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Pectin isolated from white cabbage – structure and complement-fixing activity

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This study was done to investigate whether white cabbage contained polysaccharides with immunostimulatory activity using the complement-fixing test as an indicator. The main polysaccharide isolated was of pectin nature. Methanolysis and ^{13}C-NMR showed that the polymers consisted of highly esterified α-galactopyranoside (α-Gal*p*A), significant amounts of α-arabinose furanoside (α-Ara*f*), β-Gal*p* and lesser amounts of rhamnose in the pyranose form (Rha*p*) and xylose in the pyranose form (Xyl*p*). Linkage analyses showed that the α-Gal*p*A residues were mainly 1,4-linked with small amounts of 1,3,4-linkages. The α-Ara*f* residues were mainly terminally (t)- and 1,5-linked, whereas β-Gal*p* was t-, 1,3-, 1,6-, and 1,3,6-linked. Positive Yariv reaction indicated polymers with arabinogalactan type 2 like structures. α-Rha*p* was mainly present as 1,2- and 1,2,4-linked residues and Xyl*p* was t- and 1,4-linked. The molecular weight varied greatly and was from 10 to 150 kDa. Cabbage polymers had biological activity and this complement-fixing activity was greatly affected by hydrolytic removal of Ara*f* from pectic side chains.

Keywords: Arabinogalactan / Biological activity / Complement-fixing activity (anticomplementary) / Pectin / White cabbage (*Brassica oleracea*)

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1 Introduction

White cabbage is an important vegetable in Scandinavian household [1]. In addition to nutrition, leaves from white cabbage are used in traditional medicine to treat bedsores and inflammation in skin and joints.

Pectin is one of the three main polysaccharide groups constituting the cell wall of dicotyledons. It consists of a diverse set of structural elements and is mainly composed of partly methylesterified stretches of (1→4)-α-D-Gal*p*A, with a small portion of (1→2)-α-L-Rha*p*, which together constitute the backbone in smooth regions [2]. Branched neutral side chains occur in the so-called hairy regions with mainly Rha*p* and Gal*p*A in approximately 1 : 1 ratio. The side chains are attached to C-4 of a varying number of rhamnose (Rha) units and consist mainly of Ara*f* and Gal*p* linked in various manners. There are also observed side chains on C-2 or C-3 on Gal*p*A [3], in which xylose in the pyranose form (Xyl*p*) is proposed to be linked to C-3 of Gal*p*A as a monomer [2]. C-2 and C-3 on Gal*p*A also seem to be acetylated to some degree [4]. Additionally, smaller amounts of rhamnogalacturonan type 2 (RGII), a highly complex, largely conserved megaoligosaccharide is present in minor amounts [5].

Pectic polymers from a number of plants are shown to have complement-fixing activity [6]. The complement system is an important part of innate immunity and consists of more than 20 serum proteins that take part in a cascade mechanism [6]. Several studies have shown that plant polysaccharides can have complement-fixing activity [6–9]. The possible wound healing properties of cabbage leaves may be at least partly due to this [8].

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Abbreviations: AGII, arabinogalactan type 2; **AIM,** alcohol insoluble material; **Api**, apiose; **Ara,** arabinose; **Ara*f***, arabinose furanose; **ICH_{50},** concentration of test substance giving 50% inhibition; **Fuc,** fucose; GalA, galacturonic acid; **α-Gal*p*A,** galacturonic acid in the pyranose form; **Glc,** glucose; **GlcA,** glucuronic acid; **IEC,** ion exchange chromatography; **LEAP,** late eluting acidic polysaccharides; **Man,** mannose; **MALLS,** multiangular laser light scattering; **M_n**, number average molecular weight; **M_w**, weight average molecular weight; **RGII,** rhamnogalacturonan type 2; **Rha,** rhamnose; **Rha*p***, rhamnose in the pyranose form; **Xyl,** xylose; **Xyl*p*,** xylose in the pyranose form

Despite of structural diversity, pectin like polysaccharides are common for all dicotyledon plants with a subset of building blocks compiled in a different manner [10]. Hence it is not unlikely that immunomodulatory effects of pectin is not a special feature for medicinal plants and herbs [6], but may also be a property of pectins on a more general basis.

This work reveals that pectin from white cabbage (*Brassica oleracea*) have complement-fixing activity. To our knowledge this is the second time polysaccharides from edible sources with nutritional value have shown to possess such effects. Recently polysaccharides from red peppers (*Capsicum annum*) showed potent effects on the complement system [11]. This present work also sheds new light on structural features of pectic polymers from white cabbage.

2 Materials and methods

2.1 Preparation of alcohol insoluble material (AIM)

White cabbage (*B. oleracea* var. Capitata, Bartolo cultivar) was cultivated at Vollebekk testfield, Aas, Akershus. Fresh leaves from parts of eight different cabbage heads (2 kg) were shredded (3 – 5 mm) refluxed three times for 2 h in 80% ethanol at 80°C, according to the method of Samuelsen *et al.* [8], to get AIM.

2.2 Extraction

The AIM (50 g) was extracted with distilled water (1 L) for 3 h at 50°C, filtered through four layers of gauze and then through Whatman GF/C filters. Finally the extract was dialyzed (M_w cut off 3500 Da) against distilled water. The residual AIM was subsequently extracted with boiling water and treated as described above.

The 50 and 100°C crude extracts from white cabbage were named Hw50 and Hw100, respectively. The extracts were concentrated on a rotavapor at 30°C and frozen at –20°C.

2.3 Ion exchange chromatography (IEC)

Each crude extract was passed through a 0.45 µm Millipore filter and applied to a DEAE-Sepharose Fast flow (Amersham Biosciences) column (390 mL). The column was eluted with distilled water at 1 mL/min flow rate for the isolation of a neutral fraction followed by elution with a NaCl-gradient (0 – 1 M) and a subsequent step of 2 M NaCl for isolation of the most negatively charged polysaccharides. Ten milliliters fractions were collected using an LKB-Super Frac (Pharmacia) fraction collector and pooled according to the carbohydrate elution profile [12], dialyzed (M_w cutoff 3500 Da), and lyophilized.

2.4 Analytic and preparative SEC

2.4.1 Analytical SEC

The fractions were subjected to SEC with serial Agilent Aquagel-OH (40, 50, and 60) columns and eluted with 50 mM Na_2SO_4 (0.8 mL/min) at 40°C. The eluent reduce polymer inter-/intrachain associations and hence aid correct molecular weight determinations [13]. Detection was done with a refractive index detector (Shimadzu RI6A). Pullulan with size distribution span 50 – 1600 kDa were used as standards. Weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity (D) were calculated with the PSS WinGPC software package. Fractions with a heterogeneous molecular weight distribution were subjected to preparative SEC.

2.4.2 Analytical HPSEC-multiangular laser light scattering (MALLS)

High pressure (HP) SEC combined with MALLS detection as described by Christensen *et al.* [14], was performed for some selected fractions.

2.4.3 Preparative SEC

Polysaccharide fractions were separated on a preparative Sephacryl S-400 HR (Pharmacia) column (2.6 cm × 100 cm) with an M_w fractionation range of 10 – 2000 kDa for dextrans. The column was coupled to a peristaltic pump P-3 (Pharmacia), an LKB-Super Frac (Pharmacia) fraction collector, and a Shimadzu RID-10A refractive index detector. The polysaccharide samples (50 mg) were dissolved in 0.1 M NaCl, filtered (0.22 µm Millex® GV Millipore) and applied to the column. The column was eluted with 0.1 M NaCl at 60 mL/h.

2.4.4 Total phenolic compounds

The fractions were analyzed for phenolic compounds by the method of Swain and Hillis [15], and ferulic acid was used as the standard.

2.4.5 Quantitative determination of protein content

The protein content of the fractions was determined by the protein assay of Lowry *et al.* [16], and BSA was used as the standard.

2.4.6 Monosaccharide composition

Methanolysis and GC analysis were run by a modification of the method of Chambers and Clamp [17] as described by Samuelsen *et al.* [8]. The derivatives were separated on a DB-5 fused-silica capillary column (30 m × 0.32 mm id).

2.4.7 Uronic acid determination

Uronic acids were in addition to methanolysis also quantified by the Carbazol method as the described by Sturgeon [18].

2.4.8 Starch determination

The detection of starch was performed by reaction with iodine/potassium iodine as described by Yu *et al.* [19]. Potato starch was used as the positive control.

2.4.9 Reduction

The uronic acids of polymer fractions were reduced with sodium borodeuteride in two steps according to the method of Kim and Carpita [20]. The reduction was followed by methylation and GC-MS.

2.4.10 Methylation

The polysaccharide samples were methylated according to the method of McConville *et al.* [21]. The partially methylated alditolacetates were extracted with dichloromethane. Samples were dissolved in dry methanol and analyzed by a Hewlett-Packard 5890 GC with a Varian FactorFour VF column (30 m × 0.25 mm id). Samples were run with a flow rate of 0.9 mL/min helium and detected with Hewlett-Packard Mass Selective Detector 5970. The quantitative results in linkage analysis were based on the relative distribution of the differently linked monomers. Subsequently, the distribution of each monomer from methanolysis was used to give the total amount of each monomer linkage.

2.4.11 Weak acid hydrolysis

Furanosides were removed from the polymer fractions by hydrolysis at mild conditions as described by Cartier *et al.* [22].

2.4.12 Precipitation with Yariv β-glucosyl reagent

Samples were incubated with the Yariv β-glucosyl reagent (1,3,5-tri-(4-β-D-glucopyranosyl-oxyphenylazo)-2,4,6–trihydroxybenzene) to indicate the presence of AG-type II according to van Holst *et al.* [23].

2.4.13 ^{13}C-NMR

Samples were dissolved in D_2O with 50 mM $(NH_4)_2C_2O_4$, incubated in water at 60°C for 5 min, centrifuged at 13 000 rpm for 5 min, and the supernatant was applied to an NMR tube. ^{13}C-NMR spectra were recorded with the following settings (75 mHz, 80°C, 2 s pulse delay, 1.64 s acquisition time, 85° pulse angle, sweep width 15 974 Hz, and approximately 60 000 datapoints). Chemical shifts were given relative to DMSO 39.5 ppm.

2.4.14 Testing for complement-fixing activity

Samples were subjected to an assay to test their ability to interfere with the complement system as described by Michaelsen *et al.* [24]. A pectic fraction from *Plantago major* (PMII) was used as a positive control [8].

3 Results and discussion

3.1 Characterization of the crude extracts

Water extraction of 50 g AIM gave approximately 1 g crude pectin extracts Hw50 and Hw100 (Fig. 1). The crude

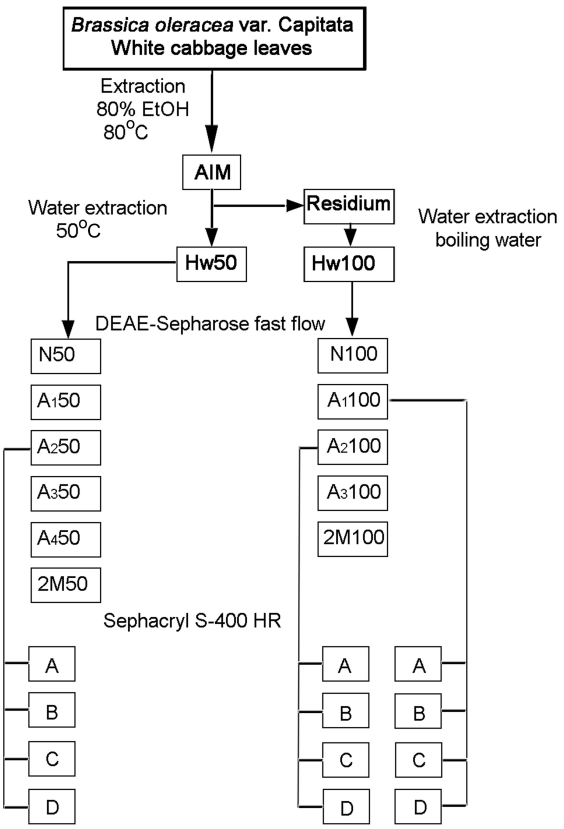

Figure 1. Isolation of polysaccharide fractions from white cabbage leaves (*B. oleracea*). Pectin from AIM was extracted at 50°C resulting in (Hw50) and residuum. The residuum was subsequently extracted with boiling water (Hw100). Hw50 and Hw100 were both subjected to IEC fractionation. The IEC fractions $A_2$50, $A_1$100, and $A_2$100 were further fractionated by SEC with Sepahcryl-400.

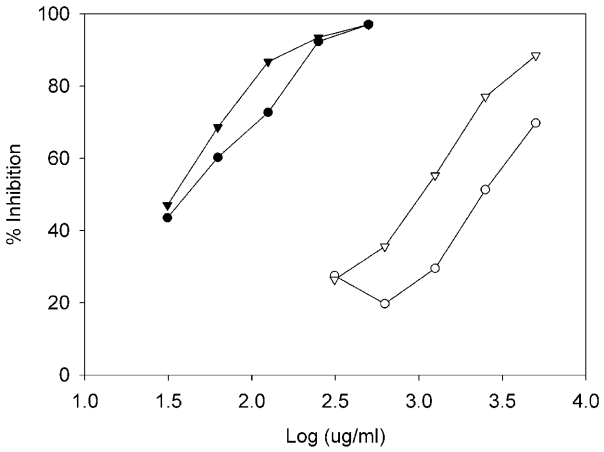

Figure 2. Complement-fixing activity of the crude extracts; Hw50 (△), Hw100 (○), and two identical controls PMII (▼, ●). Samples were run in triplicates.

extracts had some complement-fixing activity, Hw50 more active than Hw100 (Fig. 2), with ICH_{50} (concentration of test substance giving 50% inhibition) values of 1.8 and 8.6 mg/mL, respectively. The activities of the crude extracts from white cabbage were much lower than the positive control, PMII (ICH_{50} = 0.040 mg/mL).

The carbohydrate content was higher in Hw100 than Hw50 (Table 1). The crude extracts and isolated fractions were first subjected to ^{13}C-NMR analysis (Fig. 3). Spectral assignments were based on previous studies [4, 25–28], and summarized in Table 2. This basic information regarding sugar constituents, linkages and anomer configuration were confirmed and further elaborated by methanolysis and linkage analysis.

The monosaccharide distribution (Table 1) and linkage analysis observed were similar to pectins from several other sources [29], with Gal*p*A (near 60%) and lesser amounts of Ara (*ca.* 15%) and Gal (*ca.* 10%). Rha, xylose (Xyl), mannose (Man), and fucose (Fuc) were detected in minor amounts, and in addition trace amounts of glucuronic acid (GlcA). Linkage composition (Table 3) indicated a main chain of (1→4)-α-Gal*p*A and (1→2)-α-Rha*p* and side chains mainly comprising arabinogalactan type 2 (AGII) like structures attached to *O*-4 of Rha*p*. The presence of AGII like structures was also supported by a positive reaction with Yariv antigen, most evident for Hw50 (Fig. 4). The degree of Gal*p*A methylesterification was roughly estimated either by the ratio of ^{13}C-NMR resonances (Table 2) of unesterified and esterified C-6 of Gal*p*A or with similar results by the ratio of anomeric signals of unesterified and esterified Gal*p*A. GlcA and apiose (Api) are diagnostic for RGII [5], The lack of Api detection and trace amounts of GlcA, both RGII diagnostic monomers, implied that the presence of RGII must be small, if present [5].

Analytical SEC revealed that the bimodal Hw50 crude extract had higher average molecular weight than the broadly distributed unimodal Hw100 (Table 4). Crude extracts were run on an SEC-MALLS to validate polymer sizes obtained on the Schimadzu SEC system and gave average molecular weights of 55 and 40 kDa for Hw50 and Hw100, respectively. The molecular size distributions of the crude extract pectic polymers were in the lower range compared with studies of pectins from other sources [29]. The high pH (7.2) during extraction, and the fact that fractions had highly methyl esterified Gal*p*A, may have induced β-elimination during water extraction. The temperature dependence of β-elimination [30, 31] might explain the smaller size of the Hw100 polymers compared

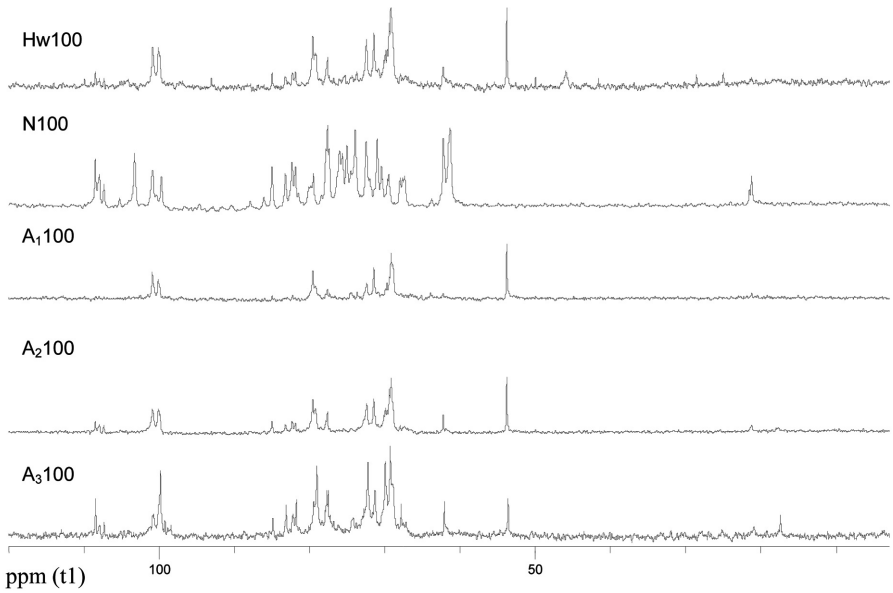

Figure 3. ^{13}C-NMR-spectra of the Hw100 extract and selected IEC fractions isolated from Hw100; N100, $A_1$100, $A_2$100 and $A_3$100.

Table 1. Monosaccharide distribution (relative w/w%) in: crude extracts, IEC fractions and SEC fractions from *B. oleracea*. Relative amounts (% w/w) of each fraction within its respective fraction series, IEC fractions from Hw50, Hw100, SEC fractions from $A_2$50, $A_1$100, and $A_2$100 are given in the column CHO (%)[a]. The total carbohydrate content (% w/w) of the fractions estimated by methanolysis is given in the CHO (%)[b] column

Fraction	Ara	Rha	Fuc	Xyl	Man	Gal	Glc	GalA	GlcA	CHO (%)[a]	CHO (%)[b]
Hw50	15.6	3.6	0.5	2.5	1.9	12.8	4.4	58.5	<0.1		40.7
Hw100	16.9	5.0	0.6	3.3	3.5	8.4	5.0	57.1	<0.1		54.7
N50	29.4	0.8	0.2	3.0	25.3	14.9	22.3	4.1	<0.1	6.3	81.3
$A_1$50	36.1	0.3	0.1	27.2	1.9	25.8	0.8	7.7	<0.1	2.1	68.9
$A_2$50	18.8	2.5	0.2	3.7	0.6	16.8	1.6	55.8	<0.1	31.2	53.8
$A_3$50	17.8	6.1	0.3	1.8	0.2	11.0	1.7	61.0	<0.1	53.2	47.5
$A_4$50	12.2	38.3	0.2	8.5	0.5	8.4	0.2	31.7	<0.1	5.3	20.1
2M50	30.6	2.4	0.3	2.4	1.2	13.2	3.4	46.4	<0.1	1.9	31.4
N100	28.0	0.2	0.3	2.3	32	11.0	24.6	1.3	<0.1	7.2	76.1
$A_1$100	12.3	0.2	0.3	11.4	0.8	8.3	2.0	64.7	<0.1	8.6	52.0
$A_2$100	18.5	6.5	0.7	1.9	0.1	8.0	1.7	62.8	<0.1	73.0	53.2
$A_3$100	19.2	13.8	0.5	2.2	0.2	8.9	1.5	53.5	<0.1	7.4	38.8
2M100	20.2	19.5	0.4	4.1	0.5	9.2	1.5	44.6	<0.1	3.9	28.5
$A_2$50A	15.4	2.4	0.3	2.0	0.3	13.4	1.3	65.0	<0.1	29.7	47.7
$A_2$50B	15.1	2.1	0.3	3.1	0.3	15.8	1.2	61.7	<0.1	70.3	58.5
$A_1$100A	9.0	1.2	0.2	10.6	0.3	8.8	1.9	67.8	<0.1	76.9	62.2
$A_1$100B	2.6	1.0	0.2	11.9	0.6	4.5	2.0	76.9	<0.1	23.1	56.9
$A_2$100A	39.3	8.7	0.2	3.3	0.6	12.5	1.4	34.0	<0.1	7.6	60.3
$A_2$100B	17.0	5.7	0.6	1.2	0.2	10.0	1.7	63.6	<0.1	67.2	95.9
$A_2$100C	7.4	5.5	0.8	1.8	0.4	5.4	3.0	75.6	<0.1	22.4	35.0
$A_2$100D	3.0	3.2	0.8	2.1	1.8	4.7	5.0	78.3	<0.1	2.8	37.5

Table 2. ^{13}C-NMR resonances of Hw50, Hw100, $A_2$50, $A_3$50, N100, $A_1$100, $A_2$100, and $A_3$100. Shifts observed are denoted * and weak signals are indicated (*)

		Shift	Hw50	Hw100	$A_2$50	$A_3$50	N100	$A_1$100	$A_2$100	$A_3$100
α-Araf	C-1	108.3–107.1	*	*	*	*	*	(*)	*	*
α-Rhap	C-1	99.1, 98.7, 98.3			*					*
(1–2)-Rha	C-6	17.5			(*)	(*)		*	*	*
(1–2)-Rha-(4–	C-6	17.3			(*)	(*)				
β-Galp	C-1	105.0, 104.7, 104.1		*	*	*	*		(*)	
β-Man/β-Glc	C-1	103.1					*			
α-Glc	C-1	100.7–99.5					*			
α-d-GalpA	C-1^U	100.7	*	*	*	*		*	*	*
	C-1	99.7	*	*	*	*		*	*	*
	C-6^E	175.7, 175.5, 175.3	*	*	*	*		*	*	*
	C-6^U									
	U*E*U	171.5	*	*	*	*		*	*	*
	U*E*E/E*E*U	171.35	*	*	*	*		*	*	*
	E*E*E	171.2	*	*	*	*		*	*	(*)
Methyl	O–CH_3	53.5	*	*	*	*		*	*	*
Acetyl	O=C–CH_3	20.9			*	*	*	*	*	(*)
	O=*C*–CH_3	174.3					*	*	(*)	(*)
% Methylesterification								55	50	30

U denotes unesterified and E esterified Gal*p*A units, the shift value refers to the methylesterified unit in the middle of the triad.

with Hw50. This is a common problem during water extractions of pectins. However, it was not an issue in this study to extract intact pectin polysaccharides, but rather to identify polysaccharides in the extract with biological effects. In traditional medicine water extracts are prepared without pH control.

The protein content was 1.3 and 1.9% in Hw50 and Hw100, which was approximately half the amount found by Stevens and Selvendran [32]. This might be due to the milder extraction protocol used. The low amount of phenolic content (less than 1%) in both extracts was expected as most of the low molecular weight phenolic compounds were removed during the preparation of AIM due to good solubility in ethanol. Phenolic compounds such as lignins and ferulic acid associated with interconnections of cell wall polysaccharides need a more rigorous treatment to be extracted [33].

Table 3. Relative distribution of linkages in IEC fractions based on methylation studies

Linkages	Hw50	N50	$A_1$50	$A_2$50	$A_3$50	$A_4$50	2M50	Hw100	N100	$A_1$100	$A_2$100	$A_3$100	2M100
T-Ara*f*	7	8	16	7	3	5	11	9	9	4	5	4	7
1,2+1,3–Ara*f*	<1	<1	1	1	1	2	4	<1	<1	n.d.[a]	<1	<1	3
1,5–Ara*f*	8	14	15	9	10	4	12	7	13	5	9	11	8
1,2,5–Ara*f*	2	4	4	2	2	<1	2	2	3	3	3	2	2
1,3,5–Ara*f*[b]	n.d.	3	n.d.	n.d.	2	<1	2	n.d.	2	<1	1	2	1
T-Xyl*p*	1	3	3	1	2	9	2	2	2	3	2	2	4
1,4–Xyl*p*	<1	n.d.	21	3	n.d.	n.d.	n.d.	<1	n.d.	9	n.d.	n.d.	n.d.
1,2,4–Xyl*p*	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<1	n.d.	n.d.
T-Rha*p*	1	n.d.	n.d.	n.d.	<1	8	<1	1	<1	<1	<1	<1	n.d.
1,2–Rha*p*	2	<1	<1	1	3	21	1	3	<1	n.d.	3	10	13
1,3–Rha*p*	<1	n.d.	n.d.	n.d.	<1	3	<1	<1	n.d.	n.d.	<1	n.d.	2
1,2,4–Rha*p*	1	n.d.	n.d.	1	1	7	<1	2	n.d.	<1	3	4	5
T-Man*p*	n.d.	<1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.
1,4–Man*p*	n.d.	21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	26	n.d.	n.d.	n.d.	n.d.
1,6–Man*p*	n.d.	<1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<1	n.d.	n.d.	n.d.	n.d.
1,4,6–Man*p*	n.d.	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5	n.d.	n.d.	n.d.	n.d.
T-Gal*p*[c]	4	6	<1	2	2	1	3	5	5	1	1	1	2
1,4–Gal*p*[c]	n.d.	6	1	n.d.	n.d.	n.d.	7	n.d.	6	n.d.	5	4	5
1,3–Gal*p*	2	<1	3	2	2	1	1	1	<1	1	<1	1	<1
1,6–Gal*p*	2	1	4	4	3	2	1	<1	<1	2	<1	2	<1
1,3,6–Gal*p*	5	2	16	9	5	4	2	2	<1	4	<1	<1	1
T-Glc*p*	<1	1	n.d.	n.d.	<1	<1	<1	<1	1	n.d.	n.d.	<1	<1
1,4–Glc*p*	4	20	<1	1	1	<1	3	4	22	2	2	1	1
1,4,6–Glc*p*	n.d.	2	n.d.	<1	n.d.	n.d.	<1	n.d.	2	<1	n.d.	n.d.	<1
T-Gal*p*A[c]	3	<1	n.d.	<1	<1	<1	<1	2	n.d.	2	1	<1	<1
1,4–Gal*p*A[c]	55	<1	8	54	59	30	44	53	1	61	59	50	42
1,3,4–Gal*p*A	<1	n.d.	n.d.	<1	<1	<1	1	2	n.d.	2	2	2	1
1,2,4–Gal*p*A	<1	n.d.	n.d.	<1	<1	<1	<1	<1	n.d.	<1	<1	<1	<1
T-Fuc*p*	<1	n.d.	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
T-Glc*p*A	<1	<1		<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

[a] n.d. = not detected.
[b] Could be 1,3,4-linked Xyl*p*.
[c] The relative difference in the diagnostic fragments 205/207 and 233/235 were used to calculate the amount of t- and 1,4-linked, respectively, Gal*p* and Gal*p*A.

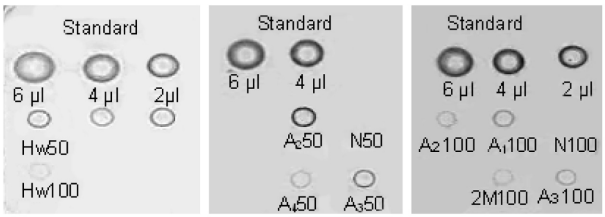

Figure 4. Precipitation of Yariv β-glucosyl antigen and polysaccharide material was performed by single radial diffusion. The following samples were tested; Hw50, Hw100, N50, $A_2$50, $A_3$50, $A_4$50, N100, $A_1$100, $A_2$100, $A_3$100, and 2M100.

3.2 IEC fractions

Crude extracts were further separated by IEC, resulting in six fractions from Hw50 (N50, $A_1$50, $A_2$50, $A_3$50, $A_4$50, and 2M50) and five fractions from Hw100 (N100, $A_1$100, $A_2$100, $A_3$100, and 2M100). The major fractions $A_2$50 and $A_3$50 constituted approximately 85% of the total carbohydrate of Hw50, and the $A_2$100 fraction constituted 73% of Hw100 (Table 1).

Table 4. Analytical SEC of IEC fractions; N50, $A_1$50, $A_2$50, $A_3$50, $A_4$50, 2M50, N100, $A_1$100, $A_2$100, $A_3$100, and 2M 100. Molecular weights were calculated with PSS WinGPC program and given in kDa relative to pullulan standards

Fraction	M_n	M_w	D
Hw50	65	125	1.9
N50	10	14	1.4
$A_1$50	14	27	1.9
$A_2$50	53	134	2.5
$A_3$50	62	131	2.1
$A_4$50	12	24	2.0
2M50	45	119	2.6
Hw100	30	70	2.3
N100	11	15	1.4
$A_1$100	12	23	1.9
$A_2$100	31	86	2.8
$A_3$100	52	145	2.8
2M100	19	92	4.8

IEC fractions tested for complement-fixing activity (Fig. 5) were, contrary to the study on *C. annuum* by Paik *et al.* [11], far more potent than the crude extracts with ICH_{50} values

from 0.1 to 0.5 mg/mL. Surprisingly, the IEC fractions obtained from Hw100 were more potent than the IEC 50 extracts, whereas vice versa for crude extracts. This could be explained by the major fraction $A_3$50, which was more active than the major fraction from Hw100 namely $A_2$100. Neither the 2M fractions nor $A_4$50 were tested due to low amount of material in these fractions.

IEC fractions were grouped into neutral (N50 and N100), early eluting acidic ($A_1$50 and $A_1$100) and late eluting acidic polysaccharides (LEAP) that include the rest of the fractions.

3.3 The neutral IEC fractions

N50 and N100 accounted for circa 5% of the total polymeric material (Table 1). The fractions were high in Ara and Gal, Man and glucose (Glc), and low in Gal*p*A. Linkage analysis (Table 3) showed that the Ara*f* residues were mainly terminally (t-), 1,5- and 1,2,5-linked, and Gal*p* was mainly t- and 1,4-linked, but also 1,3,6-linked. The Man*p* residues were mainly 1,4- and 1,4,6-linked. Glc*p* was 1,4-linked, but starch was not detected. Both fractions had unimodal peak shapes in analytical SEC with molecular weight of approximately 10 kDa (Table 4). They did not react with Yariv antigen (Fig. 4), but N100 was one of the most active complement-fixing fractions (Fig. 5) with ICH_{50} approximately 0.2 mg/mL, whereas N50 was not tested due to insufficient amount of material. This indicated that N50 and N100 contained two types of polysaccharides, possibly an arabinogalactan and a glucomannan. Ara*f* and Gal*p* could constitute an individual arabinogalactan or be degradation products from pectic side chains, as suggested elsewhere [34]. The absence of starch was in accordance with results published previously [1], still the resonance at 100.7 could be α-linked Glcp. Based on this, and linkage studies, this polymer could be similar to the glucomannan described by Hua *et al.* [35] containing a (1→4)-β-Man*p* backbone substituted with (1→4)-α-Glc*p* in C-6 of Man*p*. However, β-linked Glc*p* may give resonances at about 103 ppm and may also be present (Table 1). The acetyl peak observed at 20.9 ppm could be assigned to acetylated Glc*p* or Man*p* [35]. However, it cannot be excluded that the fraction could contain other structures like, *e.g.*, a galactomannan.

3.4 The early eluting acidic fractions

$A_1$50 and $A_1$100 (2 and 8% of total extracts, respectively (Table 1)) were different from LEAP's, with high levels of Xyl and Ara (Table 1). The Xyl*p* was mainly 1,4-linked, but also 1,2,4-linked as found elsewhere [32] (Table 3) whereas the Ara*f* was t-, 1,5- and 1,2,5-linked. $A_1$100 was significantly high in 1,4-linked Gal*p*A (61%). $A_1$50 had a bimodal distribution (Table 4) with one minor and one major peak corresponding to approximately 50 and 10 kDa, respectively. The peak of $A_1$100 (Table 4) was less than 10 kDa with a front at appoximately 40 kDa. This fraction was later subjected to preparative SEC. $A_1$100 was one of the most active fractions (Fig. 5) with ICH_{50} of 0.1 mg/mL when testing for complement-fixing activity. This fraction also showed a positive reaction with Yariv antigen (Fig. 4).

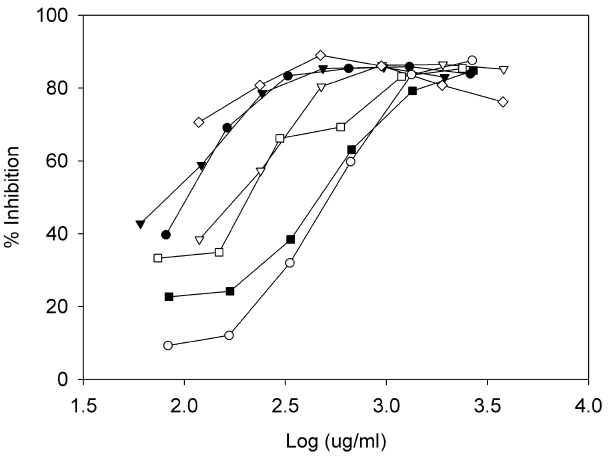

Figure 5. Complement-fixing activity of IEC fractions: $A_2$50 (■), $A_3$50 (□), N100 (△), $A_1$100 (●), $A_2$100 (○), $A_3$100 (▼), and control (PMII) (◇). Samples were run in duplicates.

Earlier studies have suggested that t-linked Xyl*p* are attached to O-3 of the Gal*p*A residues [29], and hence significant amounts of 1,3,4-linked Gal*p*A would then be present. Since mainly 1,4- and t-linked Gal*p*A were found, Xyl*p* may be a constituent of individual coeluting polymers. Stevens and Selvendran [36] detected arabinoxylan in a hemicellulose fraction after alkali treatment, and it is possible that minor amounts of arabinoxylans were extracted during water extraction. The presence of two polymers was indicated by bimodal SEC elution (chromatograms not shown). $A_1$100 was among the fractions with the highest complement-fixing activity, and the high amount of Xyl*p* might have a positive influence on the activity. An heteroxylan with Ara substituted residues from *P. asiatica* has previously been reported to have complement-fixing activity [37].

3.5 Analyses of LEAP fractions

LEAPs represent a majority of the polymeric materials, have a high portion of Gal*p*A and relatively high amounts of Ara and Gal, and some Rha and Xyl (Table 1). Linkage analysis (Table 3) showed 1,4-linked Gal*p*A residues and small amounts of 1,3,4-linkages. Ara*f* was t- and 1,5-linked

Table 5. Relative distribution of linkages in IEC fractions based on methylation studies of $A_2$50B, $A_2$100B, and $A_2$100C before and after weak acid hydrolysis

Linkages	$A_2$50B	$A_2$50B^H	$A_2$100B	$A_2$100B^H	$A_2$100C	$A_2$100C^H
T-Ara*f*	7	<1[a)]	7	<1	4	<1
1,2+1,3-Ara*f*	<1	<1	<1	<1	<1	<1
1,5-Ara*f*	6	<1	7	<1	3	<1
1,2,5-Ara*f*	<1	<1	1	<1	<1	<1
1,3,5-Ara*f*	<1	<1	1	<1	<1	<1
T-Xyl*p*	<1	2	1	<1	2	<1
1,4-Xyl*p*	2	<1	<1	<1	<1	<1
1,2,4-Xyl*p*	<1	<1	<1	<1	<1	<1
T-Rha*p*	<1	<1	<1	<1	2	<1
1,2-Rha*p*	<1	2	4	3	4	1
1,3-Rha*p*	<1	<1	<1	<1	<1	<1
1,3,4-Rha*p*	<1	<1	<1	<1	<1	<1
1,2,4-Rha*p*	2	<1	<1	<1	<1	<1
T-Gal*p*	3	3	6	2	4	2
1,4-Gal*p*	<1	<1	<1	<1	<1	<1
1,3-Gal*p*	2	1	<1	<1	<1	<1
1,6-Gal*p*	5	5	<1	<1	<1	2
1,3,6-Gal*p*	6	2	<1	<1	<1	<1
T-Glc*p*	<1	1	<1	2	<1	2
1,4-Glc*p*	<1	1	<1	3	3	5
1,2,4-Glc*p*	<1	<1	<1	<1	<1	<1
1,4,6-Glc*p*	<1	<1	<1	<1	<1	<1
T-Gal*p*A	1	5	2	8	3	7
1,2-Gal*p*A	<1	<1	<1	<1	<1	<1
1,4-Gal*p*A	59	63	59	62	70	71
1,3,4-Gal*p*A	1	<1	2	<1	2	<1
1,2,4-Gal*p*A	<1	<1	<1	<1	<1	<1
T-Fuc*p*	<1	<1	<1	<1	<1	<1
T-Glc*p*A	<1	<1	<1	<1	<1	<1
Sum[b)]	94	84	90	79	95	89

[a)] The table values <1.0 are not included in the sum.
[b)] The reduced sums for acid hydrolysed fractions are due to the hydrolytic removal of the monomers above.
H denotes samples subjected to weak acid hydrolysis.

and Gal*p* was 1,6- 1,3-, and 1,3,6-linked. Rha*p* was mainly present as 1,2- and 1,2,4-linked residues and Xyl*p* was t- and 1,4-linked. Later eluting fractions had an increasing amount of Rha*p*. Side chain lengths of 3 – 30 residues were estimated through the Ara*f*+Gal*p*/1,2,4-linked Rha*p* ratio. Previously, 20 – 40% branching on Rha*p* units have been observed [32], which was in accordance with our observations (20 – 50%). In the same study, side chains of approximately five residues were found [32]. The overall larger side chains in this study, 20 and 10 residues for IEC fractions from Hw50 and Hw100, respectively, could be due to the different extraction properties used. The Ara*f*:Gal*p* ratio of arabinogalactan side chains was roughly 3:2 and 2:1 for the Hw50 and Hw100 fractions, respectively.

LEAPs had higher molecular weight and a broader size distribution than the early eluting acidic fractions (Table 4). LEAPs of Hw100 were bimodal and broadly distributed with M_r ranging from 20 to 1000 kDa. The composition of Hw50 fractions was also broadly distributed with molecular size distribution similar to the Hw100 fractions, except $A_4$50, which had a main peak at circa 10 kDa.

The most strongly retained IEC fractions contained far less Gal*p*A than the early eluting fractions. This elution pattern was probably due to increased levels of less charged esterified Gal*p*A. This was confirmed as reduced intensity of both esterified C-6 (δ 171.5 – 171.2) and O-Me (δ 53.5) resonances in ^{13}C-NMR. The opposite seems to be true for the degree of acetylation, which when visually judged was highest in the latest eluting acidic fractions. ^{13}C-NMR also revealed that the Ara rich fractions $A_2$100 and $A_3$100 (Fig. 3) probably contained Ara*f* due to the absence of any Ara*p* signals at 105 ppm [38], which was also supported by weak acid hydrolysis as discussed below.

3.6 SEC fractions

After analytical SEC (Table 4), three fractions ($A_2$50, $A_1$100, and $A_2$100) were selected for preparative SEC. The other fractions were either in insufficient amounts for fractionation by SEC (N50, $A_1$50, $A_4$50, $A_3$100), or they showed a unimodal distribution; ($A_3$50, 2M50, N100, 2M100). Fraction A represents the highest molecular weight and D

Table 6. Monosaccharide distribution (%) before and after weak acid hydrolysis

Fraction	Ara	Rha	Fuc	Xyl	Man	Gal	Glc	GalA	GlcA	Sum
$A_2$50A	15.4	2.4	0.3	2	0.3	13.4	1.3	65	<0.1	100.1
$A_2$50A^H	<0.1	1.9	<0.1	0.9	0.3	4.9	2.6	74.5	<0.1	85.1
$A_2$50B	15.1	2.1	0.3	3.2	0.3	15.8	1.2	61.7	<0.1	99.7
$A_2$50B^H	<0.1	2	<0.1	1.5	<0.1	11	2.1	68.3	<0.1	84.9
$A_2$100B	17	5.7	0.6	1.2	0.2	10.1	1.7	63.6	<0.1	100.1
$A_2$100B^H	<0.1	4.4	<0.1	0.7	0.2	3.4	4.3	70	<0.1	83
$A_2$100C	7.4	5.5	1	1.8	0.4	5.4	3	75.6	<0.1	100.1
$A_2$100C^H	<0.1	1.9	<0.1	<0.1	<0.1	5.4	6.8	78.5	<0.1	92.6

H denotes samples subjected to weak acid hydrolysis.

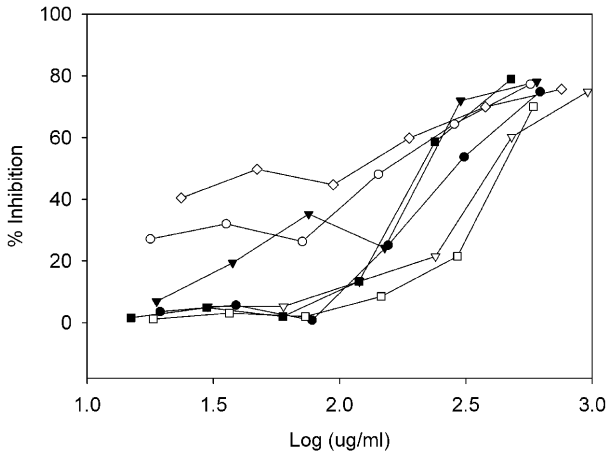

Figure 6. Complement-fixing activity of SEC fractions: $A_2$50A (■), $A_2$50B (□), $A_1$100A (●), $A_1$100B (○), $A_2$100A (▼), $A_2$100B (△), and control PMII (◇).

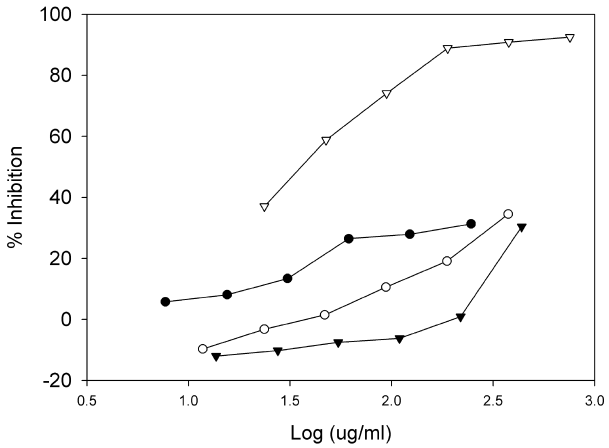

Figure 7. Complement-fixing activity of SEC fractions subject to weak acid hydrolysis; $A_2$50A^H (●), $A_2$50B^H (○), $A_2$100B^H (▼), and positive control PMII (△).

the lowest (Fig. 1). The fractions $A_2$50A and $A_2$50B; $A_1$100A and $A_1$100B; $A_2$100A, $A_2$100B, $A_2$100C and $A_2$100D were the only ones in sufficient yields to be tested further. The earliest eluting fractions in SEC had higher quantities of Ara and Gal than the later eluting fractions (Table 1). Linkage studies of three of the SEC fractions (Table 5), showed similarities to their respective source IEC fractions and complement-fixing activity was thus expectedly approximately the same for SEC fractions (Fig. 6) as for IEC fractions (Fig. 5). Only A and B fractions from SEC were tested due to insufficient amounts of the C and D fractions.

3.7 Weak acid hydrolysis of four SEC fractions

Hydrolysis of SEC fractions ($A_2$50A, $A_2$50B, $A_2$100B, $A_2$100C) markedly reduced complement-fixing activities (Fig. 7) compared with nonhydrolysed fractions (Fig. 6). The treatment resulted in a marked decrease in Gal*p* and an almost complete removal of Ara*f* residues (Table 6). The loss of predominantly t-linked Ara*f* (Table 5) indicated Ara*f* were situated mainly at the outer parts of side chains. However, there was an observed decrease in t- and 1,3,6-linked Gal*p* as well. If all Ara*f* residues were positioned more distant than 1,3,6-linked Gal*p* to the main chain, one would expect a more pronounced increase in 1,3 and 1,6-linkages due to the high amounts of t-linked Ara*f*. Hence, Ara*f* could also be positioned closer to the main chain than Gal*p*, as previously reported for soybean pectin [39]. These findings support earlier findings that complement-fixing activity is located to Ara/Gal rich segments of the pectic material [6]. Weak acid treatment of samples resulted in a small reduction in molecular weight distribution (data not shown), indicating that the side chains affect the hydrodynamic volume to some extent.

4 Concluding remarks

This study showed that polysaccharides extracted from white cabbage by hot water had complement-fixing activity. The major part had a pectic character with a predominant main chain of (1→4)-α-D-Gal*p*A, with a small portion of (1→2)-α-L-Rha*p* and side chains of AGII-like structures linked at C-4 of Rha*p*. Weak acid hydrolysis of some frac-

tions resulted in almost complete removal of Ara*f*, which also greatly decreased the complement-fixing activity. The removal of AGII- and arabinan structures indicated that one of these, or both structural moieties were of key importance for activity. However, from the fractions obtained in this study, it was not possible to elucidate whether it was one or the other, or both holding activity. The results might indicate the general feature that structural elements, often associated with pectins, possess immunological properties, regardless of the source being medicinal plants or common edible plants. Whether processing steps such as boiling, fermentation, or eating will alter these properties and their possible *in vivo* effects need to be elucidated.

This paper is dedicated to Dr. Erland Bråthen, a great collegue who died far too young. We appreciate the technical assistance from Ellen Hanne Cohen at School of Pharmacy, University of Oslo, and Finn Tønnesen for recording GC-MS spectra. In addition we would like to thank Professor Bjørn Erik Christensen at NTNU for running the SEC-MALLS analyses of crude extracts. This research was supported by the Fund for the Research Levy on Agricultural Products.

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