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Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5

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Abstract

The mechanism of action of purified apple pectin methylesterase on pectin (degree of methoxylation: DM 75) and methoxylated homogalacturonans (DM 70 and 90) was studied at pH 7.0 (optimal pH of the enzyme) and at pH 4.5 (close to the pH of apple juice). Different interchain distributions of the free carboxyl groups were obtained at pH 7.0 and 4.5: high-performance ion exchange chromatography indicated a typical single chain mechanism at pH 7.0, but a mechanism differing from the single and multiple chain ones at pH 4.5. However, the same intrachain distribution of the newly demethoxylated galacturonic acid residues was observed for both pHs by ^{1}H NMR. The high content of consecutive de-esterified or consecutive esterified galacturonic acid residues suggested that apple PME acted with a multiple attack mechanism on the pectic substrate. The degree of multiple attack of the enzyme was greater than or equal to 10–11.

Keywords: Apple; Pectin methylesterase; Mechanism of action; Interchain and intrachain distributions

1. Introduction

In the fruit juice industry, a technological problem of cloudy juices is to stabilise the cloudiness during storage. Pectin methylesterase (PME, E.C. 3.1.1.11) has been shown to induce cloud loss and texture modifications of food products from fruits (juice, nectar) by action on pectins [1]. The major structural feature of pectin is a linear chain of (1→4)-linked α-D-galacturonic acid residues, which can be partially esterified by methoxyl groups. These homogalacturonan chains, of $\overline{DP}$ > 70–100 [2], are interspersed with rhamnogalacturonan regions presenting the repeating units →4)-D-Gal*p*A-α-(1→2)-L-Rha*p*-α-(1→. Some of the rhamnose units carry side chains composed essentially of galactose and arabinose, forming 'hairy regions'. On the whole, the backbone of pectins can be estimated to be more than 500 residues long. Pectin methylesterase is the enzyme that catalyses the demethoxylation of esterified pectins. This enzyme has been purified from many fruits such as banana [3], orange [4],

Abbreviations: DM, degree of methoxylation; PME, pectin methylesterase; $\overline{DP}$, degree of polymerisation; G, de-esterified galacturonic acid; E, esterified galacturonic acid; HPIEC, high-performance ion exchange chromatography; HPSEC, high-performance size exclusion chromatography.

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tomato [5], papaya [6] and apple [7]. The physico–chemical and kinetic properties of purified enzymes such as molecular weight, isoelectric point, optimal pH, optimal temperature have been extensively investigated but little is known about the action pattern of the PMEs on their substrate [8–14].

Three action patterns are generally evoked in enzymatic transformation of polysaccharides [15]:

1. Single chain mechanism: binding of the enzyme is followed by a conversion of all contiguous substrate sites on the polymer chain.
2. Multiple chain mechanism: the enzyme–substrate complex dissociates after each reaction resulting in conversion of a single residue for each attack.
3. Multiple attack mechanism: the enzyme catalyses the transformation of a limited average number of residues for every active enzyme–substrate complex formed; the average number of processed residues has been defined as the degree of multiple attack ($\bar{N}_E$).

In the case of PME, indirect methods (enzymatic degradation methods [8] and studies of calcium binding on pectins [9,10]) have permitted identification of two mechanisms of action of PME on pectins. Acidic microbial PMEs (*Aspergillus japonicus*, *Aspergillus niger*, *Aspergillus foetidus*) catalyse a random cleavage of esterified carboxyl groups [9,12] (multiple chain mechanism). Alkaline PMEs from higher plants (tomato, orange, alfalfa) and from fungal origin (*Trichoderma reesei*) produce blocks of free carboxyls groups, i.e., they catalyse demethoxylation of pectin linearly along the chain (single chain mechanism). Solms and Deuels [11] have shown that plant PMEs need a free carboxyl group unit in the neighbourhood of a methoxylated unit to start the hydrolysis. Direct method ^{1}H and ^{13}C NMR spectroscopies suggest the third action pattern for plant PMEs on pectin [13,14] (multiple attack mechanism). Grasdalen et al. [13] and Catoire et al. [14] showed that tomato PME at pH 6.5 and two isoforms of mung bean hypocotyls PME at pH 7.6 follow this behaviour with a $\bar{N}_E \sim 7–8$ and $\bar{N}_E \sim 2–5$, respectively. Direct and indirect methods thus give confusing informations on the action patterns of plant PME at the interchain (indirect) or intrachain (direct) level.

Denès et al. [7] have shown that maximal activity of purified apple PME was obtained at pH 7.5. On the other hand, at pH 4.5 (close to apple juice pH), PME activity represents only 1% of the optimum. Nevertheless, such an activity is sufficient to destabilise the cloud of apple juices during storage. The present work describes the behaviour of purified apple PME at pH 7.0 and 4.5 by a combination of indirect (ion-exchange chromatography) and direct (^{1}H NMR spectroscopy) methods.

2. Results and discussion

Enzymatic degradation of pectin.—After incubation with apple PME at pH 7.0 for different times, pectin was injected on an ion-exchange column (Fig. 1(a)). The three populations (or peak) appearing in the chromatograms were quantified with the peak separation and analysis Peakfit™ software. The initial pectin (DM 75) showed a major peak (70%) eluting at an acetate concentration of 0.18 M (Table 1). A second population, eluted at 0.31 M, represented 23% of the total pectin. The last fraction, which had a very high charge density (eluting at 0.47 M) was eluted at a concentration close to that recorded for a pectate (DM < 4). During the enzymatic treatment at pH 7.0 (from DM 75 to 42), the first population of pectin (slightly charged) decreased from 70 to 3% (Table 1). Conversely, the highly charged population increased from 7 to 94%. The amount of the second population of the substrate increased to a maximum and decreased rapidly, while this peak shifted towards a concentration close to that of pectate (from 0.31 to 0.43 M). This was a typical behaviour of pectin demethoxylation by a single chain mechanism. In this mechanism, also described for orange, tomato and alfalfa PMEs [8,9], the enzyme catalyses the demethoxylation of pectin linearly along the chain producing two populations of polymers: the first is not modified and the second is completely demethoxylated.

The same experiment was carried out at pH 4.5 (Fig. 1(b)). The action of PME was rapidly stopped (DM 67) and was only slightly recovered by addition of the same amount of enzyme (data not shown). During the enzymatic treatment, the slightly charged fraction decreased (from 70 to 22%) and the second population increased (from 23 to 69%). This second peak widened and shifted to a higher ionic strength, indicating an increased charge density. The highly charged fraction remained constant during the hydrolysis, in contrast to the effect observed at pH 7.0.

This mechanism of action of apple PME at pH 4.5 was different from the previous one described at pH 7.0, and also different from the multiple chain mechanism observed at pH 4.5 for *A. niger* (Fig. 1(c)). In this mechanism, the proportion of the major population remained relatively constant (76–88%), but it shifted towards higher ionic strength, i.e., charge density (0.20–0.42 M). In this multiple

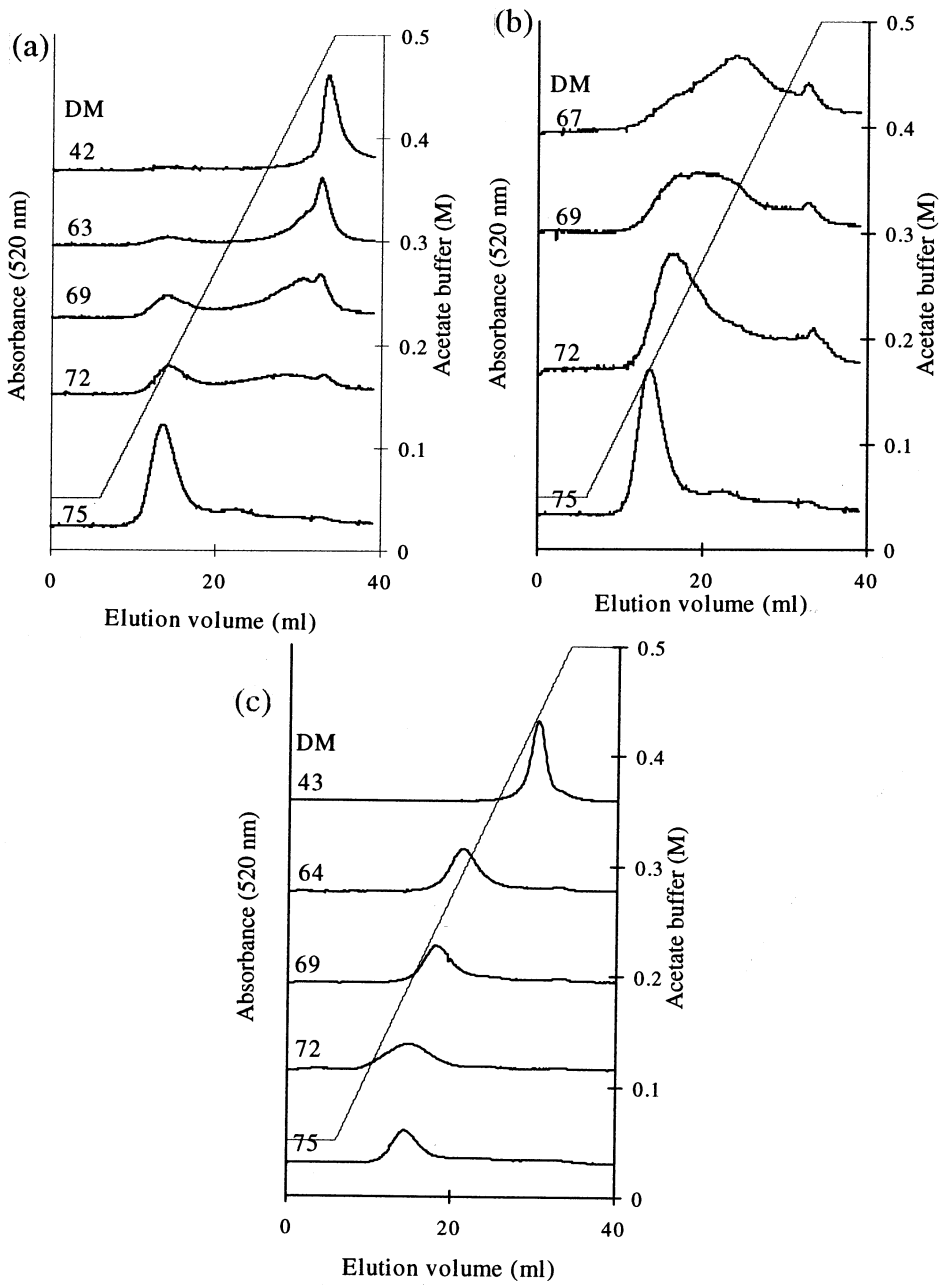

Fig. 1. HPIEC of demethoxylated pectin by purified apple PME at pH 7.0 (a) and pH 4.5 (b) and by *Aspergillus niger* PME at pH 4.5 (c). (—) acetate ammonium buffer (pH 6.0) concentration; (—) uronic acids.

Table 1
Fractions and acetate ammonium concentrations of elution of peak chromatograms at different incubation time

	Area (%) (*acetate ammonium* (M))		
DM	Peak no. 1	Peak no. 2	Peak no. 3
Apple PME: pH 7.0			
75	70 (*0.18*)	23 (*0.31*)	7 (*0.47*)
72	42 (*0.20*)	52 (*0.40*)	6 (*0.48*)
69	26 (*0.20*)	61 (*0.40*)	13 (*0.48*)
63	17 (*0.19*)	34 (*0.42*)	49 (*0.47*)
42	3 (*0.18*)	3 (*0.43*)	94 (*0.48*)
Apple PME: pH 4.5			
75	70 (*0.18*)	23 (*0.31*)	7 (*0.47*)
72	66 (*0.24*)	27 (*0.32*)	7 (*0.49*)
69	42 (*0.22*)	52 (*0.33*)	6 (*0.48*)
67	22 (*0.22*)	69 (*0.37*)	9 (*0.48*)
A. niger PME: pH 4.5			
75	76 (*0.20*)	15 (*0.34*)	9 (*0.48*)
72	87 (*0.23*)	9 (*0.34*)	4 (*0.48*)
69	78 (*0.26*)	16 (*0.34*)	6 (*0.48*)
64	94 (*0.31*)		6 (*0.48*)
43	88 (*0.42*)		12 (*0.48*)

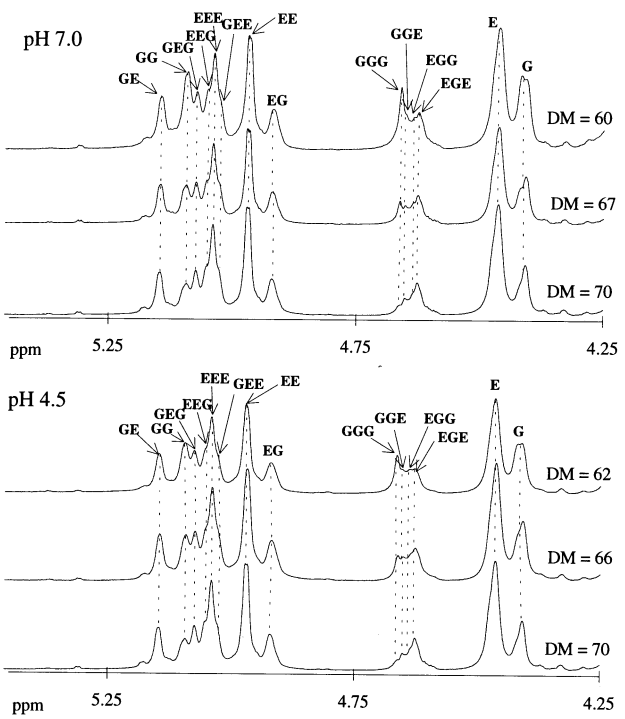

Fig. 2. ^{1}H NMR spectra of homogalacturonans (DM 70) after apple PME hydrolysis at pH 7.0 and 4.5.

chain mechanism, PME catalysed a random cleavage of esterified carboxyl groups.

Enzymatic degradation of homogalacturonans.—After incubation with apple PME at pH 7.0 and 4.5, methoxylated homogalacturonans (DM 70 and 90) were analysed by ^{1}H NMR. Homogalacturonans were chosen to avoid the interference of rhamnose H-1 signals in the NMR spectra of the pectin [16]. Fig. 2 shows the spectral region of protons H-1, H-5 and H-4 in the G (de-esterified galacturonic acid) and E (esterified galacturonic acid) residues of methoxylated homogalacturonans (DM 70) after enzyme hydrolysis. Chemical shifts (δ) of protons of E and G residues were dependent on the nature of their neighbouring units. Signals characteristics of monads, dyads and triads were assigned using the data of Tjan et al. [17], Andersen et al. [18], Grasdalen et al. [13,19], except for the G$\underline{\text{E}}$E triad, which was assumed to correspond to the signals at $\delta = 5.02$ ppm in the H-5 E region. Frequencies of dyads and triads for a random distribution of free and methoxylated GalA residues were calculated according to the formulae shown in Table 2. The parameters used were the degree of polymerisation of pectic chains ($\overline{DP}$) and the proportion of methoxyl residues (DM). Probabilities were modified with the DP of the chain because of the effect of the molecule ends.

Because of the intervention of the $\overline{DP}$ in the calculation of the Bernouillian probabilities, and because of risks of β-elimination at neutral pH and high temperature used during spectra recording, homogalacturonans (DM 70) were analysed by high-performance size exclusion chromatography (HPSEC) before and after NMR analysis (Fig. 3). The average degree of

Table 2
Formula for calculation of probabilities of dyads and triads of a Bernouillian sequential distribution [a]

Dyads	Probabilities	Triads	Probabilities
$\underline{\text{E}}\underline{\text{E}}$	$F=\frac{C_{DP-2}^{NM-2}}{C_{DP}^{NM}}$	E$\underline{\text{E}}$E	$F=\frac{C_{DP-3}^{NM-3}}{C_{DP}^{NM}}$
$\underline{\text{E}}\underline{\text{G}}$ or $\underline{\text{G}}\underline{\text{E}}$	$F=\frac{C_{DP-2}^{NM-1}}{C_{DP}^{NM}}$	E$\underline{\text{E}}\underline{\text{G}}$ or E$\underline{\text{G}}\underline{\text{E}}$ or G$\underline{\text{E}}$E	$F=\frac{C_{DP-3}^{NM-2}}{C_{DP}^{NM}}$
$\underline{\text{G}}\underline{\text{G}}$	$F=\frac{C_{DP-2}^{NM}}{C_{DP}^{NM}}$	G$\underline{\text{G}}$E or G$\underline{\text{E}}\underline{\text{G}}$ or E$\underline{\text{G}}\underline{\text{G}}$	$F=\frac{C_{DP-3}^{NM-1}}{C_{DP}^{NM}}$
		G$\underline{\text{G}}\underline{\text{G}}$	$F=\frac{C_{DP-3}^{NM}}{C_{DP}^{NM}}$

[a] NM, degree of methoxylation of pectin chains; $\overline{DP}$, average degree of polymerisation of pectin chains.

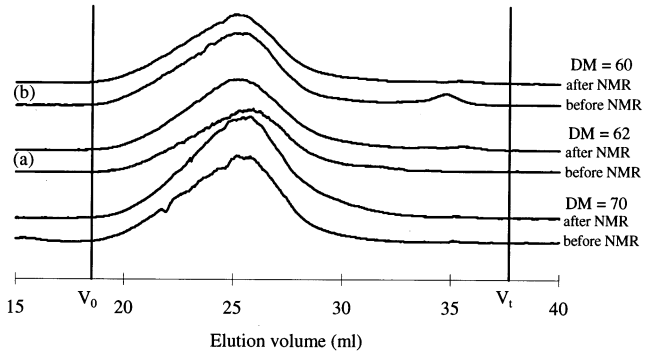

Fig. 3. HSPEC patterns before and after ^{1}H NMR of homogalacturonans with various degrees of methoxylation resulting from apple PME hydrolysis at pH 7.0 (a) and pH 4.5 (b).

polymerisation $\overline{DP}$ before ^{1}H NMR ($\overline{DP} = 64 \pm 7$, $n = 5$) was not significantly different from the value determined after ^{1}H NMR analysis ($\overline{DP} = 62 \pm 6$, $n = 5$). Thus, the average $\overline{DP}$ of homogalacturonans was determined: 63 ± 6, $n = 10$. We concluded that the reaction of β-elimination was negligible during the recording of the spectra at 80 °C and pH 6.0 in the 600 MHz spectrometer. We also concluded that apple PME did not modify the $\overline{DP}$, i.e., in accordance with previous results [7], the purified enzyme was not contaminated with depolymerase activity. The observed $\overline{DP}$ variation did not modify significantly the Bernouillian dyad and triad probabilities. For example, probabilities of the triad GGG (F_{GGG}) were 0.023 and 0.025 and of the triad EEE (F_{EEE}) were 0.338 and 0.330 for $\overline{DP}$s 57 and 69, respectively. Bernouillian probabilities were more affected for lower degrees of polymerisation due to the effect of the molecule ends. For example, the Bernouillian probability of the triad EEE was 0.264 for the methoxylated polygalacturonic acid of $\overline{DP}$ 15 used by Grasdalen et al. [13] to study tomato PME action pattern.

The frequencies of monads, dyads and triads in HGs of $\overline{DP}$ 70 and 90 (Table 3) were in good agreement with Bernouillian probabilities, indicating that the methoxylation of homogalacturonans occurred randomly, as reported by Renard and Jarvis [20]. Table 4

Table 3
Monad, dyad and triad frequencies of the initial methoxylated homogalacturonans [a,b,c]

	δ (ppm)	HG 70		HG 90	
		Bernouillian probabilities	^{1}H NMR frequencies	Bernouillian probabilities	^{1}H NMR frequencies
Monads (H-4)					
G	4.45	0.296	0.292	0.897	0.873
E	4.50	0.704	0.708	0.103	0.127
DM by ^{1}H NMR			71		87
Dyads (H-1)					
GG	5.10	0.088	0.082	0.011	0.051
GE	5.15	0.213	0.239	0.100	0.100
EG	4.92	0.213	0.208	0.100	0.088
EE	4.98	0.489	0.471	0.789	0.762
Triads (H-5)					
GGG	4.69	0.023	0.023	0.001	0.003
GGE	4.65	0.062	0.053	0.010	0.003
EGG	4.66	0.062	0.057	0.010	0.005
GEG	5.06	0.062	0.053	0.010	0.055
EGE	4.64	0.151	0.153	0.090	0.052
EEG	5.05	0.151	0.099	0.090	0.107
GEE	5.02	0.151	0.137	0.090	0.086
EEE	5.04	0.338	0.424	0.698	0.690

[a] G, de-esterified unit; E, esterified unit; HG, Methoxylated Homogalacturonans.
[b] Bernouillian probabilities were calculated with a $\overline{DP}$ of 63.
[c] DMs by ^{1}H NMR were determined using the ratio H-4 (E):(H-4 (E) + H-4 (G)) signals multiplied by 100.

Table 4
Monad, dyad and triad frequencies after apple PME hydrolysis of HG 70 and HG 90 at both pH [a,b]

	HG 70				HG 90			
	pH 7.0		pH 4.5		pH 7.0			pH 4.5
DM by analytical method	67	60	66	62	85	81	74	85
Monads (H-4)								
G	0.328	0.386	0.358	0.399	0.138	0.184	0.290	0.146
E	0.672	0.614	0.642	0.601	0.862	0.816	0.710	0.854
DM by ^{1}H NMR	67	61	64	60	86	82	71	85
Dyads (H-1)								
GG	0.097	0.161	0.084	0.110	0.061	0.090	0.152	0.054
GE	0.231	0.242	0.251	0.237	0.088	0.106	0.122	0.092
EG	0.215	0.179	0.194	0.200	0.078	0.075	0.097	0.074
EE	0.457	0.418	0.475	0.453	0.773	0.729	0.630	0.780
Triads (H-5)								
GGG	0.082	0.136	0.058	0.130	0.050	0.091	0.181	0.043
GGE	0.061	0.046	0.083	0.069	0.005	0.007	0.006	0.006
EGG	0.057	0.077	0.080	0.063	0.007	0.011	0.032	0.018
GEG	0.028	0.035	0.027	0.026	0.028	0.060	0.038	0.048
EGE	0.110	0.103	0.090	0.098	0.039	0.041	0.061	0.035
EEG	0.096	0.127	0.147	0.098	0.104	0.051	0.072	0.091
GEE	0.118	0.108	0.124	0.117	0.073	0.064	0.052	0.058
EEE	0.448	0.368	0.391	0.399	0.693	0.676	0.558	0.701

[a] DMs by ^{1}H NMR were determined using the ratio H-4 (E):(H-4 (E)+H-4 (G)) signals multiplied by 100.
[b] HG, Methoxylated homogalacturonans.

shows frequencies of monads, dyads and triads of homogalacturonans after enzyme action. DMs of homogalacturonans were recalculated by the ratio of H-4 E to H-4 E plus H-4 G signals in the NMR spectra. They were in good agreement with the values determined by chemical methods (Tables 3 and 4). The observed variations of frequency for F_{GGG} and F_{EEE} as a function of final DM following action of PME at pH 7.0 and 4.5 on homogalacturonans of DM 70 are shown in Fig. 4. Bernouillian frequencies are shown for comparison. Experimental results were not significantly different for the two pHs. When the DM decreased, the frequencies F_{GGG} were higher than the Bernouillian probabilities. This suggested a blockwise distribution and was confirmed by the experimental frequency of F_{EEE}, which was also higher than Bernouillian probabilities.

The effective numbers average block length of E residues ($\bar{N}_E$) were calculated for the two populations of homogalacturonans (DM 70 and 90) according to the formula of Grasdalen et al. [13] ($\bar{N}_E = DM/F_{EG}$) (Table 5). Initial values of $\bar{N}_E$ were 3 and 10 for homogalacturonans of DM 70 and 90, respectively. $\bar{N}_E$ values remained constant during the enzymatic reaction, suggesting that the enzyme demethoxylated at least 3 and 10–11 successive E residues at each attack on homogalacturonans of DM 70 and 90, respectively. This indicated that the degree of multiple attack of the enzyme was greater than or equal to 10–11. Grasdalen et al. [13] indicate that the degree of multiple attack of tomato PME may be greater than 7 using a highly esterified poly (D-galacturonate) (DM 92, $\overline{DP}$ 15). Catoire et al. [14] determined a $\bar{N}_E$ of 2–5 for three isoforms of mung bean hypocotyls at pH 7.6 using an apple pectin (DM 74.4, $\overline{DP}$ 50). In fact, the degree of multiple attack of the PME seemed to be equal to the average number of successive E residues on the initial substrate. Thus, the degree of multiple attack could be higher using a substrate with higher DP and higher DM containing higher average block length of E residues.

Mechanism of action of PME.—The apple PME **interchain mechanism** of action on pectin was pH-dependent. At pH 7.0 [7], the behaviour was a single chain mechanism. At pH 4.5, the behaviour was different from both the single and the multiple chain mechanism. The same pattern was observed with commercial orange PME (data not shown), suggesting that this behaviour was not specific of the apple fruit but due to the pH differences.

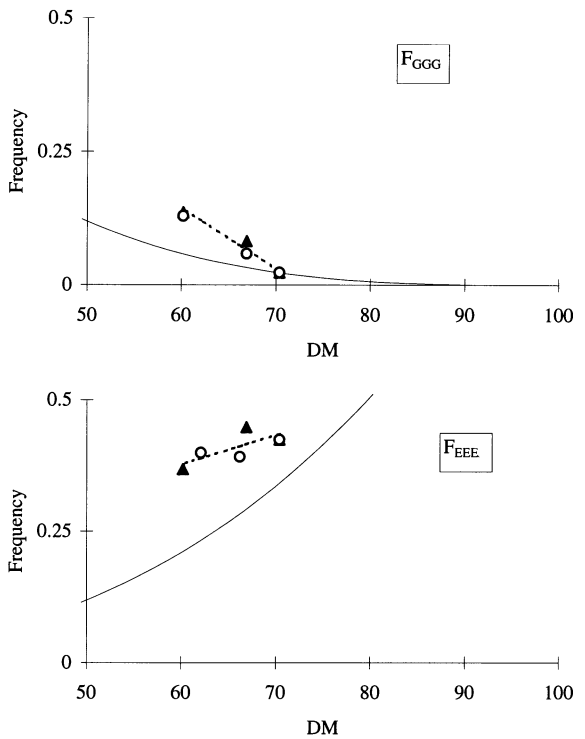

Fig. 4. Triad frequencies F_{GGG} and F_{EEE} resulting from apple PME hydrolysis of homogalacturonans (DM 70) at pH 7.0 (▲) and at pH 4.5 (○) compared with calculated Bernouillian triads probabilities (– – – – –).

Table 5
Effective number average block length of E residues ($\bar{N}_E$) during the enzymatic reaction of apple PME on homogalacturonans [a]

HG 90		HG 70			
pH 7.0		pH 7.0		pH 4.5	
DM	$\bar{N}_E$	DM	$\bar{N}_E$	DM	$\bar{N}_E$
90	10.2	70	3.4	70	3.4
85	10.9	67	3.1	66	3.4
81	10.7	60	3.4	62	3.1
74	7.6				

[a] DM, degree of methoxylation; HG, methoxylated homogalacturonans.

However, the **intrachain mechanism** of action of apple PME on homogalacturonans was the same at both pH 7.0 and 4.5. Thus, the enzyme has acted by blocks, the size of which was equal to the multiple attack degree, along the homogalacturonan chain whatever the pH. Catoire et al. [14], using ^{13}C NMR pectin analysis, also showed that the intrachain mechanism of action of mung bean hypocotyls isoforms is pH-dependent (pH 7.6: multiple attack mechanism and pH 4.6: single chain mechanism).

At pH 7.0, apple PME bound to a chain of pectin, which it completely demethoxylated by successive blocks (equal to the multiple attack degree), then acted on a second chain. Conversely, at pH 4.5, the enzyme demethoxylated a block of E residues equal to the multiple attack degree on a chain of pectin and then attacked another chain of pectin. Thus, in apple juice, the action of endogenous PME would produce a population of pectins with a globally moderate but locally high charge density. This would be sufficient to induce gel formation by the juice pectin in the presence of the endogenous calcium in spite of the low enzyme activity.

A new classification, taking into account both the intra- and interchain levels, can be proposed for the pattern of demethoxylation of pectin:

- A single chain, multiple attack mechanism leading to a **blockwise** distribution of G on a **part** of pectin population, the remaining pectins are not affected. This behaviour concerned apple PME at pH 7.0.
- A multiple chain, multiple attack mechanism leading to a **blockwise** distribution of G with shorter blocks but distributed on all pectic chains. This is the behaviour of apple PME at pH 4.5. The ion-exchange patterns (Fig. 1) showed that *A. niger* is a multiple chain enzyme. Pectin gelation studies [21] suggested that it could have a multiple attack mechanism, which still has to be confirmed by analysis of its reaction products by NMR.
- A multiple chain, single attack mechanism leading to a **random** distribution of G on all pectic chains (alkaline demethoxylation).

- A single chain, single attack mechanism has not been yet reported.

3. Experimental

Materials.—Pectin methylesterase was purified from apple (cv *Golden delicious*), as previously described [7]. *A. niger* PME (Rapidase CPE 9111) was a gift from Gist Brocades (Seclin, France). Tetrabutylammonium hydroxide (TBA-OH) was purchased from Sigma® and other reagents were analytical grade. Apple pectin (DM 75) was a gift from S.K.W. (Redon, France). Homogalacturonans were prepared from citrus pectin (S.K.W., Beaupte, France) by de-esterification (4 °C, pH > 12, 2 h), followed by acid hydrolysis (0.1 M HCl, 80 °C, 72 h) [2]. They were methoxylated by the procedure of Matricardi et al. [22] as adapted by Renard and Jarvis [20] using CH_3I as the reactant.

Chromatography.—For high-performance ion exchange chromatography (HPIEC) fractions of 200 μL of samples at ~6 μmol GalA mL^{-1} were injected onto a Progel™ TSK DEAE 5 PW (7.5 cm × 7.5 mm i.d., Sigma®-Aldrich) column equipped with a TSK DEAE 5 PW pre-column. Samples were eluted by a linear gradient of ammonium acetate (0.05–0.5 M, pH 6.0) using a Kontron Instrument system at a flow rate of 0.6 mL min^{-1}. HPSEC was carried out using a LDC system. Fractions of 200 μL of samples at ~6 μmol GalA mL^{-1} were injected on four combined Progel-TSK™ columns (G5000-G4000-G3000-G2500-PWXL; 30.0 cm × 7.8 mm i.d.) mounted in series, equipped with a TSK-XL pre-column. Columns were thermostated at 35 °C and eluted by sodium acetate (0.4 M, pH 4.0) at 0.8 mL min^{-1}. The columns were calibrated using pectins of known molecular weights, determined by viscosimetry, for $\overline{DP}$ calculation. GalA concentration was measured in the eluates of HPIEC or HPSEC by post column derivatisation using the automated *m*-hydroxybiphenyl (MHBP) colorimetric method [23].

Analytical methods.—The DMs were determined as the molar ratio methoxyl: galacturonic acid multiplied by 100. Methoxyl content was measured after saponification of homogalacturonans (2 mg mL^{-1}) in 0.04 M NaOH for 1 h at room temperature. It was extracted by steam distillation and measured by gas chromatography (HP-FFAP capillary column of 50 m × 0.32 mm i.d., 0.52 μm film thickness; Hewlett Packard, Les Ulis, France) at 70 °C using hydrogen as carrier gas. Propanol was used as internal standard.

Nuclear magnetic resonance.—^1H NMR spectra of methoxylated homogalacturonans before and after enzymatic treatments were recorded on a Bruker DRX 600 spectrometer operating at 600.13 MHz. NMR data were processed using the UXNMR program (Bruker Analytische Messtechnik) on an INDY workstation (Silicon Graphics). The length of the 90° pulse was ca. 7 μs. All 1D spectra were collected using 16 K data points, and transformed after zero-filling to 32 K data points. For integration, the spectra were baseline corrected. No other mathematic treatment was applied. All spectra were recorded at 353 °K under a careful temperature regulation. Solutions of homogalacturonans were adjusted to pH 6.0 and exchanged twice with 99.9% deuterium oxide (D_2O) before solubilisation in 0.4 mL 100% D_2O (Eurisotop, Saclay, France). Acetone (1 μL) was added in the sample as internal reference. ^1H chemical shifts were referenced to acetone assigned to 2.225 ppm.

Enzymatic degradation.—Pectin (20 mL, 2.5 mg mL^{-1}) or methoxylated homogalacturonans (25 mL, 2.5 mg mL^{-1}) were incubated at 35 ± 0.1 °C in a thermostat-controlled cell of an automatic pH-stat (Metrohm) with apple PME (0.5 μg proteins). The enzymatic hydrolysis was followed by automatic titration with 0.02 M KOH of carboxyl groups released from the pectin solution. Enzymatic hydrolysis was followed at pH 7.0 and 4.5 with NaCl concentrations of 0.1 and 0.5 M, respectively.

Chromatograms and ^1H NMR spectra analysis.—Chromatograms and ^1H NMR spectra were analysed using the peak separation and analysis software Peakfit™ version 4 for Win32 (Jandel Scientific). Chromatograms and spectra were fitted using the Gaussian and Lorentzian peak functions, respectively. The H-1 signals of spectra were split in doublets.

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