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Descriptive parameters for revealing substitution patterns of sugar beet pectins using pectolytic enzymes

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ABSTRACT

Enzymatic fingerprinting was applied to sugar beet pectins (SBPs) modified by either plant or fungal pectin methyl esterases and alkali catalyzed de-esterification to reveal the ester distributions over the pectin backbone. A simultaneous pectin lyase (PL) treatment to the commonly used endo-polygalacturonase (endo-PG) degradation showed to be effective in degrading both high and low methylesterified and/or acetylated homogalaturonan regions of SBP simultaneously. Using LC-HILIC–MS/ELSD, we studied in detail all the diagnostic oligomers present, enabling us to discriminate between differently prepared sugar beet pectins having various levels of methylesterification and acetylation. Furthermore, distinction between commercially extracted and de-esterified sugar beet pectin having different patterns of substitution was achieved by using novel descriptive pectin parameters. In addition to DB_{abs} approach for nonmethylesterified sequences degradable by endo-PG, the "degree of hydrolysis" (DH_{PG}) representing all partially saturated methylesterified and/or acetylated galacturonic acid (GalA) moieties was introduced as a new parameter. Consequently, the description DH_{PL} has been introduced to quantify all esterified unsaturated GalA oligomers.

1. Introduction

Pectin is probably the most complex and versatile polysaccharide present in plant cell walls. Pectin consists of different kinds of polymers, which are covalently attached to each other. The primary structural elements of pectin are the homogalacturonan (HG) and rhamnogalacturonan (RG-I) regions (Guillon, Thibault, Rombouts, Voragen, & Pilnik, 1989). The HG consists of a backbone of galacturonic acid moieties, which can be methylesterified at the C-6 of galacturonic acid. The RG-I subunit has repeats of alternating of α-1,4-linked D-galacturonosyl-α-1,2-L-rhamnose residues and the rhamnose units may be substituted with neutral sugar side chains (Voragen, Coenen, Verhoef, & Schols, 2009). Because of its excellent gelling and thickening properties, industrially extracted pectin is valued highly as a food ingredient. The suitability of pectin as gelling agent or stabilizer is determined by the distribution of methylesters over the homogalacturonan region, galacturonic content, average molar mass, degree of methylesterification (DM) and degree of acetylation (DA) (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004). Pectins from different sources show different gelling abilities due to variations in these parameters. At present, apple pomace and citrus peels are the main sources of commercially successful pectins and they have been extensively studied. Enzymatic degradation of the pectins with an endo-polygalacturonase (endo-PG) of *Kluyveromyces fragilis* followed by analysis of the partially methylesterified oligogalacturonides released has been used as a method to distinguish the substitution pattern within citrus pectin structures. In these studies, the Degree of Blockiness (DB) was calculated from the level of oligosaccharides released as quantified by high performance anion exchange chromatography HPAEC (pH 5), while the absolute degree of blockiness (DB_{abs}) was obtained from both capillary electrophoresis and HPAEC (pH 5) analyses of the digests (Daas, Voragen, & Schols, 2000; Guillotin et al., 2005; Ngouémazong et al., 2011; Ström et al., 2007). Although this approach has been widely used to differentiate substitution patterns of citrus pectins, the focus on nonmethylesterified sequences provides only a view on the relatively low methylesterified region of the pectin structure. Consequently, fingerprinting of highly methylesterified pectins with pectin lyase (PL) in combination with several chromatographic methods available has been used to distinguish series of differently de-esterified lemon pectins in order to study the highly methylesterified segment of pectin (Limberg et al., 2000a). Recently, the degree of blockiness (DBMe) and absolute degree of blockiness ($DB_{abs}Me$) for the methylesterified regions present in the HG region of pectin based on pectin lyase digestion was introduced (Ralet et al., 2012).

So far, the above-mentioned methods were not yet fully applied for the analysis of more complex pectins e.g. acetylated sugar

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beet pectin. Sugar beet pulp is a by-product of the sugar industry and is seen as a potential raw material of pectin. Sugar beet pectin (SBP), however, cannot compete commercially with citrus and apple pectins as gelling and thickening agent due to the high level of acetyl groups present in the HG regions of SBP (Thakur, Singh, Handa, & Rao, 1997). The GalA residue in SBP can be acetylated at positions *O*-2 and/or *O*-3 in both RG-I and HG. Similar to lemon pectins (Ralet, Dronnet, Buchholt, & Thibault, 2001), a series of de-esterified SBPs by alkali, plant PME and fungal PME has been characterized by enzymatic fingerprinting using endo and exo-PGs (Buchholt et al., 2004). However, the use of only PGs limits the analysis of SBP due to the presence of both methylesters and acetyl groups attached to the homogalacturonan region hindering PG to release sufficient levels of diagnostic oligomers.

In this research, digestion using RG-I degrading enzymes, endo-PGII and PL was carried out to generate sufficient diagnostic oligosaccharides from the acetylated SBP having different degrees of methylesterification. The enzymatic fingerprinting using both endo-PGII *and* PL simultaneously followed by HILIC coupled to ESI-MS/ELSD detection and HPAEC-PAD/$UV_{235\ nm}$ analysis was used to reveal the substitution patterns and developed new parameters for the distinction of differently prepared sugar beet pectins.

2. Experimental

2.1. Pectin samples

Sugar beet pectin, SBP6230 with a degree of methylesterification (DM) of 62 and degree of deacetylation (DA) of 30 was extracted from fresh sugar beet pulp and the pectin was further de-esterified by alkali, plant or fungal pectin esterases by DuPont (Brabrand, Denmark). Different series of pectins were used as substrates (Fig. 1 and Table 1): SBP6230 modified by plant pectin methyl esterase (p-PME) to yield the P-series of SBP (P5328, P4628, P3429) and SBP6230 modified by fungal pectin methyl esterase (f-PME) yielded the F-series (F5129, F4429, F2830). For the B-series, SBP6230 was alkali de-esterified yielding B5326, B3124, B0915 and B0100. Moreover, partial deacetylation of SBP6230 in a sodium methylate/methanol solution resulted to B′6126 while esterification of SBP under methanol in acidic solution converted SBP6230 into the high DM E7329 pectin. The chemical composition of these SBPs have been described elsewhere (Buchholt et al., 2004).

2.2. Enzymatic hydrolysis

The enzymes used in this study were *Aspergillus aculeatus* endo-galactanase (EC 3.2.1.89) (Schols, Posthumus, & Voragen, 1990), *Myceliophthora thermophila* (C1) exo-arabinase (EC 3.2.1.1), endo-arabinanase (EC 3.2.1.99) (Beldman, Searle-van Leeuwen, De Ruiter, Siliha, & Voragen, 1993; Kühnel et al., 2010), and RG-hydrolase enzyme (EC 3.2.1.B9) (Mutter, Renard, Beldman, Schols, & Voragen, 1998), pectin lyase (EC 4.2.2.10) (Schols et al., 1990) and endo-polygalacturonase II (EC 3.2.1.15) (Limberg et al., 2000b). All SBP samples were dissolved in 50 mM sodium citrate buffer pH 5 (5 mg/ml). The hydrolysis was performed at 40 °C by incubation of the pectin solution with the RG-I degrading enzymes and PL for 6 hours followed by the addition of endo-PGII and subsequent incubation for another 18 hours. Enzyme doses were sufficient to degrade theoretically their corresponding substrates within 6 hrs into monomers. Inactivation of enzymes was performed at 100 °C for 6 min and the digests were centrifuged (20,000 × *g*, 20 °C, 10 min). The supernatants obtained were analyzed by high performance anion exchange chromatography (HPAEC-PAD/UV) and UHPLC-HILIC coupled to ESI-IT-MS^n and ELSD detectors.

2.3. Characterization and quantification of the degradation products

High performance anion exchange chromatography (HPAEC). The precise level of monomeric GalA was analyzed by HPAEC. The pectin digests were diluted with Millipore water to 1 mg/ml and the analysis was performed on ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric and UV detection (HPAEC-PAD/UV) (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (ID 2 mm ID × 250 mm) and a CarboPac PA guard column (ID 2 mm × 25 mm). Both a ICS5000 ED (PAD) and an Ultimate 3000 Diode Array Detector (Dionex) were connected to detect eluting compounds. The flow rate was set 0.3 ml/min. The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C. The elution profiles were as follows: 0–60 min 20–70% B, 60–65 min 70–100% B, 65–70 min 100% B, 70–70.1 min 100–20% B and finally column re-equilibration by 20% B from 70.1 to 85 min. The injection volume was 10 μl. Galacturonic acid (Sigma–Aldrich, Steinheim, Germany) was used as a standard.

Hydrophilic interaction liquid chromatography (HILIC). Pectin digests, diluted to 1 mg/ml in 50% (v/v) acetonitrile, were analyzed using an Accela UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to an evaporative light scattering detector (Agilent 1200 series, Gen Tech Scientific Inc., NY, USA) and an ESI-IT-MS^n-detector (LTQ Velos Pro ion trap MS, Thermo Scientific). Chromatographic separation was performed on an Acquity UPLC BEH Amide column (1.7 μm, 2.1 mm × 150 mm) in combination with a Van Guard precolumn (1.7 μm, 2.1 mm × 5 mm; Waters Corporation, Milford, MA, USA). Elution was performed at a flow rate of 500 μL/min and a column oven temperature of 35 °C. The injection volume was set to 5 μL.

The composition of the three mobile phase lines were (A) 99:1 (v/v) water/acetonitrile (water/ACN), (B) 100% (v/v) ACN and (C) 200 mM ammonium formate/50 mM formic acid buffer (pH 3). For the optimal gradient and reproducibility of results, about 5% buffer (C) was constantly added throughout the elution. The following elution profile was used: 0–1 min, isocratic 80% B; 1–30 min, linear from 80% to 50% B; followed by column washing: 30–35 min, linear from 50% to 40% B and column re-equilibration; 35–45 isocratic 80% B. The eluent was split into 10:1 using an ASI flow splitter (Analytical Scientific Instruments, Richmond, CA, USA) before leading to the ELSD and the ESI-IT-MS^n detector. The drift tube temperature of the ELSD was set to 35 °C and the gain to 12. MS-detection was performed in negative mode with the ion source voltage set to −4.5 kV, heater temperature 225 °C, capillary temperature 350 °C, sheath gas 47 (arbitrary units), auxiliary gas 20 (arbitrary units) and auto-tuned on tetragalacturonic acid (*m*/*z* 721). Mass spectra were acquired over the scan range *m*/*z* 150–2000. Xcalibur software was used to process the data (Thermo). The amounts of oligomers were quantified by ELSD using GalA oligomer standards as previously described (Remoroza et al., 2012).

2.4. Determination of descriptive pectin parameters

Determination of the absolute degree of blockiness of non-methylesterified GalA regions. The amount of monomer in the digests obtained in HPAEC-PAD and nonesterified dimer and trimer as determined by HILIC-ELSD were subsequently used to calculate the absolute degree of blockiness (DB_{abs}). This mathematical expression provides information about the number of nonesterified GalA residues present in oligomers relative to all GalA residues present in the polymer (Guillotin et al., 2005). DB_{abs} is defined as mol of galacturonic acid residues present as nonesterified mono-, di- and

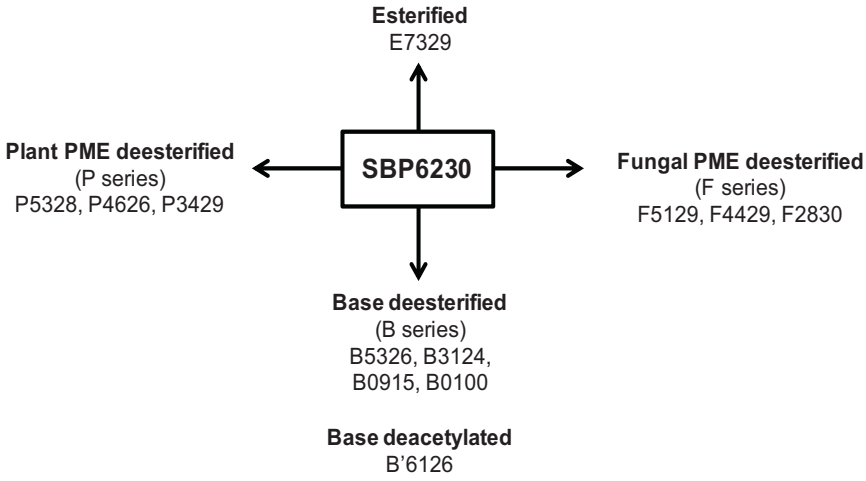

Fig. 1. Sugar beet pectins used in the study.

trimer per 100 mol of total GalA units in the polymer generated after endo-PGII digestion.

$$DB_{abs} = \frac{\sum_{n=1,2,3}[\text{saturated GalA}_n \text{ released}]_{\text{nonesterified}} \times n}{\text{total GalA in the polymer}} \times 100$$

Determination of the degree of hydrolysis by PL (DH_{PL}). Aside from the saturated nonesterified components as degraded by endo-PGII, the amount of unsaturated GalA oligomers for DP 2–8 by PL action is determined as well by HILIC. $DB_{abs}Me$ has been defined (Ralet et al., 2012) as mol of (un)saturated methylesterified GalA residues per 100 mol of total GalA units in the polymer generated after PL digestion. In this study, all (un)saturated GalA residues present in oligomers (DP 2–8) both methylesterified and/or acetylated released by PL action in SBP were quantified and expressed as degree of hydrolysis (DH_{PL}). DH_{PL} is not a new mathematical expression but an adapted terminology based of the previous concept, 'Absolute Degree of Blockiness' ($DB_{abs}Me$) for highly methylesterified stretches (Ralet et al., 2012). DH_{PL} was used rather than $DB_{abs}Me$ since according to our findings oligosaccharides released by PL may not necessarily have a sequence of methylesterified GalA residues only.

$$DH_{PL} = \frac{\sum_{n=1-8}[\text{(Un)saturated GalA}_n \text{ released}]_{\text{esterified}} \times n}{\text{total GalA in the polymer}} \times 100$$

3. Results and discussion

3.1.1. Structure elucidation of the generated oligosaccharides after enzymatic digestion

In order to fingerprint SBP6230 (DM 62, DA 30), a mixture of RG-I degrading enzymes, endo-PGII and PL was used to degrade the sugar beet pectin followed by HILIC analysis of the oligosaccharides released. As a result, 39% of the total GalA residues in the polymer was quantified, which is considered as a substantial improvement compared to the exo- and endo-PG digestion of DM ~60 SBPs, yielding <6% w/w of the GalA units in the polymer as oligomers (Buchholt et al., 2004). The HILIC elution pattern of the SBP6230 digest (Fig. 2) shows a number of oligomeric fragments originating from the RG-I region (arabinans and rhamnogalactans). However, most of the oligosaccharides detected were released from the HG backbone of pectin (saturated and unsaturated GalA oligomers). All major peaks were annotated following MS/MS analysis as exemplified in two cases. One of the products of the SBP6230 digestion was a saturated DP 5 oligomer (*m/z* 953) having one methylester and one acetyl group (5^{11}). Based on the MS^n analysis as described (Remoroza et al., 2012), two structures were shown to be present: $(GalA)_2$-$GalA_{MeAc}$-$(GalA)_2$ and $(GalA)_2$-$GalA_{Ac}$-$GalA_{Me}$-GalA.

Table 1
Characteristics of sugar beet pectin samples used in this study.

Pectins	GalA (w/w %)[a]	DM (mol %)[b]	DA (mol %)[b]	DB_{abs} (mol %)[c]	DH_{PG} (mol %)[d]	DH_{PL} (mol %)[d]	Yield (mol %)[e]
SBP6230	55	62	30	12	22	17	39
E7329	58	73	29	7	14	22	36
B'6126	56	61	26	12	24	16	40
F5129	57	51	29	10	38	n.d.	38
F4429	57	44	29	12	39	<1	39
F2830	57	28	30	15	65	n.d.	65
P5328	58	53	28	14	24	6	30
P4628	57	46	28	17	34	2	36
P3429	57	34	29	19	54	n.d.	54
B5326	58	53	26	20	64	3	67
B3124	58	31	24	29	81	<1	81
B0915	57	09	15	50	77	n.d.	77
B0100	58	01	00	90	95	n.d.	95

n.d. not detected.

[a] Theoretical galacturonic acid composition as described (Buchholt et al., 2004).

[b] Mol of methanol/acetic acid per 100 mol of the total galacturonic acid in the sample.

[c] Absolute degree of blockiness and the amount of mono-, di- and trigalacturonic acid per 100 mol of the total galacturonic acid in the sample.

[d] Degree of hydrolysis by endo-PGII (DH_{PG}): the amount of saturated galacturonic residues per 100 mol of the total galacturonic acid in the sample. Degree of hydrolysis by PL (DH_{PL}): the amount of galacturonic acid residues in unsaturated galacturonic oligomers per 100 mol of the total galacturonic acid in the sample.

[e] Yield: mol of galacturonic acid recovered after digestion of SBPs by endo-PGII and PL per 100 mol of the total galacturonic acid in the sample.

Table 2
Summary of GalA oligomers and their structures released by endo-PGII and pectin lyase after digestion of different sugar beet pectin samples as analyzed by HILIC–MS^n (Figs. 3 and 4).

Structure			SBP6230	B5326	P5328	F5129	B3124	P3429	F2830
DP 2	2^{00}		+	+	++	+	+	++	++
DP 3	3^{00}		+++	++	+++	++	++	+++	+++
	3^{01}		–	–	–	–	+	+	+
	3^{01}		–	–	–	–	+	+	+
	3^{01}		–	–	–	–	–	+	–
	$U3^{20}$		+	–	–	–	–	–	–
DP 4	4^{10}		+	+++	+	+++	+++	–	+
	4^{11}		+	–	–	–	+	+	+
	4^{11}		+	–	–	–	+	+	+
	4^{01}		–	–	–	–	+	+	+
	4^{01}		–	–	–	–	+	+	+
	$U4^{10}$		+++	+	+	–	+	–	–
	$U4^{20}$		+	+	+	–	+	–	–
	$U4^{30}$		+	–	–	–	–	–	–
	$U4^{21}$		+	–	–	–	–	–	–
	$U4^{31}$		+	–	–	–	–	–	–
	$U4^{31}$		+	–	–	–	–	–	–
DP 5	5^{10}		–	+	–	+	+	–	+
	5^{11}		+	–	–	–	+	+	+
	5^{11}		+	–	–	–	+	+	+
	5^{01}		–	–	–	–	+	+	+
	5^{01}		–	–	+	–	+	+	+
	5^{01}		–	–	+	–	+	+	+
	5^{20}		–	+	–	+	+	–	+
	5^{21}		–	–	–	+	+	–	+
	$U5^{20}$		–	+	–	–	–	–	–
	$U5^{30}$		–	+	–	–	–	–	–
	$U5^{31}$		+	–	–	–	–	–	–
	$U5^{41}$		+	–	–	–	–	–	–
	$U5^{41}$		+	–	–	–	–	–	–

Table 2 (*Continued*)

Structure			SBP6230	B5326	P5328	F5129	B3124	P3429	F2830
DP 6	6^{11}		–	–	–	+	+	+	+
	6^{11}		–	–	–	+	+	+	+
	6^{11}		–	–	–	+	+	+	+
	6^{12}		–	–	–	–	–	+	+
	6^{01}		–	–	–	–	–	+	+
	6^{01}		–	–	–	–	–	+	+
	6^{02}		–	–	–	–	–	+	–
	6^{20}		–	+	+	+	+	–	–
	6^{21}		+	+	+	+	+	+	+
	6^{21}		+	+	+	+	+	+	+
	$U6^{30}$		+	+	–	–	–	–	–
DP 7	7^{11}		–	–	–	–	+	–	–
	7^{12}		–	–	–	–	+	–	+
	7^{02}		–	–	–	–	–	+	–
	7^{20}		–	–	–	+	+	–	–
	7^{21}		+	+	–	+	+	–	+
	7^{21}		+	+	–	+	+	–	+
	7^{22}		–	–	–	–	–	+	+
	7^{31}		+	+	–	+	–	–	–
DP 8	8^{22}		–	–	–	–	+	–	+
	8^{31}		+	+	–	–	–	–	+
	8^{31}		+	+	–	–	–	–	+
	8^{31}		+	+	–	–	–	–	+
	$U8^{31}$		+	–	–	–	–	–	–

Nonesterified GalA ○ ; methylester ⬭ ; acetyl group ▲ *O*-2, △ *O*-3; unsaturated GalA ● .

The single GalA unit within a pectin oligomer having both a methylester and acetyl group confirmed previous observations by others (Perrone et al., 2002; Quéméner, Cabrera Pino, Ralet, Bonnin, & Thibault, 2003). These two GalA pentamers have acetyl substitution on *O*-2 of GalA residues as extracted from MS^2 and internal ring cleavage, which is also consistent with previous findings (Needs, Rigby, Colquhoun, & Ring, 1998; Ralet, Crépeau, & Bonnin, 2008). Additionally, Fig. 2 and Table 2 show that pectin lyase generated a wide range of unsaturated oligomers, e.g. $U8^{31}$ (*m/z* 1491) with three methylesters and one acetyl group. This unsaturated octamer was annotated as $UGalA_{Me}$-$(GalA)_2$-$GalA_{Ac}$-$GalA_{Me}$-$(GalA)_2$-$GalA_{Me}$. The location of the acetyl group was at the *O*-3 position of the fifth GalA residue from the reducing end. The GalA residues at both the reducing end and non-reducing end within the $U8^{31}$ octamer were methylesterified. Table 2 shows that PL also released unsaturated GalA oligomers (e.g. $U4^{21}$ and $U5^{20}$) that do not contain a methylester at the reducing end as has been reported already in literature (Körner, Limberg, Mikkelsen, & Roepstorff, 1998; Limberg et al., 2000a). The precise annotation of the degradation products released by endo-PGII or by PL clearly

demonstrates the wide range of GalA oligomeric structures present in the digest of parental SBP. These results show that by using endo-PGII and PL, SBP can be converted into oligomeric fragments to a large extent and the oligomers can be identified and quantified using online LC–MS approach.

Since our approach did not include any PME activity, novel oligomeric structures have been found when compared with the structures of oligomers described elsewhere (Ralet et al., 2005). The structures found in this study are summarized in Table 2. This approach, as demonstrated for parental SBP, was subsequently applied to differently prepared sugar beet pectins having different extents of methylesterification and acetylation.

3.1.2. *Fingerprinting of chemically and enzymatically modified sugar beet pectins*

To study the methylester distribution within SBP samples having the same degree of acetylation (~30) but differing in their functional properties, a series of de-esterified sugar beet pectins by alkali, p-PME and f-PME originating from SBP6230 was characterized. Because the efficiency of endo-PGII and PL to degrade SBP depends on the amount and distribution of methylesters, two series of SBP having a *methylesterification* of ~50 and ~30 were examined.

Characterization of 50% methylesterified sugar beet pectins. The generated diagnostic GalA oligomers present in B5326, P5328 and F5129 SBP digests were analyzed and quantified. Digestion of alkali treated SBP (B5326) and enzymatically treated (P5328, F5129) pectins by RG-I degrading enzymes, endo-PG and PL released 67%, 44% and 46% of the total GalA residues present in the polymer, respectively (Table 1). The observed yield of GalA oligomers released from fungal and plant PME modified SBPs already suggests that the pattern of substitution in the homogalacturonans of F5129 and P5328 is quite different compared to alkali treated B5326. To reveal the differences between the three de-esterified DM ≈ 50 SBP samples, a detailed analysis on the degradation products was performed.

The HILIC analysis of the degradation products in the P5328 digest (Fig. 3C and Table 2) shows a mixture of saturated non-esterified dimer (2^{00}) and trimer (3^{00}) as the main reaction products next to relatively low levels of methylesterified and/or acetylated GalA oligomers of DP 4–8. Similar levels of nonesterified dimers and trimers were found for B5326. Clearly, the tetramer with a single methyl ester 4^{10} is the most dominant peak in the B5326 digest. Except for penta-GalA oligomer 5^{11}, the oligosaccharides present in the B5326 digest were also present in f-PME de-esterified SBP (F5129) digest, although in different proportions (Fig. 3B). De-esterification by alkali leads to a random distribution of methylesters in the HG region of pectin (Limberg et al., 2000a). Surprisingly, several unsaturated GalA oligomers were dominantly present in the alkali de-esterified SBP (B5326) digest than in P5328, specifically oligosaccharides of DP 4–6 carrying one or more methylesters (Fig. 3A and C). Despite similar levels of DM and DA, unsaturated GalA oligomers were not detected in the F5129 digest, which supported a previously made statement that de-esterification of pectin by the random acting enzyme results in a deletion of PL cleavage sites in the homogalacturonan region of pectins (Limberg et al., 2000a). These observations are in contrast to the level of unsaturated oligosaccharides in the alkali de-esterified B5326. In addition, several oligomers (e.g. 5^{21} and 6^{20}) were found to be present in the de-esterified DM 50 SBP digests (Table 2). These oligomers were not present in the parental SBP6230 digest. Also, within the DM 50 SBP digests differences in oligomeric structures were observed.

Characterization of low methylesterified sugar beet pectins. It was expected that pectin sample having a DM close to 30% and still having a DA of ~30 favored the release of high levels of

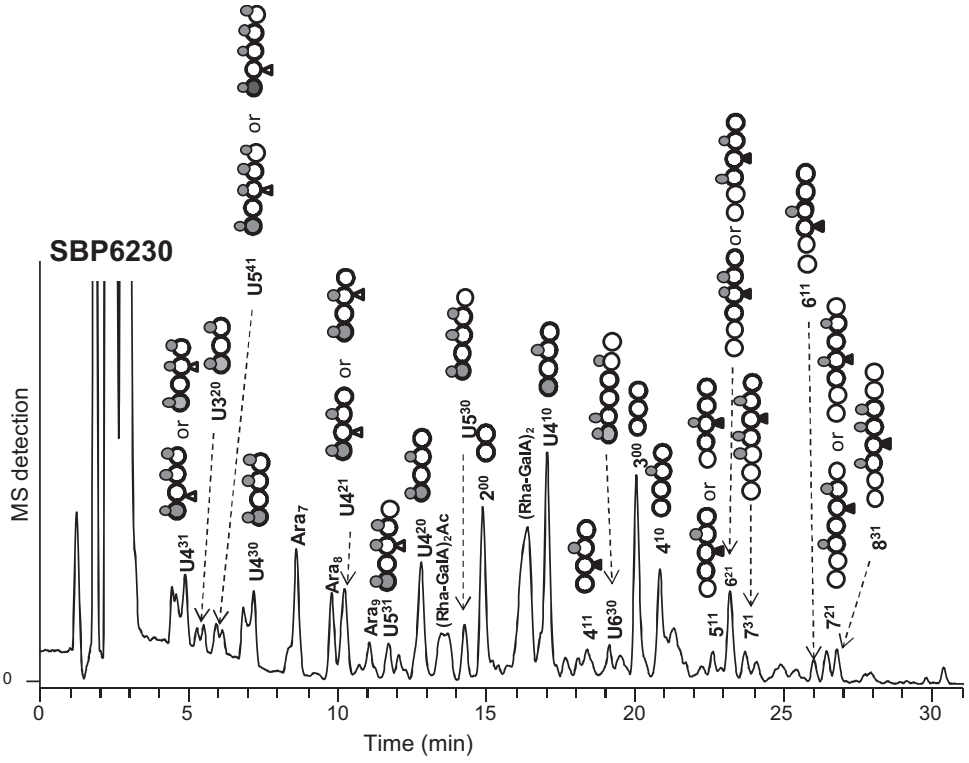

Fig. 2. HILIC-MS profile of SBPB6230 after digestion by endo-PGII and PL. Peak annotation: 5^{21}, DP 5, 2 *O*-methylester, 1 *O*-acetyl group. U: unsaturated GalA. Rha: rhamnose, GalA, galacturonic acid, Ac: acetyl group. Structure elucidation: Galacturonic acid ○; Methylester ●; Acetyl group O-3 position △; O-2 position ▲; Unsaturated GalA ◐.

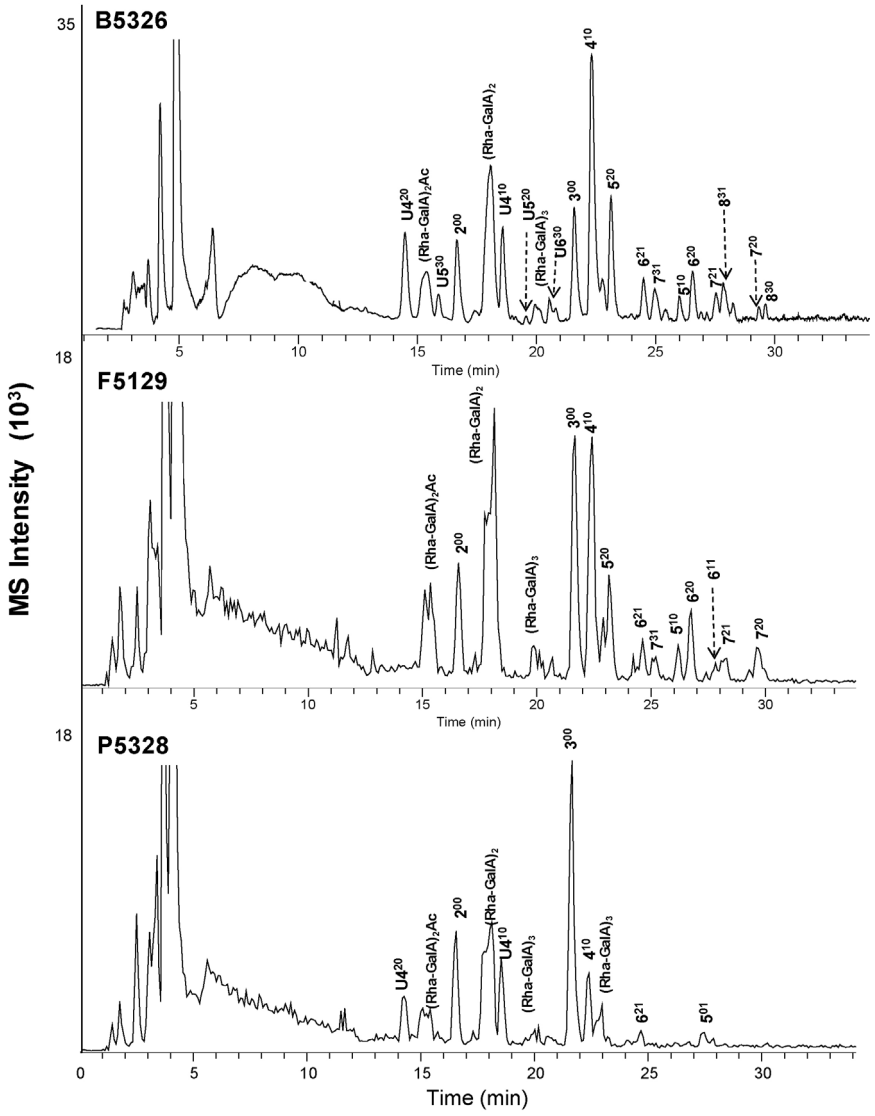

Fig. 3. HILIC-MS profiles of endo-PGII and PL sugar beet pectin digests: (A) B5326, (B) F5129, (C) P5328. Peak annotation: 5^{21}, DP 5, 2 *O*-methylester, 1 *O*-acetyl group. U: unsaturated GalA. Rha: rhamnose, GalA, galacturonic acid, Ac: acetyl group.

GalA oligomers by endo-PGII. Indeed, about 67% of the total GalA residues present in the polymers in B3124, P3429 and F2830 digests was released as oligosaccharides (Table 1). Fig. 4 shows the HILIC-MS chromatograms of alkali (B3124), p-PME (P3429) and f-PME (F2830) treated pectins and Table 2 shows the overview of both the novel and previously identified oligosaccharides present in the digests. A number of methylesterified GalA oligomers (3^{10}, 4^{20}, 5^{20}, 6^{20} and 7^{20}) were present in B3124 digest (Fig. 4A), which was not the case for B5326 and F5129. Limberg et al. (2000a) also reported the presence of these types of oligomers after endo-PGII digestion of non-acetylated commercial citrus pectin (DM 31). This indicates the presence of similar endo-PGII degradable sequences within the homogalacturonans of acetylated alkali de-esterified B3124 sugar beet pectin and non-acetylated DM 30 citrus pectin. The presence of the endo-PGII resistant hepta-GalA oligomer (7^{20}) shows the similarity in the pattern of methylesterification between the alkali de-esterified DM ~30 SBP (Table 2) and citrus pectins (Limberg et al., 2000a). The two randomly de-esterified SBPs examined, F2830 and B3124, behaved differently. Tetra-GalA oligomer (4^{10}) was the major product in the B3124 digest, while it was not observed in F2830 (Fig. 4B). In agreement to the statement of Ralet et al. (2012) that alkali treated citrus pectin (DM ~45 or lower) is not a good substrate for PL, small amounts of unsaturated GalA oligomers ($U4^{10}$, $U4^{20}$) were detected in B3124 digest ($DH_{PL} < 1$; Table 1 and Fig. 7). Despite the fact that both alkali and f-PME de-esterified SBPs have been described previously as randomly substituted pectins (Ralet et al., 2012), f-PME treated SBPs appeared quite differently from alkali treated SBPs. In P3429, endo-PGII released novel GalA oligomeric structures of singly and doubly acetylated galacturonides (4^{01} and 6^{02}) demonstrating that the P3429 HG region not only contains unsubstituted regions but also sequences with only acetyl substitution. The removal of methylesters from the GalA moieties after de-esterification of the pectin polymer by p-PME resulted in acetylated oligomers of DP 3–7 after PG/PL digestion. Obviously, the p-PME was not hindered by the acetyl groups present in SBP.

MS fragmentation analysis of the latter oligosaccharides showed that the acetyl esters were mostly on the *O*-3 position although *O*-2 acetylated GalA residues within certain oligomers were detected as well.

In Table 2 it is shown that a series of GalA oligomers present in the modified SBP digests of DM 50 and DM 30, may not necessarily be present in the parental SBP. All SBP samples mentioned in Fig. 1,

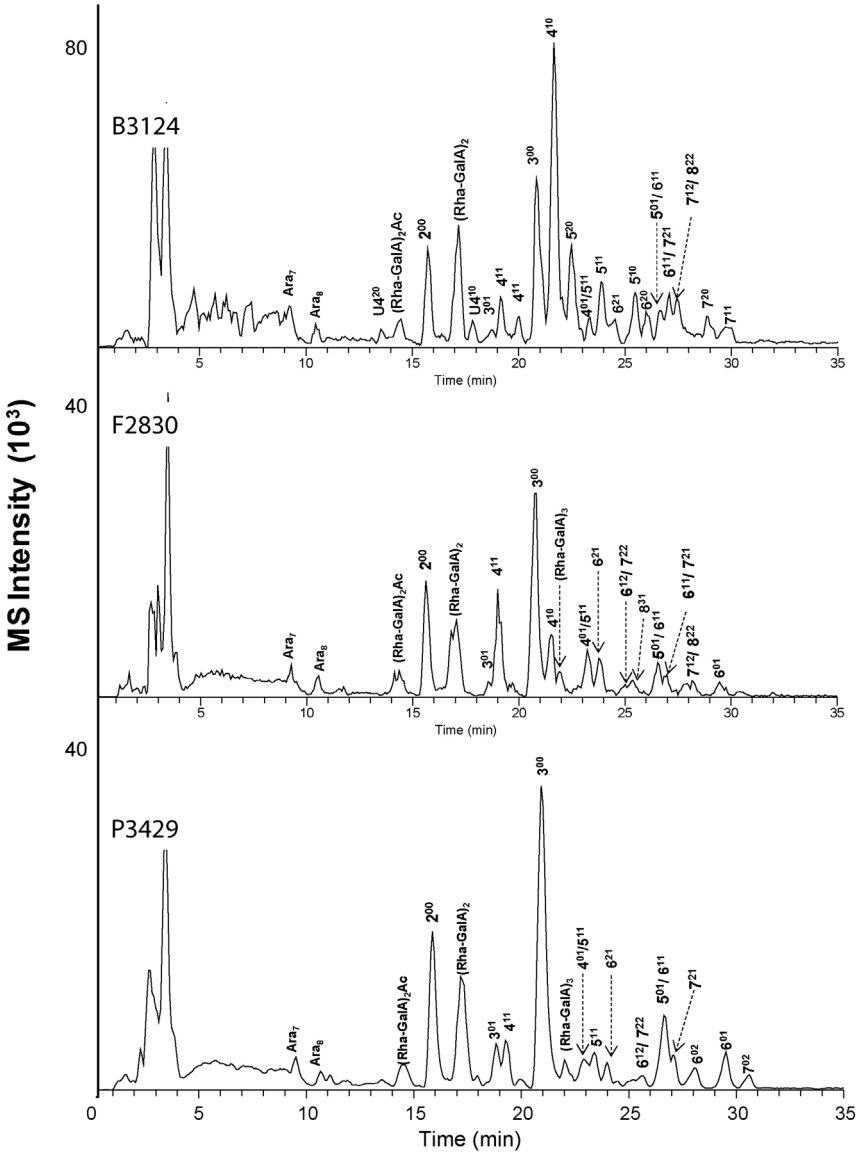

Fig. 4. HILIC-MS profiles of endo-PGII and PL sugar beet pectin digests, (A) B3124, (B) F2830, (C) P3429. Peak annotation: 5^{21}, DP 5, 2 *O*-methylester, 1 *O*-acetyl group. U: unsaturated GalA. Rha: rhamnose, GalA, galacturonic acid, Ac: acetyl group.

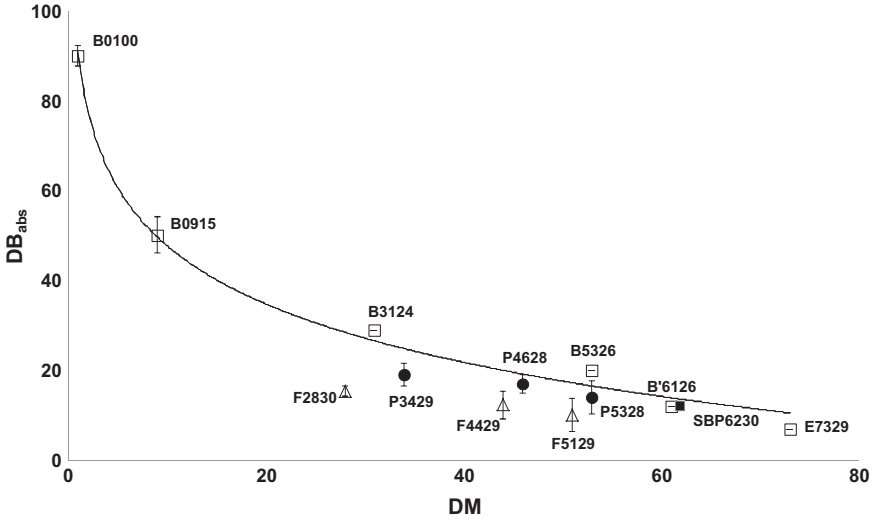

Fig. 5. Absolute degree of blockiness (DB_{abs}) versus degree of methylesterification (DM) for sugar beet pectins. Solid line indicates the correlation between DB_{abs} of alkali modified SBPs and DM. $DB_{abs} = -18.74 \ln(DM) + 90.954$ ($R^2 = 0.99$). Alkali modified pectins □; SBP6230 ■; P-series ●; F-series △.

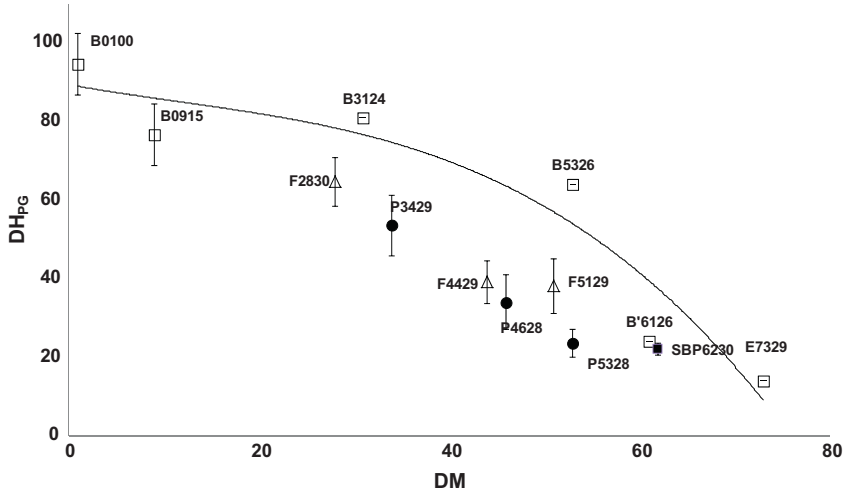

Fig. 6. Degree of hydrolysis (DH_{PG}) versus DM for different series of sugar beet pectins. The solid line visualizes the correlation between DH_{PG} of the alkali modified SBPs and DM. $DH_{PG} = -0.0002 \times DM^3 + 0.0081 \times DM^2 + 0.4372 \times DM + 89.38$ ($R = 0.91$). B-series pectins □; SBP6230 ■; P-series ●; F-series △.

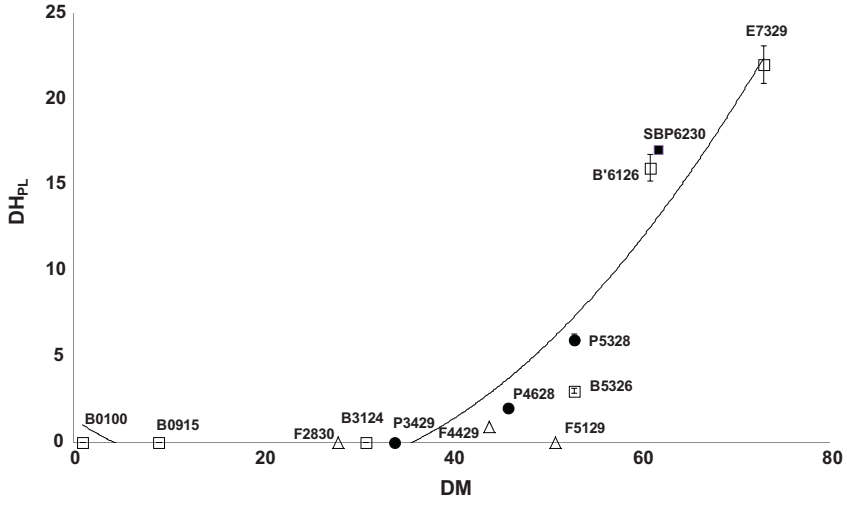

Fig. 7. Degree of hydrolysis (DH_{PL}) versus the degree of methylesterification (DM) for the different series of sugar beet pectin. Solid line indicates the relationship between the DH_{PL} for the alkali modified SBPs and DM. $DH_{PL} = 0.0009 \times DM^2 - 0.352 \times DM + 1.36$ ($R = 0.93$). B-series □; SBP6230 ■; P-series ●; F-series △.

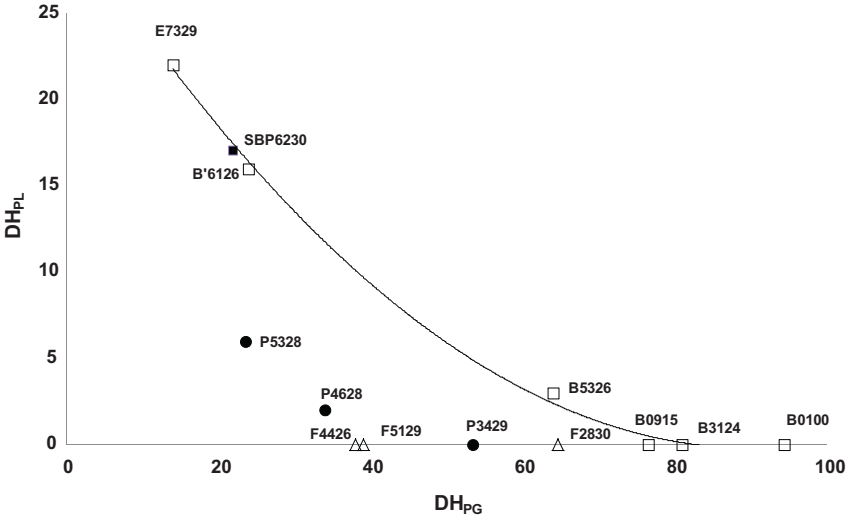

Fig. 8. Degree of hydrolysis by PL (DH_{PL}) versus degree of hydrolysis of endo-PG (DH_{PG}) for the different series of sugar beet pectin. Solid line indicates the relation for the alkali modified pectins. $DH_{PL} = 0.004 \times DH_{PG}{}^2 + 0.682 \times DH_{PG} + 30.575$ ($R = 0.99$). B-series □; SBP6230 ■; P-series ●; F-series △.

including the DM 50 and DM 30 SBP series, have been analyzed in the same way and will be described in more detail using descriptive pectin parameters.

3.1.3. Descriptive parameters for the distribution of acetylated and methylesterified SBP

Absolute degree of blockiness of nonesterified GalA residues. The determination of the total number of nonesterified GalA residues present within mono, di- and trimers is widely used to calculate the absolute degree of blockiness (DB_{abs}) for non-acetylated pectins (Guillotin et al., 2005). In this study, DB_{abs} for different SBP samples are compared by plotting the DB_{abs} values versus the corresponding DM values (Fig. 5) as suggested by Daas et al. (2000). The solid line indicates the correlation between DB_{abs} and DM as shown before for lemon pectin assuming a random removal of methylesters by alkali (Limberg et al., 2000a). Digestion of pectin B0100 by pectolytic enzymes resulted in 90% of GalA present as mono-, di- and trimers of total GalA residues in the polymer as a result of the absence of methylesters in the homogalacturonan region. Also, the alkali de-esterified pectins B0915 and B3124 have high DB_{abs} values of 50% and 29%, respectively. The amount of degradable sequences by endo-PGII decreased as expected when highly substituted pectins were digested e.g. B5326 having a DB_{abs} of 20%.

Plant PMEs are known to be processive enzymes (Guillotin et al., 2005), which give rise to the production of high level of blockwise nonmethylesterified GalA moieties in a pectin (Micheli, 2001). Despite the presence of nonmethylesterified GalA present in the homogalacturonan of enzymatically modified SBPs DM 30 (DA ~30), F2830 and P3429 pectins had relatively low DB_{abs} values than B3124. This result was most clearly observed in P3429 digest (Table 2) that has a significant amount of singly acetylated GalA oligomers (4^{01} and 5^{01}), which explains the relatively low DB_{abs} values for F2830 and P3429. Nonesterified GalA moieties were more apparent in B3124 than in P3429 and F2830 digests. Fig. 5 illustrates that chemically and enzymatically modified SBP samples having a DM of 50 and a DA of ~30, have DB_{abs} of <20%. For sugar beet pectins (DM ≥ 60), DB_{abs} values for alkali, plant and fungal PME de-esterified SBPs vary only slightly and the differences were too small to be able to distinguish between the SBP samples. Due to the presence of acetyl esters in SBP, the commonly used DB is not helpful to view the differences between SBP samples.

Degree of hydrolysis by endo-PGII (DH_{PG}). A clear overview of the homogalacturonan of SBPs may be achieved by investigating the total oligomers released by endo-PGII. Using the amounts of individual oligosaccharides available, a mathematical expression (degree of hydrolysis, DH_{PG}) has been established to calculate the amount of galacturonic acid residues present as saturated GalA monomer or oligomers of DP 2–8 in mol per 100 mol of the total GalA residues in the polymer.

$$DH_{PG} = \frac{\sum_{n=1-8}[\text{saturated GalA}_n \text{ released}]_{\text{nonesterified and esterified}} \times n}{\text{total GalA in the polymer}} \times 100$$

The solid line has been drawn for the alkali treated SBPs to visualize the correlation between the proportions of total nonesterified, methylesterified and/or acetylated GalA oligomers in SBPs and the DM (Fig. 6). For the B-series, the DH_{PG} values decrease gradually when DM > 40. The latter result could indicate the substitution homogeneity within the homogalacturonans of alkali de-esterified pectins. In contrast to B-series pectins, DH_{PG} for the F-series (F2830, F4429 and F5129) decreased as a function of DM, indicating that the substitution patterns in f-PME de-esterified SBPs have an even more regular distribution of methylesters and acetyl groups compared to the B-series preventing the hydrolysis by endo-PGII. Moreover, the P-series pectins have very low DH_{PG} values compared to the B- and F-series pectins suggesting the very low levels of endo-PGII degradable GalA sequences in the HG pectin backbone of the P-series. In addition, highly esterified chemically modified pectins (DM > 60) were also included in the series. It was observed that very low DH_{PG} values were obtained for high DM SBPs confirming that the GalA moieties within the homogalacturonans are highly substituted as confirmed by the low DB_{abs} values. Since all pectins behaved roughly similarly with respect to DB_{abs} (Fig. 5), this mean that F and P pectins lack partly methylated/acetylated sequences degradable by endo-PGII compared to pectins from the B-series.

The quantification of the total endo-PG degradable esterified sequences (Fig. 6) clearly discriminates the B-series pectin samples (DM 30–50) having high levels of DH_{PG} than the F-series pectins implying that f-PME modified pectins have different pattern of methylesterification. Both F- and B-series were predicted to have a homogenous distribution of methylesters within their homogalacturonan regions (Buchholt et al., 2004). B-series, however, appeared to have more endo-PGII degradable regions than the F-series pectins. Using the new parameter DH_{PG}, the different low DM (<50) SBPs were effectively distinguished.

Degree of hydrolysis by PL (DH_{PL}). To substantiate the differences of differently de-esterified SBPs, the data obtained after PL digestion of SBP polymers was evaluated and found useful for the distinction of highly methylesterified pectins. The adapted terminology DH_{PL}, is based on the previous concept, 'Absolute Degree of Blockiness' (DB_{abs}Me) for highly methylesterified stretches (Ralet et al., 2012). In the current work, degree of hydrolysis (DH_{PL}) was used rather than DB_{abs}Me since some unsaturated GalA oligosaccharides present in the SBP digests do not represent an uninterrupted sequence of methylesterified GalA residues, which are adjacent to each other (Table 2). Using HILIC analysis, the amounts of unsaturated GalA oligomers (DP 2–8) were calculated as the representative diagnostic oligomers for the determination of PL degradable sequences. Fig. 7 shows the correlation between the amounts of products released by PL and the DM for different SBP samples, again including a trend line between DH_{PL} and DM. A decreased DH_{PL} values was apparent for B-series than P- and F series. Clearly, B5326, F5129 and P5328 pectins showed distinct different DH_{PL} values suggesting the different proportions of methylesterified sequences degradable by PL. Digestion of P5328 by PL resulted in a relatively high DH_{PL} (DH_{PL} = 3) which indicates that alkali de-esterified SBPs have both sequences degradable by PL and endo-PGII. The absence or very low abundance of unsaturated GalA oligomers in F5129 indicates that PL resistant GalA sequences exist within enzymatically modified SBP, despite the relative high DM. Obviously, pectins with DM > 60 gave the highest DH_{PL} values confirming the high methylesterified HG regions of pectin. This is further visualized in Fig. 8 showing the correlation between DH_{PL} and DH_{PG}. The high substitution of methylesters in DM ≥ 60 pectins facilitated the PL action but is not favorable for the endo-PGII action. Apparently, B5326 behaved slightly different from other alkali and enzymatically modified SBPs. The reason for this deviation is probably originating from the preparation of this pectin. The quantitative information formulated for different SBPs clearly show the importance of using series of pectins modified in the same way to be able to accurately evaluate unknown pectin samples.

4. Conclusions

By obtaining significant levels (>40%) of diagnostic oligomers obtained after digestion via combined endo-PGII and PL action, the detailed analysis of different oligomers resulted the identification of novel GalA oligomeric structures, increased insight in different patterns of methyl distribution within the homogalacturonans of

SBPs and thereby, in the de-esterification mechanisms. Because the nonesterified GalA sequences can be distinguished from the methylesterified and acetylated GalA sequences, accurate and quantitative parameters are developed besides the well-established absolute degree of blockiness (DB_{abs}). Rather than using DB_{abs}, the parameters Degree of hydrolysis by endo-PGII (DH_{PG}) and PL (DH_{PL}) provide information on the distribution of methyl esters within differently prepared sugar beet pectins.

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References

Beldman, G., Searle-van Leeuwen, M. J. F., De Ruiter, G. A., Siliha, H. A., & Voragen, A. G. J. (1993). Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. *Carbohydrate Polymers*, *20*(3), 159–168.

Buchholt, H. C., Christensen, T. M. I. E., Fallesen, B., Ralet, M. C., & Thibault, J. F. (2004). Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers*, *58*(2), 149–161.

Daas, P. J. H., Voragen, A. G. J., & Schols, H. A. (2000). Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*(2), 120–129.

Guillon, F., Thibault, J.-F., Rombouts, F. M., Voragen, A. G. J., & Pilnik, W. (1989). Enzymic hydrolysis of the "hairy" fragments of sugar-beet pectins. *Carbohydrate Research*, *190*(1), 97–108.

Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A., & Voragen, A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*(3), 391–398.

Körner, R., Limberg, G., Mikkelsen, J. D., & Roepstorff, P. (1998). Characterization of enzymatic pectin digests by matrix-assisted laser desorption/ionization mass spectrometry. *Journal of Mass Spectrometry*, *33*(9), 836–842.

Kühnel, S., Hinz, S. W. A., Pouvreau, L., Wery, J., Schols, H. A., & Gruppen, H. (2010). *Chrysosporium lucknowense* arabinohydrolases effectively degrade sugar beet arabinan. *Bioresource Technology*, *101*(21), 8300–8307.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000a). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*. *Carbohydrate Research*, *327*(3), 293–307.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000b). 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. *Carbohydrate Research*, *327*(3), 321–332.

Micheli, F. (2001). Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. *Trends in Plant Science*, *6*(9), 414–419.

Mutter, M., Renard, C. M. G. C., Beldman, G., Schols, H. A., & Voragen, A. G. J. (1998). Mode of action of RG-hydrolase and RG-lyase toward rhamno-galacturonan oligomers. Characterization of degradation products using RG-rhamnohydrolase and RG-galacturonohydrolase. *Carbohydrate Research*, *311*(3), 155–164.

Needs, P. W., Rigby, N. M., Colquhoun, I. J., & Ring, S. G. (1998). Conflicting evidence for non-methyl galacturonoyl esters in *Daucus carota*. *Phytochemistry*, *48*(1), 71–77.

Ngouémazong, D. E., Tengweh, F. F., Duvetter, T., Fraeye, I., Van Loey, A., Moldenaers, P., et al. (2011). Quantifying structural characteristics of partially de-esterified pectins. *Food Hydrocolloids*, *25*(3), 434–443.

Perrone, P., Hewage, C. M., Thomson, A. R., Bailey, K., Sadler, I. H., & Fry, S. C. (2002). Patterns of methyl and O-acetyl esterification in spinach pectins: New complexity. *Phytochemistry*, *60*(1), 67–77.

Quéméner, B., Cabrera Pino, J. C., Ralet, M.-C., Bonnin, E., & Thibault, J.-F. (2003). Assignment of acetyl groups to O-2 and/or O-3 of pectic oligogalacturonides using negative electrospray ionization ion trap mass spectrometry. *Journal of Mass Spectrometry*, *38*(6), 641–648.

Ralet, M. C., Cabrera, J. C., Bonnin, E., Quéménér, B., Hellín, P., & Thibault, J. F. (2005). Mapping sugar beet pectin acetylation pattern. *Phytochemistry*, *66*(15), 1832–1843.

Ralet, M.-C., Crépeau, M.-J., & Bonnin, E. (2008). Evidence for a blockwise distribution of acetyl groups onto homogalacturonans from a commercial sugar beet (*Beta vulgaris*) pectin. *Phytochemistry*, *69*(9), 1903–1909.

Ralet, M.-C., Dronnet, V., Buchholt, H. C., & Thibault, J.-F. (2001). Enzymatically and chemically de-esterified lime pectins: Characterisation, polyelectrolyte behaviour and calcium binding properties. *Carbohydrate Research*, *336*(2), 117–125.

Ralet, M.-C., Williams, M. A. K., Tanhatan-Nasseri, A., Ropartz, D., Quéméner, B., & Bonnin, E. (2012). Innovative enzymatic approach to resolve homogalacturonans based on their methylesterification pattern. *Biomacromolecules*, *13*(5), 1615–1624.

Remoroza, C., Cord-Landwehr, S., Leijdekkers, A. G. M., Moerschbacher, B. M., Schols, H. A., & Gruppen, H. (2012). Combined HILIC-ELSD/ESI-MS^n enables the separation, identification and quantification of sugar beet pectin derived oligomers. *Carbohydrate Polymers*, *90*(1), 41–48.

Schols, H. A., Posthumus, M. A., & Voragen, A. G. J. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research*, *206*(1), 117–129.

Ström, A., Ribelles, P., Lundin, L., Norton, I., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium–pectin and acid–pectin gels. *Biomacromolecules*, *8*(9), 2668–2674.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin – A review. *Critical Reviews in Food Science and Nutrition*, *37*(1), 47–73.

Voragen, A. G. J., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263–275.