus was termed as RGAE.

In the present study, we purified an enzyme from *Aspergillus aculeatus* active to deacetylate beet pectin, and its sub-units AcHG and AcRG. We demonstrated that RGAE is active on the same substrates as AspacAE, but to a different extent. Thus, we showed that these acetylesterases are not specific for one or the other of the backbone subunits, but that they are active in both regions with variable efficacy.

The use of chemical or enzymatic treatments of beet pectin allowed us to obtain substrates from a unique source with various amounts of methyl and acetyl groups, and thus to investigate the effect of substrate structure towards enzymatic activity. The enzymatic deacetylation with AspacAE increased when substrates having similar contents in acetyl groups have decreasing contents in methyl groups. Similarly, the acetylesterases from mung bean hypocotyls and *Erwinia chrysanthemi* were shown to be favoured by PME (Bordenave et al., 1995; Shevchik & Hugouvieux-Cotte-Pattat, 1997). The PAE from orange peel was more active on not methoxylated substrate (Williamson, 1991). On the opposite, RGAE was insensitive to methoxylation up to DM 60. This corresponds to the results obtained on MHR since no increase in acetyl release was observed by submitting the substrate to RGAE in combination with citrus- or *Aspergillus* PME (Searle-van Leeuwen et al., 1992).

A total deacetylation of pectin was never reached. For example, native pectin such as SBP6230 or Pw5945 were deacetylated at about one third by AspacAE and one fifth by RGAE. By using a combination of AspacAE and RGAE, no synergy could be demonstrated. As acetyl groups can be carried on O-2 and O-3 positions on the GalA residue (Quémener et al., 2003), one of the hypotheses could be that one of these two positions is easier to de-esterify than the other. It was previously estimated that around 40% of acetyl groups are on O-2 and 60% on O-3 (Ralet, Crépeau, & Bonnin, 2008). However, the final DAc of the pectin never corresponds to 40% or 60% of the initial one, suggesting that the acetyl position on the GalA is not important for the enzyme activity and that AspacAE and RGAE attack the same acetyl groups and therefore are not specific for one position.

Taken together, our results indicate that *A. aculeatus* produces two different acetylesterases, meaning that it produces two proteins for an unique activity. This behaviour is rather common for fungi and particularly for *Aspergilli*. For instance, *Aspergillus niger* produces seven polygalacturonases (as reviewed by Benen & Visser, 2002) and two acetylesterases (Searle-van Leeuwen et al., 1996).

## 5. Conclusion

In this work, an interesting series of substrates was prepared corresponding to the different structural domains of acetylated pectin. Such substrates are essential to determine unambiguously the specificity of enzymes. This is illustrated by our work on AspacAE and RGAE that can be concluded not to be specific for different subunit of pectin but that are able to work regardless of the backbone constitution. Hence, we propose to give up the term RGAE and to use only the term PAE.

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