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Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger* ☆

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Abstract

A method to determine the amount of galacturonic acid in blocksequence (BS) in pectin homogalacturonan (HG) is described. The method is based on a combination of endopolygalacturonase II (endo-PG II) and exopolygalacturonase (exo-PG) digestion followed by quantification of the liberated galacturonic acid monomer. The amount of monomers released is directly related to the amount of non-esterified galacturonic acid units located between two other non-esterified galacturonic acids units on the HG chain. The amount released for exo-PG digestion only corresponds to the BS located at the non-reducing end of the polymer. The difference between total- and exo-BS was calculated to be the amount of endo-BS located either within or on the reducing end of the HG. Three series of model pectins obtained by de-esterification of a high-ester pectin with either plant pectin methyl-esterase (p-PME, P-series), fungal pectin methyl-esterase (f-PME, F-series) and chemical de-esterification using base (B-series) were analysed and compared with a fully de-esterified pectic acid sample obtained from the same raw material. Clear differences for the increase of the amounts of blocksequence could be seen between de-esterification of the P- and F-series samples supporting a blockwise and a homogenous de-esterification mechanism, respectively. f-PME and base treatment showed only minor differences in the increase of galacturonic acid units in BS, despite differences seen in their methyl-esterification pattern. Differences between the amounts of galacturonic acid located in exo- and endo-BS, provided evidence for the need of a certain start side or blocklength for p-PME to de-esterify blockwise.

Keywords: Pectin; Pectin methyl-esterase; endo-Polygalacturonase; exo-Polygalacturonase; MALDIMS; ESIMS/MS

☆ Analysis of pectin structure, Part 3. For Part 2, see Ref. [1].

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1. Introduction

Pectins are a family of complex galacturonic acid rich polysaccharides found in the primary cell wall and intercellular spaces of higher plants [2,3].

Pectins extracted from apple and citrus fruits have a long commercial tradition as a textural food ingredient. They are used as a gelling agent in marmalades and jams or as a thickening agent in other fruit-based applications [4]. More recently, pectins have been used in dairy products because of their potential in stabilising milk proteins in acid conditions [5].

The ability of pectins to crosslink via calcium bridges is the basis for several pectin-applications, including calcium gelation and milk protein stabilisation. The nature of the calcium crosslinks is believed to follow the egg box type interaction [6], in which two HG chains containing a sequence or block of adjacent non-esterified galacturonic acid units next to each other are coordinated to a middle layer of calcium ions. The exact number of non-esterified galacturonic acids necessary to be present in this blocksequence (BS) to form an interaction is unknown. Usually, figures reported vary between 8–12 units [7] for the minimum length of the BS necessary for a successful interaction.

Due to the commercial importance of this functionality of BS several attempts have been made to quantify the amount of galacturonic acid located in BS. Grasdalen and co-workers [8] used the ^{1}H NMR resonances for H-4 and H-5 of galacturonic acid, which have different chemical shifts depending on whether the galacturonic acid unit and its next neighbours are methyl-esterified or not. Using this method, the ratio of a non-esterified galacturonic acid triad sequence relative to mixed or fully methyl-esterified triads can be determined.

Enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II (endo-PG II) [9] and characterisation using anti-pectin-antibodies [1] revealed discernible differences between the methyl-esterification patterns on the model pectins produced by the action of p-PME (producing blockwise de-esterification) and f-PME and base catalysis (producing homogeneous de-esterification). Moreover there were also some differences in the methyl-esterification patterns produced by f-PME and by base catalysed de-esterification. These differences were probably due to the substrate specificity of f-PME, which yielded a certain pattern of methyl-esterification different from a random distribution, which was assumed for the base catalysed reaction.

Recently Daas and co-workers [10] also reported the use of endo-polygalacturonase for analysing methyl-esterification patterns with special focus on the presence of BS on the analysed pectins. We now describe a different approach using a combination of endo- and exopolygalacturonase (endo-PG II and exo-PG) which allows to quantify the amount of galacturonic acid units located in BS by simply measuring the amount of liberated free galacturonic acid monomer after combined digestion. Additional information about the location of the BS on the HG backbone can be obtained, if digestion with only exo-PG is used.

2. Results and discussion

Model pectins.—We recently described [9] the production and analysis of three series of model pectins. They were all obtained from one lot of commercial Grindsted™ Pectin URS 1200 (E81) by de-esterification using either plant pectin methyl esterase (p-PME) from orange peel (P-series) or fungal pectin methyl-esterase (f-PME) from *Aspergillus* (F-series) or base-catalysed de-esterification (B-series). The main analytical data for these samples are summarised in Table 1.

Analysis of enzyme specificity.—Application of enzymes for the structural analysis of pectins requires a good understanding of the catalytic activity of the applied enzyme and its preferred cleavage sites on the pectin backbone. Only this fundamental knowledge allows a realistic interpretation of product patterns to give structural information for the analysed pectin. The action of pectinases on the HG chain is shown in Scheme 1. Recent advances in mass spectrometry allow the anal-

ysis of complex mixtures of galacturonic acid oligomers, produced by enzymatic digestion of pectin without the need of any separation step using MALDIMS [11]. Methyl-esterification patterns on each oligomer in the complex mixture can be analysed using a newly developed sequencing method based on ESIMS/MS [12]. Thereby, information about the specificity of the enzyme used for analysis can be obtained. Quantification of the digestion products can be performed using the latest developments in HPAEC separation technology [13].

In Part 1 [9] of this series, we reported, on the basis of MALDIMS time-course experiments, that endo-PG II selectively attacked large continuous sequences of non-esterified galacturonic acid units on the HG backbone, before partly methyl-esterified areas were digested. Furthermore, we showed that enzymatic fingerprinting effectively revealed the differences in methyl-esterification patterns of samples de-esterified in a blockwise fashion (P-series) or in a homogeneous fashion (F- and B-series).

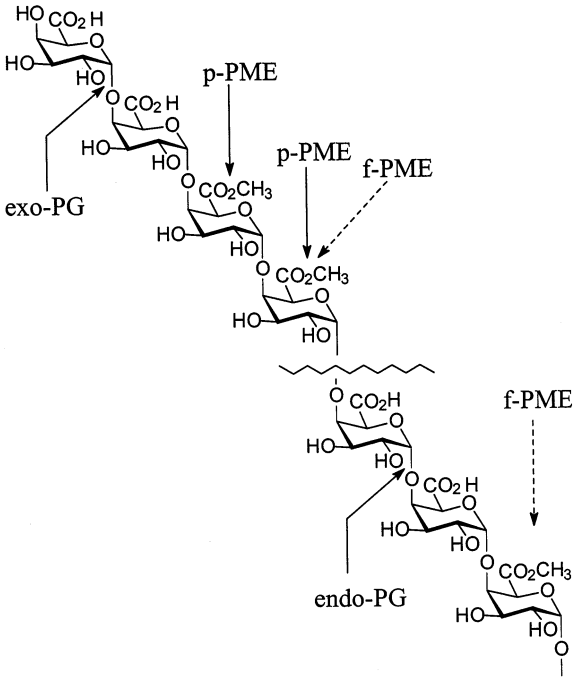
MALDIMS analysis of endo-PG II digests (Fig. 1(A)) of different pectins from the F-, P- or B-series showed variations in peak intensities for $[M-H]^-$ ions. The intensities for larger oligomers, for example, were much higher for F58 than for F31, together with much lower intensities for oligomers with only a few methyl-ester groups like 3^1, 4^1, 5^1. The hexamer with one methyl-ester group (6^1) was no longer detectable after full-length digestion with endo-PG II. There were also differences

Table 1
Analysis data for model pectins [a]

Pectin sample	% DE	% AGA		η (L/g)	av. M_w (D)	% Dry matter
		in AAIS	in sample			
Mother pectin Grindsted™ Pectin URS 1200						
E81	81.1	87.3	78.7	0.45	90000	91.0
Samples from de-esterification with p-PME						
P76	76.1	89.7	81.5	0.35	70000	93.8
P73	72.9	92.3	82.6	0.34	68000	93.2
P70	70.4	90.5	80.3	0.29	57000	93.7
P66	66.2	91.7	83.6	0.30	60000	95.5
P60	59.7	90.2	81.7	0.26	52000	96.8
P53	52.6	89.1	80.9	0.28	55000	90.8
P46	46.1	89.8	78.3	0.26	51000	93.1
P41	40.5	87.9	78.7	0.26	51000	89.6
Samples from de-esterification with f-PME						
F76	75.7	89.8	85.9	0.46	93000	95.8
F69	68.9	88.4	82.4	0.40	81000	95.8
F58	58.2	90.4	82.5	0.40	81000	94.6
F43	42.6	89.3	77.9	0.37	74000	93.0
F31	31.1	86.6	74.5	0.35	69000	92.5
F11	11.0	84.7	66.8	0.27	53000	88.3
Samples from base-catalysed de-esterification						
B71	71.3	89.5	82.6	0.40	80000	95.0
B64	63.8	88.2	83.7	0.38	75000	95.2
B43	42.9	88.1	83.5	0.22	43000	94.2
B34	33.7	89.3	85.6	0.45	89000	94.0
B15	14.7	91.8	88.2	0.31	62000	94.1
PGA	1.4	86.7	64.6 [b]	0.20	39000	85.5

[a] DE, degree of esterification; AGA, anhydro-galacturonic acid; AAIS, acid and alcohol insoluble solids; av. M_w, weight-average molecular weight.

[b] Low % AGA in PGA sample is caused by an alcohol-soluble contamination, most likely water or inorganic salts.



Scheme 1. Action of pectolytic enzymes on a partly methyl-esterified HG backbone [endo-PG II, exo-PG: endo-, exopoly-galacturonase (EC 3.2.1.15), p-PME and f-PME pectin methyl-esterase (EC 3.1.1.11) from either plant or fungal origin].

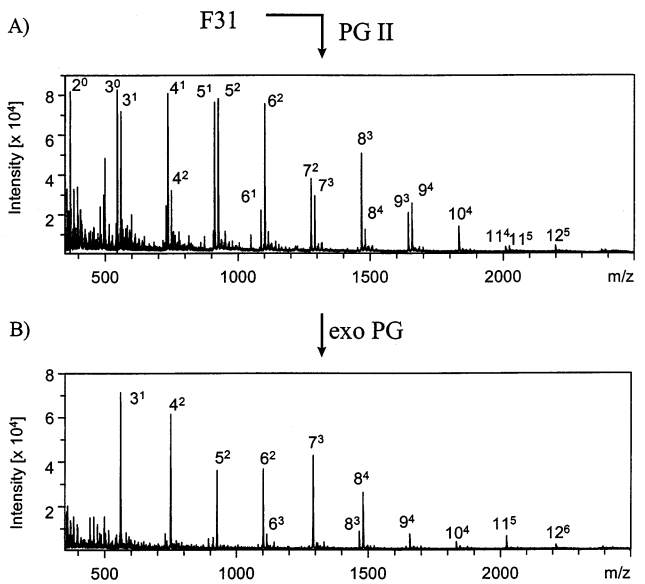


Fig. 1. (A) Digestion (20 h) of F31 with 0.04 U endo-PG II/mg pectin analysed by MALDIMS in negative ion mode. (B) Digest (A) treated with 0.01 U exo-PG/mg pectin for 20 h analysed by MALDIMS in negative ion mode. $(M-H)^-$ ions are labelled by giving the DP in normal numbers, and the number of methyl ester groups in superscript numbers.

seen between pectins of the same DE but different methyl-esterification patterns like P60 and F58 (data not shown).

However, sequence analysis using ESIMS/MS [12], revealed that the methyl-esterification pattern found on the single oligomer species were greatly conserved, regardless of which pectin was used. This indicated that endo-PG II has quite strict requirements for substrate binding. A common feature for all oligomers was that both the reducing and the non-reducing end contained a non-esterified galacturonic acid as the first unit. Endo-PG II is therefore restricted to cleave between two non-esterified galacturonic acid units.

In many oligomers the second unit from the non-reducing end was a second non-esterified galacturonic acid group whereas the second unit from the reducing end was predominantly found to be methyl-esterified (Fig. 2). Furthermore, none of the oligomers analysed contained sequences of more than three adjacent free galacturonic acid units. These triad sequences were mainly found to be located at the non-reducing end.

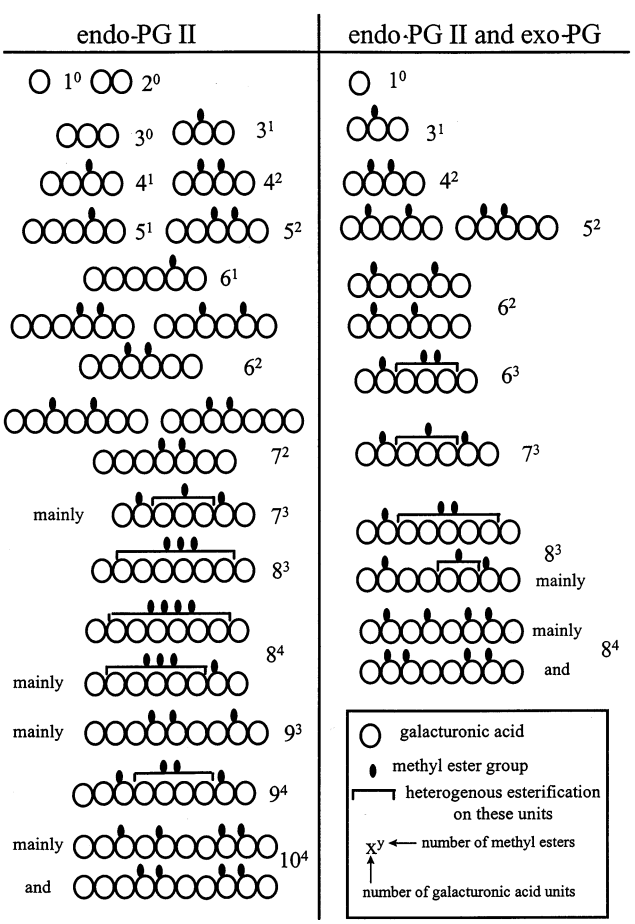


Fig. 2. The deduced oligomer sequences derived from ESIMS/MS analysis in the endo-PG II and exo-PG digests shown in Fig. 1. All oligomers are drawn with their reducing end to the right-hand-side.

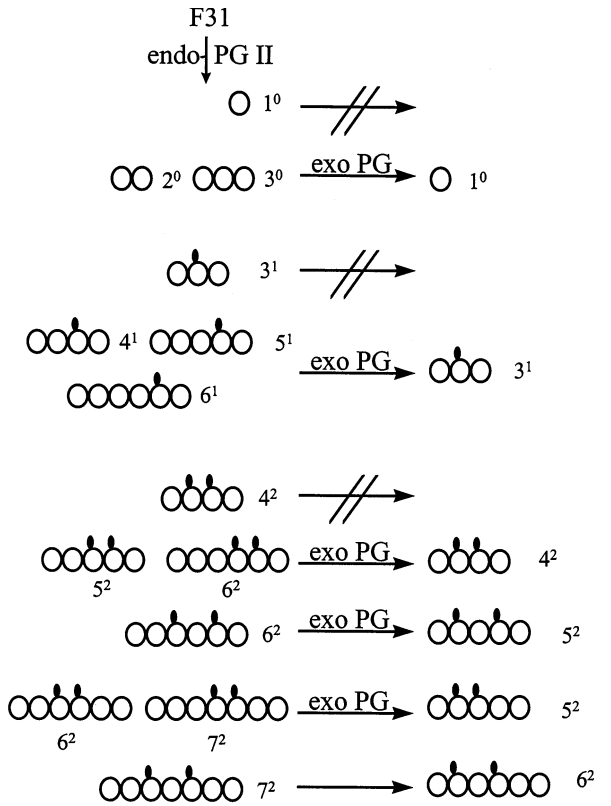


Fig. 3. Postulated reactions catalysed by exo-PG on the endo-PG II fragments. The oligomer sequences were determined by ESIMS/MS and are listed in Fig. 2.

If 0.04 U endo-PG II/mg pectin is used, low ester pectins like F31 showed a few examples for oligomer structures (Fig. 2), which contained this triad sequence on the reducing end (as in one structure for 7^2) or four acids groups joined together at the non-reducing end (as in 6^1 Fig. 2). Time-course analysis of digestion of high ester pectins, as F58 with 0.04 U endo-PG II/mg pectin showed that these oligomers were also formed in the beginning of the reaction but they were slowly digested further (data not shown), indicating that they were still substrates for endo-PG II. If larger quantities of these less-favourable substrates were produced as seen for F31 (Fig. 1(A)), the chosen pectin–enzyme ratio and incubation time was not sufficient to fully digest those substrates. For quantitative analysis, the amount of enzyme per pectin was therefore doubled from 0.04 U/mg pectin to 0.08 U/mg pectin, for pectins with a DE below 50%, to ensure completed digestion. This was realised, practically, by diluting the pectin concentration from 5 to 2.5 mg/mL for pectins with a DE below 50%, but using a constant amount of 0.2 U endo-PG II/mL solution, to ensure complete digestion in all cases. Thereby, all sequences of three or more adjacent galacturonic acids were cleaved, unless located at the non-reducing end of the formed oligomers.

Exo-PG from *Aspergillus* was earlier characterised by Visser and co-workers [14] to break down non-esterified galacturonic acid oligomers from the non-reducing end to produce monomers as the only product. We have recently reported [12] the action of exo-PG on partly methyl-esterified oligomers. The enzyme has, like endo-PG II, a strict specificity to cleave between two non-esterified acid groups only. Thus, oligomers with just one free acid group on the non-reducing end are not substrates for exo-PG.

MALDIMS analysis of the endo-PG II digest of F31 prior and after exo-PG digestion (Fig. 1(A) and (B)) showed that all oligomers in the endo-PG II digest containing no methyl-ester groups (1^0, 2^0, 3^0) were broken down to galacturonic acid monomer (1^0).

Oligomers containing one methyl-ester group in the endo-PG II digest (3^1, 4^1, 5^1 and 6^1) yielded all 3^1, which is the only detectable oligomer with one methyl-ester group present after exo-PG digestion. This could be rationalised, because the structures found for 4^1, 5^1 and 6^1 in the endo-PG II digest (Fig. 2) all carried their methyl-ester group in position 2 from the reducing end; and either two, three or four non-esterified galacturonic acid units at the non-reducing end, which were cleaved off by exo-PG to yield 3^1 as the only product. These postulated reactions are summarised in Fig. 3.

Separation of the partially methylated oligomers on Dionex HPAEC at neutral pH [13b] (Fig. 4), confirmed that the peaks assigned to be 4^1 and 5^1 had disappeared after exo-PG treatment, together with a clear increase of the concentration of 3^1. The oligomers with two methyl-ester groups (5^2, 6^2 and 7^2) in the endo-PG II digest, were digested by exo-PG to yield 4^2, 5^2 and 6^2, respectively. Thus, one species of 5^2 containing its methyl-ester groups in position 2 and 3 from the reducing end, disappeared to give 4^2, whereas another species of 5^2 containing methyl-ester

groups at position 2 and 4 from the reducing end, was produced from digestion of some 6^2 and 7^2 in the endo-PG II digest. In analogy to 3^1, a distinct increase for 4^2 could be detected by Dionex HPAEC at pH 6 (Fig. 4) after exo-PG digestion, caused by cleavage of all 5^2 and some 6^2 from the endo-PG II digest.

Quantification of blocksequence by fingerprinting with endo- and exo-PG.—We have provided evidence that treating pectin with endo-PG II digestion followed by exo-PG will cleave all sequences of three or more free galacturonic acid units to yield exclusively the galacturonic acid monomer. Product analysis after combined digestion showed that the remaining methyl-esterified oligomers contained one free-acid group on the reducing and the non-reducing end and that no sequence of more than two adjacent non-esterified galacturonic acid groups was present.

By combining all this information, we came to the conclusion that the amount of galacturonic acid monomer liberated after combined digestion with endo-PG II and exo-PG, is a good approximation of the amount of galacturonic acid groups which were located in the middle of a triad sequence of free galacturonic acid units in the original pectin. This allows us

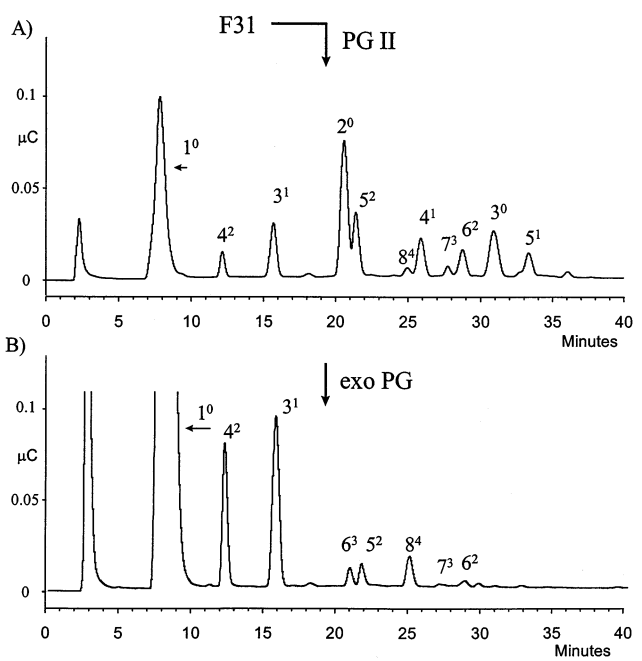


Fig. 4. HPAEC analysis at pH 6 of the products generated by treating pectin F31 with endo-PG II and exo-PG. Tentative peak assignment following retention order as published in Ref. [17]. Peaks are labelled as in Figs. 1–3.

to apply the same model calculations as described by Grasdalen and co-workers [8] to predict the theoretical amounts of contiguous galacturonic acid units in a BS generated by a blockwise or random de-esterification mechanism. In addition, we were able to differentiate between those galacturonic acid units joining in a BS located at the non-reducing end and those joining in internal or reducing end BS by using exo-PG only.

All model pectins were analysed for the amount of galacturonic acid monomer liberated by combined endo-PG II and exo-PG digestion and exo-PG digestion only. The difference between total- and exo-BS was called endo-BS, indicating that it was inaccessible by exo-PG, and therefore located either internally on the HG, or at its reducing end. All obtained data are listed in Table 2 as mg/mL concentration of galacturonic acid monomer obtained from a 5 mg/mL pectin solution by the different enzyme treatments. For samples with a DE $< 50\%$ the values obtained for a pectin concentration of 2.5 mg/mL were multiplied by 2 to allow better comparability to those run at 5 mg/mL pectin concentration. All data shown resulted from triplicate digestions to reduce the error margin to 0.01 mg/mL. In order to take the slight variations in the total content of galacturonic acid (see % AGA in Table 1) of the pectins into account, we calculated the relative amounts of galacturonic acid in BS, compared to the total amount of galacturonic acids present in the whole pectin in columns 4–7 of Table 2. Results for the completely de-esterified pectic acid (PGA) showed that only about 57% of the galacturonic acid present was accessible by the combined treatment, and thereby converted to galacturonic acid monomer. The remaining 43% cannot be released by endo-PG II and exo-PG treatment, which means they must be located in or at the border of other pectin subunits. A 17.5% amount of the total galacturonic acid could be released by exo-PG treatment only, which is about 30% of the total accessible amount, which was located at the non-reducing end. Because we were interested in the number of galacturonic acid units in BS on the HG, we calculated the amount of liberated galacturonic acid from a sample rela-

Table 2
Amount of galacturonic acid (GalA) found in BS found for model pectins [a]

Pectin sample	GalA in exo-BS (mg/mL)	GalA in endo-BS (mg/mL)	GalA in total-BS (mg/mL)	GalA in exo-BS (%* [b])	GalA in endo-BS (%*)	GalA in total-BS (%*)	GalA in exo-BS (%** [c])	GalA in endo-BS (%**)	GalA in total-BS (%**)
Mother pectin Grindsted™ Pectin URS 1200									
E81	0.045	0.060	0.015	1.1	0.4	1.5	6.5	1.0	2.7
Samples from de-esterification with p-PME									
P76	0.070	0.030	0.100	1.7	0.7	2.5	9.8	1.8	4.3
P70	0.128	0.062	0.190	3.2	1.5	4.7	18.2	3.9	8.2
P66	0.157	0.154	0.311	3.7	3.7	7.4	21.5	9.2	13.0
P60	0.210	0.255	0.465	5.2	6.2	11.4	29.4	15.6	19.8
P53	0.206	0.554	0.760	5.2	13.6	18.8	29.1	34.0	32.7
P41	0.234	0.744	0.977	5.9	18.9	24.8	34.0	47.3	43.3
Samples from de-esterification with f-PME									
F76	0.07	0.05	0.121	1.6	1.2	2.8	9.3	2.9	4.9
F69	0.099	0.110	0.209	2.4	2.7	5.1	13.7	6.7	8.8
F58	0.096	0.251	0.343	2.3	6.1	8.3	13.3	15.2	14.5
F43	0.157	0.449	0.606	4.0	11.5	15.6	23.0	28.9	27.1
F31	0.155	0.618	0.763	4.2	16.6	20.5	23.8	41.5	35.7
F11	0.419	1.342	1.761	12.5	40.2	52.7	71.7	100.6	91.6
Samples from base-catalysed de-esterification									
B71	0.077	0.03	0.107	1.9	0.7	2.6	10.7	1.8	4.5
B64	0.099	0.071	0.170	2.4	1.7	4.1	13.5	4.2	7.1
B43	0.140	0.421	0.561	3.4	10.0	13.4	19.2	25.2	23.4
B34	0.275	0.556	0.831	6.4	13.0	19.4	36.7	32.5	33.8
B15	0.317	1.272	1.589	7.2	28.8	36.0	41.1	72.2	62.8
PGA	0.565	1.290	1.855	17.5	39.9	57.4	100	100	100

[a] Columns 2–4: amounts ($\pm$ 0.01 mg/mL) of galacturonic acid monomer found in 5 mg pectin, as determined by digestion with either exo-PG (GalA in exo-BS) or endo-PG II, followed by exo-PG (GalA in total-BS) as described in Section 4. The amount of GalA in endo-BS was calculated as: endo-BS = [total-BS − exo-BS].

[b] %* values in columns 5–7 give relative amounts of released GalA from a BS per total GalA (AGA in sample). Values were calculated as: [%*] = {GalA in BS (mg/mL)/% AGA × 5 (mg/mL)} × 100%.

[c] %** values in columns 8–10 give relative amounts of GalA released from BS per released amounts found for PGA. Values were calculated as: [%**] = {%* (sample)/%* (PGA)} × 100%. The relation between % DE (Table 1) and the data in columns 8–10 is visualised in Fig. 5(A–C).

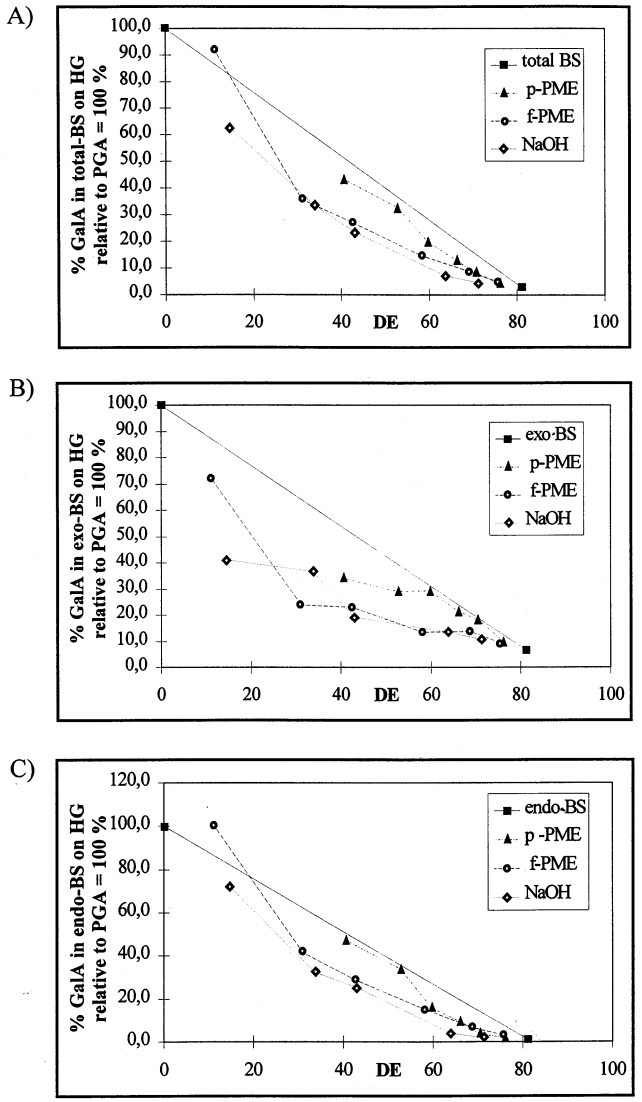


Fig. 5. % DE vs. amount of galacturonic acid found in (A) total-BS, (B) exo-BS and (C) endo-BS determined for the model pectins, relative to the amount found for PGA set as 100%. Data are listed in the last three columns of Table 2.

tive to the amount liberated from PGA set as 100%. These values are listed in the last three columns of Table 2.

In Fig. 5, the increase of galacturonic acid units in total-, exo- and endo-BS is drawn relative to the decrease of DE. Thereby, we could easily compare the effects that the different de-esterification procedures had on the formation of BS. A strictly blockwise de-esterification should lead to a linear increase from the value found for E81 to PGA (drawn lines) with decreasing DE. The increase of triad sequence for a random de-esterification process was described by Grasdalen and coworkers [8] to follow the equation $(1 - DE/100)^3 \times 100\%$ for pure partially methylated polygalacturonic acid. The latter equation seems to be in good correlation with the values found for the increase of galacturonic acid in total-BS obtained for the B-series. De-esterification using f-PME gave similar but slightly higher values as seen for base treatment until the DE drops below 20–30%. Below this level, a substantially higher increase was observed like for the last F-series pectin F11, even increasing above the theoretical value for a blockwise de-esterification mechanism. One explanation could be that, due to the enzyme specificity methyl-ester groups were only removed from the homogalacturonan (HG), whereas the determined DE of 11% refers to the average DE of all subunits. As a consequence, the DE of the HG must be substantially below the total-DE of 11%. For the P-series pectins the increase of the amount of galacturonic acid in the total-BS seemed to follow the theoretical random curve in the very beginning. The increase turned to be linear after a DE of about 70% is reached.

However, differentiation of total-BS in amounts of galacturonic acid present in the exo- and endo-BS as shown in Fig. 5(B) and (C), revealed that the increase of the non-reducing end exo-BS followed the straight line predicted for the generation of a single de-esterified blocksequence of non-esterified galacturonic acid units at the beginning of the de-esterification reaction. This process is slowed down severely after a DE of about 60% was reached. One possible explanation for this saturation effect at around DE 60%, could be a lack of suitable starting site at the non-reducing end at that stage, because all molecules containing a non-reducing end blocksequence at the beginning of the p-PME reaction might have been de-esterified until either a critical blocklength or another pectin subunit was reached. All other polymers not containing a blocksequence at the non-reducing end at the beginning of the reaction, need to be attacked randomly by p-PME first, before a certain start site for blockwise p-PME action is generated. This implicates that processive de-esterification by p-PME is working towards the reducing end and that a certain BS needs to be present before tight substrate

binding of p-PME leads to de-esterification by a single chain mechanism, which is in accordance to other reports in the literature [8,15,16]. Accordingly, it could be observed that p-PME action causes an increase in the calculated amounts of contiguous galacturonic acid units in the endo-BS, according to a theoretical random de-esterification mechanism first. After some random de-esterification, several suitable start sides were generated which allowed a blockwise de-esterification mechanism which lead to a linear increase of endo-BS. Intermediately, the increase in endo-BS formation was seen to be even steeper than expected for a blockwise de-esterification; probably due to joining several small BS to some larger BS. The de-esterification of the last methyl-esterified galacturonic acid unit between two small BS would add three galacturonic acid units to the now enlarged BS, because the two bordering units and the de-esterified unit all become internal units.

The values for galacturonic acids located in the exo-BS for the B- and F-series were both in agreement with a random de-esterification process at the non-reducing end. The endo-BS values gave slightly higher values for the F-series compared to the B-series especially at lower DE values. This could be caused by the above-discussed effects of enzyme specificity of f-PME to de-esterify HG only, whereas base catalysis probably also de-esterifies the other subunits.

3. Conclusions

We have developed a method for characterisation of pectin by determining the amount of contiguous galacturonic in BS on the HG backbone. We could show by MALDIMS and ESIMS/MS, that both endopolygalacturonase II (endo-PG II) and exopolygalacturonase (exo-PG) cleave between two free galacturonic acid units only. The remaining oligomers, after exo-PG treatment, carried a non-esterified galacturonic acid group on both the reducing, and the non-reducing end, and in general did not contain sequences of more than two adjacent non-esterified galacturonic acid units.

All galacturonic acid units, which were located in between two other non-esterified galacturonic acid groups on the HG of the original pectin, were obtained as monomers after a combined digestion with endo-PG II and exo-PG. The problem in quantifying the amount of contiguous non-esterified galacturonic acid units in BS, was thereby reduced to a simple measurement of the concentration of released galacturonic acid monomer, after a combined digestion with endo-PG II, followed by exo-PG.

The galacturonic acid units originally placed on the borders of a BS of at least three units in the original pectin are present at the reducing and non-reducing end of the partially esterified oligomers after the combined enzyme treatment.

In addition, we used exo-PG digestion to determine the ratio of galacturonic acid units located in an exo-BS at the non-reducing end of the pectin, which thereby could be differentiated from all other endo-BS on the HG or at the reducing end.

Analysis of the increase of galacturonic acid in BS during three different de-esterification procedures gave information about the different methods used for de-esterification. The values obtained for a base-catalysed de-esterification showed the best fit for a theoretically random de-esterification process. For f-PME, a slightly higher increase of BS, especially after extended treatments down to very low DE values, indicated a somewhat more ordered de-esterification process which still was much more like a random or homogeneous than a blockwise process with regards to BS amounts.

For p-PME, the expected linear increase of BS characteristic for a blockwise de-esterification process was observed; only after some initial decrease in DE. Hereby the increase seen for exo- and endo-BS were very different. The exo-BS increased linearly with decreasing DE, as expected for a blockwise de-esterification at the beginning of the reaction, but it became more like a random process after about 20% reduction in DE. However, endo-BS increased as expected for a random process in the beginning. Only after some reduction in DE, the increase became linear as characteristic for a blockwise process.

Both effects allow the conclusion that p-PME needs a certain start side to catalyse the removal of methyl-ester groups in a blockwise fashion towards the non-reducing end. If such a start side is not present, p-PME removes methyl-ester groups as expected for a random process.

4. Experimental

Purification and characterisation of exo-PG.—exo-PG was purified from a commercial *Aspergillus* pectinase preparation. The crude extract was dialysed against 50 mM MES pH 6.8 and the dialysed extract was applied on an anion exchange chromatography column (Mono Q HR 26/10, Pharmacia) equilibrated with 50 mM MES pH 6.8. The flow rate was 5.4 mL/h. After washing the column with the equilibration buffer, proteins were eluted with a linear NaCl gradient (0–0.5 M, 1000 mL). Fractions containing exo-PG activity were combined and applied to hydrophobic interaction chromatography (Phenyl Sepharose HiLoad HR 26/10) equilibrated with 1.8 M $(NH_4)_2SO_4$ in 50 mM MES pH 6.8. Bound proteins were eluted with a linear decreasing gradient of $(NH_4)_2SO_4$ (1.8–0 M, 1 L). The fractions containing exo-PG activity were collected and dialysed against 50 mM NaOAc, pH 5.0. The exo-PG was further purified by separation on a Mono Q HR 26/10, equilibrated with 50 mM NaOAc, pH 5.0. The bound exo-PG was eluted with a linear NaCl gradient (0–0.5 M, 1 L). The fractions containing exo-PG activity were pooled and concentrated using ultrafiltration (Amicon cut value 10.000). The last step in the purification procedure was the application of the concentrated sample (1 mL sample) to gel-filtration (Superose 12 HR) equilibrated with 50 mM NaOAc, 0.1 M NaCl, pH 5.0. The exo-PG activity was identified by product characterisation on Dionex HPLC, which showed that only galacturonic acid monomer was produced. The purity was investigated by SDS-PAGE, revealing a homogenous fraction with one protein band of a MW of 78 kDa.

The activity of both polygalacturonases were determined as described elsewhere [14], using PGA as a substrate. One unit of either exo- or endopolygalacturonase causes a formation of 1 μmol new reducing ends per min.

Pectins.—The raw material for the preparation of the P-, F-series and B-series pectins was from Mexican lime (*Citrus aurantifolia*) peel. A commercially extracted slow set pectin from this peel was used as parent pectin for commercial production of Grindsted™ Pectin URS 1200 (E81) by esterification in acid–MeOH medium. All pectins have been produced from one lot of this commercial product by treatment with either p-PME (P-series), f-PME (F-series) or base (B-series). Analytical data obtained for the produced pectins are summarised in Table 1. A detailed description of reaction conditions and performance of analytical methods has been given in the first article of this series [9].

Enzymatic fingerprinting using a combination of endo-PG II and exo-PG.—A pectin sample was dissolved in a 50 mM NaOAc (pH 4.2) at a concn of 5 mg/mL for pectins with DE > 50% and at a concn of 2.5 mg/mL for pectins with DE < 50%, by overnight shaking at room temperature (rt). For quantification, 0.1 mg/mL glucuronic acid was added as internal standard. This pectin soln (1 mL) was incubated with 0.05 U exo-PG for 20 h at rt. The reaction was stopped by incubating the samples in a boiling water bath for 5 min.

Another 1 mL portion of this pectin soln was first incubated with 0.2 U PG II for 20 h at rt. The reaction was stopped by incubating the samples in a boiling water bath for 5 min. Then, 0.05 U exo-PG was added to the cooled soln and the digestion carried out as described above for exo-PG digestion, only.

Both samples were centrifuged and filtered through a 0.45 μm filter prior to chromatographic analysis. Separation was carried out by HPAEC on a Dionex AI 450 system using PAD detection. 0.1 M NaOH (from 50% aq soln, Malinckroft Baker, Holland) and 1 M NaOAc (dry, E. Merck) in 0.1 M NaOH were used as eluents A and B. Eluents were degassed by sparging with helium for 15 min, and keeping under helium for the entire run. The sample (20 μL) was loaded on an analyti-

cal PA 10 (250 × 4.9 mm, Dionex) strong anion-exchange column, connected to a PA 10 guard column (40 × 4.9 mm, Dionex) equilibrated at 90% A and 10% B. Chromatography was performed at a flow rate of 0.8 mL/min with post column addition of NaOH 0.4 mL/min 0.3 M prior to PAD detection.

For separation of liberated galacturonic acid monomer from internal standard and partly methyl-esterified oligomers, a stepwise linear gradient (10–50% B in 10 min followed by 50–65% in 15 min) was used. After each run the column was washed with 100% B for 5 min and allowed to re-equilibrate with 90% A and 10% B for 10 min.

The internal standard glucuronic acid was eluting straight after the galacturonic acid monomer. The peak for mono-galacturonic acid was integrated relative to the internal standard. Standard solutions (0.01–1 mg/mL) using commercial galacturonic acid (Sigma) in 0.1 mg/mL glucuronic acid as internal standard, were used for quantification. The molar amount of galacturonic acid detected, was multiplied with its M_w (194) and is expressed in mg/mL as listed for the P-, F- and B-series pectin in Table 2. Values obtained for samples with DE < 50% were multiplied by 2 to allow direct comparability. All digestions were carried out in triplicate to reduce the average error margin to 0.01 mg/mL.

The resulting amount of mono-galacturonic acid by incubation with exo-PG only, gave the amount of galacturonic acid in the exo-BS and the amount obtained by digestion with endo-PG II followed by exo-PG gave the amount of galacturonic acid in the total-BS. The amount of galacturonic acid in the endo-BS was calculated as the difference between the amount found for the total-BS minus the amount found for exo-BS.

For qualitative analysis of methylated oligomers and changes in oligomer patterns caused by exo-PG treatment, an HPAEC method running at pH 6 was established. The peak assignment was done according to the elution order determined by Daas and co-workers [13b] for a similar method and results obtained from MALDIMS and ESIMS/MS as shown in Figs. 1–3.

All samples were prepared as described above. Separation was carried out by HPAEC on a Dionex AI 450 system using PAD detection. Water and 1 M NaOAc (dry, E. Merck) adjusted to pH 6 by addition of conc HOAc, were used as eluents A and B. Eluents were degassed by sparging with helium for 15 min, and keeping under helium for the entire run. The sample (100 μL) was loaded on an analytical PA 10 (250 × 4.9 mm, Dionex) strong anion exchange column connected to a PA 10 guard column (40 × 4.9 mm, Dionex) equilibrated at 95% A and 5% B. Chromatography was performed at a flow rate of 0.8 mL/min with a post column addition of 0.8 mL/min 1 M NaOH prior to PAD detection.

A linear gradient (5–50% B in 40 min) was used. After each run, the column was washed with 100% B for 5 min and allowed to re-equilibrate with 95% A and 5% B for 10 min.

Mass spectrometry.—MALDI time-of-flight (TOF) spectra were acquired on a Bruker Reflex II mass spectrometer (Bruker Daltonik, Bremen, Germany) in reflector mode using delayed ion extraction (delay time 400 ns). To avoid saturation of the detector by matrix ions, low-mass detector gating (cut-off at 300 Da) was used. The instrument was calibrated externally in the negative ion mode using the peptides angiotensin I and ACTH (1–17).

Electrospray MS/MS spectra were acquired on an Esquire (Bruker-Franzen Analytik GmbH, Bremen, Germany) quadrupole ion trap or on a Micromass Q-TOF mass spectrometer (Manchester, UK). The ion trap has a fundamental radio frequency (RF) of 781 kHz and was operated with an estimated helium pressure of 5×10^{-3} mbar. Ions were scanned in enhanced resolution mode with a scan speed of 2000 Da/s using resonance ejection at the hexapole resonance of one-third the RF frequency. For MS/MS experiments, precursor ions were isolated using frequency sweeps followed by collisional activation for 40 ms with resonance amplitudes between 1 and 3 V (peak-to-peak) across the endcap electrodes. The standard ESI source (Analytica of Branford, Branford, CT, USA) was replaced by a home-built nano-electrospray ion source. The spraying voltages in negative mode was −650 V. The Q-TOF electrospray

mass spectrometer was equipped with a nano-electrospray Z-spray ion source. A potential of −800 V was applied to the nano-flow tip in the ion source and the cone voltage was set to −55 V. For the MS/MS experiments, the precursor ion was selected in the first quadrupole (Q1) and fragmented in the hexapole collision cell with a collision energy varying from 20 to 40 eV. Argon was used as the collision gas at a pressure of approximately 6.0×10^{-5} mbar. The fragment ions were detected in the TOF-reflector with a resolution of about 5000.

Sample preparation for MALDIMS. 2,4,6-Trihydroxyacetophenone (THAP, HPLC grade; Fluka) was dissolved in MeOH to a concentration of 150 mg/mL. Nitrocellulose (Trans-blot transfer medium, 0.45 μm; Bio-Rad) was dissolved in acetone to a concentration of 15 mg/mL. THAP and nitrocellulose solutions were mixed in the ratio 4:1. A 0.2 μL volume of matrix was placed on the metal target. The solution spreads out rapidly, forming a thin layer of homogeneous, very fine crystals. Analyte solutions were desalted using home-made miniaturised columns containing 2 μL ammonium-loaded cation-exchange resins (50W-X8, 200–400 mesh, hydrogen form; Bio-Rad) as described earlier [11]. The analyte solution (1.5 μL) was passed over the column and spotted directly onto the matrix layer. The cation exchange resins (0.1 μL) was added to the analyte droplet and the sample was allowed to dry in air. When the sample was dry, the loose cation-exchange resin was removed with pressurised air.

Sample preparation for ESIMS. Samples were desalted as described above, and ^{18}O-labelled at the reducing end, as described previously [12], to allow the differentiation of isomeric fragment ions. ^{18}O-labelled unseparated pectin digests were analysed in pure water without any addition of organic solvents to avoid precipitation of the acidic oligomers.

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