ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 326 (2000) 120–129

CARBOHYDRATE RESEARCH

Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase☆

Piet J.H. Daas, Alphons G.J. Voragen, Henk A. Schols *

Department of Food Technology and Nutritional Sciences, Laboratory of Food Chemistry, Wageningen Agricultural University, Bomenweg 2, NL-6703 HD Wageningen, The Netherlands

Received 15 July 1999; accepted 16 January 2000

Abstract

A method was developed that enabled the study of non-esterified galacturonic acid sequences (so-called blocks) in pectin. Endopolygalacturonase of *Kluyveromyces fragilis* was used to extensively degrade pectin, and the composition of the galacturonic acid molecules produced was determined with high-performance anion-exchange chromatography at pH 5. With this technique, the amount of non-esterified mono-, di-, and trigalacturonic acid released was determined. In addition, the relative amounts of methyl-esterified oligomers — up to 10 galacturonic acid residues — could be observed. By comparing the percentages of non-esterified mono-, di-, and trigalacturonic acids released, pectins with large enzyme-degradable blocks could be distinguished from pectins with small enzyme-degradable blocks. High percentages of mono- and digalacturonic acid were found for pectins containing small non-esterified blocks. The total area of all peaks corresponding to methyl-esterified oligomers was found to be indicative for the distribution of these blocks. The higher the ratio of the methyl- to non-esterified peak areas, the more closely associated blocks are present. Randomly esterified pectins, with degrees of methyl esterification of 50 and higher, contained smaller, more clustered blocks than commercial extracted pectins of comparable degrees of esterification. The approach developed enables a very detailed study of the methyl-ester distribution of pectin to be carried out and is a very important addition in the study of the functional behavior of this complex polymer.

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

1. Introduction

Pectins are of interest both because of their structural role in plant tissue [2] and because of their excellent gelling and thickening properties [3,4]. The dominant feature of pectin is a linear chain of α-(1→4)-linked D-galacturonic acid units in which varying proportions of the acid groups are methyl esterified. This homogalacturonan backbone is occasionally interrupted by rhamnose-rich regions, which can be highly substituted with neutral sugar-rich side chains [5]. Uninterrupted homogalacturonan regions with a degree of polymerization (DP) of about 70–100 units have been isolated from various plant tissues [6,7]. Varying portions of the galacturonic acid regions are present as methyl esterified. Because the pat-

Abbreviations: DM, degree of methyl esterification; DP, degree of polymerization; endo-PG, endopolygalacturonase; HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detection.

☆ Part 2. Investigation of the galacturonic acid distribution of pectin with enzymes. For Part 1, see Ref. [1].

* Corresponding author. Tel.: +31-317-482239; fax: +31-317-484893.

E-mail address: henk.schols@chem.fdsci.wau.nl (H.A. Schols)

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00037-9

tern of methyl esterification is very important for the rheological and gel-forming properties of pectin, it has been the subject of many studies [3,4]. Previous investigations of the methyl-ester distribution of pectin have included NMR spectroscopy [8,9], calcium binding [10], enzymatic [1,7,11–14], and chemical approaches [7,15].

With purified pectolytic enzymes, detailed information on the methyl-ester distribution or the distribution of the enzyme-degradable sites over the pectic backbone can be obtained. 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 galacturonic acid content of the degradation products is required [1,7]. Over the years, pectin-degrading enzymes have been studied extensively [16]. In particular, the active site of the homogalacturonan-degrading enzyme endopolygalacturonase (endo-PG) has been characterized in detail [17,18]. For determination of the methyl and galacturonic acid content of the endo-PG degradation products of pectin, a high-performance anion-exchange chromatographic (HPAEC) method has been developed [19]. Here, the non- and partially methyl-esterified degradation products are separated at pH 5 with a sodium acetate gradient, followed by postcolumn sodium hydroxide addition to allow pulsed amperometric detection. With this technique, the elution profiles of endo-PG degradation products of pectins varying in degree of methyl esterification (DM) and methyl-ester distribution were studied [1]. The endo-PG of *Kluyveromyces fragilis* was used to extensively degrade pectin and the total amount of non-esterified galacturonic acid liberated was determined. It was demonstrated that this could be used to differentiate pectins with a similar DM and a distinct methyl-ester distribution [1]. As endo-PG requires a sequence of non-esterified galacturonic acid residues (a so-called block) to act, pectins that contain large amounts of these 'endo-PG degradable' blocks can be distinguished from pectins with only a small number of such blocks, on the basis of the release of different amounts of non-esterified galacturonic acid after enzyme action. The amount of residues released was expressed as the percentage of the total amount of non-esterified galacturonic acid residues present in pectin and called 'the degree of blockiness' [1]. In this report it will be demonstrated that with the techniques developed, pectins with large 'endo-PG degradable' blocks can also be differentiated from pectins with small degradable blocks by comparing the individual amounts of non-esterified galacturonic acid molecules released. In addition, information regarding the distribution of these blocks can be obtained from the elution profiles. Commercially extracted pectins of varying degrees of DM as well as chemically and enzymatically modified pectins with more defined methyl-ester distribution were used as substrates.

2. Results

Determination of the non-esterified mono-, di-, and trigalacturonic acid proportions in pectin endo-PG digests.—Prior to analysis, all pectins were extensively degraded with endo-PG of *Kl. fragilis*. The digests were analyzed with HPAEC at pH 5. To assure correct identification of each oligomer, the endo-PG digest of a commercially extracted DM 30 pectin (C30) of known composition was included in each series. The amount of non-esterified galacturonic acids detected was calculated as described previously [1]. Even if both the DM and the total amount of non-esterified galacturonic acid residues released were nearly identical, pectin digests could give dissimilar elution patterns. As an example, the elution profiles of the endo-PG digests of B56 and B57 (two blockwise de-esterified pectins of DM 56.4 and 57.1%, respectively) are shown in Fig. 1. In these digests large amounts of non-esterified mono-, di-, and trigalacturonic acid were detected. After response-factor correction, the total amounts of non-esterified galacturonic acid residues released were similar: 32.7 and 34.4% of the total amount of non-esterified galacturonic acid present in B56 and B57, respectively (Table 1). However, from Fig. 1 it is clear that the proportions in which the non-esterified molecules were produced differed greatly.

Varying proportions of mono-, di-, and tri-galacturonic acid were also seen in many of the other pectin digests. The individual amounts of the non-esterified galacturonic acids were determined for each pectin digest and expressed as the percentage of the total number of non-esterified galacturonic acid molecules released (Table 1). Non-esterified tetragalacturonic acid was observed, only in small amounts, in low DM pectin digests (<

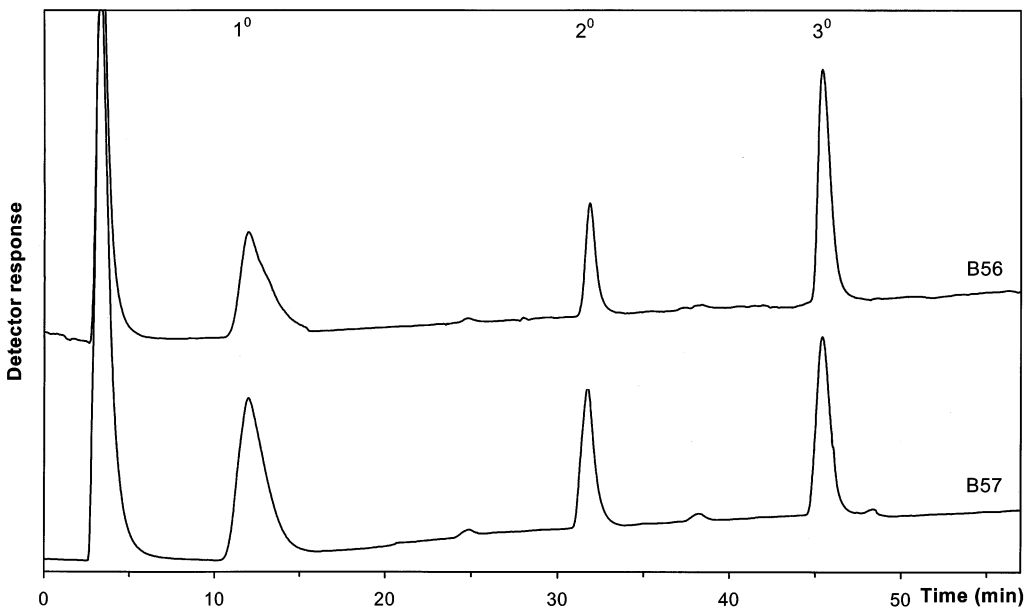

Fig. 1. HPAEC pH 5 elution patterns of B56 and B57 pectin endo-PG digests. The DM is indicated by the number of the sample code. Peaks corresponding to non-esterified mono-, di-, and tri-GalA are labeled.

Table 1
Degree of methyl esterification (DM) and the percentage of non-esterified galacturonic acid (GalA), the proportions of non-esterified mono-, di-, and tri-GalA liberated, and the ratio of methyl- to non-esterified peak areas as obtained after endopolygalacturonase degradation of pectin

Pectin [a]	DM (%)	GalA liberated [b] (%)	Mono-GalA (%)	Di-GalA (%)	Tri-GalA (%)	Methyl- to non-esterified peak area ratio
B71	70.6	10.7	32	47	21	0.11
B57	57.1	34.4	41	35	24	0.02
B56	56.4	32.7	33	30	37	0.01
C74	73.5	1.74	23	60	17	0
C69	68.5	13.2	39	32	29	0.22
C67	67.4	4.7	47	32	21	0.11
C56	56.1	7.5	28	47	25	0.60
C30	30.4	16.5	21	44	35	0.77
CR52	51.7	4.6	18	57	25	1.24
CR31	30.7	16.4	17	48	35	1.02
M85	85.0	2.1	28	47	25	0.22
M93	93.1	0				0 [c]
PGA	0.0	88.2	21	42	37	0
R70	70.2	1.0	27	67	6	0.94
R52	52.1	3.7	18	70	12	2.00
R32	31.5	16.6	19	47	34	1.10

[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 galacturonic acid percentage calculated as described in [1].

[c] Because no methyl-GalA oligomers were liberated, a value of zero was obtained.

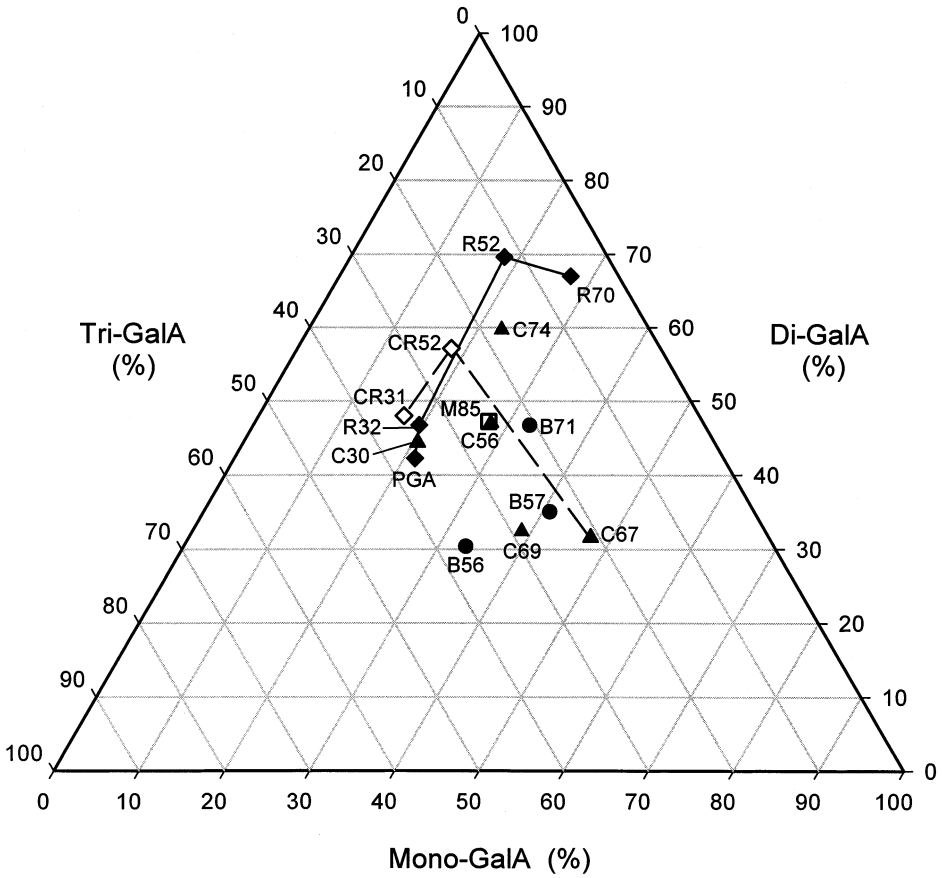

Fig. 2. Ternary graph of the percentages of non-esterified mono-, di-, and tri-GalA observed in pectin endo-PG digests. PGA, polygalacturonic acid. Commercial (C69–C30; ▲), pectin methylesterase de-esterified (B71–B56; ●) and highly esterified M85 (□) pectins are shown. The randomly esterified (R70–R32, PGA; ◆) and alkaline de-esterified (C67, CR52–CR32; ◇) pectin series are connected with lines.

35%); samples that always contained large amounts of non-esterified mono-, di-, and trigalacturonic acid. Since endo-PG of *Kl. fragilis* would exclusively degrade non-esterified tetramer to mono- and trigalacturonic acid [17], these additional amounts should be included in the mono- and trimer amounts. However, because of the small amounts of tetramer present, the inclusion of its degradation products did not significantly affect the mono-, di-, and trimer proportions and was therefore ignored. The percentage of non-esterified mono-, di-, and trigalacturonic acid produced was obtained from pectin digestions performed in triplicate (standard deviation of 3% were found). The mono-, di-, and trigalacturonic acid proportions of the various pectin digests are best compared when plotted in a ternary graph (Fig. 2). In this Figure, the data for the random esterified (R70–R32 and polygalacturonic acid) and the randomly de-esterified C67 series (C67, CR52 and CR31) are connected with lines to improve readability and to illustrate the differences more clearly.

For randomly esterified pectins, the percentage of non-esterified monogalacturonic acid was highest for the R70 digest (DM 70.2%). Other samples of this series all contained nearly identical proportions of monomer. The digalacturonic acid percentage of the R70 pectin was somewhat lower than that of pectin R52 (DM 52.1%) but this percentage quickly decreased for pectins of lower DM. The proportion of trimer increased for pectins of decreasing DM. Compared with the randomly esterified pectin R52, the alkaline de-esterified pectin CR52 (derived from the non-randomly esterified DM 67 pectin) [1] released digalacturonic acid in much lower and trigalacturonic acid in much higher proportions. The percentage of monomer was identical. The galacturonic acid proportions of pectin CR31 (DM

30.7%) were very similar to those of the randomly esterified R32 and the commercial C30 samples of comparable DM (31.5 and 30.4%, respectively). The proportions of mono-, di-, and trigalacturonic acid in the other commercial (C74–C56) and blockwise de-esterified pectins (B71–B56) differed greatly (Fig. 2). The highly esterified M85 pectin (DM 85%) released the non-esterified galacturonic acids in a proportion identical to those of the commercial pectin C56 of much lower DM (56.1%).

Determination of the methyl- to non-esterified peak area ratio.—The endo-PG action on pectins of similar DM may liberate very similar amounts of non-esterified mono-, di-, and trigalacturonic acid but very different amounts of methyl-esterified oligomers. As an example, the elution profiles of the R32 and C30 pectin digests are shown in Fig. 3. Peaks were identified as described previously [1]. In Fig. 3 it can be seen that the non-esterified components are present in nearly identical amounts and proportions (Table 1 and Fig. 2). The areas of the peaks corresponding to methyl-esterified oligomers, however, differed significantly for the R32 and C30 digests (Fig. 3). Because the PAD-response factor(s) of methyl-esterified components is not known [1], an exact quantification of these oligomers was not possible. Therefore, peak areas were used instead, although the total amount of oligomers produced may be underestimated [20]. As a means to express the (relative) amount of all esterified components liberated, the combined areas of the peaks corresponding to methyl-esterified oligomers were divided by the total area of the non-esterified galacturonic acid peaks (Table 1). The ratios were obtained from digests performed in triplicate. Standard deviations of 1% were found. In Fig. 4, the ratios are plotted versus the DM of the pectins.

For all digests, the methyl- to non-esterified peak area ratio was highest for the randomly esterified pectin series (Fig. 4). The ratio was highest for the R52 digest (DM 52.1%), whereas the ratios for the R32 and R70 digests (DM 31.5 and 70.2%, respectively) were much lower. The methyl- to non-esterified ratio for the digests around DM 55% showed the greatest diversity: values between 0 and 2 were found (Fig. 4 and Table 1). Random de-esterification of the commercial C67 pectin (DM 67.4%) resulted in a large increase in the methyl- to non-esterified ratio for sample CR52 (DM 51.7%) and in a decrease for the CR31 pectin of lower DM (30.7%). The ratio

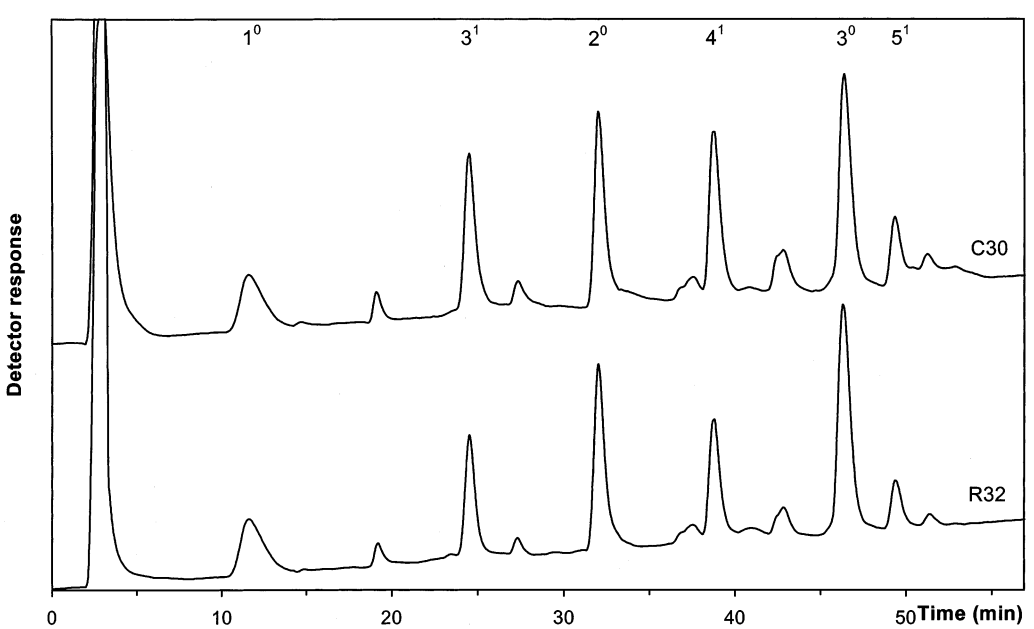

Fig. 3. HPAEC pH 5 chromatograms of C30 and R32 pectin endo-PG digests. Only the peaks corresponding to non- and mono-methyl-esterified GalA are labeled. The arabic numbers indicate the DP. The superscript number denotes the amount of methyl esters. All other peaks resulted from methyl-esterified GalA oligomers of DP 4–10 with two or more methyl esters and were identified as described in [1].

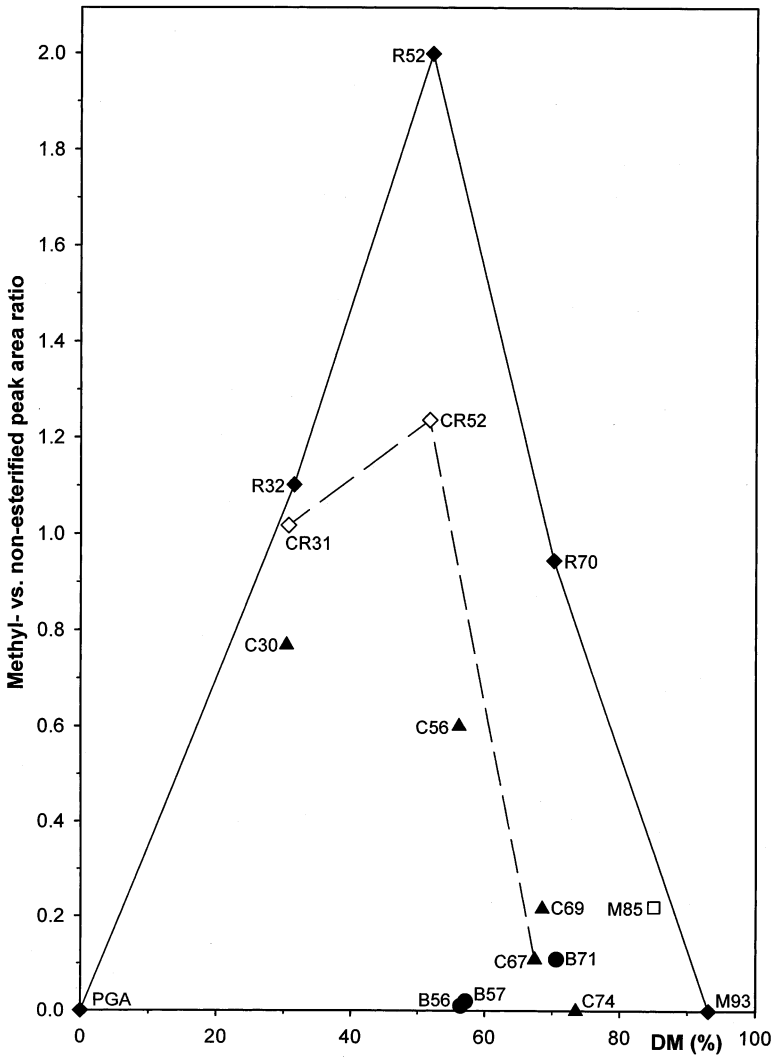

Fig. 4. Ratio of the methyl- to non-esterified GalA peak areas of pectin endo-PG digests plotted vs. the DM. Commercial (C69–C30; ▲), pectin methylesterase de-esterified (B71–B56; ●) and highly esterified M85 (□) pectins are shown. The randomly esterified (R70–R32, PGA; ◆) and alkaline de-esterified (C67, CR52–CR32; ◇) pectin series are connected with lines.

for the latter digest was nearly identical to the ratio of the randomly esterified R32 sample (DM 31.5%). The methyl- to non-esterified ratio in commercial and blockwise de-esterified pectin digests was always lower than that of the randomly esterified and randomly de-esterified pectin digests of comparable DM. The ratio of the M85 sample (DM 85%) was close to the value expected for a randomly esterified sample of similar DM. Because of the absence of methyl-esterified oligomers, the methyl- to non-esterified peak area ratio of polygalacturonic acid, M93, and C74 was zero (Table 1).

3. Discussion

The information revealed by the proportions of non-esterified mono-, di-, and trigalacturonic acid liberated after endo-PG digestion of pectin is best illustrated by the results of the randomly esterified pectin series. The higher the methyl-ester content, the larger the relative proportions of non-esterified di- and monogalacturonic acid observed (Table 1 and Fig. 2). In a previous paper, it was shown that the *Kl. fragilis* enzyme employed is 'most likely' able to cleave the chain in non-esterified galacturonic acid sequences of four

and more [1]. From the calculations described by Chen and Mort [21] it can be deduced that, for randomly esterified pectins, the occurrence of non-esterified blocks decreases with increasing DM. The average size of these blocks will also decrease for pectins of increasing DM. As a result, endo-PG will predominantly have to degrade blocks close to the limit of its action in randomly esterified, high DM pectins. For the latter pectins, this will result in the predominant release of small (di- and mono-) galacturonic acid molecules. During the degradation of polygalacturonic acid, endo-PG is obviously not hindered by any methyl ester substituents and produces mono-, di-, and trigalacturonic acid in proportions strictly determined by the mode of action of the enzyme. In Fig. 2, it can be seen that these proportions greatly differ from those observed in most of the other pectin digests. Only during the degradation of DM 30 pectins (C30, CR31, and R32) endo-PG is apparently not hindered by any methyl ester substituents. From the above, it is obvious that the proportion in which non-esterified mono-, di-, and trigalacturonic acids are released depends on the (relative) amounts of the non-esterified blocks composed of four and more galacturonic acids. The latter is illustrated in Fig. 5, in which the degradation of three DM 50 pectins with different methyl-ester distributions and different average sized blocks is shown. Though all three pectins will release exactly the same amount of non-esterified galacturonic acid residues after endo-PG action, the proportions of mono-, di-, and trigalacturonic acid released clearly differ. The smaller the average size of the 'endo-PG degradable' blocks, the smaller the galacturonic acids produced. As a consequence, pectins that predominantly release mono- and digalacturonic acid (e.g., R70, R52, and C74) will mainly contain small non-esterified blocks. Pectins with large average sized blocks will release mono-, di-, and trigalacturonic acid in proportions comparable to those of a polygalacturonic acid digest (e.g., CR31, R32, and C30). Pectins containing (a whole range of) different sized blocks will produce the non-esterified components in proportions depending on the predominance of one or more of these block sizes. Digest C56, for instance, contains more mono- and di- and less trigalacturonic acid than the polygalacturonic acid digest (Fig. 2). This indicates that apart from some large blocks, some quite small and intermediate sized blocks are present in pectin C56. The C67 digest, in contrast, contains much more mono- and somewhat less di- and trigalacturonic acid than the polygalacturonic acid digest. The latter is the result of a lot of small blocks next to some intermediate and large non-esterified blocks. It is, however, important to consider the absolute amount of non-esterified galacturonic acids produced as well [1]. For instance, the M85 and C56 pectin

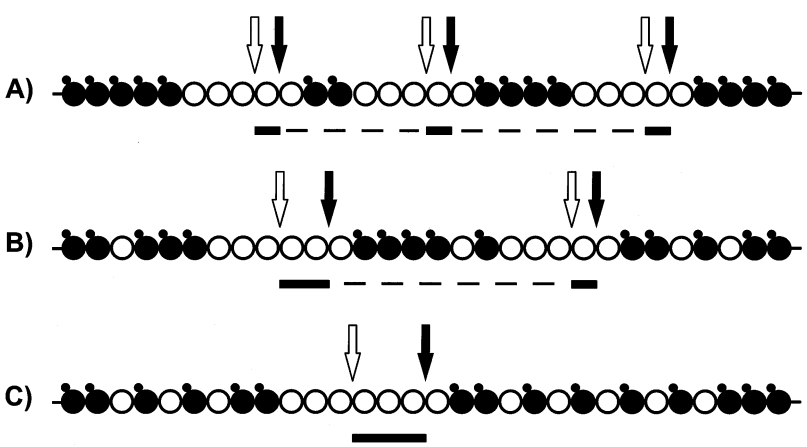

Fig. 5. Schematic representation of endo-PG action on three DM 50 pectins with different methyl-ester distributions. It is assumed that endo-PG needs four adjacent non-esterified GalA residues to act [1]. Methyl- and non-esterified GalA are represented by ● and ○, respectively. The sites of endo-PG attack are indicated by black and white arrows, showing the first and second linkage cleaved, respectively. The non- and methyl-esterified GalA molecules released are drawn with solid and dashed underscores, respectively.

digests both release mono-, di-, and trigalacturonic acid in an identical proportion (Fig. 2), revealing the same average 'endo-PG degradable' block size. The absolute amount of residues produced, however, is much higher for C56 (Table 1). This demonstrates that the latter pectin contains much more degradable blocks of similar size.

The information indicated by the methyl- to non-esterified peak area ratio obtained after endo-PG digestion of pectin is also best explained by the results of the randomly esterified pectin series. As expected, the methyl- to non-esterified area ratio will be high for pectins releasing large amounts of methyl-esterified oligomers and small numbers of non-esterified galacturonic acids. For randomly esterified pectins the occurrence of 'endo-PG degradable' blocks will increase with decreasing DM whereas, simultaneously, the chance of liberating a methyl esterified oligomer after endo-PG action gradually decreases. As a result, starting from a highly, randomly esterified pectin, the ratio of methyl- to non-esterified galacturonic acid production will increase up to a specific DM and then gradually decrease. From Fig. 4, it can be concluded that this transition occurs at a DM of around 50%. With the HPAEC method employed, esterified oligomers up to DP 10 can be detected [1]. As a result of this and the assumed active site of the enzyme, pectins with 'endo-PG degradable' blocks located at distances of more than six galacturonic acid residues from one another will not release any detectable methyl-esterified oligomers. Hence, a high methyl- to non-esterified peak area ratio is indicative of the occurrence of blocks located at distances closer than six galacturonic acid residues from one another. These observations are illustrated in Fig. 5. Of the three DM 50 pectins shown, each contains 'endo-PG degradable' blocks, but releases different amounts of methyl-esterified oligomers after enzyme action. Pectin A releases two methyl-esterified oligomers (a hexamer with two methyl esters and an octamer with four methyl esters), whereas Pectin B produces only a decamer with five methyl esters. Pectin C does not release any esterified oligomers after endo-PG action. The data in Fig. 4 reveal that pectins C74, B56, and B57 contain almost no clustered blocks. Pectins C67, C69, and B71 clearly contain some clustered 'endo-PG degradable' blocks, but much less than the randomly esterified R70 pectin of comparable DM. Pectins C30, CR52, C56, and also M85, do contain quite a few clustered blocks. Of these samples, C56 has a much lower methyl- to non-esterified ratio than pectin R52. Because C56 releases more than twice the amount of non-esterified galacturonic acid residues than R52 and in non-identical ratios (Table 1), it can be concluded that the lower methyl- to non-esterified ratio is caused by the fact that the C56 blocks are more evenly distributed over the pectic backbone than those of R52. The same, but to a lesser extent, is very likely the case for CR52 and C30. In the M85 sample, the non-esterified blocks are more or less randomly distributed. For an accurate interpretation of the methyl- to non-esterified ratio it is important to include the information obtained from the total amount of non-esterified mono-, di-, and trigalacturonic acids released, because the latter provides insight into the total amount and average size of degradable blocks present.

For the study of the methyl-ester distribution of pectin, the approach described in this report is an important addition. By analyzing a pectin endo-PG digest on HPAEC at pH 5, very detailed information on the intramolecular methyl-ester distribution of the polymer is obtained. By determining the amount of non-esterified mono-, di-, and trigalacturonic acid present, it is not only revealed to what extent the methyl-ester distribution of this pectin differs from a random one [1], but also information indicating the average size and the occurrence of blocks limiting the action of endo-PG is obtained. By determining the ratio of the methyl- to non-esterified peak areas, the extent of clustering of these blocks can be obtained. This information is a very important addition in the study of the functional properties of pectins. It is important to realize that for an exact calculation of the average 'endo-PG degradable' block size of a pectin, a detailed study of the mode of action of the enzyme on methyl-esterified substrates is essential. In addition, determination of the

PAD-response factors of methyl-esterified oligomers and the location of the methyl ester(s) on these oligomers are a prerequisite for a successful completion of these studies. With the approach described above, intermolecular differences in the methyl-ester distribution of pectin cannot be revealed. Hitherto, this aspect has been studied mainly by anion-exchange [22,23] and/or size exclusion chromatography [24]. Current research is focused on the use of endo-PG in combination with these chromatographic techniques, to reveal differences in the occurrence and distribution of 'endo-PG degradable' blocks over the various chains present in pectin.

4. Experimental

Materials.—GalA was purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). NaOH (50% solution) was purchased from J.T. Baker (Deventer, The Netherlands). Di- and tri-GalA were obtained from Sigma Chemical Co. (St. Louis, MO). Tetra-GalA was prepared and purified as described in Ref. [25]. Endo-PG was purified from a preparation of *Kl. fragilis* CBS 397, as described by Versteeg [26], with an activity of 20 μmol GalA produced min^{-1} mL^{-1} (20 units mL^{-1}). The purified enzyme was devoid of pectin methyl esterase, pectin lyase, and pectate-lyase activity. Polygalacturonic acid was purchased from ICN (Aurora, OH). Lemon pectins with DMs ranging from 30.4 to 73.5% (C30–C74) were kindly provided by Copenhagen Pectin A/S (Lille Skensved, Denmark), a subsidiary of Hercules Inc. (Wilmington, DE). The two highly esterified pectins M85 (DM 85%) and M93 (DM 93.1%) were prepared as described in Ref. [1]. Random de-esterification of the M93 pectin, by saponification with diluted NaOH, resulted in the production of a set of randomly methyl-esterified pectins (R70, R52, R32) with DMs of 70.2, 52.1, and 31.5%, respectively [9]. The commercial C67 pectin was de-esterified with NaOH to DMs of 51.7 (CR52) and 30.7%, (CR31). Tomato pectin methylesterase was used to blockwise de-esterify pectin as described previously [1]. Pectins B71, B57, and B56 (DM 70.6, 57.1, and 56.4%, respectively) were used in this study. The uronic-acid content and the DM of the pectins are summarized in Table 1. The acetyl ester content was $\leq 1.5\%$ for all pectins. Endo-PG digests were prepared as described in [1]. A mixture of partially methyl-esterified GalAs of known composition was produced by incubating the commercial DM 30.4 pectin (C30) with endo-PG [19].

Chromatographic analysis.—The methyl ester and GalA content of the *Kl. fragilis* endo-PG degradation products were determined with HPAEC at pH 5 [1,19]. Shortly before analysis, samples were thawed and centrifuged for 10 min at 16.000*g*. Amounts of 80 μL were injected. The chromatograms were integrated by peakfit software (SPSS Inc., Chicago, IL) as described in Ref. [1]. The 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. In proportion to mono-GalA, the response factors of the di-, tri-, and tetramer were 2.0, 1.0, and 2.4 for this specific HPAEC/PAD configuration. During each series, the PAD-response area of a standard amount of mono-GalA (0.103 μmol) was determined to enable accurate calculation of the non-esterified GalA concentrations of each sample in that series [1]. Usually, a peak area of about 3.5×10^8 was observed per μmol mono-GalA injected (detector range 0.2 μC). This absolute response, however, varied over time 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 [20].

General methods.—The uronic-acid content of pectin was determined after pre-hydrolysis with H_2SO_4 [27] followed by colorimetric detection with an automated *m*-hydroxydiphenyl method [28]. The methoxyl and acetyl contents were determined by high-performance liquid-chromatography (HPLC) analysis of the MeOH and HOAc released on alkaline de-esterification [29].

Acknowledgements

The authors thank Miranda Huisman and Nienke Peerlkamp for valuable suggestions.

Part of this work was financially supported by Hercules Inc.

References

[1] P.J.H. Daas, K. Meyer-Hansen, H.A. Schols, G.A. De Ruiter, A.G.J. Voragen, *Carbohydr. Res.*, 318 (1999) 135–145.
[2] A. Bacic, P.J. Harris, B.A. Stone, in J. Preiss (Ed.), *Carbohydrates*, *The Biochemistry of Plants*, Vol. 14, Academic Press, London, 1988, pp. 297–371.
[3] A.G.J. Voragen, W. Pilnik, J.-F. Thibault, M.A.V. Axelos, C.M.G.C. Renard, in A.M. Stephen (Ed.), *Food Polysaccharides and Their Applications*, Marcel Dekker, New York, 1995, pp. 287–339.
[4] C. Rolin, J. De Vries, in P. Harris (Ed.), *Food Gels*, Elsevier Science, Amsterdam, 1990, pp. 401–434.
[5] H.A. Schols, A.G.J. Voragen, in J. Visser, A.G.J. Voragen (Eds.), *Progress in Biotechnology*, *Pectins and Pectinases*, Vol. 14, Elsevier Science, Amsterdam, 1996, pp. 3–19.
[6] J.-F. Thibault, C.M.G.C. Renard, M.A.V. Axelos, P. Roger, M.-J. Crépeau, *Carbohydr. Res.*, 238 (1993) 271–286.
[7] T.P. Kravtchenko, M. Penci, A.G.J. Voragen, W. Pilnik, *Carbohydr. Polym.*, 2 (1993) 195–205.
[8] H. Grasdalen, A.K. Andersen, B. Larsen, *Carbohydr. Res.*, 289 (1996) 105–114.
[9] T.G Neiss, H.N. Cheng, P.J.H. Daas, H.A Schols, *Poly. Preprints*, 1 (1998) 688–689.
[10] R. Kohn, G. Dongowski, W. Bock, *Nahrung*, 29 (1981) 75–85.
[11] C.E. Tuerena, A.J. Taylor, J.R. Mitchell, *Carbohydr. Polym.*, 2 (1982) 183–191.
[12] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 245–258.
[13] J.A. De Vries, M. Hansen, J. Søderberg, P.-E. Glahn, J.K. Pedersen, *Carbohydr. Polym.*, 6 (1986) 165–176.
[14] H. Kiyohara, J.-C. Cyong, H. Yamada, *Carbohydr. Res.*, 182 (1988) 259–275.
[15] A.J. Mort, F. Qui, N.O. Maness, *Carbohydr. Res.*, 247 (1993) 21–35.
[16] W. Pilnik, A.G.J. Voragen, in P.F. Fox (Ed.), *Food Enzymology*, Vol. 1, Elsevier Science, Amsterdam, 1991, pp. 303–336.
[17] R. Pasculli, C. Geraeds, A.G.J. Voragen, W. Pilnik, *Lebensm. -Wiss. u. -Technol.*, 24 (1991) 63–70.
[18] J.A.E. Benen, H.C.M. Kester, J. Visser, *Eur. J. Biochem.*, 259 (1999) 577–585.
[19] P.J.H. Daas, P.W. Arisz, H.A. Schols, G.A. De Ruiter, A.G.J. Voragen, *Anal. Biochem.*, 257 (1998) 195–202.
[20] A.T. Hotchkiss Jr., K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200–206.
[21] E.M.W. Chen, A.J. Mort, *Carbohydr. Polym.*, 29 (1996) 129–136.
[22] H.A. Schols, J.C.E. Reitsma, A.G.J. Voragen, W. Pilnik, *Food Hydrocoll.*, 3 (1989) 115–121.
[23] T.P. Kravtchenko, A.G.J. Voragen, W. Pilnik, *Carbohydr. Polym.*, 19 (1992) 115–124.
[24] T.P. Kravtchenko, G. Berth, A.G.J. Voragen, W. Pilnik, *Carbohydr. Polym.*, 18 (1992) 253–263.
[25] A.G.J. Voragen, H.A. Schols, J.A. De Vries, W. Pilnik, *J. Chromatogr.*, 244 (1982) 327–336.
[26] C. Versteeg, Pectinesterases from the orange fruit — their purification, general characteristics and juice cloud destabilizing properties, *PhD Thesis*, Wageningen Agricultural University Wageningen, The Netherlands (1979).
[27] H.N. Englyst, J.H. Cummings, *Analyst*, 109 (1984) 937–942.
[28] J.-F. Thibault, *Lebensm. -Wiss. u. -Technol.*, 12 (1979) 247–251.
[29] A.G.J. Voragen, H.A. Schols, W. Pilnik, *Food Hydrocoll.*, 3 (1986) 65–70.