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# Two-step enzymatic fingerprinting of sugar beet pectin

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ABSTRACT

A two-step enzymatic fingerprinting method was introduced to analyze a highly methylesterified and acetylated sugar beet pectin having a degree of methylesterification (DM) of 62 and acetylation of 30. A cocktail of pectolytic enzymes, including endo-polygalacturonase II (endo-PGII) and pectin lyase (PL), was used for the first digestion. The endo-PGII and PL resistant pectin fragments were isolated and subjected to a second digestion using fungal pectin methylesterase and endo-PGII. After the two sequential digestions, 78% of the total GalA residues present in the parental pectin were recovered as mono- and oligomers, which were used to quantitatively describe the parental SBP. For this reason, the descriptive parameters degree of blockiness ($DB_{abs}$), degree of hydrolysis by PG ($DH_{PG}$) and degree of hydrolysis by PL ($DH_{PL}$) were established for both digestions. The first digestion revealed the presence of short blocks of nonesterified GalA residues and blocks of partly methylesterified and acetylated GalA residues in the parental SBP, in addition to blocks of highly methylesterified and acetylated GalA residues. The second digestion revealed the presence of blocks of methylesterified, partly methylesterified and/or acetylated GalA residues in a sequence not to be degradable by neither endo-PGII nor by PL. The acetyl groups were present in an blockwise manner. Application of the method to two differently prepared DM 50 SBPs showed that the two pectins differ in the ratio of blocks of nonesterified and blocks of partly methylesterified and acetylated GalA residues.



## 1. Introduction

Pectin isolated from apple pomace and lemon peel is widely used in the food industry as gelling, viscosifying, and stabilizing agent in food applications. Sugar beet is considered as a potential source of pectin. Sugar beet pectin (SBP) essentially consists of homogalacturonan (HG) and type I rhamnogalacturonan (RG-I) regions (Kravtchenko, Voragen, & Pilnik, 1992). About 90% of the total galacturonic acid (GalA) residues are present in the HG fragment of SBP and the remaining 10% GalA residues are present within the RG-I structural elements (Ralet & Thibault, 2009). RG-I is constructed of repeating units of α-(1 → 2)-linked rhamnosyl and α-(1 → 4)-linked GalA residues, in which the rhamnosyl unit can be substituted with neutral sugar side chains (Voragen, Coenen, Verhoef, & Schols, 2009). The GalA unit in both RG-I and HG can be acetylated at positions *O*-2 and/or *O*-3 (Quemener, Desire, Lahaye, Debrauwer, & Negroni, 2003). The level and distribution of esters have important commercial implications because of their effects on the functionality of the pectin (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Acid extracted commercial SBP may have a degree of acetylation (DA) and degree of methylesterification (DM) (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004).

Revealing the ester distribution patterns in SBP is complex due to the fact that the HG is highly decorated with both methylesters and acetyl groups. An enzymatic fingerprinting method has been developed (Ralet, Crépeau, & Bonnin, 2008) for the elucidation of the distribution pattern of acetyl groups in SBP. Although the methylester distribution is highly important as well with respect to functionality of the pectin, it was not addressed in that study. Recently, the simultaneous use of endo-polygalacturonase (endo-PGII) and pectin lyase (PL) to degrade a highly methylesterified and acetylated parental sugar beet pectin (DM 62, DA 30) and the subsequent analysis of the digest using LC-HILIC coupled to online ELSD/MSn was reported (Remoroza, Buchholt, Gruppen, & Schols, 2014). This enzymatic degradation of SBP6230 resulted in 40% degradation of the HG region based on the amounts of GalA residues present in monomer and nonesterified, partially methylesterified/acetylated unsaturated GalA oligomer released. Quantification of these oligosaccharides was used for the determination of the pectin descriptive parameters absolute degree of blockiness ($DB_{abs}$), degree of hydrolysis by PG ($DH_{PG}$) and degree of hydrolysis by PL ($DH_{PL}$) (Remoroza et al., 2014). These parameters were used to describe the so-called blocks of (1) nonesterified, (2) partly methylesterified, acetylated and (3) highly substituted GalA

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**Table 1**
Chemical characteristics of pectin samples used in this study.

| Mother pectin | De-esterification method | Pectin | GalA | Rha | Ara | Gal | DM[a] (%) | DA[a] (%) |
|---|---|---|---|---|---|---|---|---|
| | | | % (w/w) | | | | | |
| SBP6230 | | SBP6230 | 59 | 5 | 12 | 10 | 62 | 30 |
| | p-PME | P5328 | 58 | 5 | 11 | 10 | 53 | 28 |
| | f-PME | F5129 | 59 | 5 | 12 | 9 | 51 | 29 |

Sugar beet pectin (SBP); plant pectin methylesterase (p-PME); fungal pectin methylesterase (f-PME). Monosaccharide composition (Buchholt et al., 2004).
[a] Moles methanol or acetic acid per 100 moles of galacturonic acid.

residues in a pectin by the known modes of action of endo-PGII and PL. However, still the non-degradable part of the SBP, representing about 60% of the HG and being endo-PGII and PL resistant, was not included in the characterization.

In the present study, a two-step enzymatic fingerprinting approach is introduced to overcome the above mentioned drawback, making it possible to elucidate the distribution patterns in the highly methylesterified and acetylated pectins. In the first digestion, RG-I degrading enzymes, endo-PGII and PL are used to degrade the pectin, followed by the separation of the high molecular weight (Mw) fragments and the low Mw GalA mono- and oligomers. Subsequently, an enzyme mixture containing fungal pectin methylesterase (f-PME) and endo-PGII is used to further digest the high Mw pectin fragments. All diagnostic GalA oligomers generated were analyzed by HPLC-MS/ELSD. Using the pectin descriptive parameters the methylester and acetyl distribution over the SBP backbone is described.

## 2. Materials and methods

### 2.1. Pectin samples

Commercially extracted SBPs, SBP6230 (DM 62, DA 30), P5328 and F5129 were provided by Danisco (Brabrand, Denmark). The chemical characteristics are described in Table 1.

### 2.2. Enzymes and enzymatic hydrolysis

Purified and well characterized RG-I and HG degrading enzymes were used to hydrolyze sugar beet pectins. The enzymes used in this study were *Aspergillus aculeatus* endo-galactanase (EC 3.2.1.89) (Schols, Posthumus, & Voragen, 1990), endo-arabinase (EC 3.2.1.99) (Beldman, Searle-van Leeuwen, De Ruiter, Siliha, & Voragen, 1993), RG-hydrolase (EC 3.2.1.B9) (Mutter, Renard, Beldman, Schols, & Voragen, 1998), *Chrysosporium lucknowense* (C1) exo-arabinase (EC 3.2.1.1) (Kühnel et al., 2010), *Aspergillus niger* fungal pectin methyl esterase (fungal PME) (EC 3.1.1.11) (Van Alebeek, Van Scherpenzeel, Beldman, Schols, & Voragen, 2003), pectin lyase (EC 4.2.2.10) (Schols et al., 1990) and endo-polygalacturonase II (EC 3.2.1.15) (Limberg et al., 2000b). Fig. 1 shows the schematic diagram of the two-step enzymatic fingerprinting approach for the SBPs.

### 2.3. First digestion

SBP solution (10 mg/2 ml) in 50 mM sodium citrate buffer (pH 5.0) was digested at 40 °C for 24 h by RG-I (endo-galactanase + endo/exo arabinase + RG hydrolase) and HG (endo-PGII + PL) degrading enzymes as described elsewhere (Remoroza et al., 2014). The reaction was stopped by heating at 100 °C for 6 min. The total digest after the first digestion was freeze-dried.

### 2.4. Second digestion

The degradation products obtained after the first digestion of SBP were fractionated into high Mw fragments and low Mw oligomers by size exclusion chromatography (see below). For the second digestion, the high Mw material was dissolved in 2 ml 50 mM sodium acetate buffer (pH 5.0) and digested using *A. niger* f-PME (20 U/ml) and endo-PGII (10 U/ml) at 40 °C for 24 h. Inactivation of enzymes was performed by heating at 100 °C for 6 min. This digest was denoted as pool I digest.

Freeze-dried pectin digest (10 mg pectin) after the first enzymatic digestion was dissolved into 100 μl 50 mM sodium citrate buffer (pH 5.0) and applied onto a PD-10 column with packed bed size of 1.45 × 5.0 cm (8.3 ml) containing *Sephadex G-25 Medium* (GE Healthcare Bio-sciences Uppsala, Sweden). The PD-10 column was equilibrated with 25 ml of 50 mM sodium citrate buffer (pH 5.0) at room temperature. Subsequently, the digest was eluted with 4.90 ml of 50 mM sodium citrate buffer (pH 5.0). The fractionation allows the separation of high Mw fragments from low Mw fragments. Fractions (0.5 ml) were collected and analyzed by HPSEC, followed by pooling on the basis of the Mw of the fragments. Two pools were obtained; pool I (fractions 1–5;

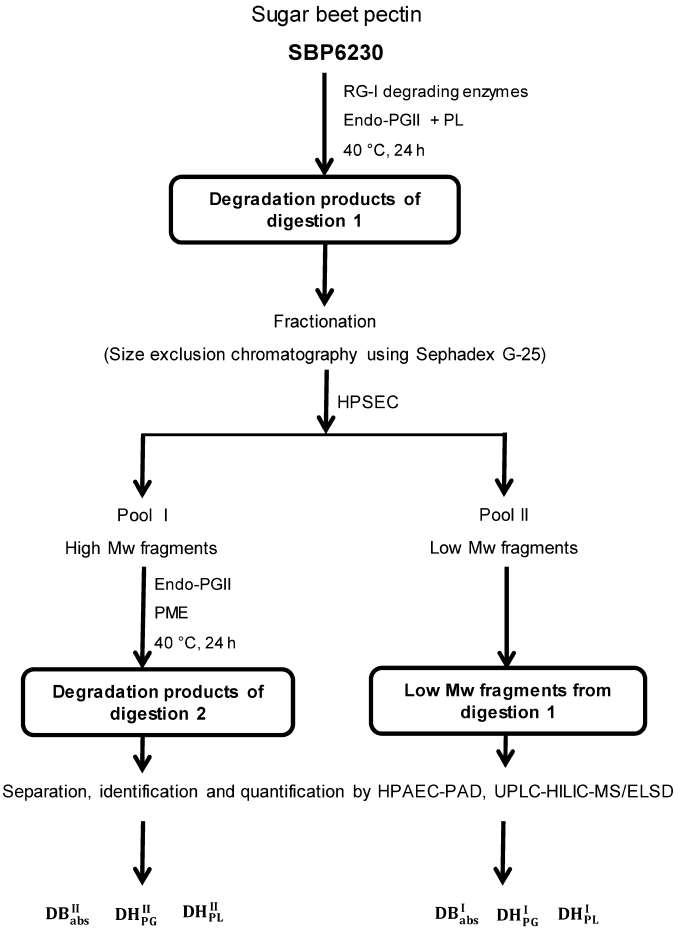


**Fig. 1.** Schematic diagram of the two-step enzymatic fingerprinting method to study the fine structure of sugar beet pectin.

high Mw material) and pool II (fractions 6–10; low Mw material) and both pools were freeze-dried. Subsequently, the freeze-dried high Mw pectin pool I was used for the second digestion (see above).

### 2.5. High performance size exclusion chromatography (HPSEC)

SBP digests were analyzed using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of four TSK-Gel super AW columns (Tosoh Bioscience, Tokyo, Japan) was used in series: one guard column (6 mm ID × 40 mm) and the columns 4000, 3000 and 2500 (6 mm × 150 mm). The column temperature was set to 55 °C. Samples (20 μL, 2.5 mg/ml) were eluted with filtered 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min and the elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan) and UV detection at 235 nm (Dionex Variable Wavelength Detection, Sunnyvale, CA, USA) as described previously (Cord-Landwehr, Leijdekkers, Moerschbacher, Schols, & Gruppen, 2012). The system was calibrated with pectins having molecular weights in the range of 10–100 kDa as have been obtained after degradation of polygalacturonic acid by yeast endo-PG (Voragen, Schols, De Vries, & Pilnik, 1982).

### 2.6. High performance anion exchange chromatography (HPAEC)

The quantification of monomeric GalA was performed by HPAEC. The SBP digests were diluted with Milli-Q water (Merck Millipore, Darmstadt, Germany) to 1 mg/ml and the analysis was performed on ICS5000 HPAEC system (Dionex) with pulsed amperometric and UV detection (HPAEC-PAD/UV) (Dionex) as described elsewhere (Remoroza et al., 2014). Galacturonic acid (Sigma–Aldrich, Steinheim, Germany) was used as standard.

### 2.7. Hydrophilic interaction liquid chromatography (HILIC-ELSD/MSn)

SBP digests, diluted to 1 mg/ml in 50% (v/v) acetonitrile, were analyzed using an Accela HPLC system (Thermo Scientific, Waltham, MA, USA) coupled to an evaporative light scattering detector (Agilent 1200 series, Gen Tech Scientific, Arcade, NY, USA) and an ESI-IT-MSn-detector (LTQ Velos Pro ion trap MS, Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed on an Acquity UPLC BEH Amide column as described previously (Remoroza et al., 2014).

### 2.8. Determination of DM

Pectin samples (≈2 mg/ml) were saponified in 100 mM NaOH for 1 hr at room temperature to determine the degree of methylesterification (DM) using colorimetric method as previously described (Guillotin, Van Loey, Boulenguer, Schols, & Voragen, 2007).

### 2.9. Determination of the absolute degree of blockiness

The amounts of monomer in the digests of pools I and II, as determined by HPAEC-PAD, and nonesterified dimer, and trimer, as determined by HILIC-ELSD, were used to calculate the absolute degree of blockiness ($DB^{I,II}_{abs}$) (Bakx, Boulenguer, Mazoyer, Schols, & Voragen, 2005). $DB_{abs}$ is defined as moles of galacturonic acid residues present as nonesterified mono-,di- and trimer generated by endo-PGII per 100 moles of total GalA residues in the polymer. The numeral in the formula refers to the results first or second of the pectin:

$$DB^{I,II}_{abs} = \frac{\sum_{n=1-3}[\text{saturated GalA}_n \text{ released}]_{\text{nonesterified}} \times n}{[\text{total GalA in the polymer}]} \times 100$$

### 2.10. Degree of hydrolysis by PG ($DH_{PG}$)

Using the amounts of individual oligosaccharides present after digestion of pool I and II by endo-PGII and PL, the degree of hydrolysis by PG, ($DH^{I,II}_{PG}$) was calculated as the number of moles of galacturonic acid residues present in the digest as saturated GalA monomer and oligomers of DP 2–8 per 100 moles of the total GalA residues in the polymer (Remoroza et al., 2014). The numeral in the formula refers to the first and second digestion of the pectin:

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

### 2.11. Determination of the degree of hydrolysis by PL ($DH_{PL}$)

All unsaturated GalA residues present in unsaturated oligomers (DP 2–8), both methylesterified and/or acetylated released by PL action from SBP, were quantified and expressed as degree of hydrolysis by PL ($DH^{I,II}_{PL}$) (Remoroza et al., 2014). The numeral in the formula refers to the first or second digestion of the pectin:

$$DH^{I,II}_{PG} = \frac{\sum_{n=1-8}[\text{unsaturated GalA}_n \text{ released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100$$

## 3. Results and discussion

### 3.1. Enzymatic digestion of SBPs

Using endo-PGII and PL, SBP6230, P5328 and F5129 (DM 51–62 and DA ~30, Table 1) were partially degraded into mono- and oligosaccharides representing 36%, 34% and 38% of the total GalA residues in the samples, respectively (Table 3). These yields were comparable with our previous results (Remoroza et al., 2014). For each SBP, the remaining not-sufficiently degraded fragments were isolated and subsequently subjected to a second digestion in order to include a larger part of the pectin for modeling its structure on the basis of the type and level of oligosaccharides released. Although all three SBPs in this study were analyzed in this way, the approach will be described in detail only for SBP6230 before discussing the outcome of the other SBPs.

### 3.2. First digestion and isolation of endo-PGII and PL resistant fractions

Fig. 2A shows the molecular weight distributions of SBP6230 before and after the first digestion. When digested, the average molecular weight of the SBP shifts from 100 kDa to lower than 3 kDa values although still material of ~43 kDa is present.

To analyze the high Mw fragments resistant to endo-PGII and PL in detail, a fractionation of the total first digest was carried out using size exclusion chromatography. Ten fractions were collected and two pools (I and II) were made based on the HPSEC analysis of all fractions (supplementary data, Fig. S1). Pool I consists of fraction 1–5 representing the high Mw fragments eluting at retention times < 11.5 min (Mw > 3 kDa), whereas pool II consists of fraction 6–10 having low Mw fragments eluting at retention times > 11.5 min (≤3 kDa) (Fig. 2B). The high signal at 13 min is typical for salt from the buffer solution.

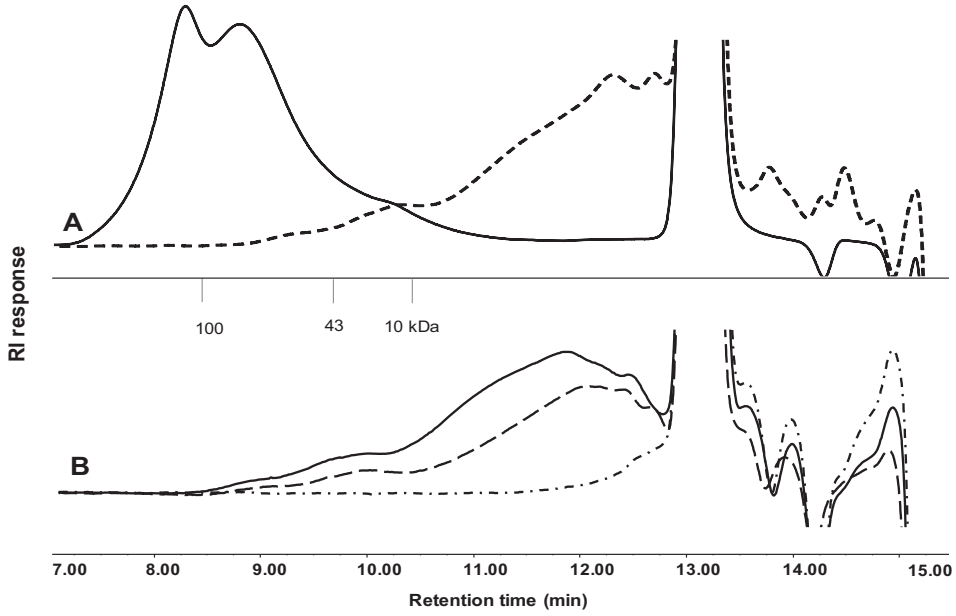


**Fig. 2.** HPSEC elution patterns of SBP6230 (DM 62, DA 30) (A) before (—) and after (- - - -) digestion with a cocktail of enzymes (RG-I and HG degrading enzymes); (B) pool I (▬); pool I + endo-PGII + f-PME (▬ ▬ ▬); pool II (- · -). Molecular weights of pectin standards (in kDa) are indicated.

### 3.3. Second digestion

To investigate the structure of the endo-PGII and PL-resistant pectin fragments, pool I was subjected to a second digestion by f-PME and endo-PGII (Fig. 1). Using this approach, f-PME randomly de-methylesterified the accessible methylesterified GalA moieties. Hence, it creates nonesterified GalA sequences that can be hydrolyzed by endo-PGII. The f-PME only released about 25% of the methylesters from the high Mw fragments of SBP6230. This is advantageous for the structure elucidation of the parental pectin as the remaining 75% of the methylesters present in the oligosaccharides still provide substantial structural information about the ester distribution in SBP. Fig. 2B presents the HPSEC elution profile after the second digestion of the high Mw fragments. It can be seen that the high Mw fragments in pool I shifted upon digestion to lower Mw values, representing oligomers that can be analyzed by HILIC-MSn/ELSD.

### 3.4. HILIC analysis of the SBP digests after enzymatic degradation

The diagnostic GalA oligomers present in pool I and II were identified and quantified by HILIC-MSn/ELSD (Fig. 3). An overview of the structures and proportions of the various oligomers of the two sequential digestions of SBP6230 is shown in Table 2. Besides the nonesterified monomer, dimer and trimer, GalA oligomers carrying 1–3 methylesters and one acetyl group, e.g. $4^{10}$, $4^{11}$, $6^{21}$, $7^{31}$ predominantly constitute pool II (first digestion). Interestingly, most of the latter oligomers have their acetyl groups on the *O*-2 position, like $6^{21}$ $GalA_2$-$GalA_{Me}$-$GalA_{Ac}$-$GalA_{Me}$-GalA. In addition, unsaturated tetramers ($U4^{20}$, $U4^{21}$, $U4^{31}$) carrying 1–3 methylesters and acetylated at either *O*-2 or *O*-3 position were also identified in pool II (Table 2).

As expected, almost no GalA oligomers were observed in pool I before the second digestion (Fig. 3B). This result exemplified the effective separation by size exclusion chromatography. The analysis of GalA oligomers in the pool I digest revealed the presence of nonesterified saturated monomer, dimer and trimer, next to partly methylesterified and/or acetylated oligomers, e.g. $7^{22}$, $7^{32}$, $8^{22}$, $9^{32}$ (Table 2). Within the GalA oligomers of DP 7–9, 2–3 methylesters and 2 acetyl groups were substituted randomly. Such saturated GalA oligomers were not present after the first digestion (pool II, Table 2 and Fig. 3C). Furthermore, the acetyl groups within these saturated GalA oligomers are located on either *O*-2 or *O*-3, while only *O*-2 acetylation was found for saturated oligomers released by endo-PGII. Unsaturated GalA oligomers (DP 4–8) were dominantly present in the pool I (Fig. 3B). The acetylation

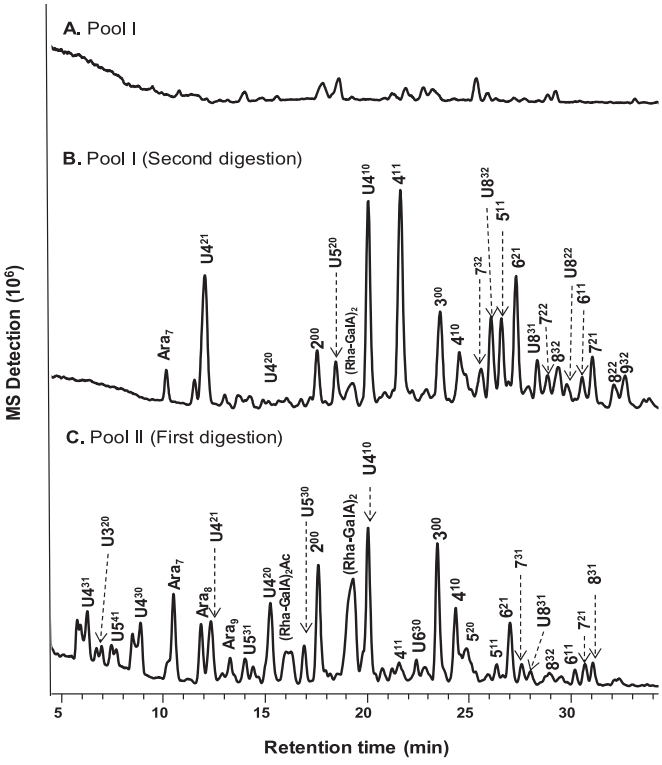


**Fig. 3.** HILIC-MSn profiles of SBP6230 digested by (A) PL + endo-PGII (first digestion; pool II); (B) pool I; (C) pool I second digestion using f-PME and endo-PGII. Peak annotation: $6^{21}$, DP 6, 2 *O*-methylesters, 1 *O*-acetyl group; U: unsaturated GalA; Rha: rhamnose; GalA, galacturonic acid; Ac: acetyl group; Ara: arabinose.

**Table 2**
Amount and structure of different GalA oligomers released by endo-PGII and pectin lyase after two sequential digestions of sugar beet pectins as analyzed by HILIC-ELSD/ESI-MSn and HPAEC-PAD.

| | | Structure | SBP6230 | | P5328 Digestion | | F5129 | |
|---|---|---|---|---|---|---|---|---|
| | | | I[a] | II[b] | I | II | I | II |
| DP 1 | $1^{00}$ |  | 4.64 | 1.46 | 4.58 | 0.98 | 3.49 | 1.55 |
| DP 2 | $2^{00}$ |  | 3.05 | 4.77 | 6.51 | 3.19 | 5.63 | 7.95 |
| DP 3 | $3^{00}$ |  | 4.86 | 6.45 | 7.92 | 5.86 | 6.37 | 7.39 |
| | $3^{01}$ |  | – | – | – | + | – | + |
| | $3^{01}$ |  | – | – | – | + | – | + |
| | $3^{01}$ |  | + | – | – | + | – | + |
| | $U3^{20}$ |  | + | – | – | – | – | – |
| | $4^{10}$ |  | 2.96 | 4.12 | 4.29 | 4.15 | 6.31 | 5.64 |
| | $4^{11}$ |  | + | 3.65 + | 3.03 | + | 4.83 | |
| | $4^{11}$ |  | + | + | + | + | + | + |
| DP 4 | $U4^{10}$ |  | 5.24 | 6.79 | 3.06 | 5.30 | – | 2.75 |
| | $U4^{20}$ |  | 2.20 | – | 2.45 | – | – | – |
| | $U4^{30}$ |  | 2.83 | – | – | – | – | – |
| | $U4^{21}$ |  | 4.26 | 3.72 | – | – | – | 3.13 |
| | $U4^{21}$ |  | + | – | – | – | – | + |
| | $U4^{31}$ |  | + | – | – | – | – | – |
| | $U4^{31}$ |  | + | – | – | – | – | – |
| DP 5 | $5^{10}$ |  | – | – | + | – | – | 2.49 |
| | $5^{11}$ |  | + | 1.36 | + | 2.65 | + | 3.13 |
| | $5^{11}$ |  | + | + | + | + | + | + |
| | $5^{20}$ |  | + | – | + | – | 2.96 | – |
| | $5^{21}$ |  | – | – | + | – | 5.24 | – |
| | $U5^{20}$ |  | + | – | – | – | – | – |
| | $U5^{21}$ |  | – | – | – | + | – | + |
| | $U5^{30}$ |  | + | – | – | – | – | – |
| | $U5^{30}$ |  | + | – | – | – | – | – |
| | $U5^{31}$ |  | + | – | – | – | – | – |
| | $U5^{31}$ |  | + | – | – | – | – | – |

Table 2 (*Continued*)

| | | Structure | SBP6230 | | P5328 Digestion | | F5129 | |
|---|---|---|---|---|---|---|---|---|
| | | | I[a] | II[b] | I | II | I | II |
| | $U5^{40}$ |  | – | – | – | – | – | – |
| | $U5^{41}$ |  | + | – | – | – | – | – |
| | $U5^{41}$ |  | + | – | – | – | – | – |
| | $U5^{41}$ |  | + | – | – | – | – | – |
| DP 6 | $6^{11}$ |  | + | + | + | + | – | + |
| | $6^{11}$ |  | + | + | + | + | – | + |
| | $6^{11}$ |  | + | + | + | + | – | – |
| | $6^{20}$ |  | – | – | + | – | 2.72 | – |
| | $6^{21}$ |  | + | 1.02 | 4.29 | 3.76 | 6.38 | 5.58 |
| | $6^{21}$ |  | + | + | + | + | + | + |
| | $U6^{30}$ |  | + | – | – | – | – | – |
| DP 7 | $7^{12}$ |  | – | – | – | – | – | + |
| | $7^{21}$ |  | + | + | + | + | 2.87 | + |
| | $7^{21}$ |  | + | + | + | + | + | + |
| | $7^{22}$ |  | – | + | – | + | – | – |
| | $7^{31}$ |  | + | – | + | – | + | + |
| | $7^{32}$ |  | – | + | – | – | – | – |
| DP 8 | $8^{22}$ |  | – | + | – | + | – | + |
| | $8^{31}$ |  | + | – | + | – | 8.31 | + |
| | $8^{31}$ |  | + | – | + | – | + | + |
| | $8^{31}$ |  | + | – | + | – | + | + |
| | $8^{32}$ |  | + | + | – | – | – | – |
| | $U8^{22}$ |  | – | + | – | – | – | – |

Table 2 (*Continued*)

| | | Structure | SBP6230 | | P5328 Digestion | | F5129 | |
|---|---|---|---|---|---|---|---|---|
| | | | I[a] | II[b] | I | II | I | II |
| | $U8^{22}$ |  | – | + | – | – | – | – |
| | $U8^{31}$ |  | – | + | – | – | – | – |
| | $U8^{32}$ |  | – | 1.40 | – | – | – | – |
| | $U8^{32}$ |  | – | + | – | – | – | – |
| DP 9 | $9^{32}$ |  | – | + | – | – | – | – |

Nonesterified GalA ○; methylester ●; acetyl group ▲ *O*-2, △ *O*-3; unsaturated GalA●.
The values indicate the moles of nonesterified and (un)saturated galacturonic residues released as mono and oligomers per 100 moles of the total GalA in the sample. + traces; – not detected.

[a] Digestion I: oligomers (low Mw) generated after the first digestion.
[b] Digestion II: oligosaccharides released from high Mw fragment after the second digestion.

**Table 3**
Characteristics of the two pools of GalA oligomers after the digestion of SBPs by two separate cocktails of pectolytic enzymes.

| Pectin | $DB^{I}_{abs}$ | $DH^{I}_{PG}$ | $DH^{I}_{PL}$ | $Yield^{I}$ |
|---|---|---|---|---|
| *First digestion* | | | | |
| SBP6230 | 13 | 22 | 14 | 36 |
| P5328 | 19 | 28 | 6 | 34 |
| F5129 | 16 | 38 | 0 | 38 |
| | $DB^{II}_{abs}$ | $DH^{II}_{PG}$ | $DH^{II}_{PL}$ | $Yield^{II}$ |
| *Second digestion* | | | | |
| SBP6230 | 13 | 25 | 17 | 42 |
| P5328 | 10 | 24 | 5 | 29 |
| F5129 | 17 | 41 | 6 | 47 |

Absolute degree of blockiness ($DB_{abs}$) and the moles of galacturonic acid (GalA) residues present as monomer, dimer and trimer per 100 moles of the total GalA in the sample. Degree of hydrolysis by PG ($DH_{PG}$): the moles of saturated GalA residues present as monomer and oligomers per 100 moles of the total GalA residues in the sample. Degree of hydrolysis by PL ($DH_{PL}$): the moles of GalA residues present as unsaturated GalA oligomers per 100 moles of the total GalA in the sample. $Yield^{I,II}$: the moles of GalA residues released as monomer and oligomers by the enzymes per 100 moles of the total GalA in the sample. The numeral in the formula refers to the first or second digestion of the pectin.

*O*-2/*O*-3 pattern was also present within the acetylated unsaturated GalA oligomers of DP 8 carrying a methylester at the nonreducing end ($U8^{22}$, $U8^{31}$, $U8^{32}$). These unsaturated DP 8 oligomers were present only in low amounts compared to the unsaturated DP 4–5 GalA oligomers. These unsaturated GalA oligomers are considered to be the products of the second endo-PGII digestion of large unsaturated GalA fragments formed during the first digestion by PL and are dominantly present in pool I after the second digestion.

During the second digestion, 42% of the total GalA residues present in SBP6230 were released as mono- and oligomers compared to 36% released as mono- and oligomers in the first digestion. Hence, in total, 78% of the GalA residues of the parental pectin can now be used for the determination of the distribution of these esters within the HG regions of SBP6230 (Table 3).

### 3.5. Methylester and acetyl group distribution in SBP

A hypothetical representation of segments within the pectin homogalacturonan backbone being differently degraded by endo-PGII or PL and the consequence for the values of the pectic parameters $DB_{abs}$, $DH_{PG}$ and $DH_{PL}$ is presented in Fig. 4. It was postulated that within pectin segments there are different types of chains. For clarity, the acetyl groups are not included in the chains A–D, although acetylation of the GalA residues may have a great impact on the degradability of endo-PGII and PL, and consequently, on the parameters. The following blocks are anticipated to be present in sugar beet pectin: (A) large blocks of nonesterified GalA sequences having high $DB_{abs}$ and high $DH_{PG}$ where $DB_{abs}$ is equal to $DH_{PG}$; (B) small blocks of nonesterified GalA sequences interrupted by short sequences of methylesterified GalA residues; (C) blocks of partly methylesterified GalA residues (with/without acetyl groups) without endo-PGII-degradable GalA sequences; (D) blocks of highly methylesterified GalA residues, low $DB_{abs}$, low $DH_{PG}$ and high $DH_{PL}$. Chain E represents a highly methylesterified and acetylated HG blocks released by PL during the first digestion, in which the distribution of both esters makes the chain resistant to endo-PG and PL action. Addition of f-PME during the second digestion removes a minor part of the methylesters (chain F). This will increase the degradability by endo-PGII and it can be seen that endo-PGII also releases nonesterified saturated DP 1–3 next to one unsaturated GalA oligomer from the segment.

Initial digestion of SBP6230 resulted in a $DB^{I}_{abs}$ value of 13% for pool II (Table 3) representing a few large blocks of nonesterified GalA residues in pectin. The $DH^{I}_{PG}$ value was 22%, representing large blocks of both nonesterified GalA residues and partly methylesterified and/or acetylated GalA residues. Table 2 shows that the proportions of $1^{00}$, $2^{00}$ and $3^{00}$ are roughly similar after the first digestion (pool II) having a ratio of $1^{00}$: $2^{00} + 3^{00} = 0.59$. This indicates the presence of small blocks of nonesterified GalA residues in the HG region of SBP (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Winning, Viereck, Nørgaard, Larsen, & Engelsen, 2007). About 9% of the saturated oligomers were present as partly methylesterified and/or acetylated GalA residues ($DH^{I}_{PG} - DB^{I}_{abs}$). The latter consists predominantly of GalA oligomers of DP 4–6 carrying 1–2 methylesters and one acetyl group at the *O*-2 position. This indicates that the endo-PGII acts close to a methylesterified GalA residue next to the neighboring *O*-2 acetylated GalA residue.

The $DH^{I}_{PL}$ was calculated to be 14%, representing the proportion of highly methylesterified GalA oligomers released by PL (Table 2). It is shown that the oligomers released by PL were partially

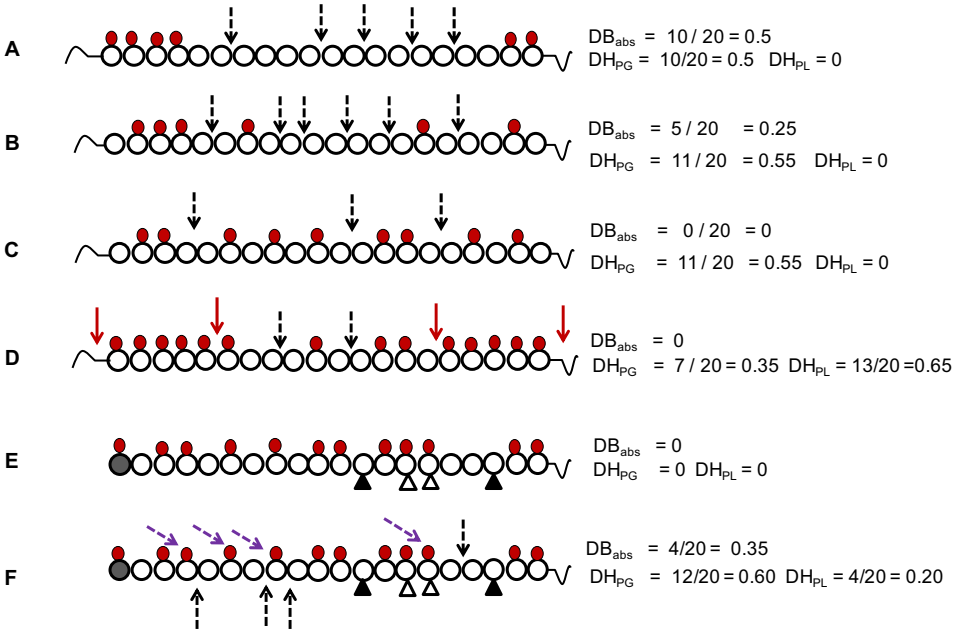


**Fig. 4.** Schematic representation of methylesterified and acetylated GalA segments postulated to be present in the homogalacturonan of SBP. (A) large blocks of nonesterified GalA sequences; (B) small blocks of nonesterified GalA sequences; (C) large blocks of partly methylesterified GalA sequences (D) large blocks of highly methylesterified sequences (E) methylesterified and acetylated pectin fragments, non-degradable by endo-PGII and PL (F) methylesterified and acetylated pectin fragments in chain E, addition of f-PME removes a part of methylesters and the chain is further hydrolyzed by endo-PGII. Galacturonic acid (GalA):○; unsaturated GalA: ●; methylester: •; acetyl group: *O*-2 ▲, *O*-3 △; endo-polygalacturonase (endo-PGII): →; pectin lyase (PL): ↓; fungal pectin methylesterase (f-PME) ↘; For clarity, the acetyl groups are not present in chains A–D.

acetylated. The predominantly present unsaturated tetramers and pentamers carrying 1–4 methylesters with one acetyl group (Table 2) indicate the presence of segments in the pectin with a few large or many small blocks of highly methylesterified and acetylated GalA residues degradable by PL. It is assumed that also the endo-PGII and PL resistant fraction could contain (high) levels of acetylation.

Table 3 shows the distinct characteristics of different GalA segments present in pool I digest: $DH^{II}_{abs}$, $DH^{II}_{PG}$ and $DH^{II}_{PL}$ being 13%, 25% and 17%, respectively. Table 2 presents the proportions $1^{00}$, $2^{00}$ and $3^{00}$ in pool I after the second digestion. The proportions of both $2^{00}$ and $3^{00}$ are about 8 times higher than the proportion of $1^{00}$. This implies that large blocks of methylesterified GalA residues (Daas et al., 1999) are present in the nondigestible fragments after the first digestion. Hence, these oligomers were present in blocks of partly methylesterified GalA residues carrying no acetyl groups, which are de-methylesterified by f-PME and further hydrolyzed by endo-PGII. Moreover, ($DH^{II}_{PG} - DB^{II}_{abs}$), being 12% represents blocks of partly methylesterified and acetylated GalA residues, which remain too highly substituted for degradation by endo-PGII. The corresponding oligomers are mainly tetramers, hexamers and heptamers having 1–3 methylesters and 1–2 acetyl groups substituted in a random manner. The unsaturated GalA oligomers (DP 4 and 8 with 1–3 methylesters and 1–2 acetyl groups at the *O*-2 and/or *O*-3) released after the second digestion represent large blocks of partly methylesterified and/or acetylated GalA residues of the pectin from the first digestion.

Although it is difficult to fully describe the acetylation patterns of the SBP, it could be deduced from the parameters and oligosaccharide structures found that following blocks exists; (1) (methylesterified) GalA sequences without acetylation (1st and 2nd digestion); (2) methylesterified GalA sequences having *O*-2 acetylation (1st digestion); and (3) partly methylesterified GalA sequences having *O*-2 or *O*-3 acetylation. The presence of these blocks further strengthen previous findings (Ralet et al., 2008) that the parental SBP has a non-random acetyl group distribution.

### 3.6. Distribution patterns in two differently prepared DM 50 SBPs

The approach discussed above for SBP6230 was also used to study two SBPs (P5328 and F5129) having a DM of ~50 and DA of ~30. These pectins have been partly characterized before (Remoroza et al., 2014). However, the level of oligomers released was not sufficiently high to draw conclusions on their ester distributions after the enzymatic fingerprinting with endo-PGII and PL. Table 2 shows the overview of the structural information and the proportions of the GalA oligomers present in the P5328 and F5129 digests. The yield of GalA residues recovered as oligomers for P5328 and F5129 after the first and second digestion were 63% and 85%, respectively (Table 3).

### 3.7. First digestion of two differently prepared DM 50 SBPs

The $DB^{I}_{abs}$, $DH^{I}_{PG}$ and the $DH^{I}_{PL}$ values for both P5328 and F5129 are presented and are similar to the values found before for these pectins (Remoroza et al., 2014). The block(s) of nonesterified GalA residues within the HG backbone represents 19 and 16% of all GalA residues in P5328 and F5129, respectively. The ratio of monomer to dimer plus trimer is quite low (~0.30) for both pectins (Table 3). This suggests that there are small blocks of nonesterified GalA residues (Daas, Voragen, & Schols, 2000). For P5328, about 9% ($DH^{I}_{PG} - DB^{I}_{abs}$) of the GalA residues was present as partly methylesterified and/or acetylated GalA residues in blocks, while this value is 22% for F5129. These blocks are represented by GalA oligomers of DP 4–6 carrying 1–3 methylesters and one acetyl group substituted in a random manner. Clearly, these blocks are predominantly present in F5129. The number of GalA residues

released by PL ($DH^{I}_{PL}$) for the two pectins is 6 and 0% for P5328 and F5129, respectively. This clearly indicates that the f-PME modified F5129 does not contain blocks of methylesterified GalA residues being degradable by PL where P5328 does. This can be explained by modification of the methylester sequences by f-PME affecting the PL degradability.

### 3.8. Second digestion of two differently prepared DM 50 SBPs

For the second digestion, the action of PME is necessary to allow endo-PGII to release 29–47% of GalA residues from the total GalA residues present in the parental pectins (Table 3). The $DB^{II}_{abs}$ values for P5328 and F5129 are 10 and 17, respectively. These values do not indicate the presence of blocks of nonesterified GalA sequences but blocks of partially methylesterified GalA residues in F5129, which are endo-PGII degradable after f-PME treatment during the second digestion. It is remarkable that these segment do not contain any acetyl groups. The ratio of monomer to dimer plus trimer (Table 2) for both pectins about 0.10 indicating that these blocks from which these fragments have been released are rather long. The values for ($DH^{II}_{PG} - DB^{II}_{abs}$) are 14 and 24% for P5328 and F5129, respectively. Obviously, the removal of part of the methylesters from the endo-PGII/PL resistant fragments from the first digestion leads to a much higher release of partly methylesterified and/or acetylated oligomers for F5129 compared to P5328. This indicates a random distribution of methylesters in F5129 as was expected, probably even slightly more random compared to the starting pectin SBP6230. The $DH^{II}_{PL}$ values for the second digestion were similar (5–6%) for both pectins and represent only the unsaturated oligomers from the large fragments released by PL during the first digestion. PME de-methylesterification in the second digestion of F5129 clearly makes the endo-PGII/PL resistant fragments more suitable for endo-PGII degradation than is the case for P5328. This confirms the blockwise distribution of nonesterified and consequently methylesterified residues in the pectin being modified by p-PME. The added value of the second digestion clearly established the differences between p-PME and f-PME modified SBPs. This can be seen in sequences of esterified GalA residues, which are not degradable by endo-PGII or PL alone. When taking into account the method of preparation of the two DM 50 pectins, several observations can be stated. Pectin P5328, which has been modified from a parental SBP (DM 62, DA 30) using p-PME is expected to have a slightly more blockwise distribution of nonmethylesterified GalA sequences. On the contrary, pectin F5129, which originated from the same parental SBP after a partial removal of the methylesters using f-PME, is expected to have a random distribution of nonmethylesterified GalA sequences. The descriptive parameters indeed confirmed significant differences in the methylester distribution between the two pectins, although less pronounced than found before for lemon pectins (Körner, Buchholt, Christensen, Roepstorff, & Mikkelsen, 2000a). Large blocks of nonesterified GalA residues are not present in P5328 since the p-PME was hindered by the presence acetyl groups forcing the enzyme to act less in a blockwise manner than would have been the case for non-acetylated pectins. A typical consequence of a blockwise removal of methylesters is that other blocks of GalA residues remain highly methylesterified. This can be recognized from the higher $DH^{I}_{PL}$ value for P5328 compared to F5129. In addition, a random de-methylesterification pattern of f-PME is recognized from the higher release of partly methylesterified GalA oligomers without acetyl groups in F5129 compared to P5328 for both enzymatic digestions. Our findings support the hypothesis (Buchholt et al., 2004) stating that due to the steric hindrance of the acetyl group present, p-PME forced to de-methylesterify SBP in a combined random (Fig. 4 chain C) and blockwise manner.

## 4. Conclusions

In general, it can be stated that the two-step enzymatic fingerprinting is able to generate additional information on the patterns of SBPs having the same DM and DA compared to the commonly used one-step enzymatic fingerprinting methods. The various proportions of different blocks of nonesterified, partly methylesterified/acetylated and highly methylesterified/acetylated sequences of GalA found for three differently prepared sugar beet pectins describe the ester distributions within the pectins in more detail than has been done before. It should be stated that the two-step fingerprinting method is laborious and, is therefore, far from suitable for routine analysis of pectins. Nevertheless, the additional information to be obtained for complex pectins is important to understand its precise chemical structure and to make the link to their functionality.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.02.052.

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