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Research Article

Release and characterization of single side chains of white cabbage pectin and their complement-fixing activity

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A mixture of single side chains from white cabbage pectin were obtained by anion exchange chromatography after applying mild chemical conditions promoting β-elimination. These pectin fragments were characterized by their molecular weight distribution, sugar composition, ^{13}C-NMR, and MALDI-TOF-MS analysis. These analyses revealed that the large oligosaccharides released by β-eliminative treatment were composed of α-1,5 linked arabinosyl residues with 2- and 3-linked α-arabinosyl side chains, and, or β-1,4 linked galactosyl side chains. Fractions were tested for complement-fixing activity in order to determine their interaction with the complement system. These results strongly indicated that there was a minimal unit size responsible for the complement-fixing activity. Neutral pectin fragments (~8 kDa) obtained from β-elimination were inactive in the complement system, although they contained a sugar composition previously shown to be highly active. Larger pectin fragments (~17 kDa) retained some activity, but much lower than polymers containing rhamnogalacturonan type 1 (RGI) structures isolated from the same source. This implied that structural elements containing multiple side chains is necessary for efficient complement-fixing activity.

Keywords: β-Elimination / Biological activity / Complement-fixing activity / Pectin / White cabbage

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1 Introduction

White cabbage is an important vegetable in Scandinavian households [1], and it is used for nutrition as well as in traditional medicine to treat bedsores [2] and inflammation in skin and joints [3]. The mechanism behind the wound healing property of plant material in not clear, but might be partly due to pectic substances interacting with the innate immune system [4, 5].

Pectin together with cellulose and hemi-celluloses are the main polysaccharide groups constituting the cell wall of dicotyledons. Pectin consists of a diverse set of structural elements. Homogalacturonan (HG) is mainly composed of partly methyl esterified stretches of α-D-1,4-linked Gal*p*A (*p*, pyranose) and has been suggested to consist of unique repeats of 80–120 α-D-Gal*p*A units [6]. Additionally, the *O*-2 and/or *O*-3 position(s) of Gal*p*A may be acetylated to some degree [7]. Some part of HG is named xylogalacturonan (XGA) [8, 9] since it contains monomeric β-D-Xyl*p* linked to *O*-3 of the Gal*p*A residue of the backbone. Rhamnogalacturonan I (RGI) is referred to as regions with 30–40 repeats of Gal*p*A and rhamnose (Rha*p*) pairs [6, 10, 11] with varying numbers of Rha*p* residues of which 20–80%

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Abbreviations: **AG,** arabinogalactan; **Ara,** arabinose; **BP,** *Brassica* pectic material; **C1q,** complement protein 1 subunit q; ***f***, furanose; **Gal,** galactose; **GalA,** galacturonic acid; **HG,** homogalacturonan; **HPSEC,** high performance size exclusion chromatography; **ICH_{50},** concentration of test substance giving 50% inhibition; **IEC,** ion-exchange chromatography; M_w, average molecular weight; **NS,** neutral sugar; ***p***, pyranose; **RGI,** rhamnogalacturonan 1; **Rha,** rhamnose **SEC,** size exclusion chromatography; **TBAF,** tetrabutylammonium fluoride; **XGA,** xylogalacturonan; **Xyl,** xylose

is branched with neutral side chains at *O*-4. These side chains consist mainly of Ara*f* (*f*, furanose) and Gal*p* residues linked in various manners, constituting polymers known as arabinogalactan I (AGI) [12] and AGII [13]. AGI is composed of a 1,4-linked β-D-Gal*p* backbone with *O*-3 substitutions of α-L-Ara*f* [14] and the Gal*p* backbone can have interspacing α-L-1,5-Ara*f* units [15]. AGII consists of highly ramified galactan with predominantly interior 1,3-linked β-D-Gal*p* with substitutions of short 1,6-linked chains exteriorly. The latter has further attachments of 1,3- and/or 1,5-linked α-L-Ara*f* [13].

Pectin has the ability to activate the complement system [16], which plays an important role in human innate immunity and consists of more than 20 serum proteins, which take part in a cascade mechanism when activated [17, 18]. Protein ligand complexes, which bind cascade initiator complement proteins, interact in different manners thus activating complement through the classical, alternative [19], and the lectin pathway [20]. Pectin has been suggested to mainly activate the complement system *via* the classical and alternative pathway [16, 21]. The classical activation pathway is initiated through the complement protein 1 subunit q (C1q), which has the shape of a bouquet of flowers with six globular heads with a range of ligand specificities [22].

It has been suggested that the structure responsible for complement-fixing activity contains 1,3- and 1,3,6-linked galactans [18, 23]. The smallest carbohydrate reported to activate the complement system is, to the best of our knowledge, an AG with an estimated size of 5 kDa [18]. Since it is unclear which specific polysaccharide structure is responsible for activating the complement system, studies are needed to characterize the specific architecture of the pectic neutral sugar (NS) side chains.

However, it has proven difficult to isolate single side chains from pectin, both by enzymatic and chemical approaches. The use of enzymes has been preferred to tailor the pectin structure, due to their ability to specifically degrade pectin under mild conditions. Structure analysis based on enzyme treatment has in many cases proven difficult due to structural variations limiting pectin degradation.

Chemical treatment of pectin has been of great interest in order to isolate single side chains. In this way, a more thorough structure annotation could be explored. The HG and RG backbone have successfully been degraded by the use of lithium in ethylenediamine to specifically cleave internal α-D-Gal*p*A linkages [24], regardless whether they are methyl esterified or not [25]. This method, however, results in undesired side chain deterioration in addition to the intended backbone cleavage. Recently, a method has been described utilizing sodium tetraborate at neutral pH and elevated temperature, which induces efficient β-elimination of premethyl esterified pectic samples [26]. However, the authors did not report whether the method is applicable for the release of longer more complex side chains. This would be of interest for both structure elucidation and for identification of functional properties of such unique structures.

This study exploits chemical treatments in order to obtain fractions containing single side chains from RGI. These structures would further be tested for complement-fixing activity. By comparing the original sample and the obtained side chains light would be shed on the mechanism behind complement-fixing activity. Observed differences would unravel structure function relationships and if macrostructure assemblies are necessary for complement-fixing activity. The method used has impact on both structural insight and prove a potential for isolating specific parts of pectin. To our knowledge, this is the first time that the potential complement-fixing activity of single side chains has been examined.

2 Materials and methods

2.1 Isolation of "prep scale" pectin from white cabbage (*Brassica oleracea*)

White cabbage (*B. oleracea* var. Capitata, Bartolo cultivar) was cultivated at the Vollebekk testfield (Aas, Akershus, Norway), and kept 1 wk postharvest at 0°C. Cabbage (200 kg) was destemmed and cut in 3–5 mm slices, immediately put into boiling water and kept for 3 h with continuous stirring. The treatment was set up to obtain a pectic product which would resemble the pectin content of blanching water used in vegetable processing. The resulting 200 L liquid "broth" was poured into 20 L buckets and cooled to 50°C. Efficient removal of protein was obtained by adding Neutrase (0.1 g/L; Novozymes, Bagsvaerd, Denmark) to the cabbage broth (pH 7.2). The protease efficiency was followed by applying samples to SDS-PAGE gels followed by subsequent silver staining [27]. The producer declare the Neutrase do not contain pectin degrading side activities. Potential undesired carbohydrase side activities was monitored by HPAEC-pulsed amperometric detection (PAD) (Dionex, Sunnyvale, USA), applying external standards of Ara, Gal, Xyl, and GalA.

Following the Neutrase treatment, plant material solids were removed from the broth by sieving. Low molecular weight sugars and color pigments were removed by ultrafiltration (Abcor membranes cut-off 10 kDa, Koch, Massachusetts, USA) continued until permeate was ≤ 1 μS. Further volume reduction of the retentate was obtained by vacuum evaporation (end volume 8 L). The concentrated solution was filtered (GF/C and F, Whatman, Kent, UK) with Celite (Sigma–Aldrich) as filter aid, prior to overnight polysaccharide precipitation at 4°C in 60% isopropanol. The precipitation was followed by two subsequent steps of washing with 60% ethanol, and finally one step of washing with pure ethanol. Centrifugation (15 min at $4000 \times g$) after each step pelletized the pectic material. Ethanol washed

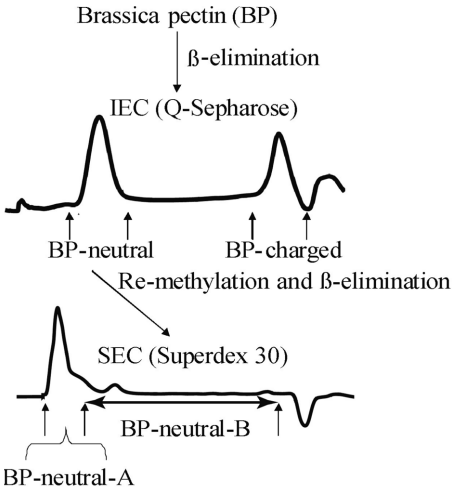

Figure 1. Flowchart describing the preparation of white cabbage pectin fractions, obtained after selective chemical degradation of BP (BP-charged, BP-neutral-A, and BP-neutral-B).

material was dried at 40°C and the resulting 280 g *Brassica* pectic material (BP) was finely grinded with a blender.

2.2 β-Elimination of *Brassica* pectin

BP (5 g) was first methyl esterified by treatment of tetrabutylammonium fluoride (TBAF, 10 g) and iodomethane (MeI, 5 mL) in wet DMSO (8% water) at 25°C. The reaction mixture was poured into three volumes of cold water (0°C) and centrifuged (4500 × *g*) to remove iodine. The supernatant was desalted by ultrafiltration (cut-off 10 kDa), against Milli-Q water which was subsequently exchanged by 0.2 M sodium borate buffer pH 7.3 (0.2 M boric acid pH adjusted by 50 mM sodium tetraborate) to a final sample concentration of 5 mg/mL. β-Elimination (Fig. 1) was carried out in sealed tubes at 125°C for 2.5 h and the reaction was terminated by immersing the sample containers in cold water, according to the Deng protocol [26]. The reaction mixture from β-eliminative treatment was desalted on a Sephadex G25 column (26 mm × 100 mm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 4 mL/min MilliQ water as eluent coupled to an RID10A refractive index detector (Shimadzu, Tokyo, Japan) and a SuperFrac (GE Healthcare Bio-Sciences AB) fraction collector. Samples (~200 mg) were applied with a 50 mL superloop (GE Healthcare Bio-Sciences AB).

2.3 Fractionating neutral sugar side chains

A 5 mg/mL solution of desalted reaction mixture from β-eliminative treatment was fractionated on a HiLoad™ Q-Sepharose 26/10 fast flow (GE Healthcare Bio-Sciences AB) at 5 mL/min by a BioLC system (Dionex) with post-column addition of 1 M NaOH (1 mL/min). The eluent was split using a custom made 1/20 splitter, before PAD. Gradient used; 0–10 min Milli-Q water, 10–20 min 0–1.0 M NaCl; 20–25 min 1.0 M NaCl; 25–30 min 1.0–0 M NaCl, and 30–35 min Milli-Q water. Two fractions, BP-neutral and BP-charged, were obtained (Fig. 1).

2.4 Preparative size exclusion chromatography (SEC) of BP-neutral

BP-neutral was remethoxylated (RME) and β-elimination was carried out a second time as described above, followed by desalting/separation on three Superdex 30 columns (1.6 mm × 60 cm, GE Healthcare Bio-Sciences AB) connected in series eluted with 1.2 mL/min MilliQ water coupled to a Shimadzu RID10A refractive index detector and a SuperFrac (GE Healthcare) fraction collector. Two fractions were collected, one of high molecular weight (BP-neutral-A) and one of lower molecular weight (BP-neutral-B).

2.5 Average molecular weight (M_w) determination

BP, BP-charged, BP-neutral-A, and BP-neutral-B were subjected to high performance SEC (HPSEC) with three PL Aquagel-OH 40–50–60 columns (Polymer Laboratories, Shropshire, UK) coupled in series and eluted at 40°C with 50 mM Na_2SO_4 (0.8 mL/min). Detection was carried out with a Shimadzu RID6A refractive index detector. Pullulans from Polymer Laboratories were used as standards (5.8, 12.2, 23.7, 48, 100, 186, 380, 853, and 1600 kDa). M_w was calculated by the WinGPC software package (PSS, Polymer Standards Service).

2.6 Monosaccharide composition

Methanolysis and GC analysis was conducted by a modification of the method of Chambers and Clamp [28] as described by Samuelsen *et al.* [4]. Polysaccharides were methanolyzed in 3 M methanolic HCl (Supelco) at 80°C for 24 h, dried under N_2, and derivatized with TMS. TMS derivatives were separated on a DB-5 fused-silica capillary column (30 m × 0.32 mm id; J&W Scientific, Folsom, USA). Samples were analyzed in triplicates. Mannitol was used as an internal standard.

2.7 ^{13}C-NMR

Samples were prepared according to Westereng *et al.* [29]. ^{13}C-NMR spectra were recorded on a Varian 300 MHz instrument (spectrometer frequency 75.44 MHz) at 40°C applying 2 s pulse delay, 1.64 s acquisition time, 85° pulse angle, a sweep width of 15974 Hz, and collection of approximately 60000 data points. Chemical shifts are given relative to TMS (δ 0) *via* DMSO (δ 39.6).

2.8 Sugar linkage analysis

Prior to linkage analysis a reduction step was performed twice according to the method described by Kim and Carpita [30], applying sodium borodeuteride to discriminate between Gal*p* and Gal*p*A in MS. The samples were methylated corresponding to the method of McConville *et al*. [31] and further hydrolyzed by 2.5 M TFA for 2 h at 100°C. The samples were reduced with sodium borodeuteride prior to acetylation. The partially methylated alditolacetates were extracted with dichloromethane, dried, redissolved in dry methanol, and analyzed by GC-MS with a Varian Factor Four VF column (30 m × 0.25 mm id) and flame ionization detection.

2.9 MALDI-TOF-MS analyses of oligosaccharides

Samples (BP-charged, BP-neutral-A, and BP-neutral-B) were desalted by adding a small spatula of Dowex 50 particles (H^+-form) to 20 µL of a 10 mg/mL sample solution, mixed, and centrifuged (13 000 × *g*, 5 min). Additionally, one part of each fraction (10 µL) was enriched with 1 µL 20 mM NaCl prior to spotting on the MALDI plate to generate primarily sodium adducts. Two microliter of a 9 mg/mL mixture of 2,5-dihydroxy-benzoic acid (DHB; Bruker Daltonics, Bremen, Germany) in 30% acetonitrile was applied to a MTP 384 target plate ground steel T F (Bruker Daltonics). One microliter sample (0.1 mg/mL) was then mixed into the DHB droplet and dried under a stream of air [32]. The samples were analyzed with an Ultraflex instrument (Bruker Daltonics) with a Nitrogen 337 nm laser beam operated in positive acquisition mode. The data were collected from averaging 250 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity.

2.10 Complement-fixing activity assay

Samples were analyzed for their ability to interfere with the complement system as described by Michaelsen *et al.* [16]. Human serum, stored in aliquots at −70°C was used as a complement source. For the analysis, a dilution of human serum was chosen, which resulted in 50% lysis of a 1% suspension of sheep erythrocytes sensitized with rabbit anti-sheep antibodies (Hemolysin, Virion, Ruschlikon, Switzerland). Human serum was incubated with polysaccharide samples, which either activate or inhibit complement proteins to various degrees. In both situations less complement proteins remain to lyze the added sensitized sheep erythrocytes. The resulting hemolysis was measured by absorbance at 405 nm. The assay was run in an isotonic buffer system (veronal buffer) containing 0.2 mM Ca^{2+} and 0.8 mM Mg^{2+} required for complement activation *via* the classical pathway. PMII, a well-characterized pectin fraction derived from *Plantago major* L. [33] was used as a positive control. Samples were run in triplicates. The activity was measured as ICH_{50}: the lowest concentration resulting in 50% inhibition of hemolysis in the test system, Method A [16]. All samples were standardized based on the amounts of sugars obtained by sugar composition analysis. Diagrams were obtained by the use of Sigmaplot 2001 software package.

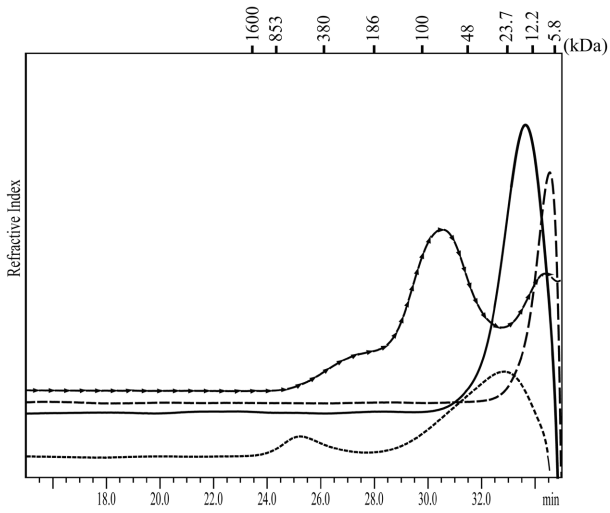

Figure 2. HPSEC molecular weight distribution profiles of BP (· · · ·), BP-neutral-A (–), BP-neutral-B (– – –), and BP-charged (→).

3 Results and discussion

To gain more information about the structural features of BP chemical release of single side chains by means of a selective β-eliminative treatment was outlined. Figure 1 gives a schematic overview of the samples and their preparation. BP was degraded according to the method of Deng *et al*. [26] and the reaction products were applied to ion-exchange chromatography (IEC) resulting in one neutral (BP-neutral) and one charged (BP-charged) fraction. The neutral fraction presumably containing intact RGI connected to the NS side chains of interest was fractionated by SEC producing two fractions containing large oligosaccharides: BP-neutral-A, and BP-neutral-B.

3.1 Molecular weight distribution of *Brassica* pectin and its β-eliminative degradation products

The BP starting material was relatively polydisperse (Fig. 2) containing two well-separated fractions, one with M_w 630 kDa and one with M_w 40 kDa. The latter contained probably oligosaccharides as a result of β-elimination during the extraction process (3 h, 100°C) [34]. The low M_w for BP-neutral-A (∼17 kDa) and BP-neutral-B (∼8 kDa) represent side chains released by the β-eliminative treatment. In the method used, conformation differences among the samples may have an effect on elution times and hence determi-

Table 1. Sugar composition (mol%) of BP and preparative scale fractions obtained from BP after β-eliminative treatment(s)

	Ara	Rha	Fuc	Xyl	Gal	Glc	GalA	% of initial BP	M_w (kDa)
BP	25	9	≤1	3	12	≤1	51		630 + 40
BP-charged	39	11	≤1	3	18	≤1	29	<1	90
BP-neutral-A	87	≤1	nd	nd	7	nd	6	<1	17
BP-neutral-B	70	3	nd	nd	22	nd	6	<1	8

nd = not detected.

nation of M_w. Thus, MALDI-TOF-MS were used as a complementary method for interpretation of molecular weights. The spectra revealed both BP-neutral-A and BP-neutral-B contained large oligosaccharides BP-neutral-A containing the largest fragments on average (see individual side chain composition section below). MALDI-TOF-signals could not be obtained for BP and BP-charged due to their high molecular masses (Table 1).

3.2 Sugar composition of *Brassica* pectin and its β-eliminative degradation products

The sugar composition of the isolated polymer fractions of the different BP samples was determined (Table 1). Both neutral fractions contained small, but clearly detectable amounts of Rha*p* and Gal*p*A presumably backbone segments connected to the side chains. The high arabinose content, observed in all fractions, indicated that the major NS side chain substitutions in RGI of water soluble white cabbage pectin are of arabinan nature. However, large amounts of galactose were observed in BP-charged and BP-neutral-B, presumably present as galactan side chains. The reduced galacturonic acid content of all chemically treated fractions in comparison to the BP pectin was due to removal of Gal*p*A residues after β-elimination and the subsequent desalting step. The IEC separation step was successful in separating the NS side chains from the charged oligosaccharides, as could be concluded from the high Ara and galactose content in the neutral fractions. In addition to typical neutral side chain elements this fraction still contains a large proportion of Gal*p*A and Rha*p*, suggesting both HGA and RG1 structures. The fraction contains a high amount of RG (22%). The relatively high amount of NSs in BP-charged indicates the method does not result in complete β-elimination. This is also supported by the observation that BP-charged and the BP-neutral fractions remained of similar size upon a second round of methylesterifications and β-elimination (data not shown). The efficiency of methylesterifications in the second step could not be monitored due to a limited amount of sample. The limited effect of the second round of β-elimination may be due to the spatial size of TBAF (planar surface of 10 Å), which act as phase transfer catalyst. The size of TBAF may limit its efficiency at Gal*p*A units, which have neutral side chains in its vicinity. Hence, sterical hindrance in the bulky hairy regions may lead to inefficient methyl esterifications at GalA *C*-6 in this region.

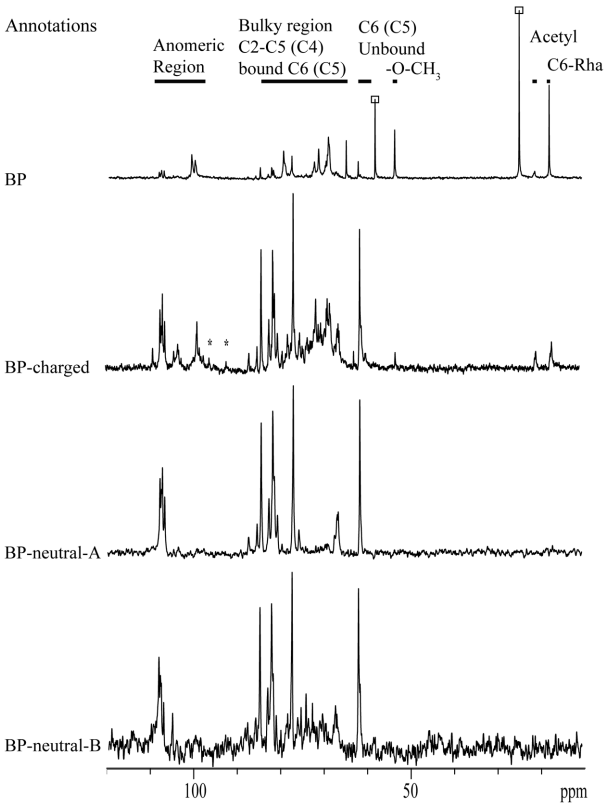

Figure 3. ^{13}C-NMR spectra obtained at 75 MHz of fractions BP, BP-charged, BP-neutral-A, and BP-neutral-B. Parenthesis in annotation line in the upper part of the figure denotes pentose signals, and * denotes anomeric carbon on α-D-Gal*p*A reducing end α- and β-configurations, whereas □ denotes signals from residual ethanol in BP.

3.3 Characterization of the neutral sugar side chains in *Brassica* pectin by ^{13}C-NMR experiments

^{13}C-NMR spectra from the different pectin fractions obtained were compared (Fig. 3). In general, anomer signals for backbone residues of α-L-Rha*p* (98.3 – 99.1 ppm),

α-D-Gal*p*A (99.8 – 100.7 ppm) and side chain elements of α-L-Ara*f* (107.0 – 108.1 ppm) [35, 36] and β-D-Gal*p* (103.2 – 105.0 ppm) [37] were identified. The ^{13}C-NMR spectra of the BP-neutral fractions revealed that side chains had a predominant Ara*f* part with a smaller degree of Gal*p* as seen from the intensities of the anomeric signals, which corresponds well with the sugar composition analysis (Table 1) and supports previous indications that arabinan side chains are present in both neutral BP fractions. BP-charged also contained RGI-rich regions with arabinan and galactan side chains, but with a much more intricate nature than BP-neutral-A and BP-neutral-B, as can be inferred from the more complex NMR spectrum. The low levels of Gal*p* indicated that in BP-neutral-B also contained AGI side chains. Additional analysis of the NMR data revealed two methyl carbon peaks at 17.3 and 17.5 ppm representing *C*-6 of α-L-1,2-Rha*p* and α-L-1,2,4-linked Rha*p*, respectively. In BP, the intensity ratio of these methyl carbon peaks indicated that mostly unsubstituted Rha*p* was present, whereas in BP-charged ~40% of Rha*p* residues seem to be branched. The observed reduction of unsubstituted *versus* substituted Rha is due to the loss of Rha-GalA, during the desalting step subsequent to the β-elimination process.

3.4 Characterizing *Brassica* pectin arabinogalactan by sugar linkage analysis

The sugar linkage analysis (data not shown) was more qualitative than quantitative, but clearly revealed that the overall structure of the BP material consisted of 1,4- and some 1,3,4-linked Gal*p*A with the interspacing α-L-Rha*p* having 1,2- and 1,2,4-linkages within the backbone structures. The side chain elements consisted predominantly of α-L-Ara*f* and β-D-Gal*p*. Most α-L-Ara*f* were terminally- and 1,5-linked with lesser amounts of 1,2,5- and 1,3,5- branching points, whereas β-D-Gal*p* was present as t- and 1,4-linked residues in addition to minor amounts of 1,3-, 1,6-, and 1,3,6-linked residues. The abundance of t-Ara, together with substantial amounts of 1,2,5- and 1,3,5-branching points indicates a highly branched arabinan structure. The sugar linkage composition demonstrates that (highly) branched arabinan is the main side chain polymer in both neutral BP fractions. Additionally in BP-neutral-B AG type I side chains polymers are present. Linkages indicative for AG type II are only present in trace levels.

3.5 Determination of the individual side chain composition

The side chain composition of BP-neutral A and BP-neutral-B was investigated by MALDI-TOF-MS. The analysis of BP-neutral-A (Fig. 4) suggested the presence of single side chains (Gal*p*-*Araf*$_n$) linked to Rha*p* of short backbone fragments; uGal*p*A-Rha*p*-Gal*p*A thereafter shortened ¤ or uGal*p*A-Rha*p* (uGal*p*A = unsaturated Gal*p*A) *via* Gal*p*. The ion at *m*/*z* 683 was interpreted to be the sodium adduct of the backbone fragment ¤ with one Gal*p* (denoted as ¤-gal in Fig. 4). The side chain composition was indicated by a homologous series of signals at *m*/*z* 683, 815, and 947; subsequent additions of 132 Da up to 4800 *m*/*z* (not shown) corresponding to a backbone fragment (*m*/*z* 683) with (Ara*f*)$_{1-30}$ additions. The predominance of pentose was in accordance with the sugar composition analysis (Table 1) and the NMR spectra (Fig. 3). This supports the hypothesis that large oligosaccharide side chains could be isolated after β-eliminative treatment of the RGI part of pectin. Three fragments weighing 44 *m*/*z* less than their ¤-Gal-Ara*f*$_n$ neighbors namely the fragments at *m*/*z* 1035, 1563, and 2092 corresponded to Gal$_1$Ara$_n$ side chain segment linked to a uGal*p*A-Rha*p* backbone. It could be argued the 44 *m*/*z* loss could be due to spontaneous decarboxylation. However, the conditions used and the fact that we did not observe a series of ions with a 44 *m*/*z* loss, this is most probably not the case.

The peak clusters observed in the spectrum (inserted zoomed spectrum in Fig. 4) result from sodium (+22 *m*/*z*) and potassium (+38 *m*/*z*) salts of the sodium adduct. The 837 (815 + 22) *m*/*z* is caused by the presence of a sodium salt of a Gal*p*A within the sodium adduct, whereas the 853 (815 + 38) *m*/*z* peak is produced by the potassium salt within the sodium adduct [38].

ESI-MS^n experiments were conducted on the following ions *m*/*z* 683, 815, and 947 to get conclusive evidence of the above-mentioned reasoning. Unfortunately, we were unable to obtain MS/MS spectra confirming the uGal*p*A-Rha*p*-Gal*p*A (¤) backbone fragment.

The BP-neutral-B contained a more complex mixture of compounds than BP-neutral-A as indicated by the MALDI-TOF mass spectrum (Fig. 5). A stretch of ions at *m*/*z* 689 – 2797 (only the section 450 – 1300 *m*/*z* is shown for simplicity) with subsequent additions of 162 Da was annotated as hexose (β-D-Gal*p*) tetra-heptadeca-mers. Furthermore, a homologous series of signals from *m*/*z* 569 – 1229 with 132 Da repeats was annotated as tetra-nonamers of Ara*f*. These series were also present in small amounts in β-eliminative degraded potato galactans and sugar beet arabinans, used as controls (data not shown). Possibly, there are linkages present within the NS side chains, which are labile for β-eliminative treatment. Alternatively, these oligosaccharides could originate from autolysis at the high temperature during the β-eliminative treatment. In addition, some fragments with repeats of 132 Da from *m*/*z* 683 – 1475 corresponded with the fragment ¤-Gal*p*$_1$-Ara*f*$_n$ additions as observed in BP-neutral-A. *m*/*z* peaks were annotated using in-house-built software to obtain the oligosaccharide composition (uronic acids, hexose, pentose, deoxyhexoses). From the sugar composition, the assumption could be made that a pentose residue represents an Ara*f* moiety, a hexose may represent a Gal*p*, and the main uronic acid Gal*p*A.

In both neutral fractions, NS chains with degree of polymerization (DP) up to 30 for BP-neutral A and 17 for BP-

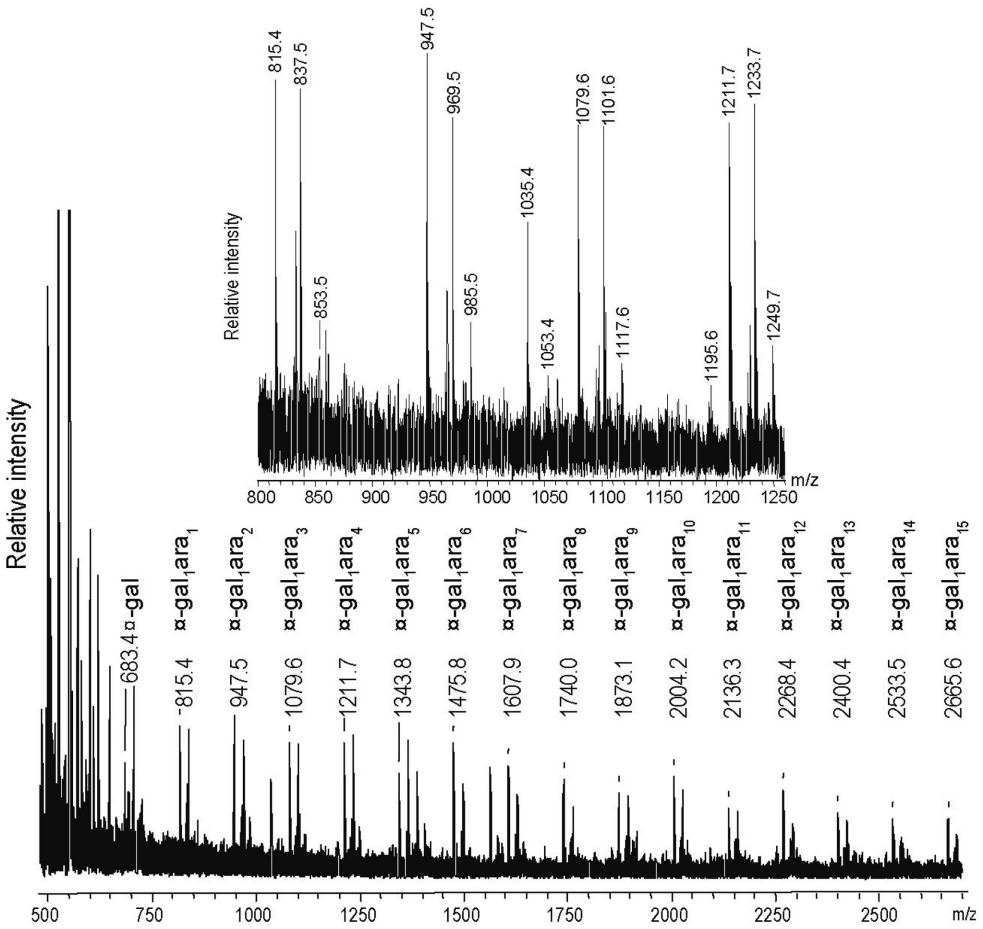

Figure 4. MALDI-TOF mass spectrum of BP-neutral-A with a zoom view of the 800 – 1250 *m*/*z* windows (¤ = uGalA-Rha-GalA^{Na+}). Based on the sugar composition, putative sugars were annotated.

neutral-B were present. The presence of both galactan and arabinan side chains in BP-neutral-B, was in agreement with the sugar composition of this pool, as well as the observed difference with BP-neutral-A (Table 1).

The 859.3 *m*/*z* signal corresponding to unsatGalA$_1$GalA$_3$Xyl$_1$ indicated the presence of XGA. The presence of XGA is currently being studied in our laboratories.

MALDI-TOF MS analysis of BP-charged yielded ions at *m*/*z* 551, 727, 903, and 1079 in the ratio 10:4:2:1 (data not shown). These masses point to α-D-Gal*p*A$_{3-6}$ fragments where the nonreducing end Gal*p*A was 4-deoxy-β-L-threo-hex-4-enepyranosyluronic acid (uGal*p*A). The occurring Δ 22 *m*/*z* representing sodium counter ions gave further evidence for the presence of uronic acids. We suggest based on the fragments observed, that the late eluting peaks in the HPSEC run of BP-charged (Fig. 2) were uronic acid containing oligosaccharides. The early eluting molecules in the predominant HPSEC peak (90 kDa) were not observed by MALDI-TOF-MS and putatively held the abundant content of Ara*f* and Gal*p* (Table 1).

The observed effects of the β-eliminative treatment indicated that this treatment promoted more reactions and involved more products than previously acknowledged [26]. It was not possible to confirm the presence of the backbone fragment, which contained a putative Gal*p*A residue at the reducing end. The mechanism of the formation of these degradation products is at present not understood.

It should be noted that sodium tetraborate used for the β-elimination process generated strong signals in MALDI-TOF-MS analysis with an alternating sequence of 162 and 176 Da mass differences (data not shown). If care is not taken to remove this salt, misinterpretation of Gal*p* and Gal*p*A may occur as these have equivalent masses. Hence, a complete removal of tetraborate is needed.

3.6 Complement-fixing activity

The complement-fixing activity of the structurally different BP and the BP-neutral fractions were compared to reveal which structural properties are important to complement-

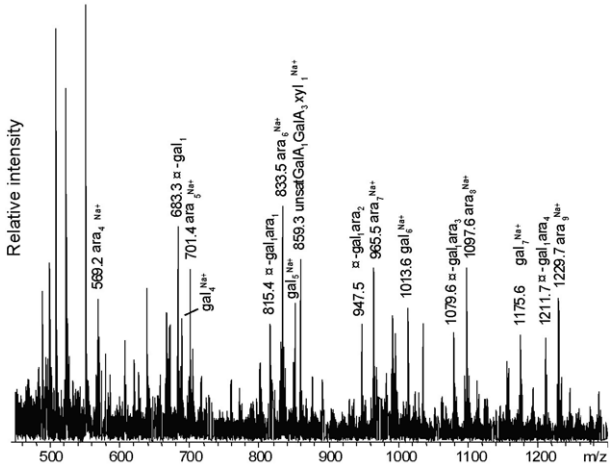

Figure 5. MALDI-TOF mass spectrum section 450 – 1300 *m/z* of BP-neutral-B with sodium adducts (H = hexose = galactose; P = pentose = Ara; ¤ = uGalA-Rha-GalA; Na^+ = sodium adduct).

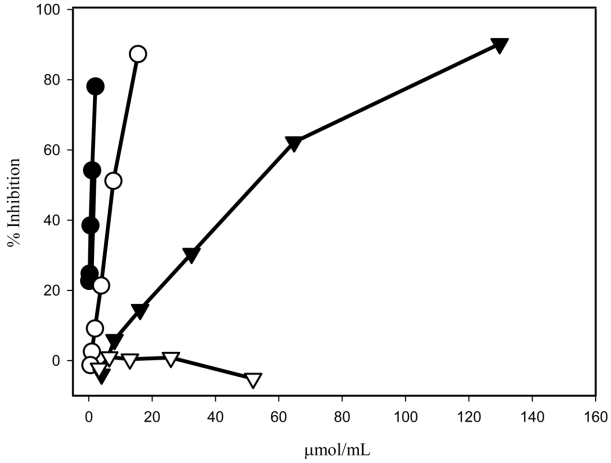

Figure 6. Complement-fixing activity (measured as% inhibition of hemolysis) of increasing concentrations (µmol/mL) of white cabbage polysaccharides; BP (o); BP-neutral-A (▼); BP-neutral-B (△); and control (PM II) (●). Samples were run in triplicates.

fixing activity. Pectic material rich in arabinans [39] and arabinans have previously demonstrated complement-fixing activity [18, 40] and small size polysaccharides might show complement-fixing activity [41]. The BP-neutral fractions contained several large arabinan containing oligosaccharides that might show activity in the complement-fixing test (Fig. 6). BP-neutral-A showed some, although very low activity (ICH_{50} = 50 µmol/mL). BP-neutral-B was expected to be an active molecule based on its sugar composition containing Gal*p* in addition to being rich in Ara residues [18, 33, 42, 43]. Still, BP-neutral-B was not active, putatively due to the molecules low molecular size (8 kDa) and three dimensional structures. Earlier results have shown it is the amount of galactose which shows the strongest correlation to activity [39]. However, if there is a minimal unit size responsible for interaction with the complement system, the oligosaccharides in BP-neutral-B, having three times the galactose content compared to BP-Neutral-A might be too small. On the other hand, the size of the oligosaccharides in BP-neutral-A was sufficient to retain some activity. Furthermore, about six times higher concentration of BP-neutral-A was needed to express the same activity as the untreated BP (ICH_{50} = 7.5 µmol/mL) and about 60 times higher concentration than the positive control PM II (ICH_{50} = 0.8 µmol/mL). BP-neutral-A and BP-neutral-B had a similar structural composition, as demonstrated by the sugar composition and ^{13}C-NMR, but varied in size (17 and 8 kDa, respectively; Fig. 2, Table 1). Complement activation of the classical pathway is triggered by binding of the recognition subcomponent C1q to the Fc part of antibodies complexed to an antigen [44]. C1q demonstrates only weak binding to nonaggregated IgG whereas, upon the presentation of multiple, closely spaced Fc regions, which are found in immune complexes, the strength of binding of C1q increases a 1000-fold [45]. This is an example how molecules which interact with the complement-fixing activity system often need to be multivalent and thus of sufficient size and three dimensional structure to accommodate multiple binding, in this case of C1q. The possibility of C1q being a target for white cabbage pectin is an open question, which needs further studies.

4 Concluding remarks

4.1 Structural findings

This study presents evidence for the successful isolation of single side chains from RGI-rich pectic structures from white cabbage by β-eliminative degradation. The degradation pattern of the β-eliminative treatment was most certain more complex than expected and the apparent degradation of side chains is currently further studied. The oligosaccharides obtained were almost pure arabinans or galactans as proven by MALDI-TOF-MS. No evidence was obtained for side chains composed of a combination of Ara with several galactose moieties and hence, no AGII/AGI structures could be positively identified. This indicates that Ara*f* and Gal*p* in side chains are present as arabinan and galactan structures rather than AG structures. Furthermore the study indicated that arabinan was present in separate clusters on RGI, but unambiguous evidence is still needed.

4.2 Biological activity of fractions

The results of complement-fixing activity strongly indicated that a minimal unit size is necessary for activity. The BP-neutral-B oligosaccharides were probably too small for expressing activity, although these sugars possessed structures previously shown to be highly active [18, 40]. BP-neu-

tral-A exhibited complement-fixing activity, but this activity was weaker compared to BP, probably due the small size of polymers present in the BP-neutral-A fraction. This implied that structural elements containing multiple side chains expressed higher complement-fixing activity. Furthermore the sugar composition of BP-neutral-A indicated that arabinan was the complement-fixing polymer.

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