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Mode of action of *Fusarium moniliforme* endopolygalacturonase towards acetylated pectin

E. Bonnin[a,*], A. Le Goff[a], G.-J.W.M. van Alebeek[b], A.G.J. Voragen[b], J.-F. Thibault[a]

[a]*Unité de Recherche sur les Polysaccharides, leurs Organisations et Interactions INRA, BP 71627, 44316 Nantes Cedex 03, France*
[b]*Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, NL-6703 HD Wageningen, The Netherlands*

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Abstract

Endopolygalacturonase from *Fusarium moniliforme* was used to degrade acetylated homogalacturonan previously prepared from sugar beet pulp. The initial velocity and the final percentage of hydrolysis decreased very rapidly with increasing degree of acetylation, showing that acetyl substitution markedly affected the enzymatic activity. MALDI-TOF mass spectrometry was used to analyse the reaction products and to show acetyl groups on the oligogalacturonates. The results demonstrated that the enzyme was able to accommodate acetyl groups in its active site cleft. The influence of acetyl groups on the mode of action of the enzyme was discussed and compared to the influence of methyl groups.

Keywords: Endopolygalacturonase; *Fusarium moniliforme*; Acetylated homogalacturonan; Sugar beet pectin

1. Introduction

Pectins are a complex family of branched heteropolysaccharides that arise from the primary cell walls of dicots. They are characterised by the presence of rhamnose and galacturonic acid (GalA) arranged as an alternance of long 'smooth' homogalacturonan, representing the major part of pectin, and short 'hairy' rhamnogalacturonan. Complex side chains of arabinose as well as galactose are attached to rhamnogalacturonan. GalA units in homogalacturonan and rhamnogalacturonan can be methyl-esterified on the carboxylic group and acetylated at position 2 and/or 3 (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Native pectins are generally highly methyl-esterified and slightly acetylated, depending on the source. The degree of methylation (DM) and the degree of acetylation (DAc) are defined as the number of moles of substituent, methanol or acetic acid, respectively, per 100 moles of GalA. In native pectins, DM may be often up to 70–80 whereas DAc is generally <15. However, acetylation may be higher in some pectin. For example, it reaches ≈ 35 in sugar beet pectins (Rombouts & Thibault, 1986).

Plant pathogenic fungi and bacteria are known to produce a wide range of cell wall-degrading enzymes, including pectin-degrading enzymes. Polygalacturonases (PG, poly[1,4-α-D-galacturonide] glycanohydrolase, EC 3.2.1.15) hydrolyse 1–4 linkages between two α-D-GalA residues in homogalacturonan. In case of a pathogenic infection, PGs release oligogalacturonides, which can act as elicitors of plant defence response (Cervone, De Lorenzo Degra, & Salvi, 1987). A large literature describes the mode of action of polygalacturonases (Benen, Vincken, & van Alebeek, 2002; Sakai, Sakamoto, Hallaert, & Vandamme 1993).

Fusarium moniliforme causes foot or stalk rot and seedling blight in a wide variety of plants (Nelson, 1992). It produces a large spectrum of hydrolytic enzymes in order to penetrate the mycellium in host tissues (Cooper, 1983). A detailed study of the PG of *Fusarium moniliforme* was carried out. We previously reported its purification and its mode of action towards GalA oligomers (Bonnin et al., 2001) and methylated pectins (Bonnin et al., 2002a). In the present paper, the action of the same enzyme was investigated on acetylated pectins.

* Corresponding author. Tel.: +33-2-40-67-50-58; fax: +33-2-40-67-50-66.
E-mail address: bonnin@nantes.infra.fr (E. Bonnin).

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Nomenclature

DAc degree of acetylation
DM degree of methylation
Dp degree of polymerisation
ESI-MS electrospray ionisation-mass spectrometry
GalA galacturonic acid
MALDI-TOF-MS matrix assisted laser desorption/ionisation-time-of-flight-mass spectrometry
PG polygalacturonase

2. Material and methods

2.1. Preparation of model pectins

Homogalacturonans were prepared by acidic hydrolysis of sugar beet pectins as previously described (Thibault, Renard, Axelos, Roger, & Crépeau, 1993).

Acetylated homogalacturonan was prepared from sugar beet pulp as follows. First, the sugar beet pulp was extruded in the conditions described in Micard and Thibault (1999). The water-soluble pectin was extracted from extrusion-cooked pulp as already described (Bonnin, Le Goff, Dolo, & Thibault, 2002b). Pectin was then solubilized in 0.05 M succinate buffer pH 4.5 and degraded by a mixture of enzymes containing orange pectin methylesterase (EC 3.1.1.11, Sigma Chemicals, L'Isle d'Abeau, France), fungal pectin methylesterase from *Aspergillus niger* (Novozymes, Copenhagen, DK), rhamnogalacturonan-hydrolase (Novozymes), endogalactanase (EC 3.2.1.89) and endoarabinanase (EC 3.2.1.55) previously purified from the commercial mixture Pectinex AR (Novozymes). After incubation, the reaction products were fractionated by anion exchange chromatography (DEAE Sepharose CL6B column, 14 × 2.6 cm; eluent, 0.05 M succinate buffer pH 4.5; flow rate, 0.7 ml/min; washing the column with 2 volumes succinate buffer; gradient, 4 column volumes up to 0.4 M NaCl in succinate buffer). Acetylated homogalacturonan eluted at 0.2 M NaCl (Bonnin et al., 2002b). It was dialyzed and freeze-dried. A series of acetylated homogalacturonans with decreasing DAc was prepared by alkaline saponification (3 h at 4 °C) of the native fraction (100 mg) by increasing volumes of 1 M KOH (50, 100, 120 μl).

GalA content was determined with the metahydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) automated by Thibault (1979). DM and DAc were calculated after chemical de-esterification of pectins and determination of methanol and acetic acid by HPLC on a C18 column (Superspher 100 RP-18, 250 × 4 mm, Merck KGaA) eluted with 4 mM H_2SO_4 (Lévigne, Thomas, Ralet, Quemener, & Thibault, 2002). The composition and molar mass of the different substrates are shown in Table 1.

Table 1
Chemical composition and molar mass of homogalacturonans

	GalA (%)	Dac	DM	Mw (g/mol)	dp
Homogalacturonan[a]	97.0	0	0	25,300	144
Ac38	58.7	38	7	16,200	84
Ac28	57.7	28	<1	13,700	73
AC18	53.7	18	<1	12,600	69

GalA: galacturonic acid; DAc: degree of acetylation; DM: degree of methylation; Mw: weight average molar mass; dp: degree of polymerisation.
[a] Values from Bonnin et al., 2001.

2.2. Purification of Fusarium PG

Fusarium PG was purified as previously described (Bonnin et al., 2001). The enzyme (PDB entry hg8) had a molar mass of 49 kDa and a specific activity of 1845 nkat/mg on polygalacturonic acid (Sigma Chemicals).

2.3. Degradation of pectins by Fusarium PG

Substrates (2 mg/ml in 0.05 M acetate buffer pH 4) were hydrolysed by adding successive amounts of PG (4 pkat/mg substrate at $t = 0$ and 100 pkat/mg at $t = 48$ h). Aliquots were withdrawn at set intervals and the reaction was stopped by boiling the samples for 5 min. The amount of reducing ends was quantified (Nelson, 1944; Sturgeon, 1990). The percentage of hydrolysis was defined as the percentage of broken linkages and was calculated as previously described (Thibault, 1983).

2.4. Determination of molar mass

Molar masses of pectins were measured by high performance size exclusion chromatography on two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, exclusion limit 1.10^6 and 4.10^6 g/mol for pullulan, respectively) eluted at 0.6 ml/min with 0.05 M sodium nitrate containing 0.02% sodium azide. The effluent was monitored using an on line Multi Angle Laser Light Scattering detector (Mini Dawn, Wyatt Technology Corp., USA) and differential refractometer (Erma 7512, Japan). Molar masses were calculated with Astra 1.4 software using refractive index increment $dn/dc = 0.146$ g/ml.

2.5. High performance anion exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex system with pulsed amperometric detection. The Carbopac PA1 column was eluted with 0.5 M NaOH, 1 M sodium acetate and water at

1 ml/min as follows: initial conditions, 20/30/50; 30 min, 20/60/20; 30–34 min, 20/60/20; 35 min, 20/30/50. Monomer, dimer and trimer of GalA (Sigma Aldrich) and oligomers of dp 4–10, purified from autohydrolysis of polygalacturonic acid (Bonnin et al., 2002a), were used as standards.

2.6. Mass spectrometry

MALDI-TOF mass spectra were acquired on a Voyager DE™-RP spectrometer (Perseptive Biosystems Inc, Framingham, MA, USA) in positive ion mode. The instrument was equipped with delayed extraction technology. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12,000 V. Hereafter, the ions were detected using the reflector mode. The lowest laser power required to obtain good spectra was used and at least 50 spectra were collected. The MALDI-TOF MS was externally calibrated using a mixture of oligoGalA. 2,4,6-trihydroxyacetophenone was used as a matrix in thin layer preparation as described before (Limberg et al., 2000). Samples were desalted prior to mass spectrometric analysis according to Körner, Limberg, Mikkelsen, and Roepstorff (1998).

3. Results

3.1. Time course of hydrolysis

Acetylated homogalacturonans were degraded by *Fusarium* PG in fixed conditions of pH, temperature and ratio enzyme/substrate. The initial step of hydrolysis was carried out with very low amount of enzyme (4 pkat/mg substrate). Fig. 1 clearly shows that the enzyme was able to degrade Ac18 and Ac28, whereas hydrolysis of Ac38 was very low. Both the initial rate and the final percentage of hydrolysis decreased with increasing DAc. When the hydrolysis was prolonged with further addition of fresh enzyme (100 pkat/mg of substrate at 48 h), the extent of hydrolysis markedly increased, except for Ac38. The final percentages of hydrolysis were 3.4, 12.3, and 21.1% for Ac38, Ac28, and Ac18, respectively, whereas it was 57% for unsubtituted homogalacturonan (Bonnin et al., 2001). These results show that acetyl groups prevent the pectins to be degraded by *Fusarium* PG.

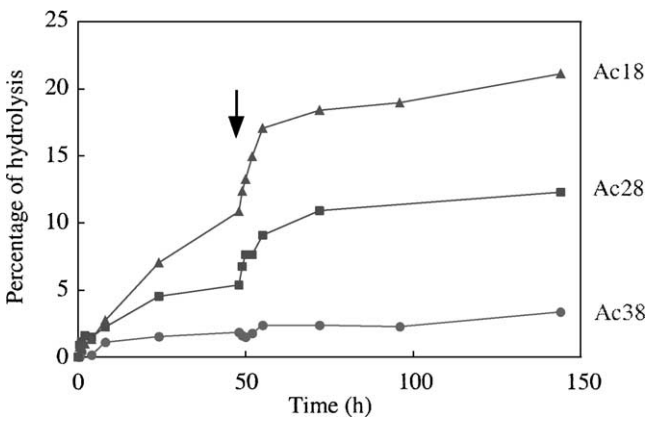

Fig. 1. Time course of the percentage of hydrolysis of acetylated homogalacturonans hydrolysed by *Fusarium* PG: Circles, Ac38; squares, Ac28; triangles, Ac18. Enzyme added at the beginning 4 pkat/mg of substrate; arrow: Addition of 100 pkat/mg of substrate.

3.2. Action pattern

The decrease in molar mass during the first step of hydrolysis was followed by HPSEC with 4 pkat of PG per mg of substrate. The initial molar masses of acetylated homogalacturonans varied from 12,600 to 16,200 g/mol (Table 1). After 8 h incubation, the molar mass of Ac18 decreased from 12,600 to 8,000 g/mol. In the same time, the percentage of hydrolysis reached 2.7%. This rapid decrease of molar mass associated with a low percentage of hydrolysis is typical of an endo mode of action, as already shown for this enzyme on homogalacturonan or methylated pectins (Bonnin et al., 2001; 2002a). The reciprocal plot of molar mass versus time (Fig. 2) for Ac18 and Ac28 varied linearly, demonstrating the multi-chain mode of attack of the enzyme. Similar results have been previously obtained with homogalacturonan or methylated pectins as substrates (Bonnin et al., 2001; 2002a).

3.3. Analysis of reaction products

Oligomers of dp up to 10 produced during the hydrolysis were analysed by HPAEC. As the analysis was performed at

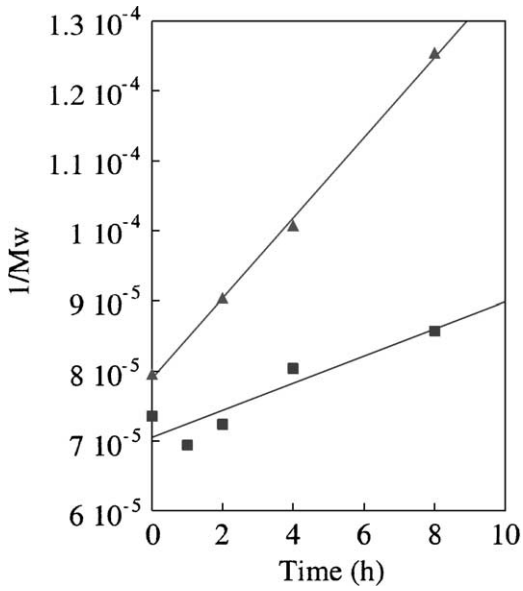

Fig. 2. Change in molar mass of acetylated homogalacturonan during the early stage of degradation of Ac18 (triangles) and Ac28 (squares) by *Fusarium* PG.

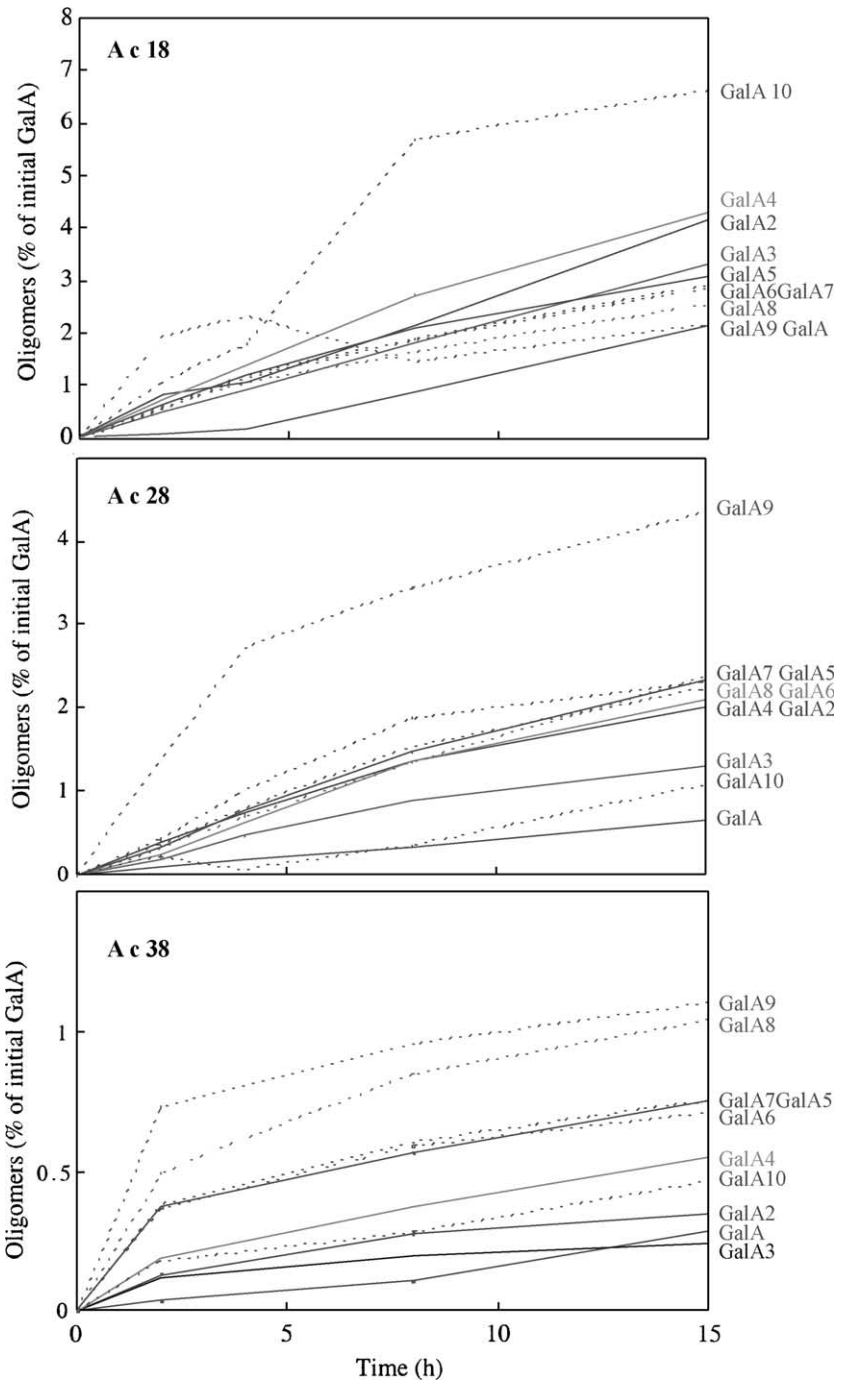

Fig. 3. Changes in oligomer concentration during hydrolysis of acetylated homogalacturonan by *Fusarium* PG. Solid lines indicate the concentration in dp 1–5, dashed lines indicate the concentration of dp 6–10.

pH 13, the oligosaccharides were completely de-esterified. The Fig. 3 shows the initial step of reaction and evidences that the first oligomers appearing in the reaction medium had high dp (GalA10, GalA9, GalA8). The shortest oligomers appeared very slowly. After 4 h incubation, the sum of dp 1–5 represented only 4.9, 2.8, and 1% of initial GalA for Ac18, Ac28, and Ac38, respectively. These results confirmed the multi-chain mode of attack of *Fusarium* PG on acetylated substrates: similar results were also observed on homogalacturonan (Bonnin et al., 2001).

The concentration of each oligomer at the final step is reported in the Table 2 for (acetylated) homogalacturonan. The sum of oligomers of dp 1–10 decreased from 98 to 18.3% when the DAc increased from 0 to 38. It decreased dramatically between DAc28 (72.9%) and DAc38 (18.3%). Thus, the amount of resistant fraction increased with

Table 2
Oligomer concentration at the final step of hydrolysis of homogalacturonan. Ac18, Ac28, and Ac38 by *Fusarium* PG

Substrate	% H	dpl	dp2	dp3	dp4	dp5	dp6	Dp7	dp8	dp9	dp10	sum
HG[a]	57.0	38.7	59.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	98.0
Ac18	21.1	13.0	23.0	7.1	21.2	9.9	4.7	3.0	5.1	1.0	0.0	87.9
Ac28	12.3	8.4	8.6	4.0	12.9	9.9	7.8	6.0	5.5	7.9	2.0	72.9
Ac38	3.4	2.5	1.4	0.6	3.6	2.0	1.6	1.7	1.5	1.9	1.5	18.3

HG: homogalacturonan; % H = Percentage of hydrolysis. Each oligmer is expressed as a percentage of initial GalA in the substrate.
[a] Values from Bonnin et al., 2001.

increasing DAc. The Ac18 digest contained 2-fold more dimer than monomer, a proportion similar to the homogalacturonan digest, suggesting the degradation of non-substituted area in the substrate. The major products were the dimer when DAc = 0, the dimer and 4-mer when DAc = 18, and the 4-mer when DAc = 28 or 38, showing a shift in the product formation towards higher dp when the DAc of the substrate increased.

The time course of oligomer production was analysed as well by mass spectrometry, in order to quantify the substituents on the produced oligomers. Only oligomers above dp3 were recorded because of the interference of

Table 3
MALDI-TOF MS analysis of final products of hydrolysis of Ac18, Ac28 and Ac38 with *Fusarium* PG

Substrat	Time (h)	H (%)	Ac	3	4	5	6	7	8	9
AC18	O	0.0	0							
	8	2.7	0	**3/0**	**4/0**	**5/0**	6/0			
			1		4/1	5/1	**6/1**			
			2			5/2	6/2			
	24	7.0	0	**3/0**	**4/0**	5/0				
			1			**5/1**	**6/1**	**7/1**		
			2			5/2	6/2	7/2	**8/2**	**9/2**
			3							9/3
	48	10.8	0	3/0	4/0					
			1	**3/1**	**4/1**	**5/1**	**6/1**	7/1		
			2		(4/2)	5/2	6/2	**7/2**	**8/2**	9/2
			3					7/3	8/3	**9/3**
	96	21.1	0	3/0						
			1	**3/1**	**4/1**	5/1				
			2		4/2	**5/2**	**6/2**	7/2		
			3				6/3	**7/3**	**8/3**	
			0			(5/0 + 1 Me)				
			1			(5/1 + 1 Me)	(6/1 + 1 Me)			
			2				(6/2 + 1 Me)	(7/2 + 1 Me)		
			3					(7/3 + 1 Me)		
Ac28	96	12.3	0							
			1	**3/1**	**4/1**	5/1				
			2		4/2	5/2	6/2			
			3			(5/3)	6/3			
			0		(4/0 + 1 Me)	(5/0 + 1 Me)				
			1		4/1 + 1 Me	**5/1 + 1 Me**	6/1 + 1 Me	7/1 + 1 Me		
			2		(4/2 + 1 Me)	5/2 + 1 Me	**6/2 + 1 Me**	**7/2 + 1 Me**		
			3				6/3 + 1 Me	7/3 + 1 Me)	**8/3 + 1 Me**	
Ac38	96	3.4	0							
			1	3/1						
			2							
			3							
			0							
			1		**4/1 + 1 Me**	5/1 + 1 Me				
			2		(4/2 + 1 Me)	**5/2 + 1 Me**	6/2 + 1 Me	7/2 + 2 Me		
			3				**6/2 + 2 Me**	**7/3 + 2 Me**		

H: percentage of hydrolysis; in bold: major product; (): minor product; nomenclature used: 4/l is a 4-mer with 1 AC, 4/1 + 1 Me is a 4-mer with 1 A_C and 1 Me.

matrix ions in the lower mass range using MALDI-TOF MS. The results are shown in Table 3. In the nomenclature used, 4/0 is the tetramer, 4/1 is a 4-mer with 1 Ac, 4/1 + 1 Me is a 4-mer with 1 Ac and 1 Me. At the beginning of the hydrolysis of Ac18, MS spectra did not reveal any oligomers. From 8 h of incubation, non-substituted 3-, 4-, 5-, and 6-mers were observed. The tetramer and pentamer were predominantly unsubstitued (in bold in Table 3), showing the hydrolysis of the unsubstituted area of the acetylated homogalacturonan. 4/0, 5/0, and 6/0 disappeared when the reaction time was prolonged. From 24 h, acetylated oligomers with dp 7 or more appeared. As they disappeared in the subsequent digests (see for example 8-mer and 9-mer), their substitution did not hinder their degradation. In other words, they were long enough to be hydrolysed although they were acetylated. In the 96 h digest of Ac18, 3/0 was the only unsubstituted oligomer. The mixture contained mainly acetylated oligomers and also some oligomers bearing both acetyl- and methyl-esters. Indeed, the initial substrates contained some residual methyl groups (cf Table 1). These oligomers are minor products in the 96 h digest and most often bore one acetyl group less than the non-methylated products. For example, the 6-mer could carry 2 or 3 acetyl groups but if it is methylated, it carried only 1 or 2 acetyl groups.

Similar oligomers were found in the end-point digest of Ac28, including acetylated and methylated oligomers. The major products with dp 5–8 all bore 1 methyl group. For Ac38, the number of oligomers formed was reduced and all the end-products contained both methyl- and acetyl-groups, except 3/1. It is not surprising that with increasing DAc the amount of acetylated and methyl-esterified oligomers increased, as Ac18 and Ac28 were prepared from Ac38 by chemical saponification, leading to concomitant release of both methyl- and acetyl-esters. The highest product dp found in this digest was 7. As the final percentage of hydrolysis of Ac38 was only 3.4% (Fig. 1), most of the reaction products were likely to have a dp higher than 7 and the products detected in mass spectrometry represented probably minor concentrations. This is suitable with the results of Table 3 where the sum of oligomers of dp 1–10 represented only 18.3% of initial GalA.

By combining HPAEC results (Table 3) and MS results (Table 3), it was possible to determine that 4/1 and 4/1 + 1 Me were the major products in Ac28 and Ac38 digests, respectively. On the opposite in Ac18 end-digest, the most abundant product was the dimer (Table 2), that was not detected in MALDI-TOF.

4. Discussion

Our previous results demonstrated that the *Fusarium* PG was specific for unsubstituted GalA residues and that it was also able to degrade methyl-esterified pectins by accommodating some methyl-groups in its active site (Bonnin et al., 2002a). From the present paper, it can be concluded that the enzyme is also able to degrade acetylated homogalacturonan. However, the enzyme hydrolyses preferentially the unsubstituted area of the substrate. With increasing DAc, the substrate exhibits a decreasing number of unesterified GalA blocks, which can be hydrolysed by PG.

A negative effect of acetyl groups on the PG degradation of pectins has been reported for a long time. Solms and Deuel (1951) hypothesized that the hydroxyl groups on O-2 or O-3 position play a key role in the formation of the enzyme-substrate complex. Rexovà-Benkovà, Mrakovà, Luknàr, and Kohn (1977) confirmed this hypothesis by showing that acetylation affected only the Km and thus the affinity of the enzyme, and not the maximum velocity of the reaction. The negative effect of acetyl groups was also observed by Pasculli, Gereads, Voragen, and Pilnik (1991) who incubated apple and sugar beet pectins with PGs from *Aspergillus niger*, *Aspergillus aculeatus* or *Kluyveromyces fragilis*. For example, they showed that the hydrolysis of apple pectins by *A. aculeatus* PG dropped down from 82.9 to 1.6% after chemical acetylation up to DAc = 180. Similar results were obtained from chemically acetylated homogalacturonan degraded by *A. niger* PG (Renard & Jarvis, 1999). While this enzyme cleaved 57% of the glycosidic bonds of non-acetylated homogalacturonan, only 8% could be cleaved at DAc = 55.

Fusarium PG shows a multi-chain mode of attack on acetylated homogalacturonan, as previously shown with homogalacturonan or methylated pectins (Bonnin et al., 2001; 2002a). Thus, the substitution with either methyl- or acetyl-groups did not modify the behaviour of the enzyme. The analysis of reaction products by MALDI-TOF MS demonstrated the presence but did not reveal the position of the substituents on the oligogalaturonates. However, the results previously obtained with methylated substrates by ESI-MS showed that the reducing and non-reducing ends of the oligomer were never methylated, demonstrating the strict requirement of the enzyme to cleave between two adjacent free GalA residues (Bonnin et al., 2002a). It could be hypothesized that the degradation occurred similarly with acetyl groups, and thus that they are never located at both ends of final products. On the other hand, the presence of oligomers with low dp and one or two acetyl groups in the final digests (such as 3/1, 4/1 or 4/2) showed that the enzyme was able to accommodate acetylated GalA in its active site cleft, as demonstrated for methyl-esterified residues in subsites -3, -2 or +2 (Bonnin et al., 2002a).

Whatever the DAc of the substrate, no fully acetylated oligomers were found in the digests.

The amount of dp3 (corresponding to 3/0 + 3/1) in the final digests was more important after digestion of acetylated homogalacturonan than after digestion of methylated pectins. Actually, dp3 represented 7.1, 4.0, and 0.6% of initial GalA for Ac18, Ac28 and Ac38, respectively, when it represented only 0.5% of initial GalA from

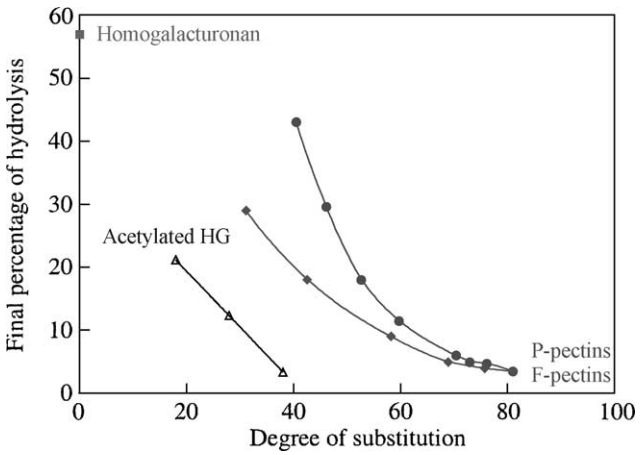

Fig. 4. Final percentage of hydrolysis of various substrates as a function of their degree of substitution: (lozenges) F-series and (circles) P-series of methylated pectins; (open triangles): Acetylated homogalacturonan; (squares) homogalacturonan.

the different pectins, whatever the initial DM (Bonnin et al., 2002a). It can be deduced from these results that the acetylated trimer was more often produced than the methylated trimer, indicating that an acetyl group could be accepted in the active site cleft of the enzyme. Another explanation could be that the trimer was converted again once it is produced if it is methylated but not converted if it is acetylated.

Fig. 4 shows the percentage of hydrolysis versus the degree of substitution for acetylated homogalacturonans (considering their DM as negligible). The final percentage of hydrolysis decreased very rapidly with increasing the DAc. On the same figure, the percentage of hydrolysis of pectins with various degrees and patterns of methyl-esterification is shown (from Bonnin et al., 2002a). The F-series corresponded to a random distribution of the methyl-esters whereas the distribution was more block-wise in the P-series. Comparison of the curves obtained for acetylated or methyl-esterified substrates shows that similar degrees of substitution allowed a larger degradation if the substituent was a methyl-ester. Thus, the acetyl groups hindered the enzymatic activity more largely than the methyl groups. Actually, the acetylated substrates used in this study are still methyl-esterified, as some end-products carried both acetyl and methyl groups. In the case of Ac38, the DM was 7 and the dp 84, meaning that on average one residue every 12 was methyl-esterified. The presence of end-products containing up to 2 methyl-groups for 6 or 7 residues, suggests that the methyl-groups were not randomly distributed on the polymer. Ac38 was extracted from sugar beet pectin by enzymatic treatment, including a fungal and a plant PMEs. The remaining methyl-esters were still present after the treatment probably because the PMEs were sterically hindered by the presence of acetyl groups. So, it is likely that any methyl-ester is located near an acetyl group. To conclude definitively on the effect of both substituents on the mode of action of *Fusarium* PG, it would be necessary to have substrates carrying only acetyl groups. Another way would be to chemically acetylate polygalacturonic acid. In this case, however, the distribution is different and some di-subtituted residues are produced from the beginning of the reaction (Renard & Jarvis, 1999). However, we have chosen to work on pectins extracted from cell wall in order to have the acetyl groups in their native position and distribution.

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