ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 327 (2000) 293–307

CARBOHYDRATE RESEARCH

Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*☆

Gerrit Limberg [a,*], Roman Körner [b,1], Hans Christian Buchholt [c], Tove M.I.E. Christensen [a], Peter Roepstorff [b], Jørn Dalgaard Mikkelsen [a]

[a] *Danisco Biotechnology, Langebrogade 1, DK-1001, Copenhagen K, Denmark*
[b] *Department of Molecular Biology, University of Southern Denmark, Odense University, Campusvej 55, DK-5230 Odense M, Denmark*
[c] *Danisco Cultor, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark*

Received 14 September 1999; received in revised form 18 February 2000; accepted 22 February 2000

Abstract

A series of pectins with different distribution patterns of methyl ester groups was produced by treatment with either plant (p-PME) or fungal pectin methyl esterases (f-PME) and compared with those obtained by base catalysed de-esterification. The products generated by digestion of these pectins with either endopectin lyase (PL) or endopolygalacturonase II (PG II) from *Aspergillus niger* were analysed using matrix assisted laser desorption ionisation mass spectrometry (MALDIMS) and high-performance anion-exchange chromatography with pulsed amperometric or UV detection (HPAEC–PAD/UV). Time course analysis using MALDIMS was used to identify the most preferred substrate for each enzyme. For PL, this was shown to be fully methyl esterified HG whereas for PG II, long regions of HG without any methyl esterification, as produced by p-PME was the optimal substrate. The blockwise de-esterification caused by p-PME treatment gave a decrease of partly methylated oligomers in PL fingerprints, which did not effect the relative composition of partly methylated oligomers. PG II fingerprints showed a constant increase of monomers and oligomers without any methyl ester groups with decreasing degree of esterification (DE), but almost no change in the concentration of partly methylated compounds. PL fingerprints of f-PME and chemically treated pectins showed decreasing amounts of partly methyl esterified oligomers with decreasing DE, together with a relative shift towards longer oligomers. PG II fingerprints were characterised by an increase of partly methylated and not methylated oligomers with decreasing DE. But differences were also seen between these two forms of homogenous de-esterification. Introduction of a certain pattern of methyl ester distribution caused by selective removal of certain methyl ester groups by f-PME is the most reasonable explanation for the detected differences.

Keywords: Pectin; Pectin methyl esterase; Pectin lyase; Polygalacturonase; MALDIMS; HPAEC–PAD/UV

☆ Analysis of pectin structure, Part 1.

* Corresponding author. Present address: Alexander von Humboldt Stiftung, Jean Paul Strasse 12, D-53173 Bonn, Germany. Fax: +49-228-833175.

E-mail address: gl@avh.de (G. Limberg).

[1] Present address: Laboratoire de Spectrométrie de Masse Bio-Organique (LSMBO), 1, rue Blaise Pascal, F-67008 Strasbourg, France.

1. Introduction

Pectins are a family of complex galacturonic acid-rich polysaccharides present in the primary cell wall and intercellular spaces of

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00067-7

higher plants [1,2]. They are composed of several subunits, which include homogalacturonan (HG) rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and xylogalacturonan [3]. Homogalacturonan (smooth region) consists of up to 200 (1→4)-linked α-D-galacturonic acid residues [4], which can be methyl-esterified at position 6 and acetylated at positions 2 or 3.

RG I has a backbone composed of the disaccharide repeating unit [α-L-Rha-(1→4)-α-D-GalA-(1→2)]. Arabinan, galactan and arabinogalactan side chains are attached to O-4 of some of the rhamnosyl residues [1]. RG II consists of a (1→4)-linked α-D-galacturonic acid backbone, which is substituted with four different oligosaccharide side chains [5], whereas xylogalacturonan contains substitution of xylose on a HG backbone [6]. The galacturonic acid units in all these subunits can be methyl esterified and acetylated as in the HG.

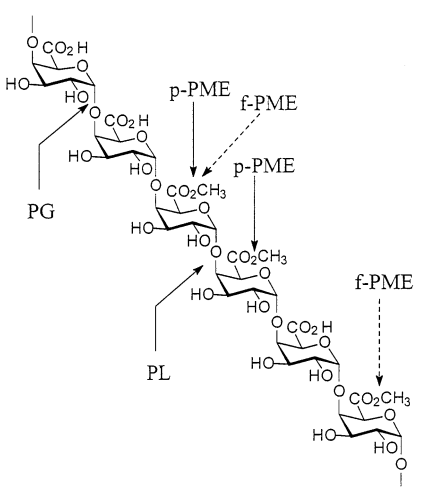
Relatively little is known about the structural basis for the functional properties of pectin, because a detailed structural characterisation is often hindered by its high degree of complexity and heterogeneity.

The availability of homogeneous enzyme preparations has increasingly contributed to the structural characterisation of pectin structure, as recently reviewed by Voragen et al. [7]. Enzymes can be used to specifically fragment the pectin polymer in certain positions and the generated products can be structurally characterised.

MALDIMS is a powerful technique for the analysis of complex mixtures of galacturonic acid oligomers, which requires minimal sample clean up [8]. Methyl esterification patterns on each oligomer in the complex mixture can be determined using a newly developed sequencing method based on ESIMS/MS [9]. Quantification of oligomers can be performed using HPAEC separation technology [10].

In the present study, we have used plant and fungal pectin methyl esterases (p-PME and f-PME) to modify pectins and endopectin lyase (PL) and endopolygalacturonase II (PG II) to analyse the enzymatically modified pectins. Furthermore, we compared the enzymatic processes with alternative chemical treatments. An overview of the enzymes used and their action on HG is given in Scheme 1.

By using PME from either plant or fungal origin, different patterns of methyl esterification will be available and compared with those obtained after chemical treatment. The different esterification patterns influence the way hydrolytic enzymes like PL or PG II cleave the HG backbone. The specificity of the enzyme is reflected in the different oligomer patterns, which were analysed both by means of mass spectrometry and high performance separation techniques.



Scheme 1. Action of pectinases on the homogalacturonan backbone (PG, endopolygalacturonase (E.C. 3.2.1.15); PL, endopectin lyase (E.C. 4.2.2.10); p-PME and f-PME pectin methyl-esterase (E.C. 3.1.1.11) from plant or fungal origin, respectively).

2. Results and discussion

Production and chemical characterisation of model pectins.—Three series of model pectins were generated by separately treating the same mother pectin with p-PME, f-PME and base. Therefore, the variations in the samples result from the de-esterification procedure used. One lot of commercial high ester lime pectin (GRINSTED™ Pectin URS 1200; E81) was chosen as the starting material because it allows greatest flexibility due to a high degree of esterification (DE, 81%). Methyl esterification patterns in the lime pectin raw material are compensated to some extent by the acid catalysed esterification with methanol, which is used in production of the mother pectin to increase the DE to 81%.

Table 1
Analysis data for model pectins [a]

Pectin sample	% DE	% AGA		η (L/g)	Av. M_w (D)	% Dry matter	pH in 1% solution	CS
		In AAIS	In sample					
Mother pectin GRENDSTEW ™ Pectin URS 1200								
E81	81.1	87.3	78.7	0.45	90,000	91.0	3.20	0.066
Samples from de-esterification with p-PME								
P76	76.1	89.7	81.5	0.35	70,000	93.8	3.17	0.061
P73	72.9	92.3	82.6	0.34	68,000	93.2	3.24	0.16
P70	70.4	90.5	80.3	0.29	57,000	93.7	3.19	0.13
P66	66.2	91.7	83.6	0.30	60,000	95.5	3.33	0.95
P60	59.7	90.2	81.7	0.26	52,000	96.8	3.34	gel.
P53	52.6	89.1	80.9	0.28	55,000	90.8	3.38	gel.
P46	46.1	89.8	78.3	0.26	51,000	93.1	4.07	gel.
P41	40.5	87.9	78.7	0.26	51,000	89.6	4.12	n.a.
Samples from de-esterification with f-PME								
F76	75.7	89.8	85.9	0.46	93,000	95.8	3.17	0.067
F69	68.9	88.4	82.4	0.40	81,000	95.8	3.10	0.057
F58	58.2	90.4	82.5	0.40	81,000	94.6	3.16	0.031
F43	42.6	89.3	77.9	0.37	74,000	93.0	3.16	0.28
F31	31.1	86.6	74.5	0.35	69,000	92.5	4.72	n.a.
F11	11.0	84.7	66.8	0.27	53,000	88.3	4.40	n.a.
Samples from base catalysed de-esterification								
B71	71.3	89.5	82.6	0.40	80,000	95.0	2.99	0.11
B64	63.8	88.2	83.7	0.38	75,000	95.2	2.65	0.12
B43	42.9	88.1	83.5	0.22	43,000	94.2	2.32	n.a.
B34	33.7	89.3	85.6	0.45	89,000	94.0	2.47	n.a.
B15	14.7	91.8	88.2	0.31	62,000	94.1	3.56	n.a.
PGA	1.4	86.7	64.6	0.20	39,000	85.5	5.15	

[a] DE, degree of esterification; AGA, anydro-galacturonic acid; AAIS, acid and alcohol insoluble solids; av. Mw, weight average molecular weight; CS, calcium sensitivity; gel., gelation of test sample; n.a., not analysed.

The action of p-PME is believed to follow a single chain mechanism [11] and to result in the consecutive removal of a number of neighbouring methyl ester groups. This gives rise to the introduction of so-called block-structures of adjacent free galacturonic acid units on the HG backbone, which allow calcium cross-linking of pectin chains. The correlation between increasing formation of the block-structures and decreasing DE due to p-PME treatment was reflected in the changes in calcium sensitivity shown in Table 1. In this way calcium sensitive high ester pectins (DE > 50%) can be produced. In addition to increased calcium sensitivity, p-PME treatment gives rise to improved protein stabilisation characteristics for high ester pectins [12].

De-esterification by f-PME is believed to result from a multiple chain mechanism, which is believed to lead to a random removal of methyl ester groups [11]. When f-PME is used for de-esterification (F-series), no increase of calcium sensitivity was observed until a DE < 50% was reached.

Chemical de-esterification using base catalysis (B-series) is — like f-PME treatment — also believed to produce a random methyl esterification pattern.

Chemical analysis data for the samples produced are summarised in Table 1. Due to the higher pH conditions used for production of the P- and B-series, these samples show reduced average molecular weight compared with the F-series pectins caused by backbone cleavages, which is due to base catalysed β-elimination reactions [13].

Analysis of enzyme specificity.—In order to use enzymes as analytical tools for analysis of intramolecular homo- or heterogeneity of pectin, knowledge about the substrate specificity of the applied enzymes is required. For both enzymes used in this work, full amino acid sequence analysis and crystallographic data [14] are available. Catalytic residues have been identified by site directed mutagenesis [15]. For PG II, the substrate binding site is made up of seven subsites [16], whereas for PL an even larger number — around 9–13 subsites — is discussed [17]. Both enzymes have been studied on either fully methyl esterified oligogalacturonides or oligomers without any methyl esterification [15,16]. Work on well defined partly methyl esterified oligogalacturonides is limited due to difficulties in obtaining pure materials in sufficient quantities [18].

We have applied our recently developed MALDIMS method [8] to analyse the products formed by enzymatic digestion of partly methyl esterified HG. Products formed during the very early stages of the reaction were compared with those present at intermediate and final stages of the enzymatic reaction. This provided important information about the preferred sites of enzyme attack on the HG and the structural requirements for a successful enzyme substrate interaction. The latter can be analysed by sequence analysis of products that accumulated during the reaction, either because of slower turn over rates or the absence of further cleavage positions on the oligomer.

During short incubation times, PL liberated mainly higher and fully methyl esterified oligomers (DP 4–15) from E81 (Fig. 1(A)) and thus has a preference to cleave in areas

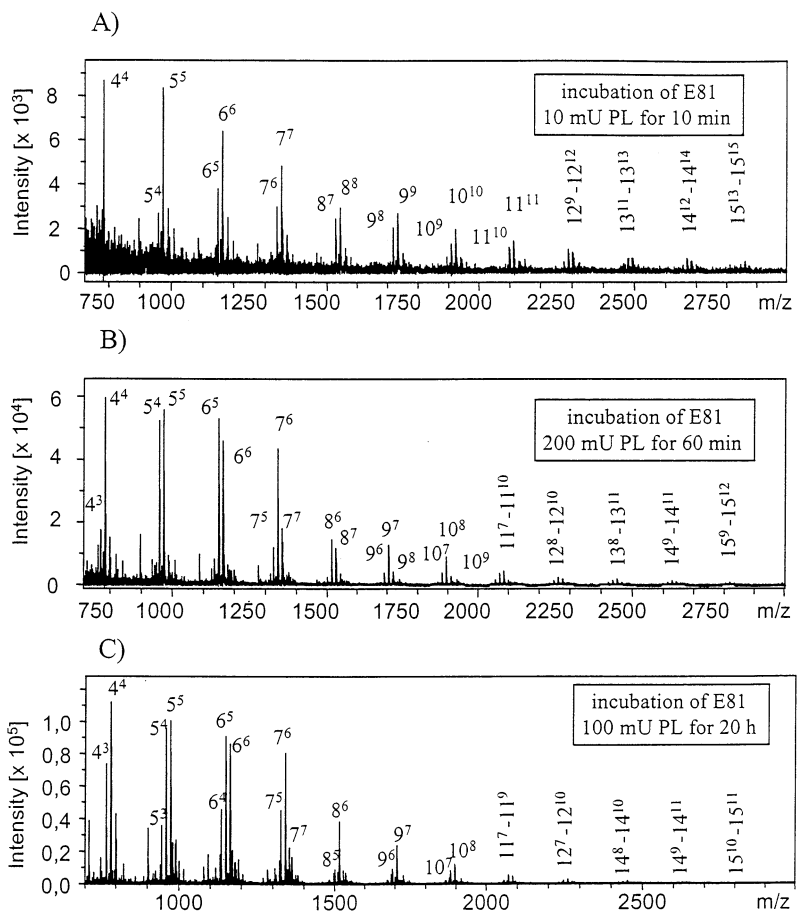


Fig. 1. Digestion of E81 mother pectin with different amounts of PL at different incubation times analysed by MALDIMS in positive mode to also detect fully methylated species: (A) start of reaction; (B) progression; (C) chosen end-point of reaction. Sodium cationised ions $[M + Na]^+$ are labelled by giving the DP in normal and the number of methyl ester groups in superscript numbers.

with 100% methyl esterification. However, if higher amounts of PL and longer incubation times were applied, some initial products were cleaved further (Fig. 1(B) and (C)), although the DE value of the accumulating oligomers was still very high. The presence of smaller fully methyl esterified oligomers (up to DP 7) indicated a weaker binding of those substrates due to an incomplete saturation of all substrate binding subsites of the enzyme. Analysis of the oligomers present at the chosen end point (20 h) showed a complex mixture of oligomers (Fig. 1(C)) with large variations in DP and DE. Fully esterified oligomers were still present up to DP 6. Kinetic studies (data not shown) showed that the cleavage rate was constantly slowed down but never reached an end point. The reaction was stopped after an incubation period of 20 h with 0.1 U PL/5 mg pectin. If at that stage an additional aliquot of enzyme was added, about 2–3% additional product formation could be observed after a second 20 h incubation period (data not shown).

Recently, we reported [9] the results obtained by ESI ion trap MS/MS sequence analysis of oligomers from extended PL digestion. We demonstrated that most oligomers were present in mixtures of several stereoisomers regarding their methyl esterification pattern and several oligomers containing a free galacturonic acid on the newly formed reducing end were detected. The cleavage point for PL is therefore not limited to two adjacent methyl esterified galacturonic acid units but can also be positioned between a free acid and a methyl esterified species. This explains the large complexity in the oligomer compositions. However, no oligomers without any methyl ester groups were detected in the PL digest. The action of PL is therefore limited to fully or partly methyl esterified regions on the HG. PGA or block-structures of adjacent galacturonic acids as present in the P-series pectins cannot be cleaved by PL.

MALDIMS spectra from PG II digests of P41 and F31 (Fig. 2) were more homogeneous, with respect to the number of different oligomers formed, compared with the data obtained from PL digests. Kinetic studies (data not shown) on P-series pectins allowed us to distinguish three reaction phases. In the first phase, a rapid digestion of the unesterified block-structure occurred. The reaction rate was similar to that observed for PGA and the products formed were oligomers without any methyl esterification (Fig. 2(A)). In the second phase, the reaction rate slowed down to levels comparable with the initial rates of PG II digestion of the F- and B-series pectins. Partially methyl esterified oligomers were seen in MALDIMS and only smaller oligomers ($DP < 6$) without any methyl ester groups were detected. In the third phase (Fig. 2(C) and (D)), these small non-esterified oligomers were cleaved further. ESIMS analysis of these sample showed that the mono-, di- and trimers were the only species present without any methyl ester groups (data not shown). We have previously described [9] that partially methyl esterified oligomers obtained from an extensive PG II digestion carry one or two free galacturonic acid units on the reducing end and one to three adjacent free galacturonic acid groups on the non-reducing end. A methyl esterified unit was never observed to be located at either end of the oligomers, indicating the strict requirement of PG II to cleave between two adjacent free galacturonic acid units.

For the F- and B-series pectins, the reaction kinetics of PG II digestion were divided into two phases corresponding to phases two and three for the P-series pectins. In contrast, PG II digestion of PGA showed two phases comparable with phases one and three for the P-series pectins.

Fingerprinting of model pectins with pectin lyase.—PL is most active on fully methylated HG and therefore shows highest activity on E81. De-esterification of E81 led to a continuous decrease of oligomer formation, as measured by the increase in OD_{235nm} after the 20 h incubation period. Thus PL generated threefold less product with P41 and B43 than E81. In contrast, PL treatment of F43 lead to a tenfold drop in product formation compared with E81. These results indicated that different methyl esterification patterns were present on the F-, B- and P-series pectins. Clearly the f-PME destroys PL cleavage sites most efficiently, whereas p-PME produces pectins with

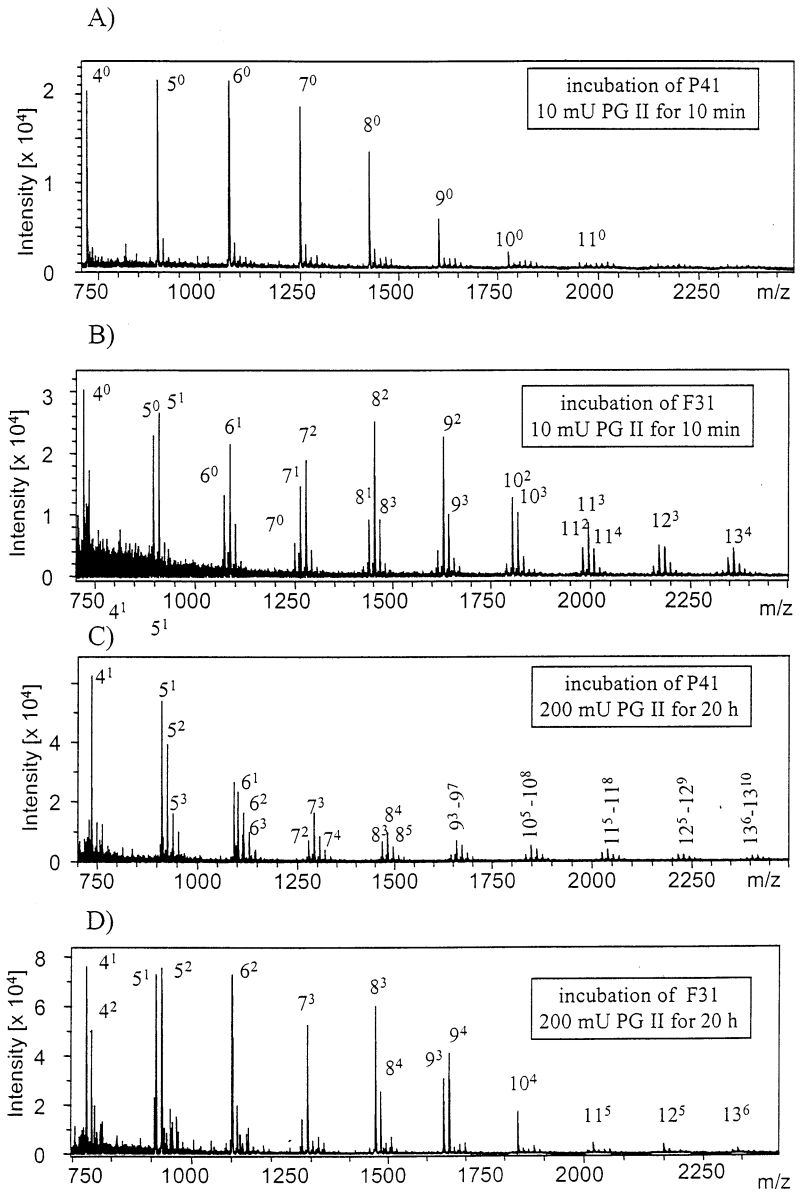


Fig. 2. Digestion of blockwise de-esterified P41 and a homogenously de-esterified F31 using different amounts of PG II at different incubation times, as analysed by MALDIMS in negative ion mode: (A) start of reaction with P41; (B) start of reaction with F31; (C) chosen end-point of reaction with P41; (D) chosen end-point of reaction with F31. $[M-H]^-$ are labelled as in Fig. 1.

the highest rate of PL degradation, indicating that areas with high DE are still present on the HG of P41.

To further analyse the nature of the oligomers formed by PL digestion, we used high-performance anion-exchange chromatography (HPAEC). Due to the complexity of the oligomer mixture (see Fig. 1) a complete separation of all partially methyl esterified oligomers was not achieved. Therefore we determined the DP distribution of the oligomers after base catalysed de-esterification at 2–3 °C. The oligomers were separated on a Mono Q anion exchange column and detected by their A_{235}, as earlier described by Endress et al. [19]. Because every oligomer contains one unsaturated unit at the non-reducing end, the DP distribution can be obtained on a molar basis directly from the relative peak integration area. Multiplication with the absolute molar concentration of double bonds and the molecular mass of the oligomer yielded the absolute concentrations of oligomers in mg/mL, as shown in Fig. 3. The P-series pectins (Fig. 3(A)) showed very similar oligomer chain length distribution together with a con-

stant decrease in oligomer concentrations with decreasing pectin DE. This shows that PL does not digest those areas modified by p-PME. These de-esterified regions contain large blocks of adjacent free galacturonic acids and are therefore not represented in the oligomer patterns. The oligomers formed by PL digestion of P-series pectins originated from essentially unchanged HG, and therefore PL showed identical cleavage patterns.

However, de-esterification using f-PME resulted in a change in oligomer chain length distribution (Fig. 3(B)). Large oligomers (DP >8) relatively increased in PL digests accompanied by a significantly higher drop in total oligomer concentrations. As a consequence, the absolute concentrations of large oligomers (DP >15) for F58 were found to be higher than for E81, F76 and F69, even so, the total oligomer concentration was significantly lower for F58 compared with E81, F76 and F69. The trimer was, in all F-series samples, the oligomer present in the highest concentration. For F31, no double bond formation due to cleavage by PL could be determined by changes in OD at 235 nm.

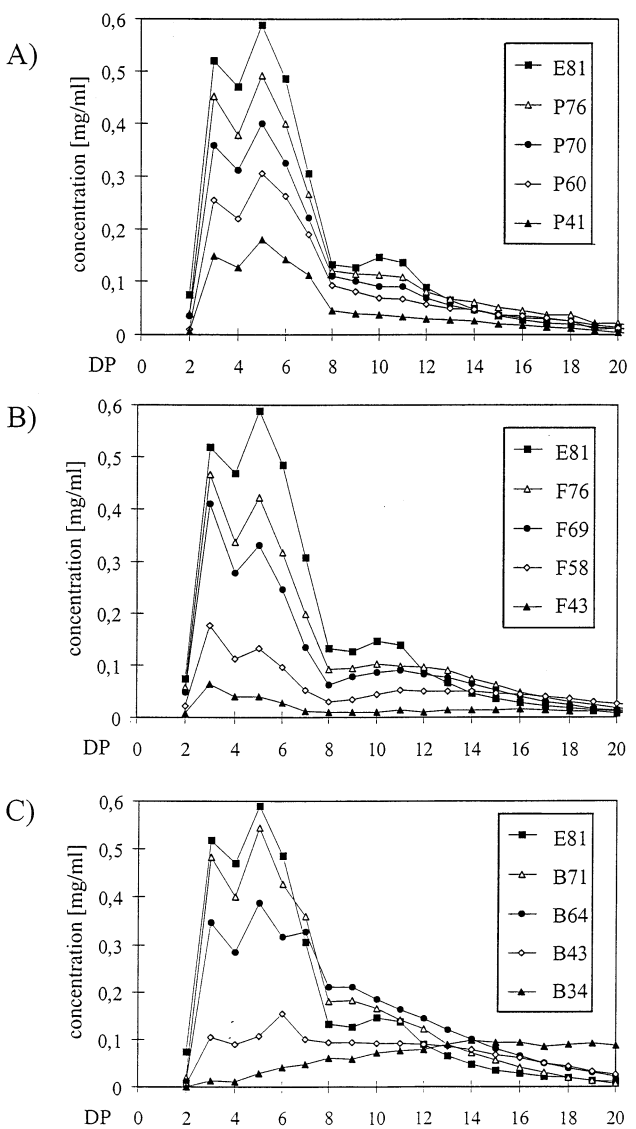


Fig. 3. Fingerprinting of model pectins using PL. The amount of oligomers formed is expressed in mg/mL, which was calculated from peak areas of Mono Q separation and increase of OD at 235 nm. For details see Section 4: (A) P-series; (B) F-series; (C) B-series pectins.

The B-series pectins showed a similar behaviour with respect to the increasing relative and absolute amounts of larger oligomers, but the drop in the total oligomer concentrations were much lower than found for the comparable F-series samples. Already for B71 the trimer was no longer the oligomer present in the highest concentration. These results underline the different methyl esterification patterns found in these two series.

To investigate the charge distribution on the oligomers formed by PL digestion, the still methyl esterified digests were separated on Mono P, as shown in Fig. 4. When fractions from this separation were analysed using MALDIMS, the basis of separation was found to be determined predominantly by the number of charges present on the oligomers. For example, some fractions contained oligomers with different DPs but always two free acid groups (data not shown). However, oligomers with the same mass over charge ration were detected in different fractions, indicating that the relative position of the acid groups also influenced retention times.

The P-series pectins (Fig. 4(A)) showed similar relative peak intensities in the area for highly esterified oligomers with lower DP (0–25 min). Parts of the new formed block-structure could now be detected as a late eluting peak at about 55 min retention time. This fraction was the only fraction that was recognised by the block-structure specific antibody PAM 1 [20]. f-PME treated pectins (Fig. 4(B)) contain, with decreasing DE, lower amounts of weakly binding high methylated oligomers and higher amounts of stronger binding oligomers containing less methyl ester groups.

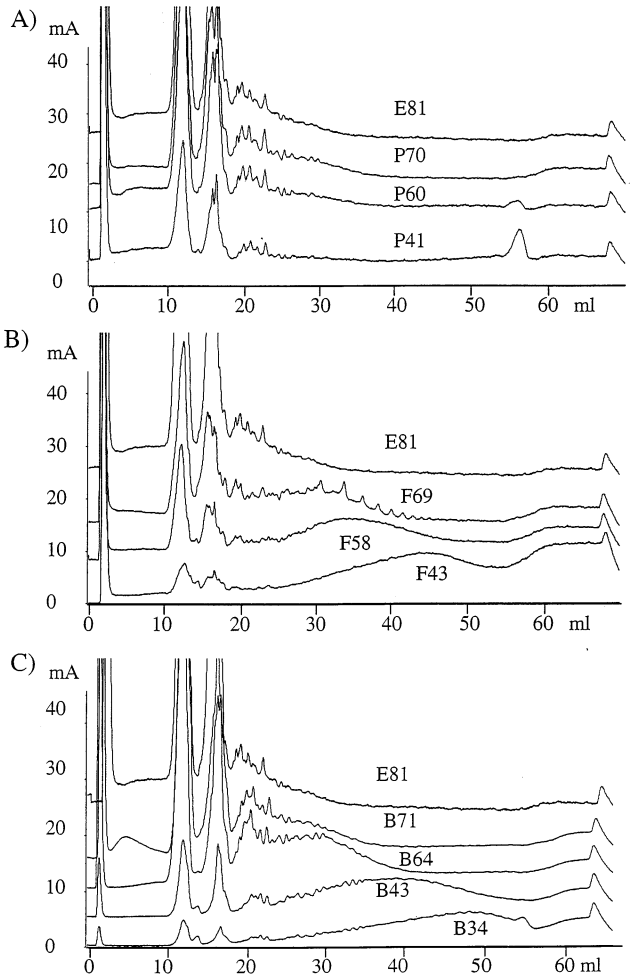


Fig. 4. Fingerprinting of model pectins using PL. Charge distribution on oligomers generated by PL digestion as retention on Mono P weak anion exchange chromatography (235 nm trace). For separation see Section 4: (A) P-series; (B) F-series; (C) B-series pectins.

This is consistent with the above mentioned observation that de-esterification with f-PME leads to a deletion of PL cleavage sites and thereby results in longer oligomers with less methyl ester groups. The overall increase in the number of charges present on each oligomer resulted in increased retention times.

The B-series pectins (Fig. 4(C)) and the F-series pectins gave similar profiles, although the shift to longer retention times for the B-series pectins was not as significant as for the F-series pectins. This can be best seen by comparison of pectins F43 and B43. This result provides additional evidence that F- and B-series pectins differ in their pattern of methyl esterification.

Fingerprinting with PG II.—The fingerprints generated by PG II digestion of the pectins were analysed for their quantitative oligomer chain length distribution and for their charge distribution by HPAEC with PAD detection [10a]. Due to the high pH of the eluent, all methyl ester groups were removed instantaneously when the material was introduced in the solvent stream and separation of the oligomers takes place according to DP. Molar response factors determined on purified oligomers were used for quantification. The complete digestion of the tetramer without methyl-ester groups (as analysed by MALDIMS) was chosen as the end point of the reaction. Further addition of enzyme and longer incubation times yielded less than 1% additional product formation. The amount of enzyme applied (0.2 U) was adjusted to allow full digestion of all pectins within the 20 h time frame.

Fingerprints on P-series pectins (Fig. 5(A)) showed that de-esterification using p-PME led to an increase in the amounts of mono-, di- and trimer, only a minor increase of the tetra- and pentamer and no measurable changes in the quantities of large oligomers. This indicates that p-PME treatment generates block-structures of adjacent free galacturonic acids that are cleaved by PG II to give mono-, di- and trimers without any methyl ester groups. The slight increase in oligomer concentrations for tetra- and pentamer from E81 to P70 could be caused by cleavages close to the new formed block-structures because no further increase can be seen after this initial drop in DE.

PG II digestion of f-PME treated pectins showed a distinctively different profile (Fig. 5(B)). The concentrations of the monomer and the oligomers with DP 2–10 were increased with decreasing DE up to F58. These stable products were no longer substrates for PG II due to their methyl esterification. The oligomer concentration for all F-series pectins peaked for the pentamer. This is probably due to a specific de-esterification pattern created by f-PME on the HG, because this phenomenon was not observed for the B-series pectins (Fig. 5(C)). Further de-esterification (F43, F31, F11) yielded additional increases in

the concentration of the monomer and small oligomers (DP 2–6), whereas concentrations of larger oligomers (DP >6) started to decrease. Furthermore, f-PME pectins showed a much higher rate of cleavage with PG II than the corresponding B-series pectins with similar DE values.

There was also an increase in concentrations of oligomers with DPs 1–12 observed following PG II digestion of the B-series pectins with decreasing DE (Fig. 5(C)). The trend to higher concentrations for smaller oligomers was clearly visible for B34 and B15, but no distinct maximum for a specific oligomer length was detectable.

Analysis using HPAEC at neutral to acidic pH [10b] confirmed that for the P-series pectins all the oligomers contributing to the increase of DP 1–3 were without any methyl ester groups. The f-PME treated samples contained high amounts of methyl esterified oligomers (Fig. 2). For example, most of the formed trimer contained one methyl ester group located on the central unit, as shown earlier by described ESIMS/MS measurements [9].

During the preparation of this manuscript, a paper by Daas et al. [21] appeared in which similar results were described. The authors have used a method that involves digestion with a different endo-PG and quantification of the oligomers without any methyl ester groups, using HPAEC at acid pH. Their results for de-esterification with another plant pectin methyl esterase from tomato and chemical de-esterification are comparable with our results for the p-PME and base treated pectins. The effect of f-PME and the difference to chemical modification was not investigated by them.

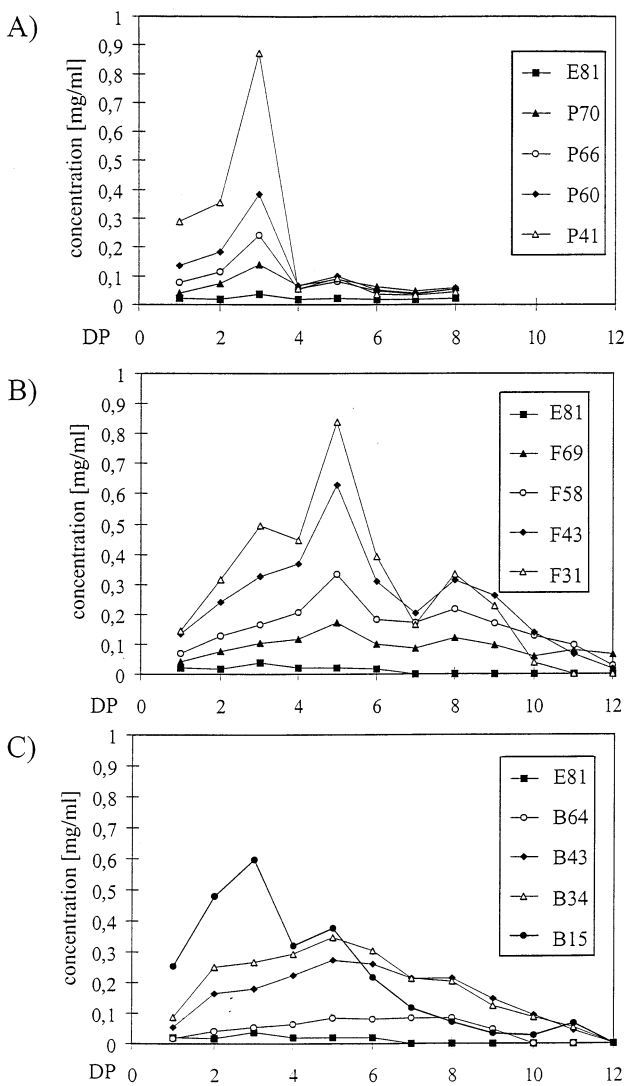


Fig. 5. Fingerprinting of model pectins using PG II. The amount of oligomers formed is expressed in mg/mL, which was calculated from PAD-response on Dionex HPLC relative to the response for glucuronic acid as internal standard. For details see Section 4: (A) P-series; (B) F-series; (C) B-series pectins.

3. Conclusions

The technique of enzymatic fingerprinting involves a combination of detailed product characterisation using MALDIMS and earlier described ESIMS/MS methods and product quantification using HPAEC with PAD or UV detection. Time course analysis of the enzymatic fragmentation of pectin allowed us to study the substrate specificity of PL and PG II on their natural pectin substrate in great detail.

Significant differences in the de-esterification mechanism of plant and fungal pectin methyl esterases (p-PME and f-PME) and a base catalysed de-esterification were shown using fingerprinting with PL and PG II. PG II has a more restricted substrate specificity than PL and therefore gave a clear differentiation between the presence of a blockwise or different forms of homogeneous de-esterification patterns. Fingerprinting using PL, however, was better able to show the differences be-

tween the two forms of homogeneous de-esterification patterns obtained by f-PME or base treatment.

De-esterification using p-PME has been identified as a process that selectively introduces block-structures of adjacent free galacturonic acid units on the HG, whereas f-PME and base treatment lead to two different forms of homogeneous methyl esterification patterns. For f-PME treated pectins, the PG II digestibility is greatly enhanced and simultaneously the PL digestibility is significantly decreased compared with pectins with similar DE obtained from base treatment. The increased fragmentation of f-PME treated pectins by PG II can be rationalised as the role f-PME plays in its natural synergy to PG II action for most efficient breakdown of plant cell walls by fungal attack. This implies that f-PME produces a pattern of methyl esterification that favours endo-PG II attack.

Additionally, enzymatic de-esterification will reduce the DE in the HG only, whereas base treatment will reduce the total DE. This effect of substrate specificity could also explain some of the observed differences between the B- and F-series pectins.

As a final conclusion, we show that three different de-esterification mechanisms for pectin can be distinguished. A blockwise de-esterification caused by the action of p-PME and two different forms of homogeneous de-esterification caused by either f-PME and base treatment. f-PME introduced a homogeneous pattern of methyl esterification that favours endo PG II attack. Whether the homogeneous methyl esterification pattern obtained by base treatment is similar or equal to a statistical random distribution needs to be further investigated.

4. Experimental

Purification of pectin methyl esterases.—Plant pectin methyl esterase (p-PME) was purified from orange peels as described [22]. f-PME from *Aspergillus niger* was purified from Pektolase™ (Danisco Ingredients, Brabrand). A 45–50 mL sample was dialysed against 50 mM MES (pH 6.8) and then applied to a DEAE-Sepharose FF column (1.5 × 30 cm). The flow rate was 100 mL/h. Unbound proteins were eluted with 50 mM MES (pH 6.8). The bound proteins were eluted with a linear NaCl gradient (0–0.5 M, 1000 mL) in the same buffer. The protein elution profile was monitored at 280 nm. Fractions of 10 mL were collected and those containing PME activity were pooled and dialysed against 50 mM NaOAc (pH 5.0) overnight. The dialysed PME fraction was further fractionated by anion-exchange chromatography on a HiLoad Q Sepharose HP column 26/10 (Pharmacia, Uppsala). The column was equilibrated with 50 mM NaOAc (pH 5.0). Proteins binding to the column were eluted with 1000 mL of a linear gradient from 0 to 0.3 M NaCl. The flow rate was 100 mL/h and fractions of 10 mL were collected. The fractions containing PME activity were pooled and applied to HiLoad Phenyl Sepharose HP 26/10, which was equilibrated with 1.8 M $(NH_4)_2SO_4$ in 50 mM NaOAc (pH 5.0). The bound proteins were eluted with 1000 mL of a linear gradient from 1.8 to 0 M $(NH_4)_2SO_4$. The flow rate was 100 mL/h. The pooled fraction containing PME activity was dialysed against 50 mM NaOAc (pH 5.8) containing 0.1 M NaCl and then fractionated by size-exclusion chromatography (SEC) on a HiLoad Superdex 75 26/60 column equilibrated with the same buffer. All operations were performed at 4 °C. After SEC the PME fraction showed only one protein band on SDS-PAGE with a M_w of 43 kDa. PME activity was determined at pH 4.5, as described by Christensen et al. [22].

Purification of pectin lyase. PL from *A. niger* was purified from Pektolase™ (Danisco Ingredients, Brabrand). Aliquots of Pectolase were dialysed and then separated on a DEAE-Sepharose FF column, followed by HiLoad Phenyl Sepharose equilibrated with 50 mM MES (pH 6.8), as described for f-PME. The fractions containing PL activity were pooled and dialysed against 50 mM triethanolamine (pH 7.5). The dialysed sample was applied to a HiLoad Q Sepharose 26/10 column. The bound proteins were eluted with linear NaCl gradient in 50 mM triethanolamine (pH 7.5) (0–0.5 M, 1000 mL). The PL fraction was

finally purified by gel filtration on a HiLoad Superdex 75 26/60 in 50 mM NaOAc, 0.1 M NaCl, pH 5.8, as described for f-PME after dialysis against the same buffer.

SDS-PAGE revealed that PL was homogeneous. PL cleaves pectin by β-elimination and generates unsaturated pectin oligomers containing a terminal non-reducing residue with a 4,5-double bond. The PL activity was followed continuously in a spectrophotometer at 235 nm using 1 mL 0.5% citrus pectin (DE 70%) in 50 mM NaOAc (pH 4.8) at 40 °C.

PG II was a kind gift from Drs Jaap Visser and Jacques Benen, Department of Molecular Biology of the Industrial Microorganism Wageningen Agricultural University, The Netherlands. Enzyme activity was determined as described before [16] using PGA as a substrate.

Synthesis of model pectins.—The raw material for the preparation of the P-, F-series and B-series pectins was lime peel from Mexican lime (*Citrus aurantifolia*). A commercially extracted slow set pectin from this peel was used as the parent pectin for commercial production of GRINDSTED™ Pectin URS 1200 (E81) by esterification in acidified MeOH.

De-esterification using p-PME (P-series). GRINDSTED™ Pectin URS 1200 (125 g) was dissolved in 4 L hot demineralised water under efficient stirring. Sodium chloride (46.6 g) was added and the solution was stirred for at least 2 h, while the temperature decreased to 40 °C. The pH was adjusted to 7.0 by addition of 1 M NaOH under efficient stirring. p-PME was added and the enzymatic de-esterification was continued at 40 °C. A pH of 7.0 was maintained by automatic addition of 1 M NaOH until the desired DE was achieved. This could be estimated from the consumption of NaOH. The de-esterification was stopped by lowering the pH to 3.0 by addition of 4 M HCl. The pectin solution was heated to 75 °C for 5 min to inactivate the enzyme. After cooling to below 40 °C, the pectin was precipitated by mixing with 4 L isopropyl alcohol and the precipitation mixture was stirred for 1 h. The precipitated pectin was filtered off and washed twice by suspension in 2 L of 3:2 isopropyl alcohol–water for 1 h and finally pressed in a cotton cloth and dried in an oven at 45 °C for 20 h. In general about 115 g of dry pectin was isolated and milled to a fine powder.

De-esterification using f-PME (F-series). GRINDSTED™ Pectin URS 1200 (125 g) was dissolved as described above. The pH was adjusted to 4.5 by addition of a solution of 20% aq Na_2CO_3. An appropriate amount of f-PME was added and the enzymatic de-esterification was continued at 40 °C and pH 4.5 by automatic dosage of a 20% aq soln of Na_2CO_3. When the desired DE was achieved the reaction was stopped as described above. After cooling to below 40 °C, the pectin was precipitated in 5 L isopropyl alcohol and stirred for 1 h. The precipitated pectin was washed and dried as described above. About 115 g of dry pectin was isolated and milled to a fine powder.

Base catalysed de-esterification (B-series). GRINDSTED™ Pectin URS 1200 (125 g) and NaCl (5.46 g) were dissolved in 4 L hot demineralised water under efficient stirring. After stirring for at least 1 h the solution was cooled in an ice bath to 2–3 °C. The temperature was kept below 4 °C, while the pH was increased to 9.0 by addition of 1 M NaOH. The mixture was stirred at that temperature and 1 M NaOH was added to maintain the pH about 9.0 until the desired DE was achieved. For those pectins having a DE above 40% the de-esterification process was stopped by lowering the pH to 2.8 by addition of 4 M HCl. The pectin was precipitated by addition to 5 L isopropyl alcohol and the precipitate was treated as described above. About 115 g of dry pectin was isolated and milled to a fine powder.

If the desired DE of the pectin was below 40%, the pectin was precipitated in 1.2 L 2 M HCl. The precipitated mixture was kept below 5 °C during the night, after which the voluminous precipitate was filtered off in a cloth and washed at room temperature (rt) by suspension for 2 h in a mixture of 800 mL MeOH and 1 L 2 M HCl. After filtration through a cotton cloth, the precipitate was further washed by suspension for 2 h in 2 L of 1:1 MeOH–water, followed by a wash with 2 L 67:33 MeOH–water. Finally, the precipitate was pressed in a cloth and dried in an oven at

45 °C for 20 h. About 105 g of dry pectin powder was isolated after milling.

Preparation of polygalacturonic acid (PGA). GRINDSTED™ Pectin URS 1200 (150 g) was dissolved in 3.8 L hot demineralised water and after dissolution the mixture was cooled to 50 °C. The pH was increased to 4.5 by addition of 20% Na_2CO_3 soln under efficient stirring. f-PME (1400 U) was added and the reaction progressed for 4 h at 50 °C, with addition of 1 M NaOH to keep the pH constant at 4.5. The mixture was stirred without further pH adjustment at rt for another 16 h. The mixture was then cooled to 2–3 °C and the pH was increased to 11 and kept there by addition of 1 M NaOH. After reaction for 4 h the temperature was increased to 40 °C for 1 h and finally the pH was lowered to 5.0 by addition of 4 M HCl and the solution was heated to 70 °C for 5 min. After cooling to 40 °C, the polygalacturonate was precipitated by pouring into 4 L isopropyl alcohol. After filtration through a cotton cloth the precipitate was further washed by suspension for 2 h in 2 L of 1:1 MeOH–water, followed by a wash with 2 L 67:33 MeOH–water. Finally the precipitate was pressed in a cloth and dried in an oven at 45 °C for 20 h. The yield of polygalacturonate was 105 g.

Characterisation of pectins.—Commercial lime pectins usually contain less then 0.2% acetylation. Therefore the degree of acetylation has not been analysed separately but is included in the degree of esterification.

Determination of DE and content in galacturonic acid (%AGA). The procedure is a modification of the method described in the Food Chemical Codex 3rd edition [23]. Approximately 3 g sample (a) was accurately weighed into a beaker and was stirred for 10 min at rt with a mixture of 3 mL concd HCl added to 60 mL of 3:2 isopropyl alcohol–water. The suspension was quantitatively transferred to a weighed fritted glas tube with the same HCl and aq isopropyl alcohol mixture as above, followed by 3:2 isopropyl alcohol–water, until the filtrate was free of chloride. The residue was finally washed with 10 mL isopropyl alcohol and dried in an oven at 105 °C for 2.5 h. After cooling the residue was weighed (b). The content of acid and alcohol insoluble solids (AAIS) was calculated as:

$$\mathrm{AAIS} = b/a \times 100\%$$

About 0.25 g of the dried material (c) was exactly weighed in a 250 mL conical flask, moistened with 0.5 mL EtOH and dissolved in 100 mL of carbonate free distilled water. The sample was titrated with 0.1 M NaOH until pH 9.0 to record the initial titre V_1. Then exactly 10 mL 1 M NaOH was added and mixed and the sample was left tightly closed for 15 min at rt for saponification of ester groups. Then exactly 10 mL of 1 L M HCl was added and stirred until the precipitated material had dissolved again. The mixture was titrated with 0.1 M NaOH until pH 9.0 to record the final titre V_2.

The DE was calculated as:

$$\mathrm{DE} = V_2/(V_1 + V_2) \times 100\%$$

The content of AGA in AAIS was calculated as:

$$\{\mathrm{AGA\ in\ AAIS}\} = (1.7607 \times V_1 + 1.9016 \times V_2)/c\%.$$

The content of AGA in the pectin sample was calculated as:

$$\{\mathrm{AGA\ in\ sample}\} = \{\mathrm{AGA\ in\ AAIS}\} \times b/a\%$$

Determination of intrinsic viscosity and average molecular weight. The intrinsic viscosity $[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c}$ is determined by extrapolating η_{sp}/c to $c = 0$. The determination of η_{sp}/c at low pectin concentration is a good approximation to the intrinsic viscosity, being particularly useful to compare the average molecular size of pectin samples.

A 1% aq sodium hexametaphosphate soln was prepared and the pH was adjusted to 4.75 by addition of a few drops of concd HCl. A pectin sample (0.09% w/v based on dry matter) was dissolved in this solution. The sample was transferred to a Hoeppler viscometer and thermostated at 21 °C. The falling time of a glass ball ($\varphi = 15.6$ mm) was measured for the test solution (t_{test}) and the sodium hexametaphosphate solution was used as reference ($t_{ref.}$). The specific viscosity was determined as

$$\eta_{sp} = t_{test}/t_{ref.} - 1$$

and the intrinsic viscosity was approximated by

$$[\eta] \approx \eta_{sp}/c \text{ where } c = 1 \text{ g/L, (0.9 g pectin dry matter} + 0.1 \text{ g moisture).}$$

The equation $M_w \approx 200{,}000 * \eta_{sp}$ was used to approximate the weight average molecular weight in the range 50,000–120,000 Da.

Determination of dry matter and pH of pectins. The content of dry matter in the pectin samples was determined by gravimetry after drying of pectin in an oven for 2.5 h at 105 °C.

The pH of the pectin samples was measured in a 1% aq pectin soln at rt.

Determination of calcium sensitivity (CS) in high ester pectins. CS is defined as the relative increase of viscosity of a pectin solution in the presence of Ca^{2+} ions. The following test method based on a modification of the procedure described by Glahn et al. [24] was used.

Pectin (4.00 g) was dissolved in about 600 mL of demineralised water. The pH was adjusted to 1.50 by addition of 1 M HCl. The sample was diluted to 666.67 g with demineralised water. This solution (290.0 g) was weighed in two beakers. To the reference sample, 10.0 mL of 1.00% aq sodium hexametaphosphate soln was added. To the other samples, 10 mL of 0.25 M $CaCl_2$ soln was added. Both samples were mixed with 50.0 mL 1 M NaOAc buffer (pH 4.70). After stirring for at least 15 min, the viscosity of the reference and the test sample were measured in a Brookfield DVII viscometer (spindle 2, 60 rpm) at 21 °C. CS was calculated as:

$$\text{CS} = \frac{\text{viscosity of test sample}}{\text{viscosity of reference sample}} - 1$$

If the test solution gels, the CS is too high to allow an accurate determination of CS.

Enzymatic fingerprinting using pectin lyase (PL).—A pectin sample was dissolved in a 50 mM NaOAc buffer (pH 5.0) at a concentration of 5 mg/mL by overnight shaking at rt. Pectin lyase (100 mU) was added to 1 mL of the above pectin solution and was incubated at rt for 20 h. The reaction was stopped by incubating the samples in a boiling water bath for 5 min. The total amount of newly formed oligomers was determined spectrophotometrically. A 50 μL treated sample was diluted with Milli Q water to 1 ml and the absorption was read at 235 nm against the untreated sample solution at the same dilution. The molar amount of new formed oligomers was calculated as

$$c = [(OD_{235}\{\text{PL treated}\} - OD_{235}\{\text{untreated}\}) /5500] \times 20 \text{ [mol/L]}$$

using the molar absorption coefficient $\varepsilon = 5500 \text{ mol}^{-1}$ for methyl esterified 4,5 anhydro-α-D-galacturonide [25].

For saponification of methyl ester groups, the samples were cooled down to 3 °C and 0.1 mL of 0.5 M NaOH per 1 mL of pectin solution was added to increase the pH to 12. The sample was shaken for 16 h at 3 °C and then it was neutralised by the addition of 0.1 mL of 0.5 M HCl per 1 mL pectin solution. The samples were centrifuged and passed over a 0.45 μm filter prior to chromatographic analysis.

An Äkta Explorer 100 (Pharmacia) with UV detection at 235 nm was used for HPLC analysis. Milli Q water and 0.75 M NH_4HCO_3 in Milli Q water were used as eluents A and B at a flow rate of 1 mL/min. Eluents were degassed prior to use. An aliquot (100 μL) of de-esterified sample was loaded onto an analytical Mono Q (HR 5/5, Pharmacia) strong anion exchange column equilibrated with 95% eluent A and 5% eluent B. After 5 min at equilibration conditions, a stepwise linear gradient (10 min 5–45% B; 30 min 45–80% B, 10 min 80–100%) was used for elution. 4,5-Unsaturated galacturonic acid oligomers were eluted in order of their DP. After each run the column was washed for 10 min with 100% B and allowed to re-equilibrated for 13 min with 5% B. The peaks were integrated and their area were calculated as relative values of the total integration areas of all peaks. The relative peak area for each oligomer peak was multiplied by the overall oligomer concentration determined by spectrophotometric measurement at 235 nm, as described above, and multiplied by its molecular mass to give the amount of oligomer expressed in mg/mL. The results for the P-, F- and B-series pectin are shown in Fig. 3.

For qualitative analysis of the PL fingerprints prior to de-esterification, an aliquot of the crude digest was centrifuged and filtered and a 100 μL sample was loaded onto an analytical Mono P (HR 5/5 Pharmacia) weak anion exchange column equilibrated with 100% eluent A. The HPLC system flow rate and eluents A and B were as described above. Fully methylated oligomers were eluted with 100% A in 5 min. Then a stepwise linear gradient (0–10% B in 20 min, 10–50% B in 30 min and 50–100% B in 5 min) was used to elute the partially methyl esterified oligomers. After each run, the column was washed for 5 min with 100% B and re-equilibrated for 15 min with 100% A. Traces obtained for the P-, F- and B-series pectins are shown in Fig. 4.

Enzymatic fingerprinting using endo polygalacturonase II (PG II).—A pectin sample was dissolved in a 50 mM NaOAc buffer (pH 4.2) at a concentration of 5 mg/mL by overnight shaking at rt. For quantification, 0.1 mg/mL of glucuronic acid was added as internal standard. This pectin solution (1 mL) was incubated with 200 mU PG II for 20 h at rt. The reaction was stopped by treating the samples in a boiling water bath for 5 min. The samples were centrifuged and filtered through a 0.45 μm filter prior to chromatographic analysis. Oligomer separation was carried out by HPAEC on a Dionex AI 450 system using PAD detection. Sodium hydroxide (0.1 M) (from a 50% aq soln, Malinckroft Baker, Holland) and 1 M NaOAc (anhyd, E. Merck) in 0.1 M NaOH were used as eluents A and B. Eluents were degassed by sparging 15 min with helium and kept under helium for the entire run. The sample (20 μL) was loaded onto 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 90% A and 10% B. Chromatography was performed at a flow rate of 0.8 mL/min with a post column addition of 0.4 mL/min 0.3 M NaOH prior to PAD detection. For elution of galacturonic acid oligomers in their DP sequence, a stepwise linear gradient (10–50% B in 10 min, followed by 50–65% in 15 min and 65–75% 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.

Due to the high pH of the eluents, all oligomers were de-esterified instantly and eluted in their DP order. The internal standard glucuronic acid eluted between the galacturonic acid monomer and dimer. Peaks were integrated relative to the internal standard. Solutions of purified oligomers with DP 1–8, obtained from Drs A.G.J. Voragen and G.-J. van Alebeek (Department of Food Chemistry, Wageningen Agricultural University) were used for quantification. For oligomers larger than 8, the molar response factor of the octamer was used. The molar amount of each oligomer was multiplied by its molecular mass and is expressed in mg/mL as shown for the P-, F- and B-series pectin in Fig. 5.

Mass spectrometry.—Matrix assisted laser desorption ionisation (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 600 Da) was used. The instrument was calibrated externally in positive and negative ion mode using the peptides angiotensin I and ACTH (1–17).

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 this matrix solution was placed on the metal target. The solution spread out rapidly forming a thin layer of homogeneous, very fine crystals. Analyte solutions were desalted using home-made miniaturised columns (2 μL volume) containing about 1.5 mg ammonium loaded cation exchange resin (50W-X8, 200–400 mesh, hydrogen form; Bio-Rad) as described earlier [8]. The analyte solution (1.5 μL) was passed over the column and spotted directly onto the matrix layer. For analysis in negative ion mode, about 0.1 mg of cation exchange resin was added to the ana-

lyte droplet and the sample was allowed to dry in air. When the sample was dry, loose cation exchange resin was removed with pressurised air. Addition of cation exchange resin on the target was omitted, if measured in positive ion mode (detection of sodium cationised ions).

Acknowledgements

We want to thank Drs J. Benen and J. Visser for supply of the enzyme PG II and Dr G.-J. van Alebeek and Professor A.G.J. Voragen for the purified galacturonic acid oligomer standards. Furthermore, Clive Phipps Walter, Karin Hartmeyer, Jytte Rasmussen, Hanne Hoberg and Anja Petersen should be acknowledged for excellent technical assistance. Financial support from the European Community within the EU-Biotechnology Program contract number ERBIO4CT960685 is gratefully acknowledged.

References

[1] M.A. O'Neill, P. Albersheim, A.G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, *Carbohydrates*, Vol. 2, Academic Press, London, 1990, p. 415.
[2] 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 Application*, Marcel Dekker, New York, 1995, p. 287.
[3] P. Albersheim, A.G. Darvill, M.A. O'Neill, H.A. Schols, A.G.J. Voragen, in J. Visser, A.G.J. Voragen (Eds.), *Pectin and Pectinases*, *Progress in Biotechnology*, Vol. 14, Elsevier, Amsterdam, 1996, p. 47.
[4] J.-F. Thibault, C.M.G.C. Renard, *Carbohydr. Res.*, 238 (1993) 271–286.
[5] V. Puvanesarajah, A.G. Darvill, P. Albersheim, *Carbohydr. Res.*, 218 (1991) 207–226.
[6] H.A. Schols, E.J. Bakx, D. Schipper, A.G.J. Voragen, *Carbohydr. Res.*, 279 (1995) 265–279.
[7] A.G.J. Voragen, P.J.H. Daas, H.A. Schols, in B.S. Paulsen (Ed.), *Bioactive Carbohydrate Polymers*, *Proceedings of the Phytochemical Society of Europe*, Kluwer, London, 2000, p. 129.
[8] R. Körner, G. Limberg, J. Mikkelsen Dalgaard, P. Roepstorff, *J. Mass Spectrom.*, 33 (1998) 836–842.
[9] R. Körner, G. Limberg, T.M.I.E. Christensen, J. Dalgaard Mikkelsen, P. Roepstorff, *Anal. Chem.*, 71 (1999) 1421–1427.
[10] (a) A.T Hotchkiss, K. El-Bahtimy, M.L Fishman, in H.-F. Linskens, J.F. Jackson (Ed.), *Modern Methods of Plant Analysis*, Vol. 17, Springer-Verlag, Berlin, 1996, p. 129. (b) P.J.H. Daas, P.W. Arisz, H.A. Schols, G.A DeRuiter, A.G.J. Voragen, *Anal. Biochem.*, 257 (1998) 195–202.
[11] R. Kohn, O. Markovic, E. Machová, *Collect. Czech. Chem. Commun.*, 48 (1983) 790–797.
[12] T.M.I.E. Christensen, J.D. Kreiberg, H. Torsøe, H.C. Buchholt, P. Rasmussen, J. Nielsen, WO 97/03574 (1997); *Chem. Abstr.* 126 (16) 211325t.
[13] C.M.G.C. Renard, J.-F. Thibault, *Carbohydr. Res.*, 286 (1996) 139–150.
[14] (a) O. Mayans, M. Scott, I. Connerton, T. Gravesen, J. Benen, J. Visser, R. Pickersgill, J. Jenkins, *Structure*, 5 (1997) 677–689. (b) Y. van Santen, J.A.E. Benen, K.-H. Schroeter, K.H. Kalk, S. Armand, J. Visser, B.W. Dijkstra, *J. Biol. Chem.*, 274 (1999) 30474–30480.
[15] S. Armand, M.J.M. Wagemaker, P. Sanchez-Torres, H.C.M. Kester, Y. van Santen, B.W. Dijkstra, J. Visser, J.A.E. Benen, *J. Biol. Chem.*, 275 (2000) 691–696.
[16] J.A.E Benen, H.C.M. Kester, L. Parenicová, J. Visser, in J. Visser, A.G.J. Voragen (Eds.), *Pectins and Pectinases*, *Progress in Biotechnology*, Vol. 14, Elsevier, Amsterdam, 1996, p. 221.
[17] F.A.E. van Houdenhoven, Ph.D. thesis, Wagneningen Agricultural University, 1975.
[18] D. Magaud, C. Grandjean, A. Doutheau, D. Anker, V. Shevchik, N. Cotte-Pattat, J. Robert-Baudouy, *Tetrahedron Lett.*, 38 (1997) 241–244.
[19] H.-U. Endress, H. Omran, K. Gierschner, *Lebensm. Wiss. u. Technol.*, 24 (1991) 76–79.
[20] W.G.T. Willats, G. Limberg, H.C. Buchholt, G.J. van Alebeek, J.A.E. Benen, T.M.I.E. Christensen, J. Visser, A.G.J. Voragen, J.D. Mikkelsen, J.P. Knox, *Carbohydr. Res.*, this issue.
[21] P.J.H. Daas, K. Meyer-Hansen, H.A. Schols, G.A. De Ruiter, A.G.J. Voragen, *Carbohydr. Res.*, 318 (1999) 135–145.
[22] T.M.I.E. Christensen, J.E. Nielsen, J.D. Kreiberg, P. Rasmussen, J.D. Mikkelsen, *Planta*, 206 (1998) 493–503.
[23] Food Chemical Codex, 3rd edition, National Academic Press, Washington, DC, 1981, p. 215.
[24] P.E. Glahn, C. Rolin, in G.O. Phillips, P.A. Williams, D.J. Wedlock (Eds.), *Gums and Stabilisers for the Food Industry*, Vol. 8, Oxford University Press, Oxford, 1996, pp. 393–402.
[25] M. Lahaye, J. Vigouroux, J.-F. Thibault, *Carbohydr. Polym.*, 15 (1991) 431–444.