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Research paper

Acetylated pectins in raw and heat processed carrots

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ABSTRACT

Heat processing results in softening of carrots, changing the pectin structure. The effect of heat processing on pectin was studied, showing that the amount of pectin in water soluble solids (WSS) and chelating agent soluble solids (ChSS) increased substantially upon heat processing of the carrots. Pectin in WSS from both unprocessed and heat processed carrot had a degree of methyl-esterification (DM) of ≈60% and a degree of acetylation (DA) of ≈20%. Enzymatic degradation released methyl-esterified galacturonic acid oligomers of degree of polymerisation ≥6 carrying acetyl groups. Mass spectrometry confirmed acetylation in highly methyl-esterified homogalacturonan (HG) regions, next to known rhamnogalacturonan (RG-I) acetylation. ChSS HGs were unacetylated.

RG-I levels of both heat processed carrot WSS and ChSS increased. Digestion of WSS with RG-I degrading enzymes showed that WSS arabinan became more linear upon heat processing resulting in the release of oligosaccharides, while in ChSS galactan became more linear.

1. Introduction

Vegetable-based products are often processed prior to consumption and the processing method used might have a big effect on the texture and firmness of the product. It was shown before that the most common processing method, thermal treatment at elevated temperature, decreased the firmness of carrots (Sila, Smout, Elliot, Van Loey, & Hendrickx, 2006). The decreased firmness can be explained by alterations in pectin structure.

Pectin is a complex mix of polysaccharides, building up the primary plant cell wall and middle lamella of vegetables. Pectin consists of galacturonic acid as the most prevailing building block, mostly present in homogalacturonan (HG) and in rhamnogalacturonan I (RG-I) structural elements. The HG backbone can be methyl-esterified at the C-6 position, and acetylated at the O-2 and/or O-3 position. The rhamnose residues in RG-I can be substituted with neutral sugar side chains, composed of arabinose and galactose (Voragen, Coenen, Verhoef, & Schols, 2009).

Softening of carrot tissue upon heat processing is mainly due to β-elimination of pectin, a pH- and temperature-dependent reaction which is highly relevant in carrots processing due to the pH of carrot tissue (≈5–5.5) (Bemiller & Kumari, 1972; Sila, Doungla, Smout, Van Loey, & Hendrickx, 2006). The molecular weight of the pectin decreases and the amount of easily extractable, water soluble pectin increases by β-eliminative depolymerisation. Due to pectins ability to form Ca^{2+}-mediated crosslinks, the degree of methyl-esterification and acetylation are also affecting firmness. It was shown that a decrease of the DM of pectins due to thermal processing promotes Ca^{2+}-crosslinking between pectin chains and hereby improves firmness (Sila, Doungla, et al., 2006).

Pectins from many sources are highly methyl-esterified, while also acetylation of RG-I is common. In addition, pectins from some specific origin, e.g. potato, sugar beet and chicory pulp are found to be acetylated on the homogalacturonan region as well (Ramasamy, Gruppen, & Schols, 2013; Ramaswamy, Kabel, Schols, & Gruppen, 2013). It is known that the functional properties of pectin depend on the distribution of methyl-esters and acetyl groups (Ralet, Crépeau, & Bonnin, 2008). Enzymatic fingerprinting methods have been established to study the distribution pattern of methyl-esters and acetyl groups in sugar beet pectin (Ralet et al., 2008; Remoroza, Broxterman, Gruppen, & Schols, 2014).

In the current study, the effect of heat processing on carrot pectin was studied by extraction of Water Soluble Solids (WSS) and Chelating agent Soluble Solids (ChSS). Subsequently, a two-step enzymatic fingerprinting of the extracted pectin in WSS and ChSS was performed using pectin degrading enzymes. Oligosaccharides formed after the second digestion were analysed to localize acetyl groups and methyl esters in WSS and ChSS.

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2. Materials and methods

2.1. Plant material

Carrots (*Daucus carota* cv. Nantaise) were obtained in Spain and frozen at −20 °C directly after purchase to avoid enzymatic activity and microbial spoilage during storage. For the processed samples, carrots were cut into cubes and subsequently heat processed.

Three different processing conditions were performed: blanching at 90 °C for 5 min followed by sterilisation at 110 °C for 6 min; blanching at 100 °C for 1 min followed by sterilisation at 110 °C for 22 min and blanching for 1 min at 100 °C followed by sterilisation at 120 °C for 33 min.

2.2. Extraction of pectin fractions

Alcohol Insoluble Solids (AIS) were obtained using a procedure as described before with slight modifications (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011), by blending frozen, peeled carrots or heat-treated carrot cubes in a 1:3 w/v ratio in 96% ethanol. The suspension was filtered using Whatman filter paper (pore size 12-25 μm) and the retaining residue was resuspended in 70% ethanol. The suspension was filtered and the residue was washed with 70% ethanol until the filtrate gave a negative reaction in the phenol-sulfuric acid test (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). In the final washing step acetone was used. AIS was dried at 40 °C and stored in a desiccator.

To extract the water soluble solids (WSS) 1 g AIS was suspended in 150 ml distilled water and held for 10 min at 99 °C. The suspension was filtered using Whatman filter paper and the filtrate was freeze-dried, yielding Water Soluble Solids (WSS).

To extract the Chelating agent Soluble Solids (ChSS), the residue from WSS extraction was suspended in 150 ml 0.05 M EDTA in 0.1 M potassium acetate for 6 h at room temperature. The suspension was filtered using Whatman filter paper, and the filtrate was extensively dialysed against potassium acetate followed by demineralised water, and freeze-dried to obtain the Chelating agent Soluble Solids (ChSS). Extraction was performed only one time. In general, the extraction procedure results in highly reproducible data with standard deviation < 3%.

2.3. Characterisation of the extracts

2.3.1. Sugar composition

To determine the pectin content of the extracted fractions, the uronic acid content was determined by the automated colorimetric m-hydroxydiphenyl method (Blumenkrantz & Asboe-hansen, 1973). Neutral carbohydrate composition was analysed after pretreatment with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). Sugars released were derivatised and analysed as their alditol acetates using gas chromatography (Englyst & Cummings, 1984), inositol was used as internal standard.

The HG to RG-I ratio was calculated by: $\frac{GalA - Rha}{2 * Rha}$ in mol%. The average side chain length of RG-I was calculated by: $\frac{Ara + Gal}{Rha}$ (Huang et al., 2017).

2.3.2. Determination of methyl-esterification and acetylation

Samples were saponified at ≈3 mg/ml in 0.25 M NaOH (3 h, 4 °C) to determine the degree of methyl-esterification (DM) using a colorimetric method as previously described (Klavons & Bennett, 1986). The same saponification procedure was used to determine the degree of acetylation (DA), by measuring released acetic acid by a Megazyme acetic acid kit (Megazyme, Wicklow, Ireland). The DM and DA were calculated as the moles of methyl esters and acetyl groups per 100 mol of GalA, respectively.

2.4. Enzymatic degradation and fractionation

2.4.1. Digestion by PG/PL

In order to study the fine chemical structure of pectin, controlled degradation by pectolytic enzymes was performed. WSS and ChSS were degraded by polygalacturonase (endo-PG) from *A. aculeatus* and pectin lyase (PL) from *A. niger*, both pure and well characterised (Limberg et al., 2000; Schols, Posthumus, & Voragen, 1990). Degradation was done at 5 mg/ml WSS or ChSS in 50 mM sodium citrate buffer pH 5 at 40 °C by head-over-tail rotation for 24 h. Enzymes were dosed to theoretically degrade all substrate to monomers in 6 h.

2.4.2. Fractionation into size-based pools

The small oligosaccharides formed by PG and PL were separated from the high Mw fragments by gel filtration according to the protocol previously described by Remoroza, Broxterman et al. (2014). The 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) was equilibrated using 25 ml of 50 mM sodium citrate buffer (pH 5.0) at room temperature. Freeze-dried PG + PL treated pectin (10 mg pectin) was dissolved into 300 μl 50 mM sodium citrate buffer (pH 5.0) and eluted with 4.70 ml of 50 mM sodium citrate buffer (pH 5.0) in fractions of 0.5 ml. HPSEC was used to determine the Mw distribution of the fractions, and to pool the digest based on the Mw into a high Mw pool (Pool I) and low Mw pool (Pool II).

2.4.3. Digestion of high Mw fraction by PG/PME/RGE

To degrade the PG- and PL-resistant polymeric material, a second enzymatic treatment was performed with high Mw material pool I obtained after the size-based separation. The second digestion was performed with pectin methyl esterase from either *A. niger* (f-PME) or *Dickeya didantii* (b-PME), endo-PG from *A. aculeatus* and RG-I degrading enzymes. The RG-I degrading enzymes (RGE) used were *A. aculeatus* endo-arabinanase (Beldman, Searle-van Leeuwen, De Ruiter, Siliha, & Voragen, 1993), endo-galactanase (Schols et al., 1990) and RG-hydrolase, and exo-arabinase from *Myceliophthora thermophila C1* (Kühnel et al., 2010).

Incubation was done in 50 mM sodium citrate buffer pH 5 at 40 °C by head over tail rotation for 24 h. Enzymes were dosed to theoretically degrade all substrate to monomers or to remove all methyl esters present (PME) in 6 h.

2.5. Structural characterisation of the extracts

2.5.1. High performance size exclusion chromatography (HPSEC)

Extracted pectin fractions before and after enzymatic digestion were analysed for their molecular weight distribution using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) coupled to a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). A set of TSK-Gel super AW columns 4000, 3000, 2000 (6 mm × 150 mm) preceded by a TSK-Gel super AW guard column (6 mm ID × 40 mm) (Tosoh Bioscience, Tokyo, Japan) was used in series. The column temperature was set to 55 °C. Samples (5 mg/ml) were injected (10 μl) and eluted with 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min. Pectin standards from 10 to 100 kDa were used to estimate the molecular weight distribution (Voragen et al., 1982).

2.5.2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF and MALDI-TOF/TOF mass spectra were recorded using an Ultraflextreme workstation controlled by FlexControl 3.3 software (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser of 355 nm and operated in positive mode.

Before analysis by MALDI-TOF MS, samples were desalted with Dowex 50W-X8 (Bio-Rad Laboratories, CA, USA) and 1 μl of sample was co-crystallised with 1 μl matrix (25 mg/ml dihydroxy-benzoic acid in

Table 1
Yield of AIS, WSS and ChSS isolated from unprocessed and heat processed carrot on dry matter basis.

	% AIS(dry matter)	mg WSS/g AIS	mg ChSS/g AIS
Unprocessed carrot	31.4	213	145
90 °C/5 min + 110 °C/6 min	51.0	321	201
100 °C/1 min + 110 °C/ 22 min	44.5	273	166
100 °C/1 min + 120 °C/ 33.5 min	45.1	321	181

50% (v/v) acetonitrile). Samples were dried under a stream of air.

For TOF/TOF mass analysis, parent and fragment ions were accelerated using a LIFT device located in the flight tube using the standard LIFT method optimized for the instrument. Maltodextrin MD 20 (Avebe, Foxhol, The Netherlands) was used for calibration.

Analysis of the uronic acid oligosaccharides in the PG/b-PME/RGE digest of the heat processed carrot samples improved after washing out the neutral oligosaccharides. This was done using centrifugal filter units with a cut-off of 3 kDa (Millipore centrifugal filter units, Merck, Billerica, Massachusetts, United States).

3. Results and discussion

3.1. Yield and composition of different pectin populations

In order to study the effect of heat treatment on carrot pectin, unprocessed and heat processed carrot cubes were subjected to analysis. AIS was isolated, and sequentially fractionated into WSS, ChSS and the residue. The effect of the different process conditions on the amount of AIS, WSS and ChSS extracted from carrots is presented in Table 1.

Comparison of the yield of alcohol insoluble solids (AIS) in the unprocessed and processed carrot samples shows that the yield increased by ≈50% after processing (Table 1). Although the yield increased, the GalA content decreased (see below, Table 2), and therefore about the same total amount of GalA was recovered by the AIS.

As well as AIS, the yield of water soluble solids (WSS) after processing increased by ≈50% compared to the unprocessed carrot sample. Next to an increased yield, the relative galacturonic acid content in the WSS fraction increased as an effect of processing (Table 2). This is assumed to be due to β-eliminative depolymerisation and subsequently enhanced extractability of pectins (Kravtchenko, Arnould, Voragen, & Pilnik, 1992). Analysis of the starch content in unprocessed WSS showed that up to 30% of glucose originates from starch and was not removed during AIS preparation.

The amount of ChSS, representing extracted calcium-bound pectin, increased after processing as well. Since the amount of calcium-bound pectin extracted into ChSS strongly depends on the DM, the yield of ChSS increased when the DM of cell wall pectin decreased upon processing due to the formation of calcium-sensitive pectin (Sila, Doungla, et al., 2006). Endogenous PME might have been active during heating of the sample, decreasing the DM, since PME activity in carrots has been reported previously (Ly-Nguyen et al., 2003). However, a decrease in methyl-esterification might be due to chemical lability of the methyl-ester as well.

It can be seen that the HG:RG-I ratio for pectin in WSS had a slightly higher relative RG-I content and pectin in ChSS a higher HG content for unprocessed when compared to processed carrot pectin.

In order to ensure that all carrot pectin was included in the analysis of processed carrots, the covering liquid in the cans in which the carrot cubes were heat processed was freeze-dried and analysed for its sugar composition. The sugar composition of this fraction was similar to WSS with respect to the sugar composition and ratios of HG and RG-I isolated, but the covering liquid contained more glucose than WSS did. This is assumed to be free glucose. However, the yield of solids in the covering liquid compared to AIS was rather low and the covering liquid material was therefore not taken into account furthermore.

The degree of methyl-esterification (DM) and acetylation (DA) are of importance for the functional properties of pectin. Both DM and DA were determined for AIS, WSS and ChSS (Table 2). The DM of the WSS fraction for the unprocessed carrot was 68%, and this corresponds with values reported previously (Massiot, Rouau, & Thibault, 1988; Sila, Doungla, et al., 2006). The DM of the pectin present in AIS decreased ≈ 50% upon heat processing as a combined effect of PME activity and thermal instability, as mentioned before.

The effect of heat processing on the decrease of DM in the WSS fractions was less pronounced than found for AIS. This has been reported before and indicates the specific solubilisation of highly methyl-esterified pectin by water extraction of the cell wall material (De Roeck, Sila, Duvetter, Van Loey, & Hendrickx, 2008). Acetylation levels of AIS were quite high, the DA was 30–40%, but since AIS also contains hemicellulose the DA might be an overestimation since calculated on basis of GalA contents. Surprisingly, quite a high DA was found for WSS, while pectin acetylation of carrot pectin was only scarcely reported before (Massiot, Rouau, & Thibault, 1988). The DA stayed rather stable upon heat processing.

Acetylation of pectin has so far mainly been related to the presence of acetylated GalA in the RG-I region (Santiago, Christiaens, Van Loey, & Hendrickx, 2016; Schols & Voragen, 1994). It can be calculated from the HG:RG-I ratio that the HG region had to be acetylated as well, since the percentage of acetyl groups was too high to be linked to the GalA residues in RG-I. Acetylation of pectin HG will be investigated and discussed below.

Table 2
Sugar composition (mol%) and degree of methyl-esterification and acetylation of AIS, WSS and ChSS of fractions isolated from unprocessed and heat processed carrots. The standard deviation (SD) is the mean of two replicates for the sugar composition, and of 3 replicates for DM and DA.

	Treatment	Mol% (SD)							DM (%) DM (SD)	DA (%) DA (SD)
		Rha	Ara	Xyl	Man	Gal	Glc	GalA		
AIS	Unprocessed carrot	2 (0.0)	8 (0.4)	2 (0.0)	3 (0.0)	11 (0.8)	36 (1.1)	38 (2.4)	71 (3.7)	41 (5.1)
	90 °C/5 min + 110 °C/6 min	2 (0.0)	7 (0.2)	3 (0.1)	4 (0.1)	9 (0.3)	43 (1.3)	32 (0.9)	40 (2.2)	42 (3.4)
	100 °C/1 min + 110 °C/22 min	1 (0.0)	6 (0.1)	4 (0.1)	4 (0.3)	8 (0.1)	49 (1.0)	28 (0.9)	27 (2.0)	30 (1.7)
	100 °C/1 min + 120 °C/33.5 min	2 (0.0)	7 (0.2)	3 (0.0)	4 (0.2)	9 (0.4)	48 (0.3)	27 (0.6)	29 (0.8)	30 (1.8)
WSS	Unprocessed carrot	1 (0.0)	3 (0.1)	0 (0.4)	9 (0.1)	5 (0.2)	11 (0.3)	40 (0.3)	68 (5.8)	18 (1.3)
	90 °C/5 min + 110 °C/6 min	2 (0.0)	10 (0.0)	0 (0.0)	2 (0.1)	15 (0.1)	10 (0.3)	62 (0.3)	60 (2.5)	18 (1.3)
	100 °C/1 min + 110 °C/22 min	2 (0.1)	11 (0.6)	0 (0.0)	2 (0.1)	18 (0.8)	10 (0.3)	57 (1.2)	52 (1.1)	18 (0.7)
	100 °C/1 min + 120 °C/33.5 min	2 (0.3)	11 (0.1)	0 (0.0)	3 (0.3)	17 (0.3)	13 (0.1)	54 (0.3)	57 (1.1)	24 (0.6)
ChSS	Unprocessed carrot	0 (0.4)	5 (0.4)	0 (0.0)	0 (0.0)	4 (0.2)	0 (0.0)	91 (0.2)	47 (5.4)	3 (0.2)
	90 °C/5 min + 110 °C/6 min	2 (0.2)	8 (0.8)	0 (0.0)	0 (0.0)	10 (1.0)	0 (0.0)	81 (2.0)	15 (3.2)	8 (1.0)
	100 °C/1 min + 110 °C/22 min	2 (0.0)	6 (0.1)	1 (0.5)	0 (0.0)	7 (0.1)	2 (2.3)	82 (3.0)	5 (2.1)	5 (1.0)
	100 °C/1 min + 120 °C/33.5 min	2 (0.1)	9 (0.1)	0 (0.0)	0 (0.0)	12 (0.1)	2 (0.4)	75 (0.3)	7 (1.2)	9 (1.9)

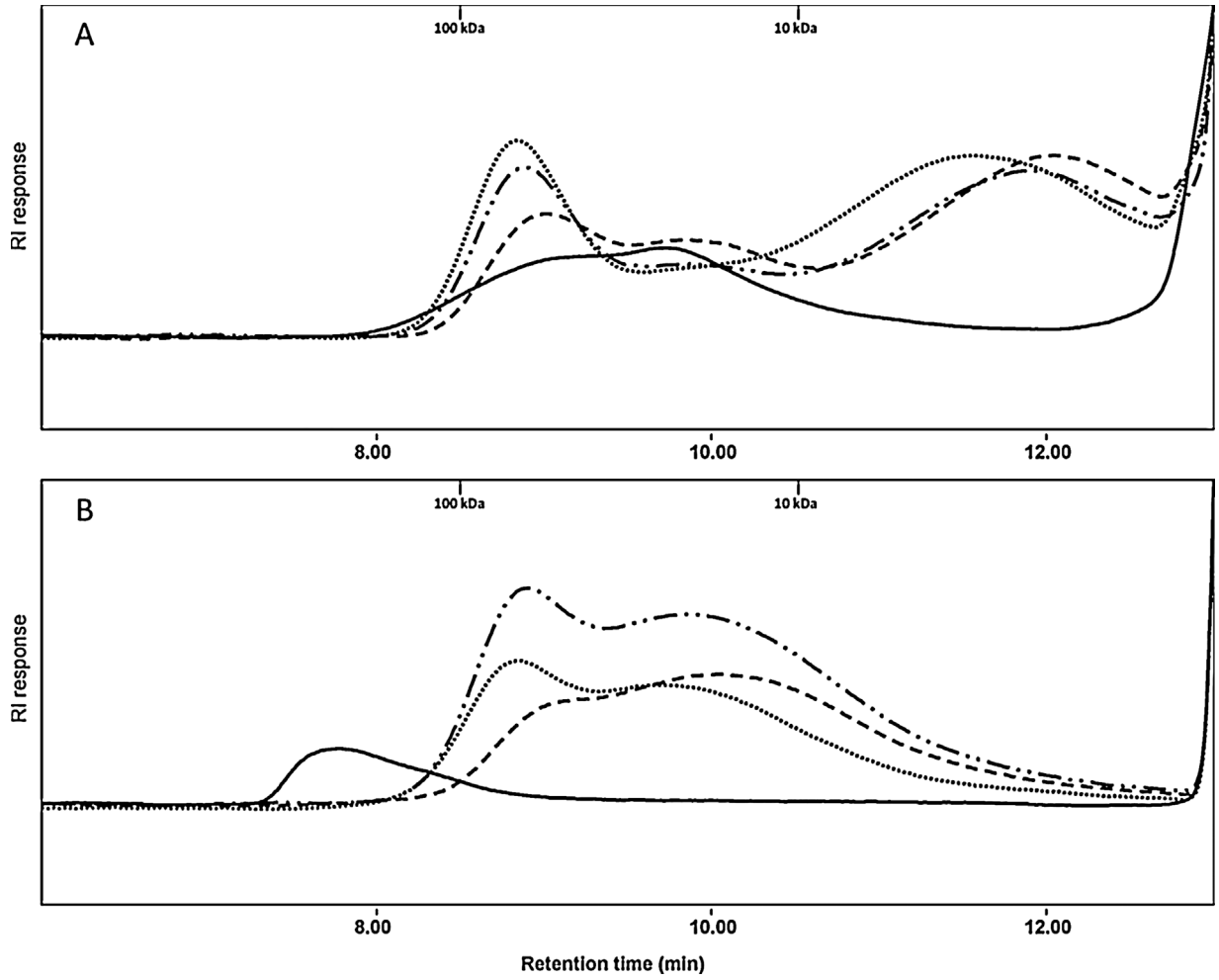

Fig. 1. HPSEC elution pattern of (A) Water Soluble Solids (WSS) and (B) Chelating agent Soluble Solids (ChSS) of unprocessed and heat processed carrots. Unprocessed (—); 90 °C/5 min + 110 °C/6 min (···); 100 °C/1 min + 110 °C/22 min (—); 100 °C/1 min + 120 °C/33.5 min (—··). Molecular weights of pectin standards (in kDa) are indicated.

Comparing the DM and DA of unprocessed with heat processed carrot samples showed that the DM and DA were lower in ChSS compared to WSS. Lower values were not surprising since the EDTA extracted ChSS pectin was typically bound by calcium, requiring long stretches of non-esterified GalA residues (Braccini & Pérez, 2001).

3.2. Effect of heat treatment on pectin molecular weight

The molecular weight distributions of the extracted fractions WSS and ChSS was determined by HPSEC and are presented in Fig. 1. The molecular weight (Mw) distribution of both WSS and ChSS changed, indicating depolymerisation after processing. The increase in area in comparison to the unprocessed carrot sample indicates e.g. an improved solubility or an increased pectin content of the extract after processing.

Upon processing, pectin populations with a high Mw were solubilised. Surprisingly quite some high Mw material was not depolymerised, even after processing carrots for 33 min at 120 °C. Besides solubilisation of high Mw pectin populations, extensive depolymerisation into pectin with a lower Mw distribution is visible.

The extracted pectin populations as present in ChSS were less extensively depolymerised than WSS in comparison to the unprocessed carrot sample, and no additional high Mw pectin was released (Fig. 1B). In carrot it is expected that an increase in temperature during processing increases the rate of β-elimination more than the rate of de-esterification (Kravtchenko et al., 1992). However, the low DM (5–15%, Table 2) clearly limited the rate of β-elimination in ChSS.

3.3. Enzymatic fingerprinting of WSS

3.3.1. Degradation of WSS by PG/PL and fractionation into size-based pools

In order to study the distribution of methyl esters and acetyl groups in pectin in WSS, an enzymatic degradation method was used (Remoroza, Broxterman et al., 2014).

Water soluble pectin was degraded with PG from *A. Aculeatus* and PL from *A. Niger*. The oligosaccharides in the digest were analysed by MALDI-TOF MS, showing the presence of unsaturated oligosaccharides in the unprocessed carrot WSS PG/PL digest (Fig. 2A). This was expected for pectin with DM ≈ 60% digested by PG and PL since PG releases saturated uronic acid oligomers from non-esterified pectin and PL releases unsaturated uronic acid oligomers from high methyl-esterified pectin. Pectic oligosaccharides were present having varying levels of esterification, e.g. from $U4^{10}$ to $U4^{40}$, meaning unsaturated GalA tetramer carrying 1–4 methyl-esters and no acetyl groups. It was shown that the heat processed carrot PG/PL digests show mainly unsaturated oligosaccharides but saturated oligosaccharides are found as well (Fig. 2B). The decrease in DM when comparing the unprocessed to heat processed carrot pectins (Table 2) was reflected in the oligosaccharides found by MALDI-TOF MS. Oligosaccharides in the unprocessed carrot PG/PL digest were more methyl-esterified than oligosaccharides in the heat processed carrot PG/PL digest. For both the unprocessed and the heat processed carrot PG/PL digest, oligosaccharides from DP 3–9 showed the same pattern and DP 4 and 5 were selected to exemplify the oligosaccharides present.

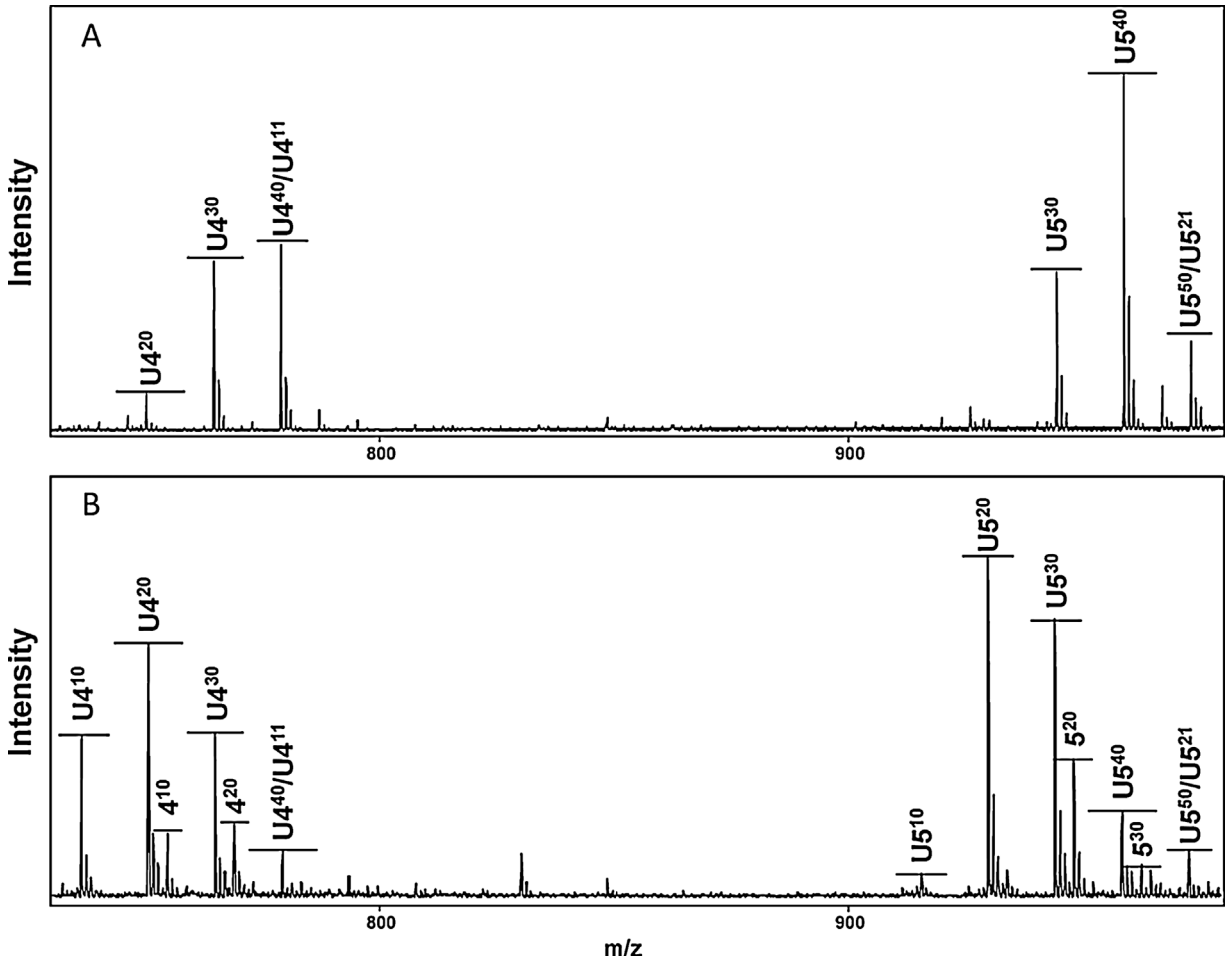

Fig. 2. MALDI-TOF mass spectrum of the PG/PL digest of (A) unprocessed carrot WSS and (B) WSS after heat treatment (100 °C/1 min + 120 °C/33.5 min). Peak annotation: $U4^{20}$, unsaturated GalA DP 4, 2 methyl-esters, 0 acetyl groups.

It has to be noted that in mass spectrometry $U4^{40}$ and $U4^{11}$ could not be differentiated since the mass of 3 methyl esters equals 1 acetyl group. Since the pattern of oligosaccharides formed showed the presence of unsaturated oligosaccharides containing 1, 2 and 3 methyl esters, the presence of $U4^{40}$, carrying 4 methyl-esters, was more likely than $U4^{11}$, carrying 1 methyl-ester and 1 acetyl group. The same applied for $U5^{50}/U5^{21}$.

The DA of all WSS fractions was relatively high, and therefore it was expected that acetylated oligosaccharides were formed after digestion with PG/PL. However, only methyl-esterified and no acetylated oligosaccharides were present in both the unprocessed and processed carrot PG/PL digests.

To confirm the presence of acetyl groups on HG and to get more structural information, the remaining polymeric pectin fragments had to be degraded to oligosaccharides. In order to perform a second, more efficient second enzymatic degradation step, the polymeric pectin was separated from the smaller oligosaccharides by fractionation of the PG/PL digest into a high Mw pool (pool I) and a low Mw pool (pool II) (data not shown). After fractionation of the PG/PL digest, ≈ 33% of all uronic acids present was recovered as oligosaccharides in the low Mw pool for both the unprocessed and heat processed WSS fraction. This indicates that the majority of the pectic material indeed was not degraded by PG/PL.

After isolation of the polymeric pectin, the DA of Pool I was determined. As shown in Table 3, the DA of pool I increased when compared to the DA of WSS. This confirms that acetylated polymeric pectin was separated from non-acetylated oligosaccharides. Taking the ratio HG:RG-I into account (Table 2), it also confirms that acetylation occurs in distinct HG regions. Highly acetylated HG is therefore a unique characteristic of carrot pectin since so far acetylation has only been described in detail for sugar beet pectin (Ralet et al., 2005).

Table 3
Degree of acetylation of WSS and of Pool I after size-based fractionation of unprocessed and heat processed carrots. The standard deviation (SD) is the mean of two replicates.

	Water soluble solids	
	DA WSS (SD) (%) DA WSS (SD)	DA WSS Pool I (SD) (%) DA WSS Pool I (SD)
Unprocessed carrot	18 (1.3)	38 (1.5)
90 °C/5 min + 110 °C/6 min	18 (1.3)	28 (1.9)
100 °C/1 min + 110 °C/ 22 min	18 (0.7)	23 (3.1)
100 °C/1 min + 120 °C/ 33.5 min	24 (0.6)	27 (2.0)

3.3.2. Enzymatic degradation of high Mw WSS Pool I by PG/b-PME/RGE

To confirm the presence of acetylation in the HG-region and to characterise the distribution of methyl esters and acetyl groups, a second enzymatic degradation step was performed on the acetylated polymeric pectin in high Mw Pool I originating from unprocessed carrot. This was done using polygalacturonase (PG), bacterial pectin methyl esterase (b-PME) and RG-I degrading enzymes (RGE). The effect of RG-I degrading enzymes on pectin degradation after heat processing will be discussed below in 3.5.

A part of the methyl-esters was removed from the pectin by b-PME,

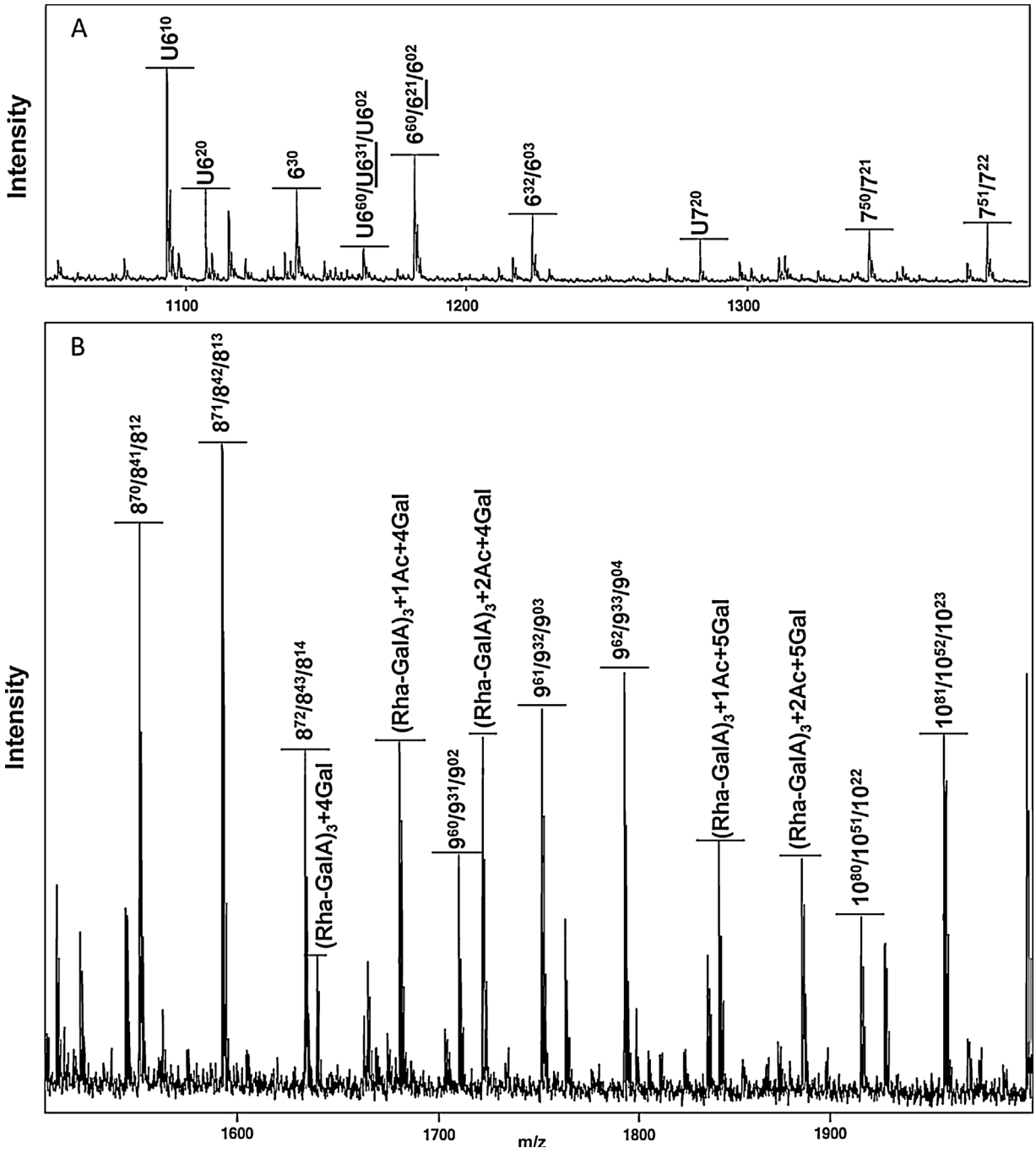

Fig. 3. MALDI-TOF mass spectrum of PG/b-PME/RGE digest of WSS from the unprocessed carrot, showing (A) DP 6–7 and (B) DP 8–10 oligomers. Peak annotation: 8^{41}, saturated GalA DP 8; 4 methyl-esters, 1 acetyl group. Gal, galactose; Rha, rhamnose; Ac, acetyl group. The most logical structure per isomer is underlined.

enabling PG to better degrade the HG-region and to release partially methyl-esterified and acetylated oligosaccharides. It was calculated from the DM before and after b-PME treatment that roughly 40% of methyl-esters was still present after incubation of carrot pectin with b-PME from *D. Dadantii*. Although part of the information on the methyl-ester distribution is lost when using b-PME, still valuable information on the methyl-ester distribution in relation to acetyl groups can be obtained. RG-I degrading enzymes were used to degrade RG-I. Analysis of the RG-I oligosaccharides was done to check for acetylation in RG-I regions as well.

Analysis of the PG/b-PME/RGE digest by MALDI-TOF-MS showed the presence of partially acetylated uronic acid oligomers of DP ≥6 in the unprocessed carrot sample, confirming acetylation in the HG region (Fig. 3A). The distribution of acetyl groups must have been quite distinct, since hardly any oligosaccharides ≤DP 5 were acetylated (data not shown). Although it is assumed that ionization efficiency decreases with increasing size and that the MS signal intensity can therefore not be used quantitatively, the MS intensity shows that the relative abundance of DP 3–5: 6–7: 8–10 is approximately 5:2:1 (data not shown). It can therefore be concluded that a quite substantial part of all pectin degraded to oligosaccharides is acetylated.

As mentioned before, 40% of the methyl-esters was still present after incubation of unprocessed carrot pectin with b-PME. Partially methyl-esterified oligomers would therefore be expected after digestion with PG/b-PME/RGE. As can be observed in Fig. 3, several oligosaccharides are tentatively suggested for one single *m/z* value. The

mode of action of PG and the presence of partially methyl-esterified fragments indicate that, e.g., the hypothetical structures of fully methyl-esterified oligomer 6^{60} and the acetylated, non-methyl esterified oligomer 6^{02} as presented in Fig. 3 as possible annotation of the oligomers are not likely to be dominant structures, whereas 6^{21} will be dominant. Following the same reasoning, also for DP 7–10, it can be concluded that both methyl-esterification and acetylation exist within the these oligomers. In order to differentiate between tentative structures, fragmentation spectra in Section 3.3.3 will provide additional information. The oligosaccharides shown in Fig. 3B confirm acetylation in the RG-I region after enzymatic digestion with PG/b-PME/RGE. It was shown before that digesting the pectins with PG/b-PME/RGE yielded partially acetylated RG-I oligosaccharides (Remoroza et al., 2012). However, acetylation of the HG region is with certainty confirmed as well since the majority of the oligomers ≥DP 6 are partially acetylated.

Determination of the degree of blockiness would be the most preferable way to quantitatively describe the distribution of acetyl groups. Remoroza, Broxterman et al. (2014) successfully used HILIC-ELSD-MS to characterise and quantify GalA oligosaccharides since in sugar beet pectin also smaller oligosaccharides were acetylated (Remoroza, Buchholt, Gruppen, & Schols, 2014). The acetylated oligosaccharides in carrot pectin digests were larger (≥DP 6) and not observed on HILIC-ELSD-MS, due to poor solubility under these chromatography conditions and/or due to poor ionization. It was therefore only possible to describe the structures in a qualitative way by using MALDI-TOF MS.

3.3.3. Fragmentation of acetylated oligosaccharides

In order to differentiate between tentative structures in the unprocessed pectin in WSS, acetylated oligomers were fragmented by MALDI-TOF/TOF mass spectrometry.

Fragmentation of the parent ion *m/z* 1751 ($9^{61}/9^{32}/9^{03}$) is shown in Fig. 4. It can be seen that fragments from parent ion from both the reducing and the non-reducing end were found (mass difference of 18), e.g. 4^{20} and 4^{21}. Most fragments formed from the parent were partially acetylated and partially methyl-esterified (Fig. 4). The fragments found, in combination with the mode of action of PG, showed that the major structures for *m/z* 1751 were 9^{61} and 9^{32}. Fragmentation of other oligosaccharides ≥DP 6 showed similar patterns with partially acetylated and partially methyl-esterified fragments. This confirmed that acetylation is present in distinct methyl-esterified regions. However, in order to more precisely describe these 2 major structures, additional separation prior to MS, or additional MS fragmentation is needed. This is also true for the other acetylated GalA oligomers present in the digest, since due to the structural variation in differently substituted pectin populations within WSS, the complexity was too high to reach complete sequencing of the oligosaccharides. Labelling of the reducing end to simplify interpretation of the fragmentation pattern, was not successful.

Degradation by PG and fungal PME (f-PME) from *A. niger* did not lead to the formation of acetylated oligosaccharides visible by MALDI-TOF MS, whereas degradation by PG and bacterial PME (b-PME) from *D. dadantii* did. These degradation patterns give information about the distribution of the methyl-esters and acetyl groups. It was reported before that b-PME acts in a blockwise manner, whereas f-PME acts randomly (Seymour & Knox, 2002). Although a substantial part of the methyl-esters was removed by b-PME, it can be seen that most oligomers are still partially methyl-esterified. This indicates that acetylation is present in highly methyl-esterified regions since acetylated oligomers are only released after removing part of the methyl-esters from these regions. The fact that only a blockwise-acting b-PME allowed PG to

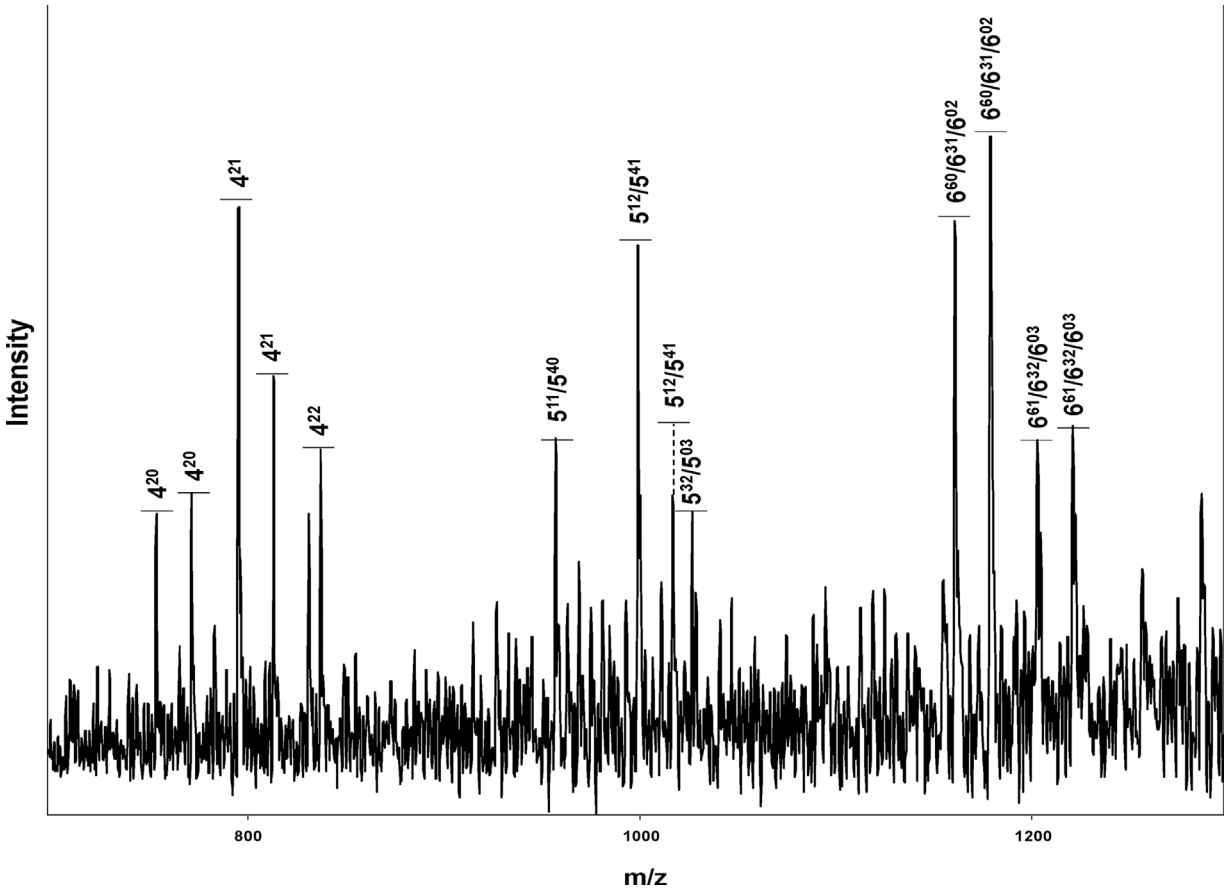

Fig. 4. Selected zoom of MALDI-TOF/TOF mass spectrum of OS with parent *m/z* 1751 from the PG/b-PME/RGE digest of WSS from unprocessed carrot. Parent ions are tentatively annotated as $9^{61}/9^{32}$.

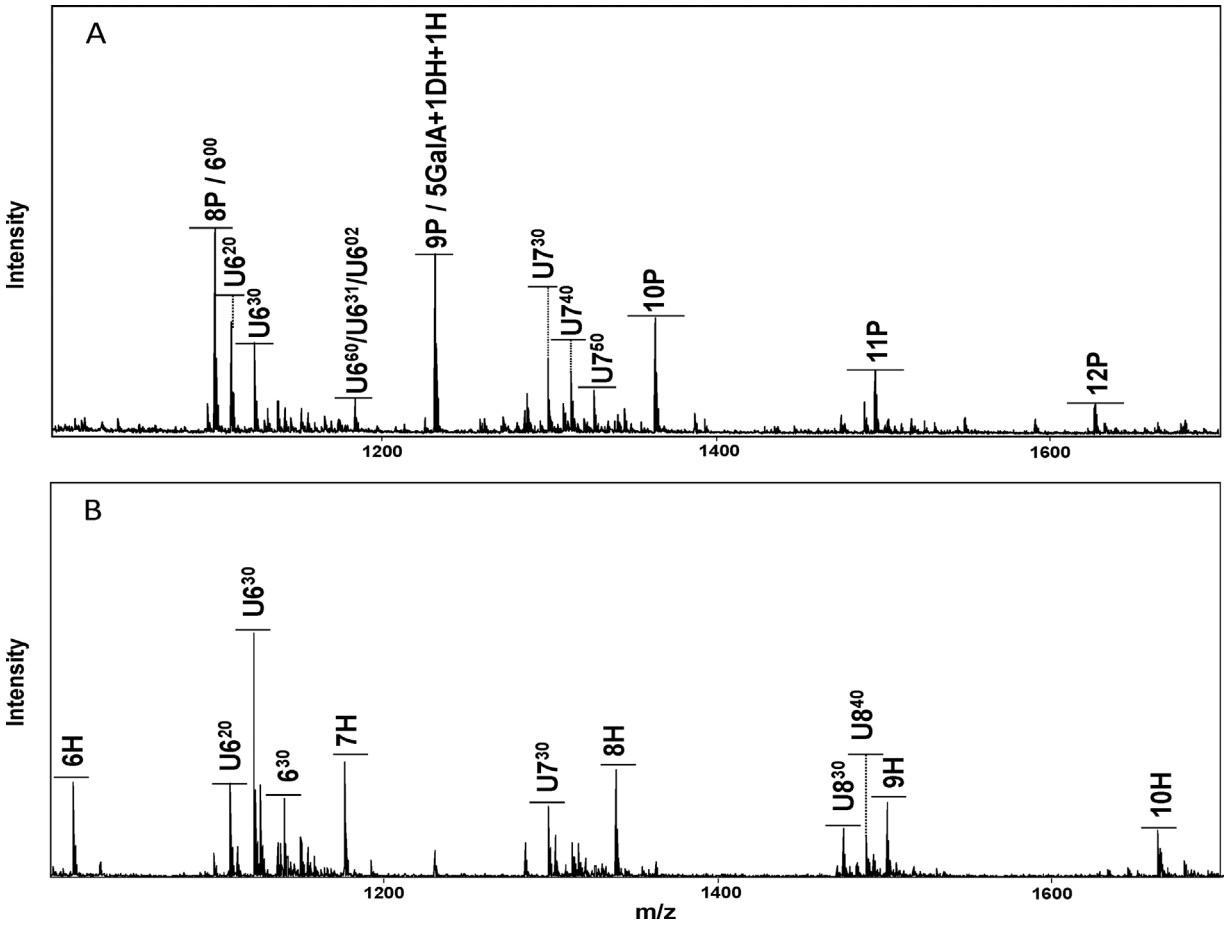

Fig. 5. MALDI-TOF mass spectrum of PG/b-PME/RGE digest of (A) WSS and (B) ChSS, both from heated carrot (90 °C/5 min + 110 °C/6 min). Peak annotation: 6^{00}, saturated GalA DP 6; 0 methyl-esters, 0 acetyl group; U: Unsaturated GalA; P, pentose; DH, deoxy hexose; H, hexose.

release oligosaccharides therefore shows that the GalA residues in acetylated regions are highly methyl esterified, possibly even fully methyl-esterified. Furthermore, our results indicate a certain tolerance of b-PME towards acetylated GalA since b-PME was able to deesterify in acetylated regions whereas f-PME was not, which was previously observed for b-PME from other sources as well (Remoroza et al., 2015).

3.3.4. Effect of heat treatment on HG in WSS

Whereas the unprocessed carrot WSS after the second incubation with PG/b-PME/RGE showed highly acetylated oligomers, these were not observed in the heat processed carrot WSS PG/b-PME/RGE digest. As can be observed in Fig. 5A, a mix of GalA and neutral oligomers was formed in the heat processed carrot PG/b-PME/RGE digest. The release of neutral oligosaccharides will be discussed below in paragraph 3.5. Since higher DP uronides certainly were expected based on the DA of heat processed WSS but could not be recognized in the mass spectrum, it was assumed that neutral oligomers ionise more easily than acidic oligomers. To test this hypothesis, neutral oligomers were washed away by filtration using a 3 kDa filter. Analysis of the retentate showed that the same acetylated oligosaccharides as found in the unprocessed carrot WSS PG/b-PME/RGE digest were indeed present in the heat processed carrot PG/b-PME/RGE digest (data not shown). This confirms that acetylation of GalA residues is not affected by heat processing.

3.4. Characterisation of HG in ChSS after PG/b-PME/RGE digestion

The same enzymatic degradation methods as described above were also used to characterise the effect of processing on detailed pectin structure in ChSS. Unprocessed and heat processed carrot ChSS were degraded by PG/PL to characterise the distribution of the methyl-esters and acetyl groups. Like in WSS, no acetylated oligosaccharides were found by MALDI-TOF MS in the PG/PL digest (data not shown). However, also the subsequent fractionation and degradation by PG/b-PME/RGE did not reveal the presence of acetylated oligosaccharides in unprocessed and heat processed carrot ChSS. With a DA of 3% for the unprocessed carrot ChSS and 5–9% for heat processed carrot ChSS, the acetyl groups are most probably exclusively present in RG-I and HG is not acetylated.

3.5. Effect of heat treatment on RG-I in WSS and ChSS

In Section 3.3 it was demonstrated that acetylation was present on HG and RG-I.

As can be observed in Fig. 3A and B, the digestion of unprocessed carrot WSS with PG/b-PME/RGE did not yield neutral oligosaccharides. Consequently, it was quite surprisingly that PG/b-PME/RGE digestion of heat processed carrot WSS and ChSS yielded neutral oligosaccharides originating from RG-I (Fig. 5). However, the oligosaccharides formed in both fractions were quite different, being enriched in pentose and hexose residues for WSS and ChSS respectively as will be discussed below.

Calculation of the ratio of RG-I to HG based on the sugar composition (Table 2) already showed that the ratio of RG-I to HG in WSS changed as an effect of processing, and the heat processed carrot WSS contained relatively more RG-I than the unprocessed carrot WSS. In order to understand the effect of heat processing and the enzyme accessibility of RG-I, it was of importance to identify all oligomers that were released. Based on the sugar composition in Table 2, the only

pentose sugar present in WSS is arabinose. In the mass spectrum of heat processed carrot WSS digest, *m/z* 1229 (Fig. 5A) corresponds with 9P or 5GalA + 1DH + 1H. Due to the presence of 8P, 10P, 11P and 12P, it was assumed that *m/z* 1229 corresponds with 9P. This was confirmed by incubation with another set of arabinofuranosidases (Rombouts et al., 1988). The presence of arabinose-based oligosaccharides after PG/b-PME-RGE digestion in heat processed WSS might indicate that arabinan was already partially modified by heat processing, and therefore more easily accessible for arabinanases. Partial modification of the pectic arabinan side chain by heat treatment might be explained by the fact that arabinose linkages are most labile amongst the glycosidic linkages in pectin (Thibault, Renard, Axelos, Roger, & Crépeau, 1993).

Digestion of the heat processed carrot WSS with only *endo*-arabinanase did not degrade the oligomers, indicating that the arabinose oligomers are highly branched, as found for arabinans from most primary plant cell walls (Dey & Harborne, 1997).

Like in WSS, the ratio of RG-I to HG in ChSS is higher in heat processed carrots than in unprocessed carrots (Table 2). Although ChSS contain both galactose (major) and glucose (minor) constituents, the hexose sugar present in the oligomers of the ChSS digest is most likely galactose, since released by galactanases present.

Comparison of the oligosaccharides formed by PG/b-PME/RGE digestion in unprocessed and heat processed carrots shows that the galactanase can be more active on ChSS in heat processed carrots. Galactan in ChSS from heat processed carrots is probably less branched with galactose or arabinose than galactan in WSS, and therefore more easily degradable by endo-galactanase. This shows that the galactan extracted into ChSS has a different structure from the galactan in WSS. Furthermore it also shows that not only the pectic arabinan side chains but also the galactan side chains are modified by heat processing. In unprocessed WSS and ChSS PG/b-PME/RGE digests no neutral oligosaccharides were formed.

4. Conclusions

The study of the effects of processing on pectin structure revealed new characteristics of carrot pectin. Processing increases the yield of water soluble pectin by β-eliminative depolymerisation, and increased solubilisation of RG-I rich pectin. For the first time the presence of acetylated HG in unprocessed carrot tissue was shown evidently. The lower DM and higher DA in pectin from processed and non-processed carrots showed that acetyl groups are more stable during processing than methyl-esters.

After a controlled enzymatic degradation and fractionation procedure it was found that acetyl groups are present in both HG and RG-I. Acetylation in HG was found to be present in distinct, highly methyl-esterified regions. Depending on the type of pectin, heat processing partially modifies the arabinans and galactans found in RG-I, and enhances enzyme accessibility and subsequent degradability of the more linear structures.

These findings indicate the extended complexity of carrot cell wall pectins including acetylation on HG and RG-I. The findings also encourage studies to learn more about the functionality of acetylation within the carrot cell wall, and about the variability of structural elements in raw plant material and its processed equivalents.

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