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Effects of extraction conditions on the chemical structure and biological activity of white cabbage pectin

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Abstract

The purpose of this study was to isolate and perform chemical analyzes as well as biological testing of pectic material from white cabbage isolated by sequential aqueous ionic solutions (SEQAIS) or a simple pure water extraction (PW). Water extraction was aimed at yielding water-soluble pectins only, while the harsher conditions in SEQAIS aimed at extracting proto pectin as well. The pectic material resulting from the various extraction steps was characterized and tested, in order to determine whether structural and biological activity were influenced through different isolation procedures. The SEQAIS fractions obtained were one water-soluble and six partly water-soluble extracts, whereas PW yielded two water-soluble extracts. Sugar composition analysis, linkage analysis, HPSEC molecular weight distribution, HPAEC and ^{13}C NMR were run to obtain structural characteristics of the extracted material. Both extraction procedures resulted in degradation of pectin. Pectin containing highly methyl esterified Gal*p*A probably underwent β-elimination due to neutral pH during PW, while hydrolysis of Ara*f* occurred in the first step of SEQAIS in 50 mM acetic acid pH 4.5. Water-soluble extracts were tested for complement-fixing activity and acidic extracts with degraded side chains showed reduced activity. Authors suggest that extraction conditions at neutral pH should be used in order to withhold side chain structure and immuno-activity.

Keywords: Pectin; White cabbage; *Brassica oleracea* var. Capitata; Complement-fixing activity; Anti-complementary activity; Biological activity; HPSEC; HPAEC; Linkage analysis; β-Elimination; Methanolysis; ^{13}C NMR; Rhamnogalacturonan I (RG-I); Rhamnogalacturonan II (RG-II); Homogalacturonan (HGA); Arabinogalactan II (AG-II)

1. Introduction

White cabbage is an important vegetable in the Scandinavian household (Wennberg, Engqvist, & Nyman, 2002) and a wide range of cultivars are available. The late maturing Bartolo is the major variety used in Norway. Besides being important in nutritional aspects, cabbage leaves are also traditionally used for healing bedsores. The mechanism governing the wound healing property of plant material is not clear, but might be partly due to pectic substances triggering the immune system (Hart et al., 1988; Samuelsen et al., 1995). The complement system is an important part of the innate immune system and consists of more than 20 serum proteins acting through a cascade mechanism (Yamada & Kiyohara, 1999). The polysaccharide influence upon the human complement system is measured by the complement-fixing test, a test that is used as an indicator of immune modulating activity.

Pectin is among the most complex macromolecules found in nature. Except from the definite structure of rhamnogalacturonan II (RG-II) as reviewed previously (O'Neill, Ishii, Albersheim, & Darvill, 2004), pectin is not known to have a conserved definite structure. The function of the vast pectin diversity is not clearly understood, but several key roles in the plant cell wall have been suggested and documented. It has been suggested to regulate the structure of the pores in

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the cell wall (McCann & Roberts, 1995), being an important element in regulation of plant cell wall growth (O'Neill, Ishii, Albersheim, & Darvill, 2004) and wall integrity through non-covalent interactions with cellulose (Zykwinska, Ralet, Garnier, & Thibault, 2005), to mention a few. Pectin putatively builds a matrix enclosing the load-bearing network of cellulose and cross binding glycans (Carpita & Gibeaut, 1993) and is of great importance for the integrity of cell wall structure (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996). Pectin structure is to a great extent general for all dicotyledons (Albersheim et al., 1996) and consists of mainly two structural elements, rhamnogalacturonan I (RG-I) and homogalacturonan (HGA) (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). RG-I contains a backbone of alternating (1 → 4)-linked α-D-Gal*p*A and (1 → 2)-linked α-L-Rha*p* with neutral side chains attached to *O*-4 of Rha*p*. The side chains consist of three different polymers; arabinans, type I arabinogalactan (AG-I) and type II arabinogalactan (AG-II). AG-I consist of a (1 → 4)-linked α-D-galactan with α-L-Ara*f* attached to *O*-3 (Perez, Mazeau, & Herve du Penhoat, 2000). The Ara residues are mainly (1 → 5)-linked α-L-Ara*f* with α-L-Ara*f* attached to *O*-2 and *O*-3 (Perez et al., 2000). AG-II is highly ramified galactan with predominantly interior (1 → 3)-linked α-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* (Darvill, McNeil, & Albersheim, 1978). HGA is a long linear chain of (1 → 4)-linked α-D-Gal*p*A with a varying degree of methyl esterified carboxyl groups. Some of the GalA units may be acetylated at *O*-2 and/or *O*-3. Some GalA units are also reported to have xylose attached to *O*-3, depending on the pectin source (Thibault & Ralet, 2001).

There are a number of different extraction protocols for isolating pectic material, which most often are done in a sequential manner. Most procedures use a first step of water/buffered solution, followed by a step with Ca^{2+}-chelators such as EDTA (Chambat, Barnoud, & Joseleau, 1984; Redgwell & Selvendran, 1986), CDTA (Jarvis, Hall, Threlfall, & Friend, 1981), ammonium oxalate (Stevens & Selvendran, 1984) or imidazol (Mort, Moerschbacher, Pierce, & Maness, 1991). The first and second steps are important to perform at a pH between 4 and 5 in order to obtain a non-degradative extraction (Voragen et al., 1995). Subsequent to chelator treatment, the material is usually treated with an alkali solution containing $NaBH_4$ which prevents alkali peeling from the reducing end at ambient or refrigerated temperature (Voragen et al., 1995). An optional last extraction step with hot diluted acid may be performed (Rombouts & Thibault, 1986). Enzyme aided pectin extraction have also been reported (Schols, Posthumus, & Voragen, 1990). When investigating for biological effects, the plant material is traditionally extracted with pure hot or cold water. Thus, such extracts are obtained without buffer control usually resulting in a pH during extraction at approximately 7, with the exception of some fruit tissues. This leaves the pectin highly vulnerable for β-elimination and removal of methyl esters (Albersheim, Neukom, & Deuel, 1960). On the other hand industrial isolation of pectin takes place at acidic conditions (pH 1.5–3) resulting in an inevitable and uncontrolled removal of neutral sugar side chains and hydrolysis of ester links (Voragen et al., 1995).

The aim of this study was to determine how the final structure and biological activity of pectin from cabbage was affected through the use of different extraction protocols. The structure and biological activity of pectin were changed in different ways depending on the isolation procedure chosen.

2. Materials and methods

2.1. Preparation of alcohol insoluble solids (AIS)

White cabbage (*Brassica oleracea* var. Capitata, Bartolo cultivar) was cultivated at Vollebekk testfield in Aas, Akershus-N and stored for 6 weeks at 0 °C. Two kilograms of cabbage was cut in 3–5 mm slices and treated with 80% ethanol at 80 °C in a Buchi reflux system for 2 h, three times repeatedly, according to Samuelsen et al. (1995). Residual plant material was dried overnight at ambient temperature to give alcohol insoluble solids, AIS. This step removes low molecular carbohydrates, color pigments and other organic compounds and denatures enzymes. The ethanol 'waste' used for AIS preparation was condensed with a rotavapor, the extract was lyophilized (EtOH) and saved as an individual extract.

2.2. Extraction of pectic material

2.2.1. Extraction by sequential aqueous ionic solutions (SEQAIS)

AIS (50 g) was sequentially extracted at a concentration of 10 mg/mL according to Fig. 1. Each fraction was filtered with Whatman GF/C filter in a Nutch funnel using vacuum and filtered under pressurized air with a Millipore stainless pressure holder unit with GF/F filter to remove particles. Polysaccharide containing liquid was adjusted to 60% isopropanol and placed overnight at 4 °C for precipitation of polysaccharides. The fractions were centrifuged at 4500*g* for 15 min and the pellet was washed with 60% isopropanol before a final centrifugation step at 4500*g* for 20 min. The resulting pellet was dissolved in water and dialyzed extensively (10–12 kDa M_w cutoff, Medicell Int. Ltd.) against distilled water at 4 °C before lyophilization. Each extract in Fig. 1 originates from the supernatant after centrifugation, whereas the pellet was subsequently treated according to next step in the sequence.

The residuum (pellet) remaining after the HCl step (Fig. 1) was treated for a possible release of pectic material with stronger cell wall associations. Boiling water (1.5 h) released a residual wall (RWa) extract and subsequently cellulase (50 mM CH_3COONH_4, pH 4.5, 3 h, 0.075 U cellulase, Sigma, *Aspergillus niger* E.C 3.2.1.4)/mg material,

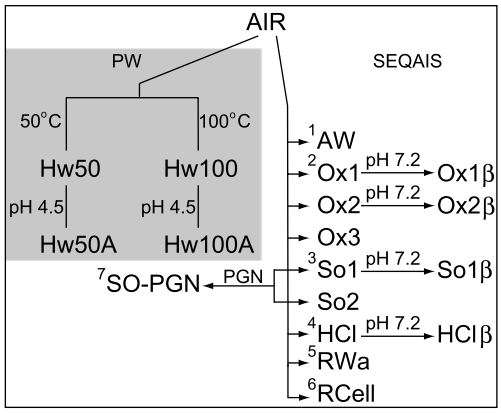

Fig. 1. Flowchart of sequential (SEQAIS) and pure water (PW) extraction of white cabbage AIS. [1]pH 4.5, 20 h (pH adjustment with HCl). [2]The three Ox treatments; 0.05 M $(NH_4)_2C_2O_4$, pH 4.5, 80 °C, 1 h with thermamyl SC (Novozymes) [3]The two soda treatments; 0.05 M Na_2CO_3 with 20 mM $NaBH_4$, ambient temperature for 2 h. [4]HCl; 0.05 M HCl, 85 °C, 2 h. [5]RWa (residual wall extract); was obtained by boiling HCl residuum suspended in 200 ml dH_2O, 100 °C 1.5 h. [6]RCell (residual wall cellulase treatment); 50 mM CH_3COONH_4, pH 4.5, 3 h, 0.075 U cellulase (Sigma, *Aspergillus niger* E.C 3.2.1.4)/mg material, 40 °C. [7]SO1 and SO2 (5 mg/mL) dissolved in ammonium acetate, pH 5.0, incubated for 6 h at 30 °C with 0.1 U/mg sample endopolygalacturonase, *A. niger* 3.2.1.15.

40 °C) released the fraction (RCell) and the corresponding residium (RPel). Extracts were dialyzed towards distilled water and lyophilized. The cellulase was tested for any potential pectic side activity by treating 5 mg of AW fraction analogously to the treatment above, with the same proportional U/amount material.

2.2.2. Pure water extraction (PW)

AIS (50 g) was extracted with distilled water at 50 and 100 °C as described elsewhere (Westereng, Yousif, Michaelsen, Knutsen, & Samuelsen, 2006) yielding two *h*ot *w*ater extracts, Hw50 and Hw100. pH measured at ambient temperature, were 7.2 before and after extraction treatment (Fig. 1).

2.2.3. Evaluation of imidazol extraction efficiency

This imidazol extraction was checked as an alternative to ammonium oxalate (Section 2.2.1) to examine the efficiency of a more readily dialyzable Ca^{2+}-chelator (Mort et al., 1991). AIS (5 g) was suspended in 500 mL 10 mM acetic acid, pH 4.5 (20 °C, 48 h). The subsequent extractions were; hot water (80 °C, 2 h) containing thermamyl; 50 mM imidazol, pH 7.0 (20 °C, 2 h); 50 mM sodium carbonate containing 20 mM $NaBH_4$ (20 °C, 2 h), and finally in 50 mM HCl, (85 °C, 2 h). Each step was performed twice and solid material was each time separated from extracts by centrifugation (4000*g*).

2.3. Treatment of selected extracts at different pH

2.3.1. Acidic water treatment of PWs

Hw50 and Hw100 were treated with distilled water adjusted with 1 M HCl to, pH 4.5 (no buffer control, pH 5.0, at end of treatment), kept at ambient temperature for 24 h to simulate the treatment of the first extract from sequential extraction, and finally dialyzed extensively in distilled water and lyophilized. The resulting samples were called Hw50A and Hw100A.

2.3.2. Treatment of various SEQAIS in hot neutral solvent

Forty milligrams of Ox1, Ox2, SO1 and HCl extracts were boiled in 10 mL 50 mM NaH_2PO_4, pH 7.2, for 3 h to simulate water extract treatment, a possible β-eliminative (β) condition. The samples were dialyzed extensively in distilled water and lyophilized yielding extracts Ox1β, Ox2β, SO1β and HClβ.

2.3.3. Polygalacturonase treatment to improve solubility

SO1 was dissolved in ammonium acetate, pH 5.0 (pH adjustment with acetic acid), incubated for 6 h at 30 °C endopolygalacturonase (0.1 U/mg sample), *A. niger* 3.2.1.15 (Sigma) and lyophilized.

2.4. Size exclusion chromatography (SEC)

2.4.1. Analytical SEC

The fractions were subjected to size exclusion chromatography (SEC) with three PL aquagel-OH (40, 50 and 60) columns (Polymer Laboratories) coupled in series and eluted at 40 °C with 50 mM Na_2SO_4 (0.8 mL/min) to reduce inter/intra-molecular interactions (Draget, Skjåk-Bræk, Christensen, Gåserød, & Smidsrød, 1996). Detection was obtained by refractive index detection (Shimadzu RID6A). Pullulans from Polymer Laboratories Ltd. (5.8, 12.2, 23.7, 48, 100, 186, 380, 853 and 1600 kDa) were used as standards. Weight-average molar mass (M_w), number-average molar mass (M_n) and polydispersity factor M_w/M_n were calculated by the WinGPC program (PSS. PSS WINGPC, 2000).

2.4.2. Analytical SEC–MALLS

High-performance (HP) size exclusion chromatography (SEC) combined with multi angular laser light scattering (MALLS) detection as described by Christensen et al. (2001), was performed on selected fractions. The samples were run on both a system with TSK-G 4000 and 3000 PWXL, or TSK-G 6000 and 5000 PWXL (TosoHaas) coupled in series, 0.8 mL/min 50 mM Na_2SO_4 with 0.1 M Na_2EDTA at ambient temperature.

Chromatograms were rendered by Adobe Illustrator 10.0.

2.5. High-performance anion-exchange chromatography (HPAEC)

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio LC system with a (2 × 250 mm) PA100 column run at 0.3 mL/min controlled by the Chromeleon 6.7 software package. A gradient of 1 M NaOAc in 100 mM NaOH was used with the

following program; 0–15 min, 15 mM NaOH; 15–20 min, 15–100 mM NaOH; 20–70 min 0–1 M NaOAc in 100 mM NaOH; 70–75 min, 1 M NaOAc in 100 mM NaOH; 75.1–76 min 1–0 M NaOAc in 100 mM NaOH; 76.1–90 min 100–15 mM NaOH. Free Arabinose was identified by an external Arabinose standard (Sigma).

2.6. Preparative SEC

Preparative SEC was performed with a Pharmacia P50 pump delivering 4 mL/min 100 mM ammonium acetate eluent, 1 g of sample was applied with a 50 mL superloop (Pharmacia) to a Sephacryl 400 column (5×100 cm, $d \times h$), refractive index detection (Shimadzu RID10A) and fractionated by a SuperFrac (Amarsham Bioscience).

2.7. Quantitative determination of protein content

The protein content of the fractions was determined by the protein assay of Lowry, Rosebrough, Farr, and Randall (1951), modified by Peterson (1979). Bovine serum albumin (BSA, New England Bio Labs Inc.) was used as a standard.

2.8. Monosaccharide composition

Methanolysis and GC analysis was run by a modification of the method of Chambers and Clamp (1971) as described by Samuelsen et al. (1995). 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 i.d). The estimates were based on triplicates ± standard deviation (*SD*).

2.9. Starch determination

The possible presence of starch was quantitatively investigated by an assay for total starch (Megazyme; AOAC Method 996.11). The detection limit of this assay was <1% starch.

2.10. Sugar linkage analysis

Prior to linkage analysis a reduction step was performed twice according to the method described by Kim and Carpita (1992), including sodium borodeuteride to discriminate between Gal*p* and Gal*p*A in MS. The samples were methylated corresponding to the method of McConville, Homans, Thomas-Oates, Dell, and Bacic (1990) and further hydrolyzed by 2.5 M TFA for 2 h at 100 °C. Samples were reduced using sodium borodeuteride prior to acetylation. The partially methylated alditolacetates were extracted with dichloromethane, dissolved in dry methanol and identified by GC–MS with a Varian Factor Four VF column (30 m × 0.25 mm i.d.) and flame ionization detection. The quantitative results in linkage analysis were based on the relative distribution of the differently linked monomers. The distribution of each monomer from methanolysis was subsequently used to give the total amount of each monomer linkage. The discrimination of Gal*p* and Gal*p*A was based on the ratio of their respective diagnostic fragments; terminal-Gal*p*/Gal*p*A (205/207 fragments) and (1 → 4)-Gal*p*/Gal*p*A (233/235 fragments).

2.11. ^{13}C NMR

Samples were prepared and ^{13}C NMR spectra recorded on a Varian 300 MHz instrument at 80 °C according to Westereng et al. (2006).

2.12. Complement-fixing activity assay

Samples were subjected to an assay that determines their ability to interfere with the complement system as described by Michaelsen, Gilje, Samuelsen, Hogasen, and Paulsen (2000). Samples were run in quadruplicates. The activity was given as the lowest concentration showing 50% inhibition (ICH_{50}) in the test system (Michaelsen et al., 2000). All samples were standardised based on the amounts of sugars obtained by sugar composition analysis. All comparisons of samples throughout the text are thus related to absolute sugar content in each sample.

2.13. Statistical calculations

One-way analysis of variance (ANOVA) were run using the MINITAB software package to test whether or not there was a significant difference ($P < 0.05$) between monosaccharide distribution before and after treatment of water extracts according to the first step of sequential extraction.

3. Results and discussion

3.1. Extraction of pectic material

The white cabbage in this study constitutes 9.7% dry material, which is similar to data reported elsewhere (Wennberg, Ekvall, Olsson, & Nyman, 2006). Thirty percent of the dry material was alcohol insoluble solids (AIS) representing 2.9% of fresh cabbage. The yields of extracts from SEQAIS (Table 1) are presented as sugar amounts obtained from sugar composition throughout the text. AIS contained ~20% pectic material (Table 1). This is about half of what has been obtained elsewhere using CDTA as a chelator (Stevens & Selvendran, 1984). The difference in extraction product could be due to higher efficiency of CDTA or residual CDTA influencing yield as suggested elsewhere (Mort et al., 1991).

The yield from PW was approximately 1/5 of the extracts from all SEQAIS extracts. The isolation of PW, which was performed without pH adjustments (pH ~ 7.2), is likely to result in β-elimination as well as significant deesterification (Albersheim et al., 1960). Thus,

Table 1
Relative monosaccharide distribution (wt%) in extracts from SEQAIS of *Brassica oleracea*

	EtOH	AW σ^2	Ox1 σ^2	Ox2 σ^2	Ox3 σ^2	So1 σ^2	So2 σ^2	HCl σ^2	RWa σ^2	RCell σ^2	RPel σ^2	Sum
Ara	2.7 ± 0.15	8.8 ± 0.16	15.4 ± 0.85	18.5 ± 4.70	21.2 ± 0.17	35.1 ± 0.85	38.9 ± 0.15	17.3 ± 0.12	26.8 ± 2.90	8.8 ± 0.20	4.0 ± 0.50	
Rha	n.d.	2.5 ± 0.03	4.1 ± 0.11	5.2 ± 1.59	6.0 ± 0.09	8.7 ± 0.11	8.9 ± 0.03	12.9 ± 0.05	13.7 ± 1.10	2.5 ± 0.00	4.7 ± 0.40	
Api	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Fuc	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0.21	n.d.	n.d.	0.3 ± 0.50	0.0 ± 0.00	1.0 ± 1.70	
Xyl	n.d.	1.5 ± 0.02	1.5 ± 1.16	2.5 ± 0.57	3.1 ± 0.16	5.1 ± 0.91	7.0 ± 0.57	2.6 ± 0.33	3.2 ± 0.10	1.5 ± 0.00	40.6 ± 3.00	
Man	0.4 ± 0.013	0.0 ± 0.00	n.d	0.6 ± 0.05	0.5 ± 0.13	n.d.	n.d.	0.4 ± 0.05	0.3 ± 0.40	0.0 ± 0.00	2.6 ± 4.50	
Gal	3.1 ± 0.37	8.0 ± 0.11	4.6 ± 0.20	6.7 ± 1.06	7.5 ± 0.45	10.3 ± 0.36	13.7 ± 0.17	28.1 ± 0.30	31.5 ± 1.10	8.0 ± 0.10	23.8 ± 1.40	
Glc	93.6 ± 0.85	0.5 ± 0.45	1.9 ± 0.07	6.3 ± 0.63	5.2 ± 0.07	0.3 ± 0.02	0.3 ± 0.03	1.3 ± 0.03	4.5 ± 0.40	0.5 ± 0.50	13.7 ± 0.10	
GalA	0.3 ± 0.5	78.7 ± 0.33	72.4 ± 1.97	60.2 ± 8.56	56.4 ± 0.18	40.3 ± 1.71	31.2 ± 0.73	37.6 ± 0.53	19.8 ± 2.90	78.7 ± 0.30	9.5 ± 0.70	
Yield (%)		0.4	1.2	0.4	0.3	1.8	0.7	0.5	0.1	0.2		5.7
Yield (mg)		610	1930	640	580	3080	1240	860	240	290		9470

σ^2 denotes standard deviation of triplicate samples.
Yield (mg) is determined based on the carbohydrate content (%) determined by sugar composition analysis multiplied by the weighed material of freeze dried extracts.
Yield (%) is relative to dry material, AIS constitutes 30% of dry material, i.e. 50 g AIS = 50 × 30/100 = 167 g dry material. 9.5/167 g × 100% = 5.7%.

formation and subsequent removal of oligomeric Gal*p*A during dialysis may explain the reduced proportion of Gal*p*A in PW compared to material extracted with chelators. Pectin with stronger cell wall association is more effectively extracted by the sequential method due to its different steps of weakening of pectin–cell wall interactions such as Ca^{2+}-bridges, ionic bonds of deprotonated carboxyl groups of GalA, and removal of cell wall components associated with pectin. A more efficient isolation of Gal*p*A-rich elements may as well explain the higher amount of Gal*p*A when utilizing chelators.

Extraction with imidazol as a chelator was tested as well. This chelator is according to Mort et al. (1991) easy to remove by dialysis, but due to a very low yield (0.6 mg/g AIS) this method was considered unsuitable in this study.

The starch content of the extracts was less than 1% when assessed by the Megazyme kit. This is in accordance with results presented elsewhere (Wennberg et al., 2006).

3.2. Sugar composition

The sugar composition of SEQAIS (Table 2) contained typical pectic components with increasing amounts of Ara and Gal, which are typical side chain residues, and a corresponding reduction in GalA throughout the extraction sequence. The HCl extract is an exception with slightly less GalA and much less Ara than the previous SO2 extract. The decrease of Ara in the HCl extract compared to SO2 could be due to hydrolysis of acidic labile Ara*f* residues (Voragen et al., 1995), or a result of other polysaccharides extracted.

The RWa extract (Fig. 1) contained some additional pectic material (approximately 2.5% of all extracted pectic material) high in Ara and Gal, and Rha/GalA ratio. The composition of the RCell extract showed that cellulase treatment released some pectic polymers high in GalA. The final residuum (RPell) after cellulase treatment contained high amounts of Xyl, Gal and lesser amount of Glc putatively of hemicellulose origin. The cellulase was tested for potential pectolytic activity with AW as substrate. Since negligible amounts of monosaccharides were released during cellulase treatment (observed by HPAEC), the pectolytic activity was low.

Table 2
Relative monosaccharide distribution (%) in crude extracts from water extraction of *Brassica oleracea* before (Hw50 and Hw100) and after treatment in water adjusted with HCl to pH 4.5 (Hw50A and Hw100A)

	Hw50 σ^2	Hw50A σ^2	Hw100 σ^2	Hw100A σ^2
Ara	21.9 ± 1.54	15.2 ± 0.14*	19.2 ± 1.15	15.0 ± 0.27*
Rha	4.5 ± 0.10	4.1 ± 0.12	5.5 ± 0.08	4.8 ± 0.14
Fuc	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Xyl	1.9 ± 0.07	1.9 ± 0.07	2.8 ± 0.07	2.2 ± 0.03
Man	2.2 ± 0.02	1.6 ± 0.01	4.4 ± 0.39	3.4 ± 0.08
Gal	11.8 ± 0.29	10.8 ± 0.37	8.1 ± 0.52	5.8 ± 0.09*
Glc	4.1 ± 0.50	1.4 ± 0.01	3.8 ± 0.16	2.8 ± 0.03
GalA	53.7 ± 2.39	64.9 ± 0.45	56.3 ± 2.23	66.1 ± 0.23

σ^2 denotes standard deviation of triplicate samples.
* Significantly ($P < 0.05$) different from the water extracts.

The AW extract (Table 1) from SEQAIS had a much lower Ara content than the PW extracts (Table 2). The marked different distributions of monomers observed, indicated there could be different pectic populations extracted. However, it is well known that Ara*f* is labile to acid hydrolysis at pH 1–3 at elevated temperatures (Voragen et al., 1995) and Ara*f* could possibly have been hydrolyzed even at such mild conditions as pH 4.5 used in SEQAIS. A significant ($P < 0.05$) reduction in Ara*f* was observed after treatment of the water extracts at pH 4.5 (Table 2). The occurrence of monomer Ara*f* released over time course (Fig. 2), confirmed that Ara*f* had been partly hydrolyzed during this treatment. To our knowledge this has not been shown previously. A slight decrease of galactose levels also appeared in Hw100A. This indicated that Ara*f* residues were mainly removed from the outer part of the side chains.

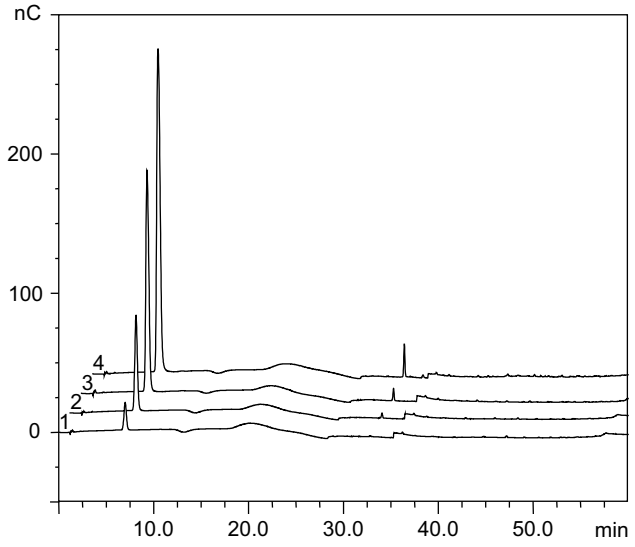

Fig. 2. HPAEC chromatogram of a time course release of Ara*f* of incubation of Hw100 at pH 4.5 at 37 °C at 0, 2, 5.5 and 21 h, curve 1–4 in respective order. Chromatograms are displaced parallel to each other at the time scale.

3.3. Linkage analyses

Prior to methylation analysis all samples underwent the reduction steps twice making the reaction approximately 90% complete. Analysis of the percentage distribution of different linkages (Table 3) revealed that the extracts from both extraction protocols contain mainly pectic material with major amounts of (1 → 4)-linked Gal*p*A residues. The amount of (1 → 2,4)-Rha*p* was higher in the extracts later in the extraction series of SEQAIS, indicating a higher abundance of branches. Those extracts also had a small amount of substitutions at *O*-3 of Gal*p*A, indicating the presence of xylogalacturonan as reported previously (Aspinall, Craig, & Whyte, 1968; de Vries, den Uijl, Voragen, Rombouts, & Pilnik, 1983). Xyl almost exclusively consist of terminal residues, which might be a part of xylogalacturonan (de Vries et al., 1983).

There were negligible amounts of terminally- and (1 → 3)-linked Fuc and terminal Glc*p*A units in most of the samples. Additionally some (1 → 4)-Glc*p* was observed. The constituent sugars of the side chain residues indicated there were mainly arabinan and AG II side chains. The

Table 3
Relative distribution (%) of linkages in fractions derived from sequential extraction and water extraction

Linkage	AW	Ox1	Ox2	Ox3	SO1	SO2	HCl	Hw50	Hw100
(→1)-Ara*f*	5	5	6	8	14	15	8	8	9
(1 → 2) + (1 → 3)-Ara*f*	n.d.	1	1	1	2	2	1	1	0
(1 → 5)-Ara*f*	3	7	8	9	15	16	7	10	7
(1 → 3,5)-Ara*f*	<1	1	1	1	2	3	<1	1	<1
(1 → 2,5)-Ara*f*	n.d.	1	1	2	3	3	1	2	2
(→1)-Xyl*p*	1	1	2	3	5	7	3	<1	2
(1 → 4)-Xyl*p*	<1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	<1
(→1)-Rha*p*	1	<1	<1	n.d.	n.d.	n.d.	n.d.	1,0	<1
(1 → 2)-Rha*p*	<1	3	4	4	7	6	7	2	3
(1 → 3)-Rha*p*	<1	<1	<1	<1	<1	<1	<1	<1	<1
(1 → 3,4)-Rha*p*	n.d.	<1	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
(1 → 2,4)-Rha*p*	<1	<1	<1	<1	2	3	5	1	2
(→1)-Gal*p*	3	<1	1	2	2	2	7	2	2
(1 → 4)-Gal*p*	n.d.	2	3	4	7	9	17	2	5
(1 → 3)-Gal*p*	1	<1	<1	<1	<1	<1	1	1	<1
(1 → 6)-Gal*p*	2	<1	<1	<1	<1	<1	2	3	<1
(1 → 3,6)-Gal*p*	2	<1	<1	1,0	<1	<1	1	4	<1
(→1)-Gal*p*A	2	1	1	1	<1	<1	1	3	3
(1 → 2)-Gal*p*A	<1	<1	<1	<1	<1	<1	<1	<1	0
(1 → 4)-Gal*p*A	74	70	59	54	38	29	33	49	50
(1 → 3,4)-Gal*p*A	1	<1	<1	<1	1	1	2	1	2
(1 → 2,4)-Gal*p*A	<1	<1	<1	<1	<1	<1	<1	<1	<1
(→1)-Glc*p*	<1	<1	1	1	<1	<1	<1	0	0
(1 → 4)-Glc*p*	<1	2	5	4	<1	<1	<1	4	4
(1 → 2,4)-Glc*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
(1 → 4,6)-Glc*p*	n.d.	<1	<1	<1	n.d.	n.d.	n.d.	<1	<1
(→1)-Fuc*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
(1 → 3)-Fuc*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
(→1)-Glc*p*A	<1	<1	<1	<1	<1	<1	<1	<1	<1
Sum	97	94	94	95	97	97	96	96	90

n.d., not detected.

presence of arabinan was indicated by a large amount of (1 → 5)-Ara with some Ara*f* substitutions at *O*-2 and *O*-3 (Table 3), which is typical linkages of arabinan (Perez et al., 2000). The pectic material contains (1 → 3)- and (1 → 6)-Gal*p* linkages and (1 → 3)- and (1 → 5)-Ara*f* linkages, indicating the presence of AG II. These materials positive reaction with Yariv (Westereng et al., 2006) further supports this. The presence of AG I is more debatable due to the fact that we did not observe the normally present (1 → 3,4)-Gal*p* (Hinz, Verhoef, Schols, Vincken, & Voragen, 2005; Perez et al., 2000).

The presence of linkages typical for RG-II (O'Neill et al., 1996) could not be positively identified, however, they might be present in minute amounts as reviewed previously (Voragen et al., 1995).

3.4. The relative molecular weight distribution

The elution profiles of the samples vary greatly (Fig. 3a and b). The estimated molar masses are given in Table 4. Under the extraction conditions used in the later SEQAIS steps, deesterification would be favored compared to β-elimination (van Buren & Pitifer, 1992). However, it has been shown that some β-elimination occur at 20 °C (Vollmert, 1950). This could explain the low M_w part of soda fractions. The later steps of the sequential extraction had much larger M_w than the first step, hence, pectin association in the cell wall is related to the polymer size.

The two extraction protocols isolated pectic material with different molecular weight distribution. The water extracts Hw50 and Hw100 (125 and 70 kDa) were approximately 1/3 of the Ox1–3 extracts (300, 330 and 345 kDa, respectively). The evidently inferior amount of Gal*p*A observed in water extracts as opposed to AW (Tables 2 and 3) could be due to release of shorter GalA oligomers during β-elimination and subsequent removal upon dialysis (Mort et al., 1991). β-Elimination and deesterification are competing reactions at neutral and even weakly acidic conditions (Albersheim et al., 1960) and it has been demonstrated that high temperatures and low pH (5–6) favour β-elimination (Kravtchenko, Arnould, Voragen, & Pilnik, 1992). Additionally various anions; citrate, malate and phytate (Keijbets & Pilnik, 1974) and also EDTA (Vu, Smout, Van Lowy, & Hendrickx, 2006) increase β-elimination efficiency through withdrawing stabilizing Ca^{2+}-ions (Keijbets & Pilnik, 1974). Four samples (Ox1, Ox2, SO1 and HCl) were treated with NaH_2PO_4, pH 7.2, at 100 °C and analysis demonstrated that β-elimination occurs during isolation in neutral hot solvents as in the water extraction (Albersheim et al., 1960). This explains the lower size of PW extracts as opposed to the initial extracts from SEQAIS, and the lower M_w of Hw100 than Hw50 may simply be due to more efficient β-elimination at elevated temperature. The treatment was also done as an attempt to improve solubility of these samples in veronal buffer to be able to test for complement-fixing activity. The treatment yielded evident degradation of the highly methyl esterified samples Ox1 and Ox2 (Fig. 3b). A control was added similar amount of NaH_2PO_4, pH 7.2 (Ox1k), and immediately analyzed by HPSEC (Fig. 3b), and no β-elimination occurred. The pectin in the HCl extract was highly methyl esterified (section below), but was not sensitive to β-elimination. It has been observed previously that a high neutral sugar:Gal*p*A ratio as found in the HCl fraction promotes a low degree of degradation (Kravtchenko, Penci, Voragen, & Pilnik, 1993). The lesser sensitivity of the HCl fraction may simply be the far lower amount of homogalacturonan compared to Ox1 and Ox2. Hence, it may be

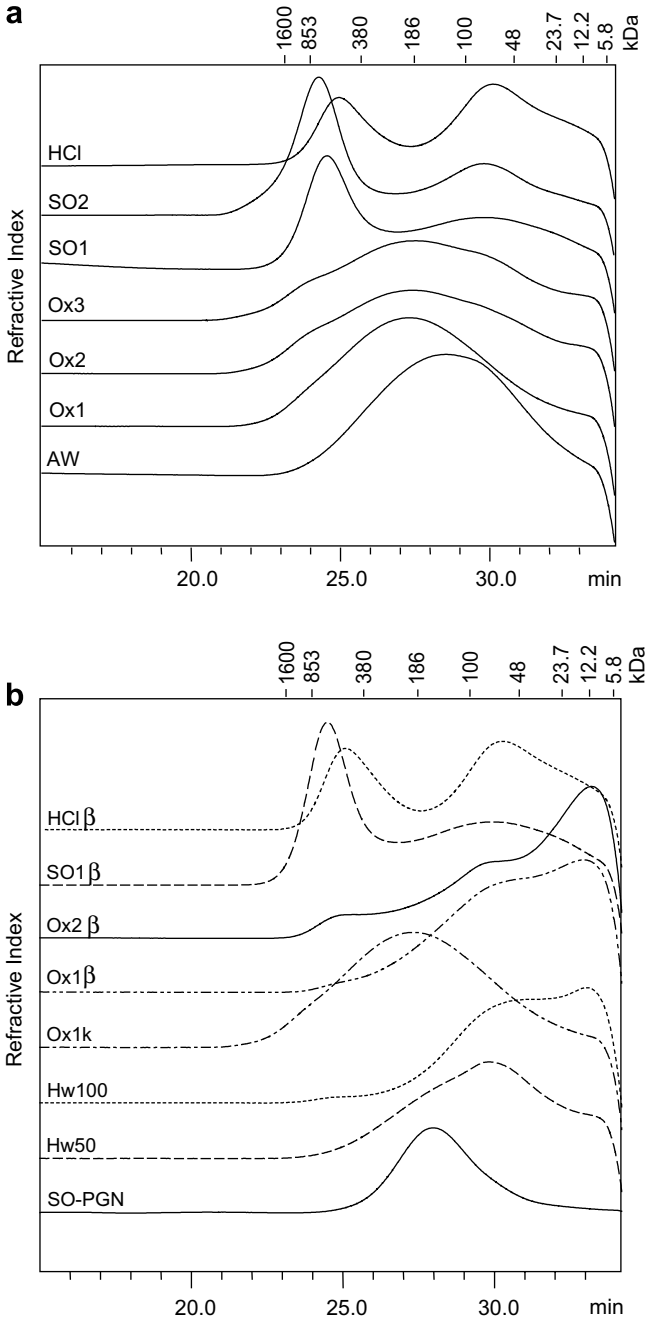

Fig. 3. (a) HPSEC elution profiles of SEQAIS fractions. (b) HPSEC elution profiles of PW extracts, β-eliminative treated samples and control and SO-PGN.

Table 4
Weight-average molar mass (M_w), number-average molar mass (M_n) and polydispersity factor (M_w/M_n) were determined for various extracts by HPSEC relative to pullulan standards

	M_n (kDa)	M_w (kDa)	M_w/M_n
AW	80	190	2.4
Ox1	120	300	2.5
Ox2	120	330	2.8
Ox3	110	345	3.1
SO1	80	385	4.8
SO2	120	720	6.0
HCl	65	230	3.5
Hw50	65	125	1.9
Hw100	30	70	2.3
Ox1k	140	350	2.5
Ox1β	30	85	2.8
Ox2β	25	105	4.2
SO1β	80	400	5.0
HClβ	55	210	3.8
SO-PGN	140	200	1.4

that a different choice of buffer and other conditions could have improved degradation efficiency and improved solubility. On the other hand one of the tasks in this study was to have conditions close to PW conditions to see if that would give similar degradation effects on the AW extract. The SO1 was not degraded under the same treatment, which was as expected due to the absent methyl esterification.

The three fractions tested for complement were subjected to MALLS for exact molecular weight determination yielding 40 and 55 kDa (Westereng et al., 2006), and 62 kDa for Hw50 and Hw100, and AW fractions, respectively. This indicated that the HPSEC system used with refractive index detection overestimated M_w to some degree.

3.5. Structural assignments of fractions by ^{13}C NMR

The SEQAIS and PW extracts were subjected to ^{13}C NMR analysis in order to determine the α/β configuration and collect information about acetylation and methyl esterification. Assignments of signals were aided by sugar composition and linkage analyses (Sections 3.2 and 3.3) and literature (Catoire, Goldberg, Pierron, Morvan, & du Penhoat, 1998; Cui, Eskin, Biliaderis, & Marat, 1996; Perrone et al., 2002; Samuelsen, Paulsen, Wold, Knutsen, & Yamada, 1998; Westerlund, Åman, Andersson, & Andersson, 1991). For a qualitative comparison of the fractions the structural information is summarised in Table 5. Complete spectra of Hw50 and Hw100 have been presented elsewhere (Westereng et al., 2006).

The (1 → 2)-Rha*p*:(1 → 2,4)-Rha*p* ratio of the *C*-6 resonances (δ 17.5 and 17.3, respectively), decreased in later steps of SEQAIS. This was in accordance with linkage analysis (Table 4) and indicated that more densely ramified regions had stronger associations within the remaining cell wall constituents.

The occurrence of the sharp 53.5 ppm resonance is diagnostic for methyl esterified Gal*p*A. The matching resonances at 100.8 and 99.7 ppm were assigned to *C*-1 of esterified (E) and unesterified (U) α-Gal*p*A, respectively. The close to 1:1 ratios of the anomeric signals for U and E residues of the Ox fractions indicated approximately 50% methyl esterification, whereas the U:E ratio of the HCl fraction was 2:3. The degree of methyl esterification (DE) could not be determined unambiguously in these regions due to overlapping signals with *C*-1 resonance from α-linked D-Glc*p* and Gal*p* if present, and the close proximity to the signals from Rha*p*. The corresponding resonances of *C*-6 of the carboxyl groups in Gal*p*A occurred in two regions (Catoire et al., 1998; Westerlund et al., 1991).

Table 5
^{13}C-Resonances observed in the extracts obtained from the SEQAIS and PW extractions

Linkage	Carbon	Resonance (δ)	AW	Ox1	Ox2	Ox3	SO1	SO2	HCl	Hw50	Hw100
α-Ara*f*	*C*-1	108.3–107.1	*	*	*	*	*	*	*	*	*
α-Rha*p*	*C*-1	99.1, 98.7 and 98.3	*		*		*	*			
(1-2)-Rha	*C*-6	17.5	*	*	*	*	*	*	*		
(1-2)-Rha-(4-β)-Gal*p*	*C*-6	17.3	*	*	*		*	*			
	C-1	105.0. 104.7 and 104.1	*				*	*			
α-Gal*p*A	*C*-1 E	100.7		*	*	*			*	*	*
	C-1 U	99.7	*	*	*	*	*	*		*	*
	C-6 U	175.7, 175.5 and 175.3	*	*	*	*	*	*		*	*
	C-6 E										
	U<u>E</u>U	171.5	*	*	*	*			*	*	*
	U<u>E</u>E/E<u>E</u>U	171.35		*	*	*			*	*	*
	E<u>E</u>E	171.2		*	*	*			*	*	*
Methyl	O—<u>C</u>H3	53.5	*	*	*	*			*	*	*
Acetyl	O=C—<u>C</u>H3	20.9 + 21.2		*	*	*			*		
	O=<u>C</u>—CH3	174.3		*	*	*			*		
% Methyl esterification			13	34	43	42			69	56	56

U denotes unesterified and E esterified Gal*p*A units. The observed nucleus is underlined.
*denotes qualitatively identified peaks.

The broad peaks of unesterified *C*-6 downfield at 175.7, 175.4 and 175.1 ppm and methyl esterified *C*-6 upfield at 171.5 (U<u>E</u>U), 171.3 (U<u>E</u>E and E<u>E</u>U) and 171.2 (E<u>E</u>E) ppm, where observed residue is underlined (Table 5). DE values (Table 5) were estimated by the *C*-6 Gal*p*A resonances utilizing the formula: $\int E/(\int E + \int U) \times 100\%$.

Finally the resonances at 174.3 and 21.2 + 20.9 ppm corresponded with the carbonyl and methyl carbons of acetyl substituents (Westerlund et al., 1991). These resonance areas, as well as the methyl ester resonances of the uronic acids in hot water extracts, were compared in the different SEQAIS extracts. It was indicated that the hot water extracts had lost acetyl and methyl ester groups during extraction. The latter was due to stripping of methyl ester groups when treated in hot neutral conditions (Albersheim et al., 1960).

3.6. Biological activity by complement-fixing assay

AW was less active than the PW extracts (Fig. 4a). This test was run twice with consistent results. The unexpected high inhibitory activity of AW at high concentration is difficult to explain. It might be that this particular sample had some physico-chemical effects influencing the complement system. Only the AW extract from SEQAIS extraction was fully soluble in the veronal buffer, which contains 0.2 mM calcium ions, and this causes a solubility problem to calcium sensitive pectins. When pectin samples were dissolved in ammonium oxalate the solution turned pale due to precipitation of calcium oxalate, thus the reduced solubility of this sample in veronal buffer could be due to an extensive intermolecular network of calcium bridges in the pectin solution. Several of the samples became gels when they were attempted dissolved in veronal buffer and made a notable, differently sized pellet upon centrifugation. In order to determine the biological effects of pectic extracts, solubility in the veronal buffer is of key importance so the other extracts could not be tested for complement-fixing activity. Insoluble extracts were therefore attempted treated with β-eliminative treatment to enhance solubility. This treatment, however, did not result in extracts soluble in veronal buffer. Samples subjected to β-eliminative treatment can hence, due to the loss of methyl groups, still be calcium sensitive regardless of lower molecular weight.

Since the SO1 fraction was resistant to beta eliminative treatment endopolygalacturonase (PGN) treatment was outlined as another attempt to obtain feasible veronal solubility. The sample was fractionated by Sephacryl 400 (see Section 2.6) and the high M_w fraction (SO-PGN) was still not soluble in veronal buffer, despite of evident reduction in size (Fig. 3b) compared with SO1 (Fig. 3a).

The complement-fixing assay was attempted run with other solvents than veronal buffer, as an alternative approach to circumvent the solubility problems, but this was not successful. Good solvents for pectin, such as ammonium oxalate and imidazol have an influence on the complement system by chelating calcium and thus destabilizing the C1s/C1r C1q complex, which is ion bridged with Ca^{2+}, and are therefore unsuitable for the assay.

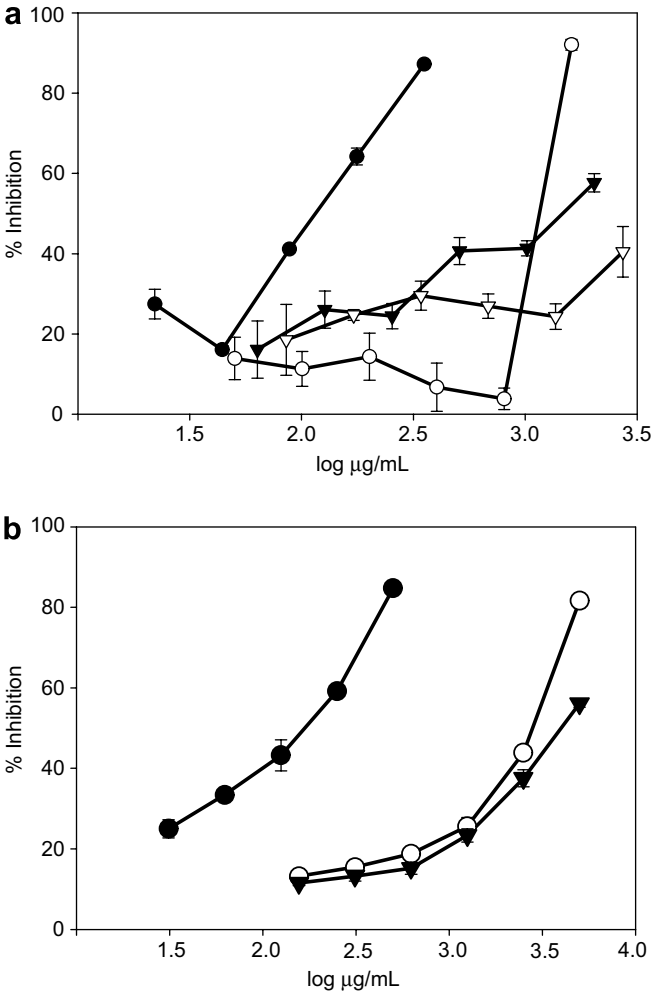

Fig. 4. (a) Complement-fixing activity of crude extracts from sequential and water extraction; Hw50 (▼), Hw100 (∇), AW (○) and control (PMII) (●) with standard deviation shown in brackets. Samples were run in quadruplicates. (b) Complement-fixing activity of Hw100 fraction with and without AW treatment conditions; Hw100 (○), Hw100A (▼) and control (PMII) (●) with standard deviation shown in brackets. Samples were run in triplicates.

Water extraction yielded pectin with moderate biological activity compared with the positive control, whereas the AW extract only had minor complement-fixing activity (Fig. 4a). Previous studies have indicated that the Ara-Gal-rich parts of pectin show the most potent activity and that homogalacturonic acid stretches seem to be of little importance for activity (Westereng et al., 2006). It has been shown previously that when Ara*f* residues are removed from pectin the complement-fixing activity is diminished (Westereng et al., 2006). The lower level of Ara*f* in AW as compared to PW extracts might be explained by hydrolysis of Ara*f* residues occurring during extraction with resulting reduction in complement-fixing activity by the former. Hence, Hw100 was treated according to AW with

subsequent dialysis to remove mono- and oligosaccharides and subjected to complement analysis. The treatment resulted in a significant ($P < 0.05$) reduction in activity (Fig. 4b). Authors like to comment that the higher degree of acetylation in AW as compared with PW was not positively correlated with complement-fixing activity. Previous analysis of complement-fixing activity have not shown any improved activity related with methyl esterification content.

It cannot be excluded that in addition to the side chain architecture, the size distribution of polymers could have an influence on activity. Since long chained polymers may have a number of side chains that might be sterically inaccessible to the complement-fixing proteins, degradation of the polymer resulting in shorter polysaccharide fragments could result in a higher number of available sites for attachment/binding. On the other hand such a degradation may disrupt the active parts of the polymer eliminating the possibility of a concertive binding of complement. The importance of a multivalent concertive binding of the initiator molecule of the classical pathway has been demonstrated previously (Kishore & Reid, 2000). Further experiments have to be carefully chosen in order to reveal whether polymer size is a descriptive property of complement-fixing activity.

4. Conclusions

Two different isolation protocols have been used to extract pectins with different characteristics. Firstly, weak acidic conditions during extraction degrade pectic side chains which reduce biological activity and this nature has to be considered when choosing extraction method. Secondly, SEQAIS extracts with longer stretches of highly methyl esterified Gal*p*A underwent β-elimination when treated with hot neutral solutions.

When preparing samples increasing calcium sensitivity of the pectins solubility problems could occur in the calcium containing veronal buffers and a subsequent problem in analyzing complement-fixing activity. Polygalacturonase treatment or addition of methyl esters at Gal*p*A could be done to reduce the calcium sensitivity prior to biological testing.

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