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Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase☆

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Abstract

A method was developed that enabled the quantification of non-methyl-esterified galacturonic acid (GalA) sequences in pectin using enzymes. Endopolygalacturonase of *Kluyveromyces fragiles* was used to degrade pectin and the mono-, di-, and oligogalacturonides liberated were analyzed with high-performance anion-exchange chromatography at pH 5. With this technique non-methyl-esterified GalA residues could be distinguished from partially methyl-esterified oligomers. Non-esterified mono-, di-, and tri-galacturonic acid were predominantly released. The total amount of non-esterified GalA liberated was expressed as the percentage of the total number of non-esterified GalA present in pectin. For this percentage the term 'degree of blockiness' is introduced. Pectins that were sequentially de-esterified with tomato pectin methylesterase released large amounts of non-esterified GalA. The more methyl esters removed with pectin methylesterase, the more non-methyl-esterified GalA were liberated by endopolygalacturonase. Random methyl-esterified pectins liberated the lowest amounts of non-esterified GalA, even when the degree of methyl esterification was relatively low. The method reveals clear differences between pectins having the same degree of methyl esterification and different functional behavior.

Keywords: Pectin; Methyl ester; Distribution; HPLC; Endopolygalacturonase

1. Introduction

Pectin is a complex polysaccharide found in the primary cell walls and intercellular regions of higher plants. Its structure is important in determining plant cell-wall strength and flexibility [1]. Because of its excellent gelling, thickening, and stabilizing properties, the polymer is extensively utilized in the food industry [2,3]. The dominant feature of pectin is a linear chain of α-(1→4)-linked D-galacturonic acid (GalA) units in which varying proportions of the acid groups are

Abbreviations: DM, degree of methyl esterification; DP, degree of polymerization; Endo-PG, endopolygalacturonase; HPAEC, high-performance anion-exchange chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAD, pulsed amperometric detection; PME, pectin methylesterase.

☆ Investigation of the galacturonic acid distribution of pectin with enzymes, Part 1.

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methyl-esterified. This homogalacturonan backbone is occasionally interrupted by rhamnose-rich regions which can be highly substituted with neutral sugar-rich side chains [4]. Pectins display a large polydispersity with varying levels of methyl esterification and neutral sugar content [2].

The pattern of methyl esterification has been the subject of many studies because of its effect on the rheological and gel-forming properties of pectin [2,3]. Previous investigations of the methyl ester distribution of pectin have included NMR spectroscopy [5,6], calcium binding [7], enzymatic [8–12], and chemical studies [8,13]. For native apple pectin, a random distribution was found [10], whereas a non-uniform distribution was reported for some commercially extracted lemon and apple pectins [8,11]. Acid and alkaline de-esterified pectins were found to be randomly esterified [5,11].

With purified pectolytic enzymes, detailed information on the methyl ester distribution—or better, the distribution of the enzyme degradable sites—over the pectic backbone should be obtained. However, for a correct interpretation of the results, sufficient knowledge of the enzyme's mode of action as well as detailed information on the methyl ester and GalA content of the degradation products is essential [8]. Only when both conditions are met, can information be obtained that will allow for a reliable reconstruction of the methyl ester distribution of the starting material. Pectin-degrading enzymes have been studied for several decades, providing adequate information on their mode of action and other more general characteristics [14]. Especially the active site of the homogalacturonan-degrading enzyme endopolygalacturonase (endo-PG) has been studied in detail [15,16]. For determination of the methyl and GalA contents of the endo-PG degradation products of pectin, two methods have recently been described [17]. The first is high-performance anion-exchange chromatography (HPAEC) at pH 5, which is capable of separating non- and partially methyl-esterified oligomers with a sodium acetate gradient, followed by postcolumn sodium hydroxide addition to allow pulsed amperometric detection (PAD). The other method is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In this report, both techniques are used in the study of the methyl ester distribution of pectin with endo-PG. Endo-PG of *Kluyveromyces fragiles* is used to extensively degrade pectin and the composition of the GalA oligomers liberated is subsequently determined. Commercially extracted pectins of varying degrees of methyl esterification (DMs) as well as chemically and enzymatically modified pectins with more defined methyl ester distributions were used as substrates.

2. Results

Optimization of HPAEC at pH 5.—HPAEC separation at pH 5 of an endo-PG digest of a commercially extracted DM 30 pectin (C30; Table 1) resulted in the complex elution profile shown in Fig. 1(A). Previous research proved that the majority of the peaks observed resulted from partially methyl-esterified GalA oligomers and demonstrated the elution principle of these oligomers [17]. Though some of the peaks could be allocated to specific oligomers, i.e., (non-esterified) mono-, di-, tri-, and tetragalacturonic acid eluting at 10.5, 31, 45, and 58.5 min, respectively, correct assignment of the methyl and GalA content of the other peaks was not completely possible for the HPAEC separation [17]. Therefore, the elution sequence of the GalA oligomers by HPAEC at pH 5 was determined. In the absence of postcolumn sodium hydroxide addition and PAD, fractions of 0.25 mL were collected during separation of 80 μL of the C30 endo-PG digest. To verify whether the oligomers were separated correctly in the absence of postcolumn detection, two identical runs—one before and one after fractionation—were performed in the presence of postcolumn sodium hydroxide addition and PAD detection. The elution profiles of both runs were identical and the profile of the first run is shown in Fig. 1(A). After Dowex 50WX8 treatment of the fractions to remove the large excess of sodium ions resulting from the buffer [17], all fractions were

meticulously analyzed with MALDI-TOF-MS. Apart from monomeric GalA, which could not be detected because of matrix ion interference in that mass region, non- and partially methyl-esterified GalA oligomers of degree of detection (DP) 2–10 were observed (data not shown). No GalA oligomers were found in the unbound fraction and after the retention time of (non-esterified) tetragalacturonic acid. Though quantification of oligomers was not possible with MALDI-TOF-MS [18], the on- and offset of each component could clearly be discerned. The elution position of monomeric GalA (10.5 min) was confirmed by HPAEC analysis of this component. MALDI-TOF-MS analysis of the fractions eluting around 11 min confirmed that, apart from a pentamer with three methyl esters at 13.5 min, no other components of DP ≥ 2 were present.

Because accurate determination of the peak area of the components present in the complex HPAEC pH 5 elution profile (Fig. 1(A)) was impossible with the standard software of the chromatography system, the peak finding and fitting software program PEAKFIT was used. With this program the complex chromatograms could be resolved and integrated. Hidden peaks (as indicated by the MALDI-TOF-MS data) were revealed by looking at the remaining residuals after first fitting the obvious maxima. All peaks were best fitted by an exponentially modified Gaussian lineshape. The position of the integrated peaks correctly corresponded with the on- and offset of the components as observed with MALDI-TOF-MS analysis of the fractions. The combined MALDI-TOF-MS and integrated peak data are presented in Fig. 1(B). In this Figure, the size of the oligomers corresponding to each peak is indicated by an arabic number. The amount of methyl esters is indicated as a superscript.

To enable quantification of the non-esterified mono-, di-, tri-, and tetragalacturonic acid amounts detected, the PAD-response factors of these components had to be determined [19]. First, the peak areas of various amounts of di-, tri-, and tetramer (0.05–2.5

Table 1
Uronic acid content, degree of methyl esterification (DM), and the percentage of non-esterified GalA liberated after endopolygalacturonase degradation of pectin

Pectin [a]	Starting pectin	Uronic acid content (%)	DM (%)	GalA liberated [b] (%)
B71	C77	79.2	70.6	10.7
B67	C72	88.0	67.3	20.2
B63	C77	79.7	62.7	19.3
B62	C72	82.1	61.9	26.8
B57	C77	78.8	57.1	34.4
B56	C72	83.5	56.4	32.7
C77		79.3	77.3	2.9
C72		75.4	72.2	13.1
C68		86.6	68.3	14.8
C67		80.5	67.4	4.7
C56		79.0	56.1	7.5
C30		78.5	30.4	16.5
C23		80.2	22.9	34.6
CR52	C67	84.5	51.7	4.6
CR31	C67	81.9	30.7	16.4
M85	C67	81.4	85.0	2.1
M93	M85	79.2	93.1	0.0
PGA		86.0	0.0	88.2
R70	M93	79.2	70.2	1.0
R52	M93	79.2	52.1	3.7
R32	M93	79.2	31.5	16.6

[a] B, tomato pectin methylesterase de-esterified; C, commercially extracted; CR, alkaline de-esterified; M, additionally methyl-esterified; PGA, polygalacturonic acid; R, random methyl-esterified.

[b] Non-esterified GalA percentage calculated from the formula given in the text.

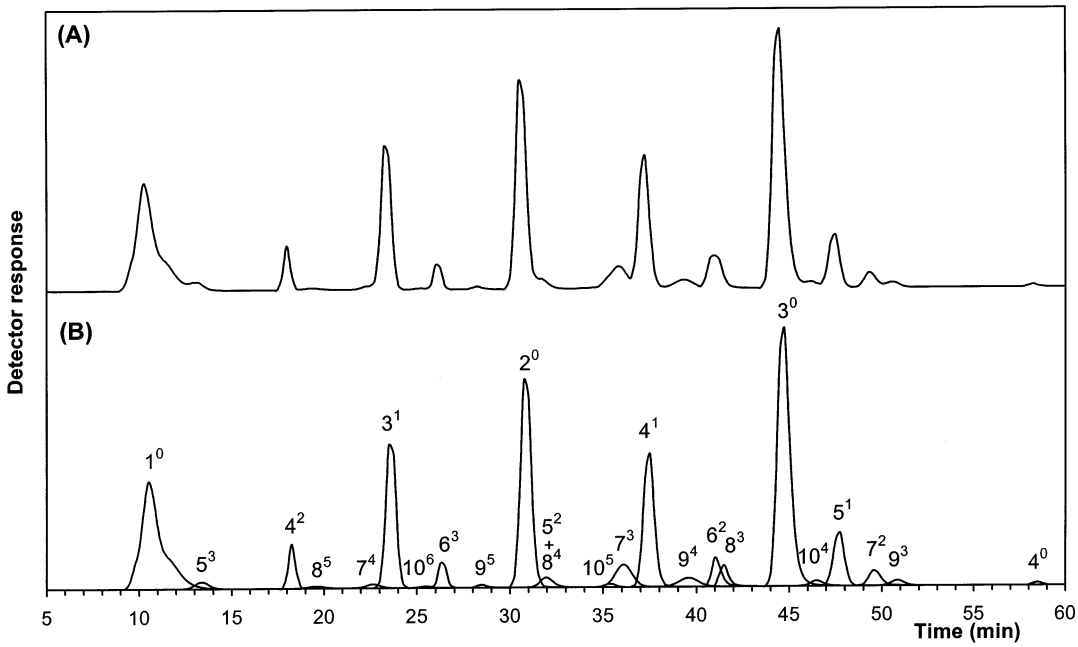

Fig. 1. Separation of the endo-PG digest of the C30 pectin (DM 30.4%) with HPAEC at pH 5. Elution profiles (A) before and (B) after integration with PEAKFIT software are shown. The location and the composition of the GalA oligomers were verified with MALDI-TOF-MS. The arabic number indicates the DP. The superscript number denotes the number of methyl ester groups.

μmol) were obtained and compared with those of identical amounts of monogalacturonic acid. The relative response factors were 2.0, 1.0, and 2.4 for di-, tri-, and tetragalacturonic acid, respectively, and were quite close to the figures found for the high pH HPAEC system (described in Section 4). A standard amount of monomeric GalA (0.206 μmol) was included in each HPAEC series to enable accurate calculation of the mono- to tetragalacturonic acid concentrations of each sample in that series. A peak area of about 7×10^8 was observed per μmol monogalacturonic acid injected. The response factors of the methyl-esterified GalA could not be determined because these oligomers were not available in a pure form.

With HPAEC at high pH the mono- to tetragalacturonic acid composition of the pectin endo-PG digests was also investigated. As a result of the high pH employed (12.7), all methyl esters were rapidly hydrolyzed [8], resulting in much simpler elution profiles [17]. HPAEC at pH 5 had revealed that mono- and digalacturonic acid solely occurred in a non-esterified form (Fig. 1(B)). Therefore, only the absolute amounts of these two components could be accurately determined with HPAEC at high pH. The high pH and pH 5 HPAEC results for mono- and digalacturonic acid were identical. The tri- and tetramer amounts as determined with the high pH system were usually much higher than the pH 5 HPAEC amounts because the methyl-esterified forms of these oligomers were rapidly de-esterified during high pH HPAEC analysis and, as a result, were included in the tri- and tetrameric peaks.

Determination of the total amount of non-esterified GalA observed in pectin endo-PG digests.—Prior to analysis, all pectins were extensively degraded with endo-PG of *K. fragiles*. The digests were subsequently analyzed with HPAEC at pH 5. To assure correct identification of each oligomer, the C30 endo-PG digest of known composition (Fig. 1(B)) was included in each series. Apart from monogalacturonic acid, the methyl ester content and GalA composition of the components as obtained by pH 5 HPAEC analysis were identical to those provided by MALDI-TOF-MS analysis. No GalA oligomers other than those found in the C30 endo-PG digest (Fig. 1) were observed in the other digests. Within a series of HPAEC analysis, retention times only varied slightly (< 0.5 min). The resolution,

however, somewhat decreased over time. Between different series, retention times could vary somewhat more (at most 4 min for the last eluting oligomers), probably as the result of small differences in the composition of the sodium acetate buffer.

Pectin digests of similar DM could give very dissimilar elution patterns (Fig. 2). The pectins B71–B57 and B67–B56, obtained by tomato pectin methylesterase (PME; EC 3.1.1.11) de-esterification of two commercial pectins (C77 and C72, respectively), gave remarkably simple and easy to interpret elution profiles. The chromatograms of the B71, B63, and B57 and the starting pectin C77 are shown in Fig. 3. In the de-esterified samples, large amounts of non-esterified mono-, di-, and trigalacturonic acids can be observed. The more prolonged the plant PME treatment, the more of these non-esterified GalA were liberated. Tetragalacturonic acid was hardly ever present in these pectin digests. The random methyl-esterified pectins R70 and R52 only released small amounts of non-esterified GalA (Fig. 4). This in contrast to the R30 pectin of that series which liberated much more oligomers after endo-PG digestion.

After response factor correction of the peak areas, the amount of non-esterified mono-, di-, tri-, and tetragalacturonic acid per 80 μL digest was obtained. The total number of non-esterified GalA residues liberated per gram of pectin was calculated from these mono-, di-, tri-, and tetramer amounts. The number of GalA residues liberated was expressed as the percentage of the total number of non-esterified GalA residues present per gram of pectin. The latter was done because it represents the maximum number of GalA that could (theoretically) be liberated by endo-PG. By expressing the number of non-esterified GalA residues liberated in this way, the effect of endo-PG action became somewhat more apparent for the high methyl-esterified pectins (see below). The whole calculation is summarized by the following equation:

$$\begin{aligned}&\text{GalA (\%)}\\&= \{[\text{area } 1^0 + (\text{area } 2^0 \cdot 2 \cdot \text{RF}2^0)\\&\quad + (\text{area } 3^0 \cdot 3 \cdot \text{RF}3^0)\\&\quad + (\text{area } 4^0 \cdot 4 \cdot \text{RF}4^0)] \cdot \mu\text{mol/area } 1^0\}\\&\quad \times [(\mu\text{mol GalA/gram pectin})\\&\quad \times (1 - (\text{DM}/100))]^{-1} \times 100\end{aligned}$$

where GalA is the non-esterified GalA, RF is the response factor, DM is the degree of methyl esterification, and x^0 is the non-esterified GalA fragment of one to four GalA residues.

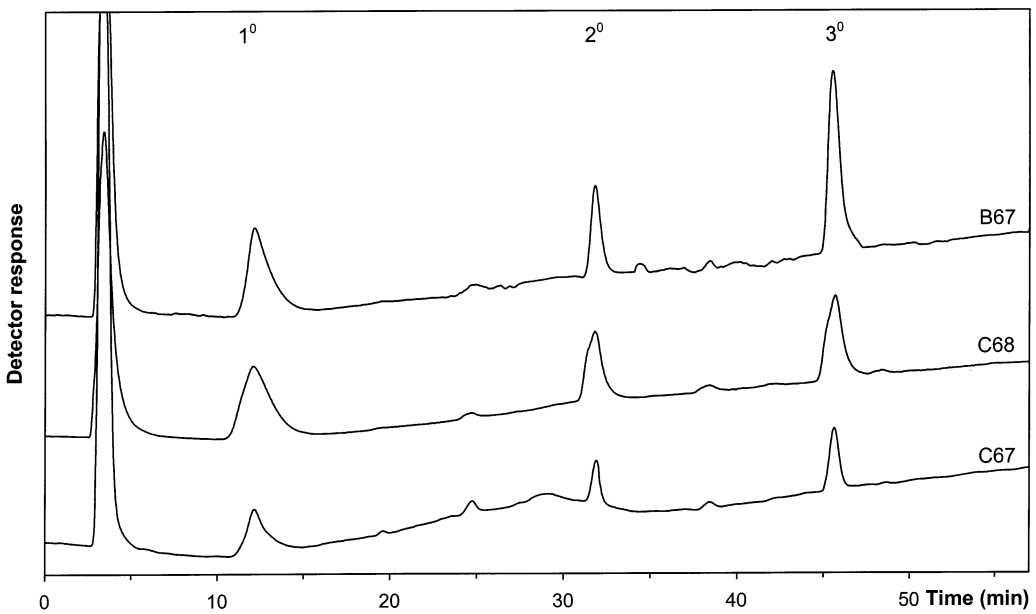

Fig. 2. HPAEC pH 5 elution patterns of three pectin endo-PG digests with an almost identical DM (~ 67%), as indicated by the number of the sample code.

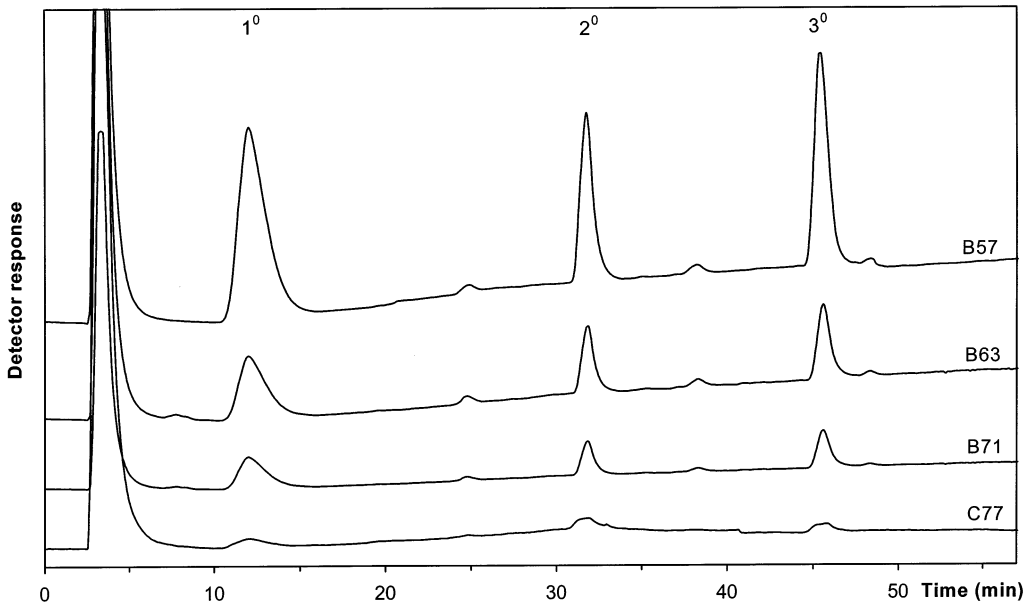

Fig. 3. HPAEC pH 5 chromatograms of C77, B71, B63, and B57 pectin endo-PG digests. The B-coded pectins were produced by tomato PME de-esterification of the C77 pectin. The DM is indicated by the number of the sample code.

The percentage of liberated GalA residues for all pectins is listed in Table 1. The results were obtained from endo-PG digestions performed in duplicate. A standard deviation of 1% was found for the percentage of GalA residues liberated. In Fig. 5, these percentages are plotted versus the DM of the pectins. The results obtained for specific pectin series, such as the tomato PME de-esterified pectins, are connected with lines to improve readability. The random methyl-esterified pectins obtained by alkaline de-esterification of the DM 93 pectin (R70–R32) liberated the lowest amounts of non-esterified GalA in comparison with pectins of similar DM (Fig. 5). The alkaline de-esterified pectins CR52 and CR31 (derived from the commercial DM 67 pectin) and the commercial C30 and C23 pectins released amounts nearly identical or close to the values expected for random methyl-esterified pectins of such DM values. The commercial extracted pectins C56 and C68 liberated much more non-esterified GalA upon endo-PG digestion (Fig. 2). For the tomato PME de-esterified pectins and their starting materials (C72, B67–B56 and C77, B71–B57 series) the percentage of GalA liberated almost linearly increased with decreasing DM (Fig. 5). Polygalacturonic acid digestion released the highest amount of non-esterified components (86%) but not all GalA was liberated. Because polygalacturonic acid is eventually obtained after total de-esterification of pectin, this polymer was included in the random de-esterified DM 93 pectin series (Fig. 5). Methyl esterification of the commercial DM 67 pectin resulted in the production of a DM 85 pectin (M85) and this treatment also reduced the percentage of GalA liberated. Endo-PG digestion of the DM 93 pectin did not release any GalA (Figs. 4 and 5). However, high-performance size-exclusion chromatography analysis revealed that the enzyme was able to decrease the molecular weight of the highly esterified polymer slightly (data not shown). The implications of the percentage of GalA liberated in relation to DM and the methyl ester distribution of pectin is extensively discussed below.

3. Discussion

From Fig. 1, it is evident that with HPAEC analysis at pH 5 and PEAKFIT assisted integration the non-methyl-esterified GalA present in pectin endo-PG digests can be completely differentiated from the methyl-esterified components. Furthermore, all peaks but one could be assigned to specific methyl-esterified oligomers (Fig. 1(B)). In comparison with the

preparative anion-exchange Source 15Q data published before [17], the CarboPac PA1 column of the pH 5 HPAEC system was better capable of separating the high methyl-esterified oligomers [17].

The information indicated by the total amount of non-esterified GalA liberated by endo-PG digestion of pectin is best illustrated by the tomato PME-treated pectin results. The more prolonged the tomato PME treatment, the larger the amount of non-esterified GalA liberated (Fig. 3). Since tomato PME de-esterified pectins in a sequential fashion [5,20], the enzyme concomitantly increased the size and/or amount of non-esterified GalA present in blocks: the preferred substrate for endo-PG [15,16]. Extensive degradation of these homogalacturonan sequences will eventually result in a mixture of non-esterified mono-, di-, tri-, and some tetragalacturonic acid [15]. The latter oligomer will only be occasionally observed because the tetramer can still be degraded by the enzyme though it is close to the limit of endo-PG action [15]. The amount of non-esterified GalA liberated by the enzyme thus indicates the presence of sequences of non-methyl-esterified GalA (so-called blocks) in pectin which are large enough to be degraded by the enzyme. Endo-PG degradation of the random methyl-esterified R70 to R32 pectins only liberated small amounts of non-esterified GalA (Figs. 4 and 5). The latter indicated the occurrence of only small, poorly endo-PG degradable GalA blocks, which was confirmed by NMR analysis [6]. The commercial extracted pectins of DM 77–56% contained considerably more blocks than random methyl-esterified pectins of comparable DM. Evidently, a whole range of different methyl ester distributions (blockwise, random, and in between) can be discerned in this way. Extensive endo-PG degradation of polygalacturonic acid only liberated 88% of the total amount of GalA. Since this polymer did not contain any methyl or acetyl groups, which could hinder the enzyme, the amount not removed could only result from GalA associated with or present in the rhamnogalacturonan part of this polymer [2]. Esterification of the commercial DM 67 pectin resulted in the production of a DM 85 pectin with a non-random methyl ester distribution (Fig. 5). Subsequent esterification of the DM 85 pectin produced a DM 93 pectin which did not liberate GalA upon incubation with endo-PG. However, size-exclusion chromatography analysis revealed that at least a few small blocks were present that the enzyme could still act upon, obviously without releasing GalA.

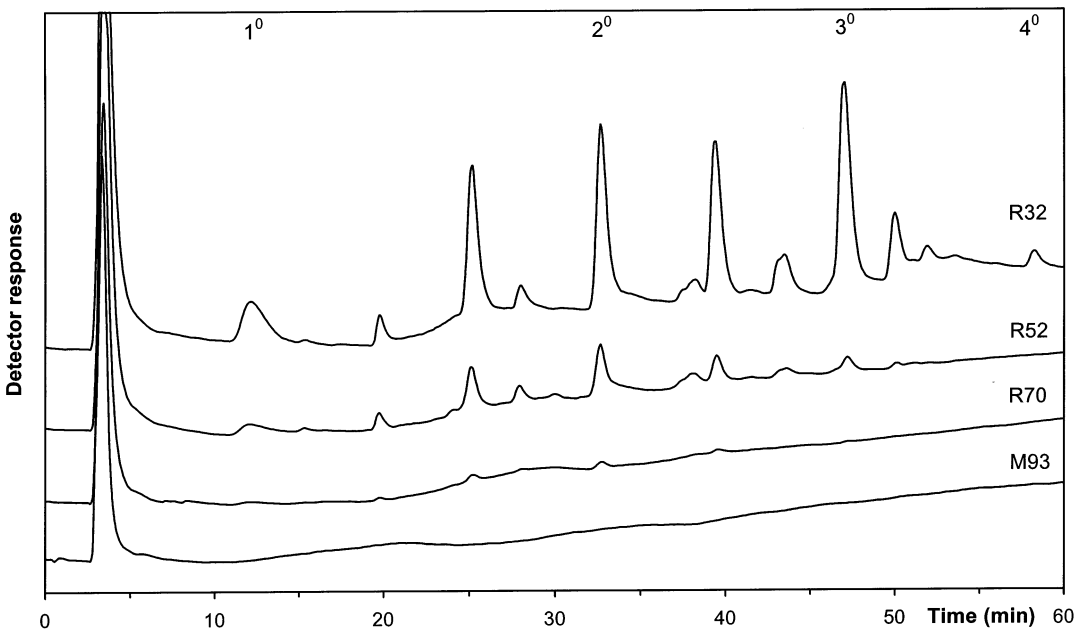

Fig. 4. HPAEC pH 5 elution profiles of M93, R70, R52, and R32 pectin endo-PG digests. The R-coded pectins were produced by alkaline de-esterification of the M93 pectin. The DM is indicated by the number of the sample code.

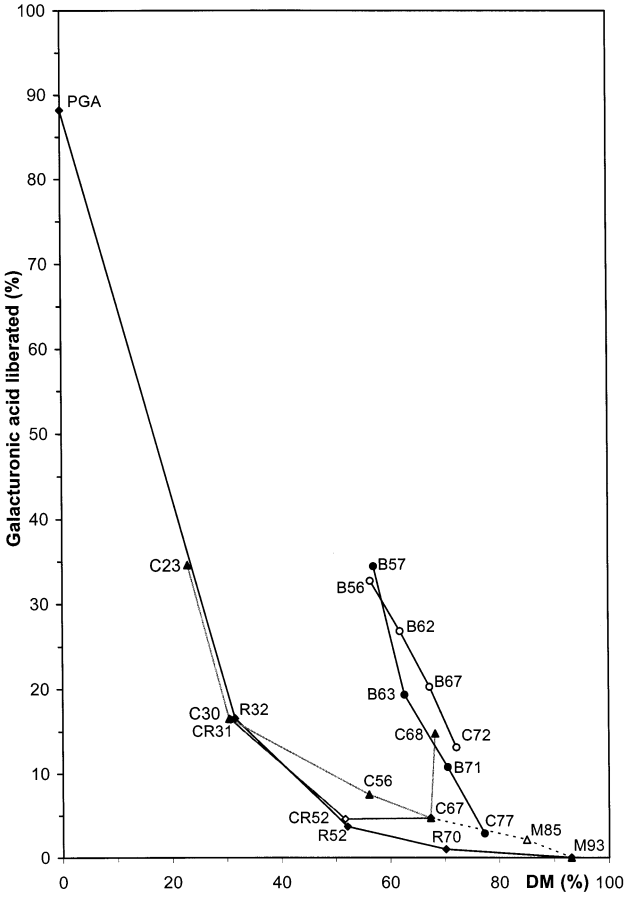

Fig. 5. Percentage of non-esterified GalA residues liberated by endo-PG vs. the DM of pectin. The following pectins are shown in series: random methyl-esterified (M93, R70–R32, PGA; ◆), alkaline de-esterified (C67, CR52, CR32; ◇), commercial (C68–C23; ▲), additionally methyl-esterified (C67, M85, M93; △), and tomato PME de-esterified C77 (C77, B71, B63, B57; ●) and C72 (C72, B67, B62, B56; ○).

For the study of the methyl ester distribution of pectin the novel method described in this report is a very important addition. Because the non-esterified GalA can be distinguished from the methyl-esterified ones, finally accurate data are obtained for the endo-PG degradation products of pectin [8,21]. With a range of random methyl-esterified pectins as a reference, the new method readily reveals to what extent the overall methyl ester distribution of a particular pectin differs from a random one. As such, it will also be very useful in the study of pectin functional properties. As a result of the use of an enzyme a limit is put on the size of the blocks detected. Because the non-esterified GalAs liberated are quantified, no information on the occurrence of GalAs in blocks smaller and equal to 'the smallest degradable block' is obtained. This in contrast to NMR which detects all GalAs in blocks [5,6]. The method is unable to discriminate between the methyl ester distribution of two pectins (of similar DM) of which one contains a few very large and the other more but smaller (degradable) non-esterified blocks if both release the same amount of non-esterified GalA upon enzyme degradation. Intermolecular differences in the methyl ester distribution of pectin are also not distinguished with this technique. Perhaps anion-exchange chromatography of pectin [22] and subsequent endo-PG degradation and pH 5 analysis of the fractions obtained could assist with this problem. For the percentage of non-esterified GalA residues liberated after endo-PG digestion of pectin we suggest the term 'degree of blockiness'.

Extensive endo-PG digestion of pectin eventually results in the total degradation of all non-methyl-esterified GalA sequences equal to and larger than the active site of the enzyme. Preliminary results of computer simulation studies—in which the number of subsites of the enzyme employed was varied—revealed that random degradation of all non-esterified GalA blocks ≥ 4 could accurately explain the amount of non-esterified GalAs liberated for the random methyl-esterified R70 to R32 pectins. These results confirm the subsite composition of four suggested for endo-PG of *K. fragiles* [15]. Assuming the enzyme strictly requires four successive non-esterified GalAs, the smallest methyl-esterified oligomer produced would be a pentamer with one methyl ester on an internal GalA residue. However, mono-esterified trigalacturonic acid is the smallest esterified oligomer observed (Fig. 1(B)). This implies that the current model of the active site is not completely correct and suggests that at least one of the subsites of the enzyme employed in our study is able to interact with a methyl-esterified GalA. Clearly, a more detailed study of the active site of endo-PG of *K. fragiles* is needed to explain accurately the composition of the methyl-esterified oligomers observed. For this, however, the PAD-response factors of each methyl-esterified oligomer as well as the location of the methyl ester(s) needs to be determined. When

these conditions are met, very detailed information on the methyl ester distribution of pectin can be obtained with endo-PG of *K. fragiles* or any other endo-PG with known subsite composition and mode of action.

4. Experimental

Materials.—Dowex 50WX8 (50–100 mesh), HOAc (ACS grade), and GalA were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). Isocarbostyril and MeCN were provided by Aldrich (Milwaukee, WI) and NaOH (50% solution) was purchased from J.T. Baker (Deventer, The Netherlands). 2,5-Dihydroxybenzoic acid, tomato PME, and di- and tri-GalA were obtained from Sigma (St. Louis, MO). Tetra-GalA was prepared by partially degrading polygalacturonic acid with endo-PG (EC 3.2.1.15) of *K. fragiles* and purified by anion-exchange chromatography as described before [23]. Endo-PG was purified from a preparation of *K. fragiles* CBS 397, as described by Versteeg [24], with a specific activity of 20 μmol GalA produced/min/mL (20 units/mL). The purified enzyme was devoid of PME, pectin lyase, and pectate lyase activity.

Polygalacturonic acid was purchased from ICN (Aurora, OH). Lemon pectins, with DM values ranging from 22.9 to 77.3% (C23–C77), were kindly provided by Copenhagen Pectin A/S (Lille Skensved, Denmark), a subsidiary of Hercules Inc. (Wilmington, DE). An 85% methyl-esterified sample (M85) was prepared by MeOH–H_2SO_4 treatment of a commercial DM 67.4 pectin (C67) according to the procedure of Heri et al. [25]. Subsequent re-esterification of the M85 sample resulted in the preparation of a DM 93.1 pectin (M93). Random de-esterification of this M93 pectin was achieved by saponification with diluted NaOH as described by Chen and Mort [21]. This procedure resulted in the production of a set of random methyl-esterified pectins (R70, R52, R32) with DM values of 70.2, 52.1, and 31.5%, respectively. The random methyl-esterification pattern was confirmed by NMR analysis [6]. The commercial C67 pectin was also de-esterified with NaOH to DM values of 51.7 (CR52) and 30.7%, (CR31). Tomato PME was used to de-esterify two commercial DM 72.2 (C72) and 77.3 (C77) pectins as described in Andersen et al. [20]. The C77 pectin was de-esterified to DM values of 70.6 (B71), 62.7 (B63), and 57.1% (B57). The DM 72 pectin was de-esterified to DM values of 67.3 (B67), 61.9 (B62), and 56.4% (B56). The uronic acid content and the DM of the pectin samples are summarized in Table 1. The acetyl ester content was $\leq 1.5\%$ for all pectins.

Enzymatic degradation of pectin.—Endo-PG of *K. fragiles* was used to degrade pectin. Pectin (20 mg) was dissolved in 4 mL of 0.05 M NaOAc (pH 5) and 0.18 units endo-PG was added. The solute was incubated for 24 h at 30°C. After incubation, samples were frozen immediately and stored until further use. Shortly before analysis, samples were thawed and centrifuged for 10 min at 16,000*g*. A mixture of partially methyl-esterified GalA of known composition was produced by incubating the commercial DM 30.4 pectin (C30) with endo-PG [17].

Chromatographic analysis and fractionation of GalA oligomers.—The pectin digests obtained after endo-PG treatment were analyzed with HPAEC at pH 5 as described before [17]. Samples of 80 μL were injected. Postcolumn NaOH addition was used to allow for PAD detection. Chromatograms were recorded with PC1000 software (Thermo Separation Products). The chromatograms were base line corrected, integrated and fitted by PEAKFIT software from SPSS Inc. (Chicago, IL). Peaks were identified by comparing their retention times with those of mono-, di-, tri-, and tetra-GalA and the partially methyl-esterified oligomers observed in the C30 endo-PG digest. To determine the exact elution position of the non- and partially methyl-esterified GalA oligomers during HPAEC analysis at pH 5, C30 endo-PG digest (80 μl) was applied to the CarboPac PA1 column of the HPAEC system and fractions were collected. Apart from the absence of postcolumn NaOH addition and the PAD detector, the apparatus and the gradient applied were identical to those described before [17]. To the column outlet, a SuperFrac fraction collector (Pharmacia, Uppsala, Sweden) was connected and 170

fractions of 0.25 mL were collected during the entire run of 85 min. All fractions were analyzed by MALDI-TOF-MS.

MALDI-TOF-MS.—This was performed with a Perseptive Biosystems Inc. Voyager-DE RP with a nitrogen laser of 337 nm wavelength and 3 ns pulse width. The mass spectrometer was operating in the positive-ion mode with a delayed extraction time of 200 ns. Ions were accelerated to an energy of 12 kV before entering the TOF mass spectrometer. The minimum laser power to obtain a good spectrum was used and 20–50 spectra were accumulated for each run. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg of isocarbostyril in 1 mL of a 7:3 mixture of water–MeCN. For analysis, 10 μL enzyme digest was added to 90 μL of matrix solution and ca. 0.05 g Dowex 50WX8 (H^+) was added. Samples were thoroughly mixed and centrifuged for 30 s to pellet the Dowex material. From the clear supernatants, aliquots of 1 μL were applied to the MALDI plate and dried in a gentle stream of air at room temperature. Prior to analysis of the HPAEC fractions, each fraction was transferred to an Eppendorf tube and ca. 0.1 g Dowex 50WX8 (H^+) was added. After thorough mixing and centrifugation for 20 s, 1 μL of supernatant was applied to the MALDI plate. On the plate, 1 μL of matrix was added and carefully mixed. The mixtures were dried in a gentle stream of air at room temperature. The mass spectrometer was calibrated externally with the GalA oligomers observed in the C30 and a polygalacturonic acid endo-PG digest [17].

General methods.—The uronic acid content of pectin was determined after H_2SO_4 hydrolysis [26] by an automated colorimeteric *m*-hydroxydiphenyl method [27]. The methoxyl and acetyl contents were determined by HPLC analysis of the MeOH and HOAc released on alkaline de-esterification [28]. The DP of GalA oligomers was determined with HPAEC at high pH on a CarboPac PA-100 column [17]. Because of the separation at high pH (12.7), all methyl esters were rapidly hydrolyzed and no longer affected the separation [8]. Detection and integration was with PC1000 software (Thermo Separation Products). Retention times were compared with those of the mono-, di-, tri-, and tetramer of GalA, and a partial degraded endo-PG digest of polygalacturonic acid. In proportion to mono-GalA, the response factors of the di-, tri-, and tetramer were 1.8, 1.0, and 2.4, respectively, for a specified HPAEC/PAD configuration and should be determined for each individual system. During each series, the PAD-response area of a standard amount of mono-GalA (0.051 μmol) was determined to enable accurate calculation of the mono-, di-, tri-, and tetra-GalA acid concentrations of each sample in that series. Usually, a peak area of about 2.3×10^8 was observed per μmol mono-GalA injected (detector range: 3 μC). This absolute response, however, varied over time, probably as the result of variations in the individual condition of the gold working electrode and/or the reference electrode of the PAD cell and small variations in the buffer composition [19].

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