The effect of structure on the dilute solution properties of branched polysaccharides studied with SEC and AsFlFFF

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Auditorium 1041, Viikki, on 16 December 2011, at 12 noon.

Department of Food and Environmental Sciences
Chemistry and Biochemistry / Food Chemistry

Helsinki, Finland 2011

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ISBN 978-952-10-7350-2 (paperback)
ISBN 978-952-10-7351-9 (PDF; http://ethesis.helsinki.fi)
ISSN 0355-1180

Unigrafia
Helsinki 2011

Pitkänen L. 2011. The effect of structure on the dilute solution properties of branched polysaccharides studied with SEC and AsFlFFF (dissertation). EKT-Series 1536. University of Helsinki. Department of Food and Environmental Sciences. 88 pp.

Abstract

Cereal arabinoxylans, guar galactomannans, and dextrans produced by lactic acid bacteria (LAB) are a structurally diverse group of branched polysaccharides with nutritional and industrial functions. In this thesis, the effect of the chemical structure on the dilute solution properties of these polysaccharides was investigated using size-exclusion chromatography (SEC) and asymmetric flow field-flow fractionation (AsFlFFF) with multiple-detection. The chemical structures of arabinoxylans were determined, whereas galactomannan and dextran structures were studied in previous investigations.

Characterization of arabinoxylans revealed differences in the chemical structures of cereal arabinoxylans. Although arabinoxylans from wheat, rye, and barley fiber contained similar amounts of arabinose side units, the substitution pattern of arabinoxylans from different cereals varied. Arabinoxylans from barley husks and commercial low-viscosity wheat arabinoxylan contained a lower number of arabinose side units. Structurally different dextrans were obtained from different LAB. The structural effects on the solution properties could be studied in detail by modifying pure wheat and rye arabinoxylans and guar galactomannan with specific enzymes.

The solution characterization of arabinoxylans, enzymatically modified galactomannans, and dextrans revealed the presence of aggregates in aqueous polysaccharide solutions. In the case of arabinoxylans and dextrans, the comparison of molar mass data from aqueous and organic SEC analyses was essential in confirming aggregation, which could not be observed only from the peak or molar mass distribution shapes obtained with aqueous SEC. The AsFlFFF analyses gave further evidence of aggregation. Comparison of molar mass and intrinsic viscosity data of unmodified and partially debranched guar galactomannan, on the other hand, revealed the aggregation of native galactomannan. The arabinoxylan and galactomannan samples with low or enzymatically extensively decreased side unit content behaved similarly in aqueous solution: lower molar mass samples stayed in solution but formed large aggregates, whereas the water solubility of the higher-molar-mass samples decreased significantly. Due to the restricted solubility of galactomannans in organic solvents, only aqueous galactomannan solutions were studied.

The SEC and AsFlFFF results differed for the wheat arabinoxylan and dextran samples. Column matrix effects and possible differences in the separation parameters are discussed, and a problem related to the non-established relationship between the separation parameters of the two separation techniques is highlighted. This thesis shows that complementary approaches in the solution characterization of chemically heterogeneous polysaccharides are needed to comprehensively investigate macromolecular behavior in solution. These results may also be valuable when characterizing other branched polysaccharides.

Acknowledgements

This study was carried out at the Department of Food and Environmental Sciences, Chemistry and Biochemistry Division, at the University of Helsinki. The work was funded by the University of Helsinki Research Funds, the Glycoscience Graduate School, the Finnish Cultural Foundation, and the Academy of Finland. I am very grateful for financial support of my work. COST action D29 is also thanked for funding the research visit at the Institute of Wood Technology and Wood Biology (vTI) in Hamburg, Germany.

I owe the deepest gratitude to my supervisors, Docent Päivi Tuomainen and Professor Maija Tenkanen. You always encouraged me during these years and your endless enthusiasm for research has motivated my work. I am very grateful for all the time we have spent by discussing and trying to resolve the challenges of polysaccharide characterization. I want to thank you for excellent supervision; you made this thesis possible.

I wish to thank Professor Vieno Piironen for leading me into the world of food chemistry. You got me interested in the chemistry and research.

My warm thanks go to Professor Harry Gruppen and Associate Professor Antje Potthast for the careful pre-examination of my thesis. Your constructive comments and suggestions were of great help.

I am grateful for my co-authors, Docent Liisa Virkki, Dr. Vladimir Aseyev, Dr. Kirsi Mikkonen, Dr. Sami Heikkinen and Ndegwa Maina. I have been fortunate in having a chance to work with such a great group of scientists. Your wide expertise in different fields of chemistry and help in preparing manuscripts have been invaluable.

I wish to express my gratitude to the hemicellulose research group and all my colleagues in D-building. Because of you, the working atmosphere has always been pleasant and supportive.

Finally, I want to thank my friends and my family for your love, support and encouragement.

Helsinki, November 2011

List of original publications

This thesis is based on the following publications, which are referred to in the text by the Roman numerals **I-VI**:

I Pitkänen L, Tuomainen P, Virkki L, Aseyev V, Tenkanen M. 2008. Structural comparison of arabinoxylans from two barley side-stream fractions. J Agric Food Chem 56:5069-77.

II Pitkänen L, Virkki L, Tenkanen M, Tuomainen P. 2009. Comprehensive multidetector HPSEC study on solution properties of cereal arabinoxylans in aqueous and DMSO solutions. Biomacromolecules 10:1962-9.

III Pitkänen L, Tuomainen P, Virkki L, Tenkanen M. 2011. Molecular characterization and solution properties of enzymatically tailored arabinoxylans. Int J Biol Macromol 49:963-9

IV Pitkänen L, Tenkanen M, Tuomainen P. 2011. Behavior of polysaccharide assemblies in field-flow fractionation and size-exclusion chromatography. Anal Bioanal Chem 399:1467-72.

V Pitkänen L, Tuomainen P, Mikkonen KS, Tenkanen M. 2011. The effect of galactose side units and chain length on the macromolecular characteristics of galactomannans. Carbohydr Polym 86:1230-5.

VI Maina NH, Pitkänen L, Heikkinen S, Tuomainen P, Virkki L, Tenkanen M. 2011. Macromolecular characterization of high-molar mass dextrans by size-exclusion chromatography, asymmetric flow field-flow fractionation and diffusion-ordered NMR spectroscopy. Submitted.

Contribution of the author to papers I to VI:

I-V The author planned the study together with the other authors and performed most of the experimental work. She had the main responsibility of interpreting the results and she was the corresponding author of the paper.

VI The author planned the study together with the other authors and performed the SEC and AsFlFFF analyses. She interpreted the SEC and AsFlFFF data and had the main responsibility of writing the SEC and AsFlFFF parts of the manuscript.

Abbreviations

α-L-Ara*f*	α-L-arabinofuranosyl
A_2	second virial coefficient
AsFlFFF	asymmetric flow field-flow fractionation
AXH	arabinoxylan arabinofuranohydrolase
AXH-d3	α-L-arabinofuranosidase (release the (1→3)-linked α-L-Ara*f* unit from disubstituted β-D-Xyl*p* residues)
AXH-m	α-L-arabinofuranosidase (release the (1→2)- and (1→3)-linked α-L-Ara*f* unit from monosubstituted β-D-Xyl*p* residues)
β-D-Xyl*p*	β-D-xylopyranosyl
BFAX	barley fiber arabinoxylan
BHAX	barley husk arabinoxylan
c^*	critical overlap concentration
D	diffusion coefficient
DLS	dynamic light scattering
DP	degree of polymerization
DS	degree of substitution
FFF	field-flow fractionation
GC	gas chromatography
GH	glycoside hydrolase
$[\eta]$	intrinsic viscosity
HDC	hydrodynamic chromatography
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HPSEC	high-performance size-exclusion chromatography
LAB	lactic acid bacteria
LALS	low-angle light scattering
LC	liquid chromatography
L_p	persistence length
LS	light scattering
MALS	multi-angle light scattering
M_n	number-average molar mass
M_w	weight-average molar mass
M_w/M_n	dispersity index
NMR	nuclear magnetic resonance
RAX	rye arabinoxylan
R_g	radius of gyration
R_h	hydrodynamic radius
RI	refractive index
R_T	thermodynamic radius
R_η	viscometric radius
S	sedimentation coefficient
SAXS	small-angle x-ray scattering

SEC	size-exclusion chromatography
SLS	static light scattering
UV	ultra violet
VISC	viscometry/ viscometric detection
WAX	wheat arabinoxylan

Contents

1 Introduction

Arabinoxylans and galactomannans are plant cell wall polysaccharides classified as hemicelluloses due to their close association with cellulose and polyphenol component lignin. Arabinoxylans are abundant especially in cereals and grasses and act together with (1→3)(1→4)-β-D-glucans as a health-promoting dietary fiber component consumed in a daily diet. Galactomannans are used industrially as stabilizers and thickeners in various food and non-food products. Dextrans are α-glucans produced by lactic acid bacteria (LAB). They have many non-food applications, but could also be utilized in food hydrocolloids (AACC 2001; Heinze et al. 2006; O'Donoghue and Somerfield 2009). All of these branched polysaccharides are biopolymers that could be exploited more efficiently in the future. The demand for new, bio-based polymeric materials in various industrial applications, including pharmaceuticals, food, and cosmetics, has increased. To develop new applications, however, the chemical composition and physical properties of biopolymers need to be thoroughly studied.

The solution characterization of structurally heterogeneous polysaccharides is challenging. At present, versatile tools, such as size-exclusion chromatography (SEC) and field-flow fractionation (FFF), are commercially available for (bio)polymer analysis. Both SEC and FFF are elution-based separation techniques invented around half a century ago. These methods, equipped with multiple detectors, are mainly used for estimating the molar mass and size distribution of polymeric samples. In SEC, the development of column packing materials, which can withstand relatively high pressures, led to the modern high-pressure SEC routinely used nowadays (Striegel et al. 2009b). FFF techniques can be divided based on the external field applied as a separation force. Among thermal, sedimentation, and flow fields, flow FFF is the most universal technique. The power of the flow field is described by the inventor of FFF, J. Calvin Giddings (2000) in *Field-flow Fractionation Handbook* (2000): "The universality arises from the fact that displacement by flow (acting as a field) is universal; a moving fluid is capable of displacing every unattached object in its path, from molecules to battleships."

Although various powerful characterization techniques are available, they are mainly developed and used for synthetic polymers. From polysaccharides, cellulose and its derivatives, starch, and glycogen are the mostly studied. Thus, it is essential to investigate the macromolecular characteristics (molar mass, size) and molecular association, and develop methods for polysaccharides that have been less studied.

In the present study, the solution properties of three structurally different polysaccharides, arabinoxylans, galactomannans, and dextrans, were investigated. The literature review of this

thesis presents the main structural features of specific polysaccharides and their potential applications. The focus is on representing the characterization techniques SEC and FFF with multiple-detection. In addition, the existing knowledge on the macromolecular properties of arabinoxylans, galactomannans, and dextrans was reviewed. The experimental part summarizes the data published in the attached papers **I-VI,** in which the effect of the chemical structure on the solution properties of each polysaccharide was studied and the use of different solvents and characterization methods were evaluated. The results are collectively discussed based on the experimental observations.

2 Literature review

2.1 Cereal arabinoxylans

Arabinoxylans and (1→3)(1→4)-β-D-glucans are the two major non-starch polysaccharides in cereals. These polysaccharides are present in the cell walls of plant tissues. Arabinoxylans consist of a (1→4)-linked β-D-xylopyranosyl (β-D-Xyl*p*) backbone with α-L-arabinofuranosyl (α-L-Ara*f*) substituents attached at position *O*-2, *O*-3, or both (Figure 1). In addition to α-L-Ara*f*, arabinoxylans may contain some 4-*O*-methylglucopyranosyluronic acid, acetyl and feruloyl substituents, and disaccharide substituents in which the α-L-Ara*f* unit is further substituted at position *O*-2 of the β-D-Xyl*p* residue. The amount and degree of substitution of arabinoxylans vary according to the cereal species and the part of the plant (Aspinall 1959; Aspinall 1980). In general, arabinoxylans of the kernel are more substituted than husk and straw arabinoxylans. Table 1 summarizes the arabinoxylan contents and arabinose-to-xylose ratios of the most common Finnish cereals: wheat, barley, rye, and oat.

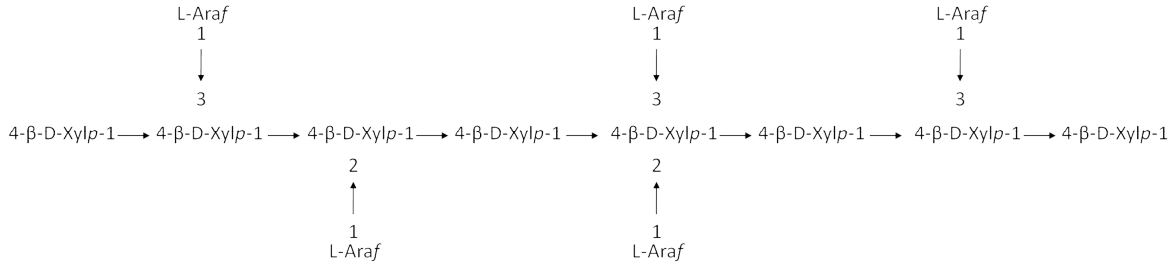


Figure 1. Schematic presentation of cereal arabinoxylan structure. Most commonly, β-D-Xyl*p* units are unsubstituted or carry an α-L-Ara*f* substituent at position *O*-3, *O*-2, or *O*-2 and *O*-3.

Table 1. Arabinoxylan content and arabinose-to-xylose ratios (Ara/Xyl) of various cereal materials.

Arabinoxylan source	Arabinoxylan content (weight %)	Ara/Xyl	Reference
Barley (whole grain)	6.6	0.42	Henry 1987
Barley (endosperm)	1.4	0.67	Henry 1987
Barley (aleurone cells)	85	0.58	McNeil et al. 1975
Barley (husks)	46	0.22	Höije et al. 2005
Oat (endosperm)	0.7	0.79	Henry 1987
Oat (bran)	5	0.86	Westerlund et al. 1993
Oat (spelt)	35-40	0.09-0.17	Saake et al. 2004; Pastell et al. 2009; Hettrich et al. 2006
Rye (whole grain)	7-12	0.56-0.68	Saastamoinen et al. 1989; Bengtsson et al. 1992b; Vinkx and Delcour 1996
Rye (endosperm)	4	0.56-0.60	Henry 1987
Rye (bran)	9	0.10-0.30	Hromádková and Ebringerová 1987
Wheat (whole grain)	6.6	0.45-0.97	Henry 1987; Izydorczyk and Biliaderis 1993
Wheat (endosperm)	2.3	0.56	Henry 1987
Wheat (bran)	6.5	0.57-1.07	Shiiba et al. 1993
Wheat (straw)	33	0.12-0.23	Lawther et al. 1995; Sun et al. 1996

In addition to β-D-Xyl*p* and α-L-Ara*f* contents, the substitution pattern also varies between cereal species. For instance, the arabinoxylans from wheat flour contain more disubstituted than monosubstituted β-D-Xyl*p* residues, whereas in rye flour the situation is reversed (Ordaz-Ortiz and Saulnier 2005; Ragaee et al. 2001). In water-extractable wheat arabinoxylan, 60-65% of the β-D-Xyl*p* groups are unsubstituted, 12-20% are monosubstituted, and 15-30% are disubstituted (Ordaz-Ortiz and Saulnier 2005). In rye, 23-34% of the β-D-Xyl*p* groups are unsubstituted, 49-59% are monosubstituted, and 15-28% disubstituted (Ragaee et al. 2001). Most of the monosubstituted β-D-Xyl*p* residues carry the α-L-Ara*f* unit at the position of *O*-3, but a minor proportion of (1→2)-linked α-L-Ara*f* has been reported to exist in rye arabinoxylan (Vinkx et al. 1995). The contents of monosubstituted and disubstituted β-D-Xyl*p* residues are almost equal in alkali-solubilized arabinoxylans from hull-less barley flours in which 48.5% of the xylose units are substituted, 47% of these with *O*-2 and *O*-3 disubstituted β-D-Xyl*p* residues, 25% with *O*-3 monosubstituted β-D-Xyl*p* residues, and 28% with *O*-2 monosubstituted β-D-Xyl*p* residues (Trogh et al. 2005).

The distribution of α-L-Ara*f* units along arabinoxylan chains is not necessarily random, and thus arabinoxylans may be heterogeneous in their chemical structure. Bengtsson et al. (1992a) stated that arabinoxylan from rye grain contains two structurally different molecular

populations; the main fraction consists of xylan chains with monosubstituted β-D-Xyl*p* residues and a minor, heavily substituted fraction with mainly disubstituted β-D-Xyl*p* units. Thus, it should be noted that characteristic values, such as arabinose-to-xylose ratios, are average numbers and might not always give a realistic idea of the actual structures.

2.2 Galactomannans

Galactomannans are polysaccharides existing in the endosperm of certain leguminous seeds. Additionally, some yeast and fungi produce galactomannans (Dea and Morrison 1975). Seed galactomannans are composed of a β-(1→4)-linked D-mannopyranosyl (β-D-Man*p*) backbone that is substituted with α-(1→6)-linked D-galactopyranosyl (α-D-Gal*p*) residues (Smith 1948) (Figure 2).

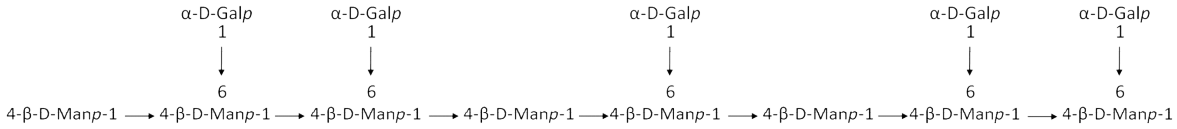


Figure 2. Schematic presentation of the seed galactomannan structure.

The most commercially important galactomannas include guar gum from guar seeds and locust bean gum from carob seeds. The galactose-to-mannose ratio for guar galactomannan ranges from 0.50 to 0.73, while the locust bean gum is known to have a wider range of the galactose-to-mannose ratios from 0.19 to 0.83 (Dea and Morrison 1975). The distribution of α-D-Gal*p* along the β-D-Man*p* backbone has been under debate, but Daas et al.'s (2000) results suggest that in guar galactomannan the distribution is blockwise whereas locust bean gum may contain random, blockwise, and ordered distributions.

2.3 Dextrans

Dextrans are exopolysaccharides produced by LAB, and they are synthesized from sucrose extracellularly by glucansucrases (Monchois et al. 1999). The most common dextrans (class 1) are α-glucans consisting of mainly α-(1→6)-linked D-glucopyranosyl (α-D-Glc*p*) units with varying amounts of α-(1→2), α-(1→3), and α-(1→4) branched linkages (Robyt 1986). Thus, dextrans are homopolysaccharides consisting only of α-D-Glc*p* units (Figure 3). Other

types of microbial α-glucans include alternans (class 2) and mutans (class 3). Alternans consist of an α-D-Glc*p* backbone with alternating α-(1→3) and α-(1→6) linkages and α-(1→3) linkages as branching points. Mutans have an α-(1→3) linked backbone with α-(1→6) branched linkages. The most important LAB genera that produce dextrans are *Leuconostoc*, *Lactobasillus*, *Weissella*, and *Streptococcus*. The most industrially important is the dextran from *Leuconostoc mesenteroides* B-512F, which is also the most studied dextran (Monsan et al. 2001).

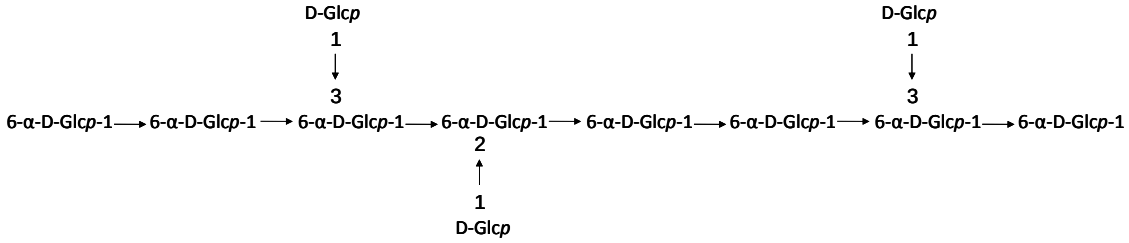


Figure 3. Schematic presentation of the class 1 dextran structure. Additional α-(1→4) branched linkages may exist. At least some of the α-(1→3) branches are elongated.

Although the exact structural features of dextrans are not known, various studies indicate that at least some of the (1→3) branched linkages attached to the main chain are elongated (Ioan et al. 2000; Maina et al. 2011), whereas the (1→2)-linked branches are terminal (Maina et al. 2008). The number of branching points varies among different dextrans. Thus, dextrans can be nearly linear or branched with various extents. The proportion of α-(1→6) linkages from all glycosidic linkages has been reported to vary between 50% and 97% (Jeanes et al. 1954). The cultivation conditions as well as the producing strain affect the branching of dextrans (Côté and Leathers, 2005). The amounts of each glycosidic linkage for dextrans from different LAB are presented in Table 2.

Table 2. The proportions of branched linkages (%) for class 1 dextrans from different microbial origins.

Dextran	**α-(1→6) %**	**α-(1→3) %**	**α-(1→2) %**	**Reference**
Leuconostoc mesenteroides B-512F	95	5		Monsan et al. 2001
Leuconostoc mesenteroides B-1299	66	7	27	Monsan et al. 2001
Leuconostoc mesenteroides B-1396	86	4	10	Seymour et al. 1979
Leuconostoc citreum E497	85.5	3.5	11	Maina et al. 2008
Weissella confusa E392	97.3	2.7		Maina et al. 2008

2.4 Industrial applicability of arabinoxylans, galactomannans, and dextrans

When arabinoxylans, galactomannans, and dextrans are compared in terms of industrial applicability, galactomannans and dextrans are used in various applications, whereas arabinoxylans are much less utilized. Although isolated arabinoxylans as such are not used industrially, their role as a health-promoting dietary fiber component in the daily diet is well-known and widely studied (Lu et al. 2000; AACC 2001; Collins et al. 2010; Zhang and Hamaker 2010). An arabinoxylan-rich diet has been shown to decrease the postprandial glucose response compared with a control meal without arabinoxylan fiber (Lu et al. 2000). The exploitation of underused hemicellulose resources, including arabinoxylans, has attracted more interest especially in the field of biodegradable packaging.

The potential of wheat and rye arabinoxylans in film formation has been studied in recent investigations (Sternemalm et al. 2008; Höije et al. 2008; Zhang et al. 2011). Höije et al. (2008) modified arabinoxylans from rye enzymatically to produce a sample set with different arabinose side unit content and used the tailored samples for film formation studies. The chemical structure of arabinoxylans was found to affect film properties. In addition to the brittleness of polysaccharide films, the significant challenge in developing arabinoxylan-based films is their moisture sensitivity and relatively poor barrier properties against water vapor (Mikkonen et al. 2009a; Mikkonen et al. 2010).

Galactomannans are used widely as thickeners and stabilizers in various food products such as dairy products, ice cream, desserts, and bakery items (O'Donoghue and Somerfield 2009). Because most galactomannans are edible, they are especially suited for food purposes (Dea and Morrison 1975). The E number (European Union number code for food additives) for guar galactomannan is E 412 and for locust bean gum E 410. Although gum arabic is the most well-known emulsion stabilizer, guar galactomannan and locust bean gum are also used as industrial stabilizers (Wu et al. 2009). Mikkonen et al. (2009b) recently studied the effect of the galactomannan structure on the stabilizing properties of oil-in-water beverage emulsions and found that adding galactomannans with high and on the other hand low degrees of polymerization (DP) formed more stable beverage emulsions. The stabilizing effect of galactomannans with intermediate chain length was poorer. Galactomannan from guar has also been used for film formation studies. The DP and degree of substitution (DS) of guar galactomannan was modified enzymatically to investigate the effect of galactomannan structure on film properties (Mikkonen et al. 2007). As in the case of arabinoxylans, the structure affected film formation.

Dextrans have various food and non-food applications. In the food industry, dextrans are used as gelling agents, thickeners, and emulsion stabilizers. Dextrans have also been used in cosmetic products, as blood-plasma substitutes (Grönwall and Ingelman 1948), and standards in size-exclusion chromatography (Heinze et al. 2006). *In situ* production of dextrans in sour dough baking has been recently studied (Lacaze et al. 2007; Katina et al. 2009). The production of dextrans during LAB fermentation improves the quality of bread without food additives.

2.5 Specific enzymatic modification of polysaccharides

The structural diversity of naturally occurring polysaccharides, which could be exploited more efficiently, is extensive. Instead of extracting structurally different polysaccharides from plants, specific enzymatic modifications can be used to produce molecules with varying chemical structure.

Branched polysaccharides can be modified with endoglycanases that hydrolyze the backbone and accessory enzymes acting on the branches. Endo-(1→4)-β-D-xylanases (EC 3.2.1.8) belong to the glycoside hydrolases (GH), which hydrolyze the bond between (1→4)-linked β-D-Xyl*p* units in the middle of the arabinoxylan chain (CAZy - Carbohydrate Active enZymes; Enzyme Commission (EC) numbers). The catalytic property of xylanase depends on the GH family. GH10 xylanases (e.g., commercial Shearzyme preparation) are able to hydrolyze the xylan chain near the α-L-Ara*f* substituent and thus produce short oligosaccharides (Biely et al. 1997; Rantanen et al. 2007). The DP of the hydrolysis products depends, however, on the enzyme dosage and hydrolysis time. GH 10 xylanases can also be used for mild reduction of DP and thus production of polymeric arabinoxylans with reduced chain length (Mikkonen et al. 2011). α-L-arabinofuranosidases (EC 3.2.1.55) hydrolyze the terminal α-L-Ara*f* units from arabinoxylans or arabinoxylooligosaccharides. The enzymes acting on polymeric arabinoxylan (arabinoxylan arabinofuranohydrolase, AXH) are divided into two groups based on their substrate specificities. AXH-m acts on the (1→2)- and (1→3)-linked α-L-Ara*f* substituents in monosubstituted β-D-Xyl*p* residues, whereas AXH-d3 hydrolyzes the (1→3)-linked α-L-Ara*f* units that are attached in disubstituted β-D-Xyl*p* residues (van Laere et al. 1997) (Figure 4). These α-L-arabinofuranosidases enable the specific tailoring of arabinoxylan structures. The other enzymes acting on arabinoxylan substituents include α-1,2-glucuronidases (EC 3.2.1.131), acetyl xylan esterases (EC 3.1.1.72), and feruloyl esterases (3.1.1.73) (Borneman et al. 1992; Enzyme Commission (EC) numbers; Tenkanen 2004).

Galactomannan chains can be similarly modified with endo-(1→4)-β-D-mannanase (EC 3.2.1.78) and α-D-galactosidase (EC 3.2.1.22). Endo-(1→4)-β-D-mannanase cleaves bonds in the middle of the (1→4)-linked β-D-Man*p* chain, and α-D-galactosidase (EC 3.2.1.22) acts on the terminal α-D-Gal*p* units (Tenkanen 2004). These enzymes have been successfully used to specifically tailor guar galactomannans (Mikkonen et al. 2007; Hannuksela et al. 2002).

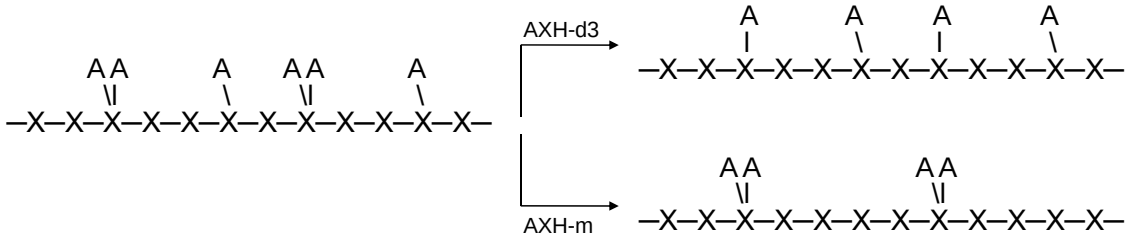


Figure 4. The actions of α-L-arabinofuranosidases AXH-d3 and AXH-m on wheat arabinoxylan. A = α-L-Ara*f*, X = β-D-Xyl*p*, \ = 1→3 bond, ׀ = 1→2 bond.

2.6 Chemical characterization of polysaccharides

Information on the chemical structures of polysaccharides can be obtained by degrading polymeric samples either completely or partially and further analyzing the building blocks using various chromatographic and spectroscopic methods. For the monosaccharide composition analysis, the polysaccharide sample is degraded to monosaccharides using acid or enzymatic hydrolysis or methanolysis, and the monosaccharides formed are separated and identified with gas chromatography (GC) or liquid chromatography (LC) methods. In acid hydrolysis, commonly used acids include sulfuric acid and trifluoroacetic acid (Brummer and Cui, 2005). Methanolysis is also applicable for acidic polysaccharides. In methanolysis, the samples are degraded with hydrochloric acid in anhydrous methanol (Sundberg et al. 1996). Enzymatic hydrolysis is a gentle method, but requires a mixture of enzymes acting on all different types of linkages of a polysaccharide (Virkki et al. 2008).

Analysis of oligosaccharides from partially degraded polysaccharides, either with acid or with enzymes, provides more information on the fine structure. Oligo- and monosaccharides can be analyzed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Lee 1990, 1996) or with mass spectrometry (Reinhold et al. 1995). Identifying and quantifying oligosaccharides is complicated by the lack of commercial oligosaccharide standards, and thus standards prepared in-house are often used. Linkages between monosaccharide units can be quantitatively determined with NMR spectroscopy

from the pure oligosaccharides or directly from the polysaccharides. The other common method for linkage analysis of polysaccharides is methylation (Ciucanu and Kerek 1984).

2.7 Methods for characterizing dilute polymer solutions

This chapter concentrates on the characterization methods used for dilute polymer, especially polysaccharide, solutions. Due to the broadly polydisperse nature of polysaccharides, methods including separation are emphasized. A dilute polymer solution is a solution with a concentration below the critical overlap concentration c^*. In concentrations above c^*, polymer chains begin to interact with each other. c^* depends on the molecular architecture and can be defined for random coil polymers based on the second virial coefficient A_2 and weight-average molar mass M_w with the following equation (Burchard 1999):

$$c^* = \frac{1}{A_2 M_w} \qquad (1)$$

The simpler approximation for c^* is based on the intrinsic viscosity $[\eta]$:

$$c^* = \frac{1}{[\eta]} \qquad (2)$$

Several methods have been employed to characterize polysaccharide solutions, including ultracentrifugation, capillary viscometry, off-line static and dynamic light scattering, and size-exclusion chromatography (SEC) and field-flow fractionation (FFF) with various detection techniques (Wang and Cui 2005). The typical methods used to characterize dilute polysaccharide solutions are collected in Table 3. The following sections discuss the principles of multi-detection SEC and FFF.

Table 3. Typical methods for characterizing dilute polysaccharide solutions.

Method	Parameters obtained	Other information
Static light scattering (SLS)	M_w, R_g, A_2, R_T	R_g can be obtained without calibration
Dynamic light scattering (DLS)	R_h, D	
Viscometry	$[\eta]$	Molar mass via Mark-Houwink relationship
Membrane osmometry	M_n	
Ultracentrifugation (sedimentation)	M_w, S	
Small-angle x-ray scattering (SAXS)	M_w	
End-group analysis	M_n	
SEC with RI/(MA)LS/VISC/DLS detection	M_w, M_n, R_g, R_h, R_η, $[\eta]$	Distribution for mass, radii, and intrinsic viscosity M_w and M_n can also be obtained with conventional calibration
AsFlFFF with RI/(MA)LS/DLS detection	M_w, M_n, R_g, R_h, R_η, D	Distribution for mass, radii R_h also from the retention time M_w and M_n can also be obtained with conventional calibration

RI = refractive index, MALS = multi-angle light scattering, VISC = viscometry, AsFlFFF = asymmetric flow field-flow fractionation, M_w = weight-average molar mass, M_n = number-average molar mass, R_g = radius of gyration, D = diffusion coefficient, S = sedimentation coefficient, R_h = hydrodynamic radius, R_η = viscometric radius, R_T = thermodynamic radius, A_2 = second virial coefficient, $[\eta]$ = intrinsic viscosity. Definitions for different molar mass averages, radii, etc. can be found in Burchard's (1999) review. The table was created using the following references: Burchard 1999; Wang and Cui 2005.

2.7.1 SEC with multiple-detection

SEC is an elution-based method for separating macromolecules according to their size, or more precisely, volume, in solution. Modern high-performance SEC (HPSEC) columns are packed with small and rigid porous particles that can withstand relatively high pressures. The SEC columns may contain particles of a certain size or particles of various sizes (mixed-bed columns with better calibration linearity). The larger molecules elute before the smaller molecules because they have less penetration into the pores of column packing material (Figure 5). Traditionally, SEC has been used to prepare fractions, estimate molar mass, and determine the association constant for macromolecules from the biological origin. Obtaining molar mass distribution is the most significant advantage of SEC compared with molar mass determination techniques without separation. As mentioned, the analytes separate in SEC according to their size. The definition of "size" is not, however, unambiguous because of the conformational variety of macromolecules. Thus, a straightforward relationship between the size and molar mass cannot be obtained. This should be taken into account when calibrating the columns for molar mass determination (Striegel et al. 2009b).

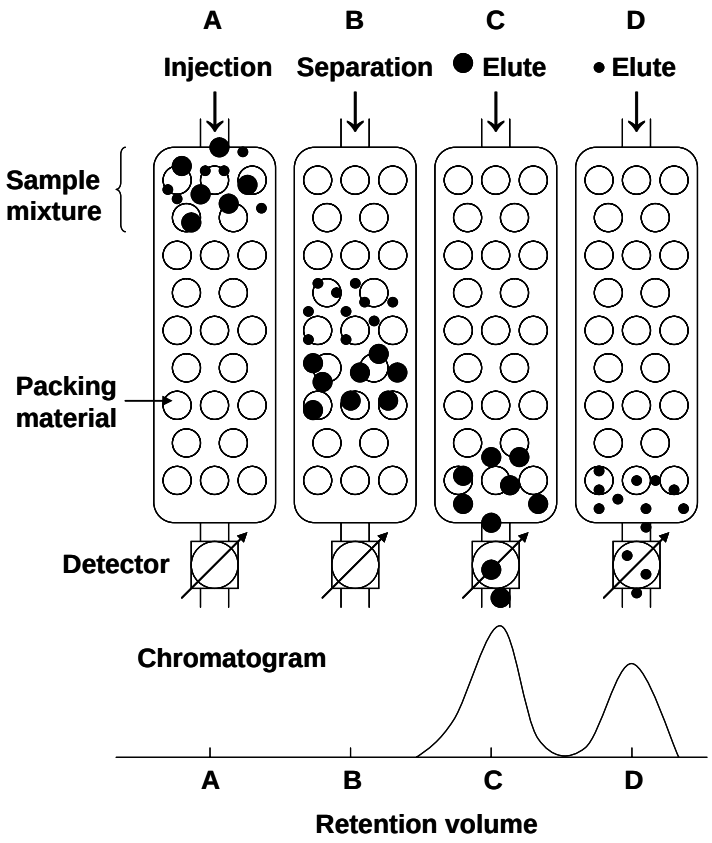


Figure 5. Separation of analytes in SEC based on size in solution.

In addition to conventional calibration of SEC columns with molar mass standards, molar mass distribution of a sample can be obtained with static light-scattering (LS) and/or viscometric detection (VISC) coupled to the SEC system. For absolute molar mass determination with LS or LS/VISC detection, a concentration sensitive detector (for polysaccharides, commonly a refractive index, RI) is needed to calculate the concentration in each elution point. The scattered light from a dilute polymer solution at the given angle θ is related to the M_w as follows (basic light scattering equation, Kratochvíl 1987):

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad (3)$$

$$K = \left(\frac{4\pi^2 n_0^2}{\lambda_0^4 N_A}\right)\left(\frac{dn}{dc}\right)^2 \qquad (4)$$

In equations 3 and 4, c is the solution concentration, R_θ is the intensity of light scattered at the angle θ, A_2 is the second osmotic virial coefficient, K is the optical constant, n_0 is the refractive index of the solvent, dn/dc is the refractive index increment, and λ_0 is the

wavelength of the incident light. At infinite dilution and zero angle, the angular dependence of the scattered light $P(\theta) = R_\theta/R_{\theta=0} = 1$ and Equation 3 yields M_w.

Various LS techniques have been coupled to SEC. When multi-angle light scattering (MALS) detection is used, the scattered light is measured with several angles, and the data is extrapolated to zero angle to yield M_w. Using MALS, R_g is obtained from the angular dependence, because the scattered light depends on not only molar mass but also molecular size at angles other than zero. If low-angle light scattering (LALS) detection is used, M_w can be calculated using a single angle accurately when the effect of angular dependence is minimized. In commercial LALS instruments, an angle of 7° is used (Viscotek 2004). The LS/VISC method, which is also called the triple-detector method (SEC^3), includes LS detection with a single angle, differential viscometry, and refractive index detection. In this method, an estimate of M_w is determined using a basic light-scattering equation assuming that $P(\theta) = 1$ and $A_2 = 0$. M_w and R_g are then calculated using Flory-Fox and Ptitsyn-Eizner relations, in which $[\eta]$ is employed to estimate R_g. The equations for the LS/VISC method and comparison of LS/VISC and MALS approaches for characterizing corn arabinoxylans were presented by Fishman et al. (2000).

The advantage of coupling the viscometer to SEC in addition to LS and the RI is the direct structural information obtained in the form of $[\eta]$ (Table 4) and the viscometric radius (R_η). R_η can be calculated from the intrinsic viscosity using the following equation (Burchard 1999):

$$R_\eta \equiv \left(\frac{3[\eta]M}{10\pi N_A}\right)^{\frac{1}{3}} \qquad (5)$$

R_η is reported to give an approximation from the hydrodynamic radius (R_h), which is obtained with dynamic light scattering (DLS) (Mourey 2004), and in many contexts, these two radii are used interchangeably. If viscometer and RI detectors are used without LS detection, the molar mass for each elution slice can be calculated from $[\eta]$ using the Mark-Houwink relation (Burchard 1999):

$$[\eta] = kM^{\alpha} \qquad (6)$$

In the equation, k and α are empirical constants that depend on the type of polymer, solvent, and temperature. For polysaccharides, k and α mainly depend on the geometry of interresidue linkages. The exponent α is usually in the range of 0.5 to 0.8 for linear random coil polysaccharides. For polysaccharides with extended solution conformation, the value for α

can be higher. Solvent quality (good, theta, poor) and intramolecular interactions also affect the α value. For instance, in the case of the formation of compact aggregates, the α value is low. In general, low α values indicate more compact conformation, whereas high values more extended conformation. The values for *k* follow the similar trend with the α value (Wang and Cui 2005).

Table 4. Effect of polymer structure on the intrinsic viscosity.

Structural or conformational change	**Effect on density**	**Effect on [η]**	**References on polysaccharides**
Increase the chain length of the linear molecule	Decreases	Increases according Mark-Houwink equation	Ioan et al. 2000
Increase the length of branches	Increases	Decreases	Ioan et al. 2000
Increase the stiffness of chains	Decreases	Increases	
Increase in branching density	Increases	Decreases	Ioan et al. 2000
Collapse of chain conformation	Increases	Decreases	
Formation of compact aggregates	Increases	Decreases	Dhami et al. 1996
Formation of large aggregates (microgel)	Decreases	Increases	

Although SEC, especially when coupled with multiple detectors, is a powerful tool for bio(polymer) characterization, separation in SEC is based on size or according to the theory of universal calibration hydrodynamic volume V_h (Grubisic et al. 1996; Hamielec and Ouano 1978). An exact theory for SEC separation is not known. Because of the separation based on the volume and not molar mass, coelution of molecules with the same volume but different molar mass may occur. This coelution of molecules with different mass is probable in the case of branched polymers, because the assumption of universal calibration is valid only for linear molecules. When the branching density or the length of the branches is increased, the molecule becomes more compact (Table 4). The structural heterogeneity of branched polymers and polysaccharides may then cause errors in the molar mass distribution obtained with SEC. The problem of "imperfect resolution" in SEC has been reported for various molecules with long-chain branching, such as poly(vinyl acetate), polyacrylates, and starch (Hamielec et al. 1978; Gaborieau et al. 2008; Gidley et al. 2010).

2.7.2 AsFlFFF with multiple-detection

Field-flow fractionation (FFF) is an elution-based technique for separating macromolecules, colloids, and particles. FFF techniques can be divided based on the separation field, and three different types for FFF are currently commercially available: thermal field-flow fractionation, sedimentation field-flow fractionation, and flow field-flow fractionation. Among these, flow field-flow fractionation, especially asymmetric flow field-flow fractionation (AsFlFFF), is the separation method most commonly used for macromolecules and polymers (Giddings 2000; Roessner and Kulicke 1994).

In AsFlFFF, the separation occurs in the thin channel, under laminar flow conditions with a parabolic flow profile. The flow velocity is the highest at the center and the slowest at the walls. When transported to the channel, polymers/particles arrange themselves in different mean layer thicknesses from the channel bottom (also called the accumulation wall) and elute from the channel with different velocities. An external cross-flow, which is perpendicular to the separation axis, is transported to the channel to contribute to the separation of analytes. The sample components that interact more strongly with the cross-flow and/or have a lower diffusion coefficient elute later than the components with less interaction with the cross-flow and/or higher diffusion coefficient. In general, smaller molecules have a higher diffusion coefficient than larger ones and thus elute before large molecules (Figure 6). Therefore, the elution order is reversed compared to that in SEC (Wahlund 2000; Williams and Lee 2006).

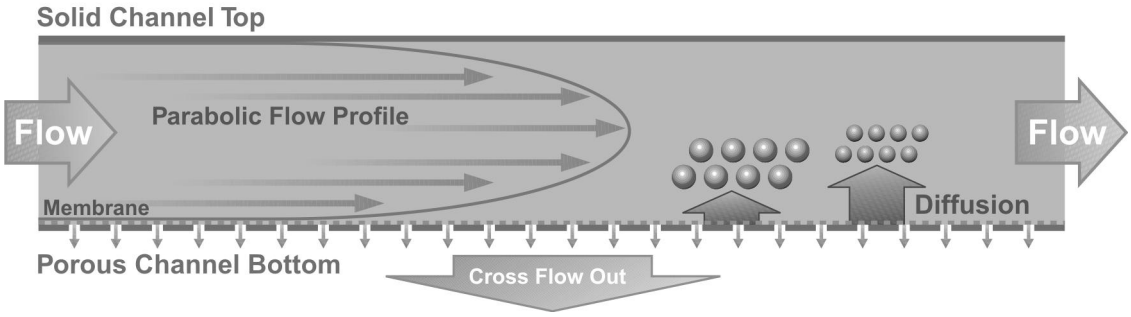


Figure 6. Separation principle and channel construction of asymmetric field-flow fractionation (AsFlFFF). The figure is presented courtesy of Postnova Analytics.

The AsFlFFF channel consists of a bottom block with a porous frit inset, a membrane and a spacer on top of the frit, and a top plate with flow outputs. The spacer defines the channel thickness and is often a trapezoid so that the channel breadth decreases toward the outlet.

Membranes with different materials and cut-off values (300-100 kDa) are available; regenerated cellulose is used most often. The other commonly used materials include polyethersulfone, ceramic, and polyvinylidene difluoride. Due to the channel design with a porous frit, undesired small molecules can be purposely removed by selecting the appropriate membrane cut-off that lets the small material go through but retains the analytes. On the other hand, because of the open channel assembly, sample material may be lost that affects the representativeness of the results (Otte et al. 2010).

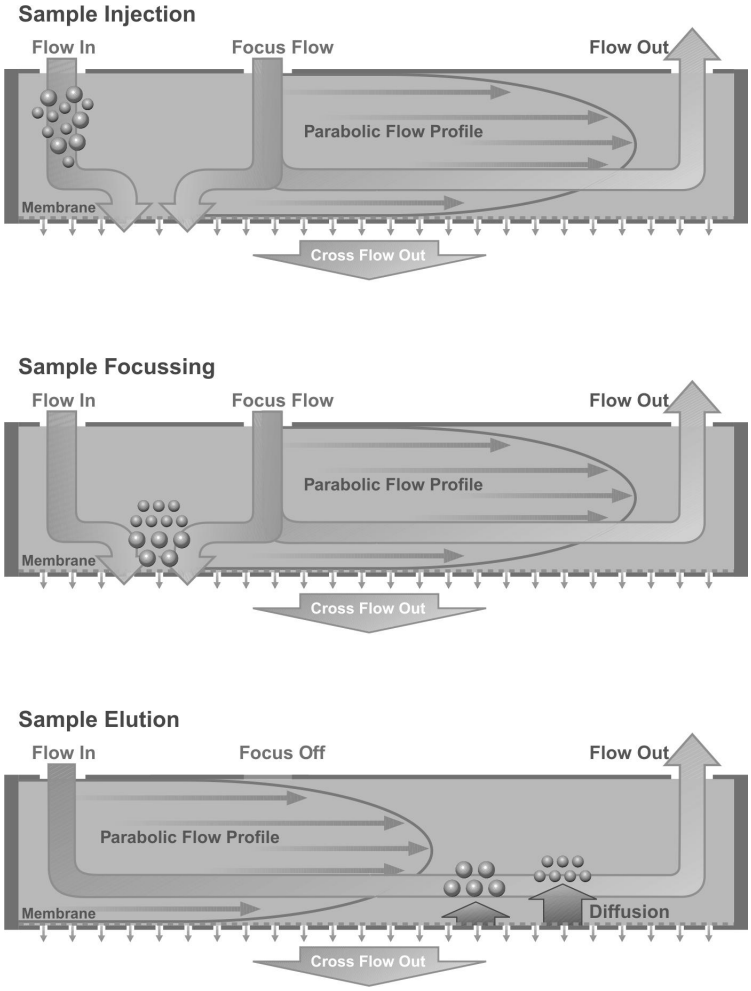


Figure 7. Sample injection, focusing, and elution in AsFlFFF. The figure is presented courtesy of Postnova Analytics.

The AsFlFFF analysis can be divided in the three steps presented in Figure 7. First, the sample molecules are injected into the system and transported to the channel by injector flow. The focusing flow should be initiated before injection. Relatively low flow rates (0.1-0.2

ml/min) should be used for injection to avoid disturbing the focusing flow. Sample focusing is an important step to relax the sample, to narrow the width of the sample zone, and to prevent band broadening. The focus flow is pumped into the channel in the direction of the channel outlet (Wahlund 2000). In the elution step, the focus flow is switched off to allow the analytes to elute. Separation can be enhanced by the cross-flow gradients. The cross-flow goes through the membrane and frit as seen in Figures 6 and 7.

In AsFlFFF, the size (R_h) of the analyte can be calculated directly from the retention time (Litzen 1993). The Stokes-Einstein yields the diffusion coefficient (D) of a molecule:

$$D = \frac{kT}{6\pi\eta_0 R_h} \qquad (7)$$

In the equation, k is the Boltzmann constant, T the temperature, and η_0 the viscosity of a solvent. The diffusion coefficients in AsFlFFF can be estimated from the retention time (t_r) with the following equation:

$$D = \frac{t^0 w^2 \dot{V}_c}{6 t_r V^0} \ , \qquad (8)$$

where t^0 is the void time, w is the channel thickness, $\dot{V}_c$ is the volumetric cross-flow rate (channel inlet flow minus channel outlet flow), and V_0 is the void volume of the channel. By combining equations 7 and 8, R_h can be calculated:

$$R_h = \frac{kTV_0 t_r}{\pi\eta w^2 t^0 \dot{V}_c} \qquad (9)$$

The above-mentioned equations for R_h calculation are valid only when the cross-flow is constant. R_h can also be calculated when decaying cross-flow is used, but it is more difficult as the flow-field decreases with time (Nilsson et al. 2006).

In AsFlFFF, as well as in SEC, the on-line coupling of the instrument with MALS and RI detectors yields the distributions for molar mass and R_g (Roessner and Kulicke 1994). Viscometry is not commonly used as a detection method with AsFlFFF, due to the high sensitivity of the differential viscometer to the pressure changes caused by the flow gradients. Additionally, other detectors, such as, DLS, UV, or fluorescence, can be coupled with AsFlFFF as with SEC.

Because separation in AsFlFFF occurs in an open channel, the method is applicable for the broad range of macromolecules with M_w of 500-10^{12} g/mol and particle size of 1 nm-100 µm (Giddings 1993). Thus, the AsFlFFF technique has been employed for various biopolymers and fragile bioparticles, such as proteins, protein aggregates, cells, and virus particles (Yohannes et al. 2011). AsFlFFF has also been used for (high-molar mass) polysaccharides. The utilization of AsFlFFF has been focused on characterizing industrially important polysaccharides, such as starch (Roger et al. 2001; van Bruijnsvoort et al. 2001; Rolland-Sabate et al. 2007; Rolland-Sabate et al. 2011), pullulan (Wittgren and Wahlund 1997), and dextran (Roessner and Kulicke 1994; Wittgren and Wahlund 1997).

2.8 Macromolecular characteristics of branched polysaccharides

Arabinoxylans and galactomannans are structurally similar molecules: they consist of a linear backbone substituted mainly with side units of only one monosaccharide residue length. Some dextrans, on the other hand, are known to have more elongated side chains. This structural difference between arabinoxylans and galactomannans containing short-chain branches and dextrans with long-chain branches dictates largely the solution behavior of branched polysaccharides.

2.8.1 Arabinoxylans and galactomannans with short-chain branching

Cereal arabinoxylans

The conformation and flexibility of arabinoxylans in aqueous solution have been investigated using several different experimental approaches, and the results reported over the past five decades are partly contradictory. In 1969, Cole suggested that arabinoxylans assume random coil conformation that is somewhat rigid due to the arabinose side chains. According to Andrewartha et al. (1979), arabinoxylans adopt a more extended rigid rod conformation in solution. The later investigations of Dervilly-Pinel et al. (2001a) suggest that arabinoxylans assume a semi-flexible conformation. These conclusions are mainly based on the chain persistence length data (persistence length L_p for arabinoxylans 6.9-9.6 nm) calculated from the SEC results. Picout and Ross-Murphy (2002) analyzed Dervilly-Pinel et al.'s (2001a) data using an alternative model for calculating L_p and obtained results that are in the range of 3-5 nm. Lower values for L_p appear more feasible because early modeling studies on arabinoxylan conformation indicate that interactions higher than the second neighbor xylose unit in the xylan backbone are negligible (Sundararajan and Rao, 1969). Regardless of the variation in

the L_p values obtained for arabinoxylans in different studies, arabinoxylans can be considered fairly flexible, random coil polysaccharides. Flexibility as such is not an unambiguous measure; in general, for flexible molecules L_p is lower than for rigid molecules.

Although molar mass and size determinations for various arabinoxylans from different cereal fractions have been accomplished, only in a very few studies has SEC with multiple detection been employed (Table 5). For many studies on barley, wheat, and rye arabinoxylans, the molar mass estimate has been obtained with relative calibration using pullulan (Trogh et al. 2005; Annison et al. 1992; Izydorczyk et al. 1998a; Izydorczyk et al. 1998b; Nilsson et al. 2000) or dextran (Gruppen et al. 1993; Hughes et al. 2007) standards. Because arabinoxylans are regarded as water-soluble molecules, in most studies aqueous (salt) solutions have been used for dissolution and eluent. As seen in Table 5, generally the molar mass for the outer part of the grain (husks, spelts) is lower than for the inner parts. Molar mass distribution for arabinoxylans is usually broad (M_w/M_n, dispersity index ≥ 1.5).

Table 5. Macromolecular characteristics of cereal arabinoxylans obtained by SEC with multiple detection (LS/VISC/RI).

Arabinoxylan source and Ara/Xyl ratio	$M_w \times 10^{-5}$ **(g/mol)**	M_w/M_n	$[\eta]$ **(ml/g)**	R_g **(nm)**	**Reference**
Barley (flour) 0.62	1.77[a]	2.0	392	26	Dervilly-Pinel et al. 2001b
Barley (husks) 0.22	0.36[b]	2.4	48		Höije et al. 2005
Oat (spelt) 0.13	0.23[b]	1.8	58		Saake et al. 2001
Rye (flour) 0.52	2.56[a]	1.5	998	42	Dervilly-Pinel et al. 2001b
Wheat (flour) 0.63	3.00[a]	1.65	530	44	Dervilly et al. 2000
Wheat (bran) 0.77	3.90[c]	1.7			Bergmans et al. 1996

M_w = weight-average molar mass, M_w/M_n = dispersity index, $[\eta]$ = intrinsic viscosity, R_g = radius of gyration
For the table, the unfractionated and/or purest arabinoxylan sample is selected from the references.
[a]Analyzed in H_2O + 0.05 M $NaNO_3$
[b]Analyzed in DMSO:H_2O (90:10)
[c]Analyzed in 0.4 M NaOAc buffer, pH 3

The most comprehensive SEC-MALS/RI/VISC studies on cereal arabinoxylans were conducted by Dervilly-Pinel et al. (2000, 2001a, 2001b). They isolated water-extractable arabinoxylans from wheat, barley, rye, and triticale flours and characterized the chemical structures as well as the physico-chemical properties of these extracts. Furthermore, the isolated arabinoxylan from wheat flour was fractionated using ethanol precipitation in order to

produce chemically more homogeneous fractions that were analyzed with SEC-MALS/RI/VISC using 0.05 M $NaNO_3$ and 2 N NaOH for dissolution. Molar masses in NaOH were lower, which was explained as arising from the disruption of the ferulic acid cross-links between the arabinoxylan chains. According to Dervilly-Pinel et al.'s data on persistence length and R_g/M relationship, the structure of arabinoxylan had no effect on the solution conformation. Despite thoroughly studying the solution properties of arabinoxylans, they used a relatively high sample concentration of 5 mg/ml that might exceed the overlap concentration and skew the molar mass, size, and intrinsic viscosity data.

Off-line SLS has also been used for characterizing cereal arabinoxylans. Ebringerova et al. (1994) analyzed the arabinoxylan from rye bran with SLS using different solvents. This arabinoxylan with a low degree of substitution had a high tendency to aggregate even in DMSO and in complexing solvents, such as cuoxam and cadoxen. In any case, SLS without separation cannot be considered an optimal molar mass and size determination method for arabinoxylans due to the significant emphasis of aggregates that might be present only in low quantities.

α-L-Ara*f* side units are known to influence the water solubility of arabinoxylans (Andrewartha et al. 1979). The wheat flour arabinoxylan with the arabinose-to-xylose ratio of approximately 0.5 is water-soluble, but when the α-L-Ara*f* content is decreased the water solubility decreases. Andrewartha et al. (1979) reported the lowered solubility for wheat flour arabinoxylans with an arabinose-to-xylose ratio below 0.42. According to Köhnke et al.'s (2011) recent studies, the α-L-Ara*f* substitution pattern also influences the water solubility of arabinoxylans.

Galactomannans

Studies on macromolecular characterization of guar galactomannan in dilute aqueous solution, including off-line viscometry, off-line SLS, and SEC-MALS/RI/VISC studies, have been reported by several groups (Robinson et al. 1982; Wientjes et al. 2000; Picout et al. 2001). Galactomannan chains are regarded as coil-like molecules confirmed by extensive experimental data on their macromolecular characteristics, such as Mark-Houwink parameters and L_p values (Picout et al. 2001; Picout and Ross-Murphy 2007). Picout et al. (2001) obtained the L_p of 4 nm for guar galactomannan using the Burchard-Stockmayer-Fixman method. Variation in these characteristics can be found in the literature due to different extents of solubilization. Evidence of aggregates present in the aqueous solution of native guar galactomannan has been reported by Cheng et al. (2002) and Picout et al. (2001). Their

[η] and molar mass data indicate the presence of aggregates in native guar galactomannan solutions and that lowering the chain length reduces the aggregation tendency. The α-D-Gal*p* side units are known to enhance the water solubility of galactomannans, whereas the polymannose chain itself is water-insoluble (Robinson et al. 1982). The effect of α-D-Gal*p* content on the solution properties of galactomannans, however, has not been studied. Molar mass, [η], and R_g reported for galactomannan depend on the author, the analysis method, and the sample used. The macromolecular characteristics for native guar galactomannan and locust bean gum obtained in aqueous solution are presented in Table 6. Cheng et al.'s (2002), Picout et al.'s (2001), and Richardson et al.'s (1998) molar mass data presented in Table 6 are obtained with SEC, and the other data with off-line LS and VISC methods. Mark-Houwink parameters are defined by analyzing the set of samples with different DP.

Table 6. Macromolecular characteristics for galactomannans.

Galactomannan	$M_w \times 10^{-6}$ **(g/mol)**	M_w/M_n	**[η] (ml/g)**	R_g **(nm)**	$k \times 10^4$	α	**Reference**
Guar galactomannan, LJX-2 (Rhone-Poulenc Inc.)	1.98	1.65	1600		3.04	0.747	Cheng et al. 2002
Guar galactomannan, M150 (Meyhall Chemicals)	1.87		1270	133		0.70	Picout et al. 2001
Guar galactomannan (Unipectin A.G.)	1.65		1250		3.8	0.723	Robinson et al. 1982
Locust bean gum (Meyhall Chemicals)	0.812	1.17	1379				Richardson et al. 1998

M_w = weight-average molar mass, M_w/M_n = dispersity index, [η] = intrinsic viscosity, R_g = radius of gyration, k = Mark-Houwink constant, α = Mark-Houwink exponent

2.8.2 Dextrans with long-chain branching

The dextran from *Leuconosctoc mesenteroides* B-512F is the most used commercially and thus the most studied in terms of dilute solution properties. Off-line light-scattering and viscometry techniques are employed in many rather comprehensive studies on the solution properties of commercially available dextrans (Nordmeier 1993; Ioan et al. 2000), but SEC-MALS/RI/VISC (Porsch and Sundelöf 1994; Chmelik et al. 1997; Ioan et al. 2000) and FFF-MALS/RI (Roessner and Kulicke 1994; Wittgren and Wahlund 1997) have also been used to characterize dextrans. The molar mass and size of dextrans are widely investigated especially because they are commonly used as standards in SEC.

The exact branching topology of dextrans is not well-known, but they are considered hyperbranched polymers with branches of various chain lengths. Characteristic features for hyperbranched polymers include the narrow molar mass distribution and that gelation can never occur (Burchard 1999). Ioan et al. (2000) characterized the aqueous, dilute solution of seven dextrans, from *L. mesenteroides* (Sigma), with different molar masses (M_w 9000 – 2.7 × 10^6 g/mol) using SLS, DLS, capillary viscometry, and SEC-MALS/RI/VISC. In addition, the sample with the highest molar mass was degraded via controlled acid hydrolysis to produce fractions with various molar masses. Their data suggest that lower-molar-mass dextrans behave in solution as linear chains, whereas the molecules in the high-molar-mass region show hyperbranched behavior. This was explained with the increased branching density (branching units per macromolecule). The generalized ratio of R_g/R_h at high molar masses approaches a constant value of 1.09, which is close to the 1.22 expected for hyperbranched polymers. Even for lower-molar-mass dextrans, R_g/R_h was below the 1.5 that is expected for linear, monodisperse random coil chains (Burchard 1999). The dimensionless ratio of R_g/R_h gives special information on the architecture of macromolecules. This value decreases when molecular density increases. Intrinsic viscosity data supported the size data: the expected power law behavior was observed only in the low-molar-mass region of the Mark-Houwink relationship, but in the higher-molar-mass region the plot is non-linear.

Only a very few studies can be found in the literature describing the dilute solution characterization of (high-molar-mass) dextrans produced by LAB other than *L. mesenteroides* B-512F. Tieking et al. (2003) screened more than 100 LAB strains from which four (two strains of *Lactobacillus reuterii*, *Lactobacillus sp.*, *Weissella confusa*) were found to produce a high-molar-mass (molar mass >10^6 g/mol), glucan-type exopolysaccharide. Their rough molar mass estimation was based on SEC with RI detection. Son et al. (2008) characterized the dextran from *Leuconostoc citreum* S5 using the same method and reported the molar mass was higher than 2 × 10^6 g/mol. Alternan (class 2 dextran) produced by *L. mesenteroides* B-1355 has been recently characterized with SEC-MALS/RI using neat DMSO as a solvent (Striegel et al. 2009a; Isenberg et al. 2010). Additionally, hydrodynamic chromatography with MALS/RI detection and off-line MALS were used for comparison because of the evidence on the shear degradation of alternans during SEC analysis. These polysaccharides had ultra-high molar mass ranging from 44.1 × 10^6 g/mol to 59.8 × 10^6 g/mol and R_g from 47 to 63 nm depending on the producing strain and analysis method. Such low values of R_g for ultra-high molar mass polysaccharides indicate highly compact conformation in solution.

3 Aims of the study

The overall aim of this thesis was to study the solution properties and macromolecular characteristics of structurally different polysaccharides, namely, arabinoxylans, galactomannans, and dextrans. Two methods, SEC and AsFlFFF with multiple-detection, were employed in characterization. The chemical structures of arabinoxylans were determined with monosaccharide composition analysis, ^{1}H NMR spectroscopy, and HPAEC-PAD analysis of oligosaccharides after enzymatic hydrolysis. Galactomannan and dextran structures have been studied in previous investigations.

The individual objectives were as follows:

- To study the solution properties of arabinoxylans from barley, rye, and wheat (**I-IV**)

- To compare organic and aqueous solvents for arabinoxylans and dextrans in SEC analyses (**II-III**, **VI**)

- To investigate the effect of arabinose side units on the macromolecular behavior of arabinoxylans (**III**)

- To study the effect of the degree of polymerization and degree of substitution on the solution properties of galactomannan from guar (**V**)

- To compare the two separation methods, SEC and AsFlFFF, for characterizing branched polysaccharides (**IV-VI**)

4 Materials and methods

This section summarizes the materials and methods presented in more detail in original publications **I-VI**.

4.1 Materials

The barley materials, husks (BH) and fiber (BF), used for isolating arabinoxylans in paper **I** were obtained from Altia Corporation (Koskenkorva, Finland). Both husks and fiber were by-products of the industrial process producing potable ethanol and barley starch. D-xylose, D-arabinose, D-glucose, D-galactose, D-mannose, and L-rhamnose from Merck (Darmstadt, Germany) and (1→4)-β-D-xylobiose and (1→4)-β-D-xylotriose from Megazyme (Bray, Ireland) were used as standards in the monosaccharide and oligosaccharide analyses (**I-III**). The anhydrous methanol used for the methanolysis reagent was from Fluka (Buchs, Switzerland) and acetyl chloride from Merck. Pyridine was obtained from Sigma-Aldrich Chemie (Steinheim, Germany) (**I**). The enzymes used in the arabinoxylan extraction process were Termamyl 120L α-amylase (Novozymes, Bagsvaerd, Denmark), pepsin (Merck), and pancreatin from swine pancreas (P-7545, Sigma, Steinheim, Germany). Barium hydroxide was from Merck and sodium borohydride ($NaBH_4$) from Fluka (**I**). The deuterium oxide (D_2O) used in NMR spectroscopy (**I-III**, **VI)** was from Merck. HPLC-grade dimethyl sulfoxide (DMSO, Lab-Scan, Dublin, Ireland), lithium bromide (LiBr, Sigma), and sodium nitrate ($NaNO_3$, Merck) were used in SEC and AsFlFFF analyses. Water was purified with a Milli-Q-Plus system (Millipore Corp., Billerica, MA, USA).

4.1.1 Polysaccharides

The polysaccharides used in studies **I-VI** are listed in Table 7. Arabinoxylans from wheat and rye and galactomannan from guar were commercial products. Barley arabinoxylans and dextrans from *Weissella confusa* and *Leuconostoc citreum* were extracted in our laboratory. The polysaccharides used as standards are presented in Table 8.

Table 7. Polysaccharides used in studies **I-VI.**

Material	Abbr.	Supplier	Study
Arabinoxylan from barley husks[a]	BHAX		**I**
Arabinoxylan from barley fiber[a]	BFAX		**I**
Wheat arabinoxylan (high viscosity)	WAX-HV	Megazyme (Bray, Ireland)	**II-III**
Wheat arabinoxylan (medium viscosity)	WAX-MV	Megazyme	**II**, **IV**
Wheat arabinoxylan (low viscosity)	WAX-LV	Megazyme	**II**, **IV**
Rye arabinoxylan (high viscosity)	RAX-HV	Megazyme	**II-III**
Galactomannan from guar		Sigma (Munich, Germany)	**V**
Dextran from *Leuconostoc* spp.		Sigma	**VI**
Dextran from *W. confusa*[b]			**VI**
Dextran from *L. citreum*[b]			**VI**

[a]Extracted in this study
[b]Extracted in our laboratory previously (Maina et al. 2008)

Table 8. Polysaccharides used as standards in SEC.

Polysaccharide standard	Supplier
Pullulan[a]	Hayashibara (Okayama, Japan)
Pullulans with nominal M_w 11 800-788 000 g/mol	Polymer Laboratories (Shropshire, UK)
Dextrans with nominal M_w 11 600-11 900 000 g/mol	Fluka (Buchs, Switzerland)
	Polymer standards service (Mainz, Germany)

[a]Used for d*n*/d*c* measurement

4.1.2 Enzymes used for polysaccharide modifications

The commercial endo-(1→4)-β-D-xylanase preparation Shearzyme 500 L (Novozymes, activity 49075 nkat/ml) was used for hydrosis of arabinoxylans in studies **I-II**. Two α-L-arabinofuranosidases AXH-d3 (Megazyme, activity 3340 nkat/ml) and AXH-m (Novozymes, activity 9245 nkat/ml), which differ in their substrate specificities, were used for specific tailoring of the α-L-Ara*f* content of wheat and rye arabinoxylans (**III**) and for enzymatic fingerprinting in studies **I-II.** AXH-d3 removes the (1→3)-linked α-L-Ara*f* units from disubstituted β-D-Xyl*p* residues, whereas AXH-m liberates the (1→2)- and (1→3)-linked α-L-Ara*f* units from monosubstituted β-D-Xyl*p* residues. In study **V**, the chain length of guar galactomannan was modified with endo-(1→4)-β-D-mannanase purified from *Trichoderma reesei* (VTT, Finland) (Stålbrand et al. 1993) and the degree of substitution with α-D-galactosidase (Biogalactosidase, Quest, The Netherlands) (Mikkonen et al. 2007).

4.2 Experimental

4.2.1 Characterization of barley husks and barley fiber (I)

The monosaccharide composition of BH and BF was determined with anion exchange chromatography after sulfuric acid hydrolysis (Puls 1993). The hydrolysis residue was determined gravimetrically with a procedure similar to the Klason lignin procedure.

The total starch was measured using a Megazyme kit (AOAC Methods 996.11), which is based on enzymatic hydrolysis of starch to glucose with thermostable α-amylase and amyloglucosidase. The content of cellulose and (1→3)(1→4)-β-D-glucan was calculated by subtracting the starch content from the total amount of anhydroglucose. The sum of anhydroxylose and anhydroarabinose presents the arabinoxylan content.

The amount of lipids was measured gravimetrically using Soxhlet extraction. The Kjehldal method was used for protein determination (6.25 × *N*). The ash content was measured gravimetrically after ashing the samples at 550 ºC in a temperature-programmed muffle oven (Nabertherm, Germany).

4.2.2 Extraction of barley arabinoxylans (I)

Water-insoluble arabinoxylans were extracted from barley husks and fiber using a method in which starch and proteins were digested enzymatically and arabinoxylans further extracted with saturated barium hydroxide (Figure 8) (Gruppen et al. 1991; Virkki et al. 2005). Barium hydroxide is known to selectively dissolve arabinoxylans from the cereal matrix (Gruppen et al. 1991).

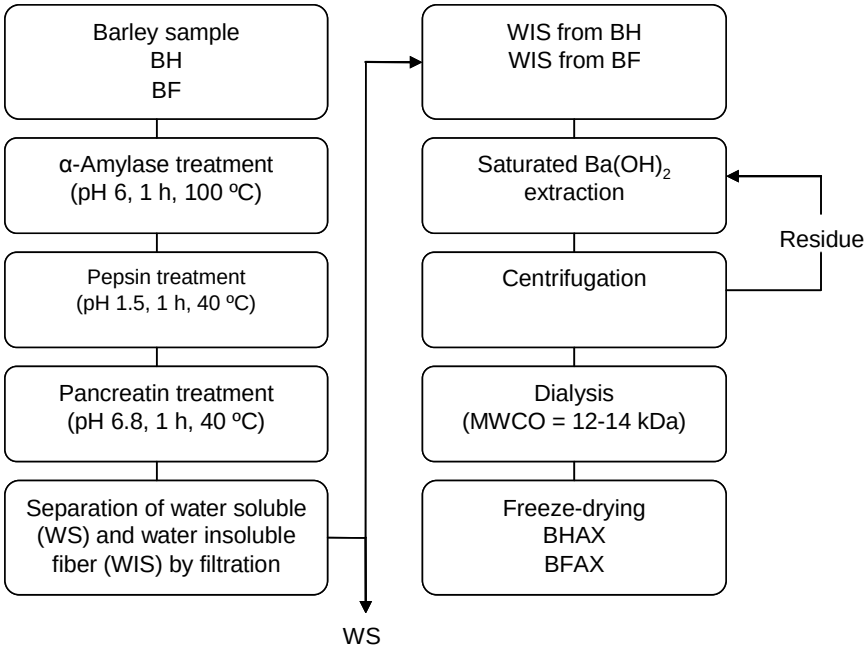


Figure 8. Schematic diagram of the isolation of arabinoxylans from barley husks (BH) and barley fiber (BF).

4.2.3 Monosaccharide composition of polysaccharides

4.2.3.1 Acid methanolysis for arabinoxylans (I)

In acid methanolysis, the arabinoxylans were degraded to monosaccharides with hydrochloric acid in anhydrous methanol. The samples were kept at 100 °C in pear-shaped flasks for 3 hours and neutralized with pyridine. After methanolysis, the samples were trimethylsilylated before GC analysis was conducted. The monosaccharide standards were treated with acid methanolysis in the same way as the arabinoxylan samples and diluted in order to make a calibration curve. The areas of all the peaks resulting from different anomers of each monosaccharide were summed for quantification. The GC instrument consisted of a Hewlett-Packard 5890 Series II GC system with flame ionization detector (FID), HP 7673 series injector, and autosampler (Hewlett-Packard, Palo Alto, CA, USA). The column used was HP-5 (30 m × 0.32 mm × 0.25 μm; Agilent Technologies, Foster City, CA, USA). The temperature program was as follows: oven temperature 70 °C, 2 °C/min to 175 °C, 12 °C/min to 290 °C. The injection volume was 0.5 μl and the split ratio 1:30.

4.2.3.2 Complete enzymatic hydrolysis of arabinoxylans (III)

The monosaccharide composition of the wheat and rye arabinoxylan samples was determined using GC after complete enzymatic hydrolysis. The previously developed enzyme-based method for water-soluble arabinoxylans was used in hydrolysis (Virkki et al. 2008).

4.2.4 Structural characterization of arabinoxylans

4.2.4.1 Enzyme-assisted profiling of arabinoxylan structures (I-II)

In studies **I**-**II,** cereal arabinoxylans were degraded to oligosaccharides and analyzed with HPAEC-PAD to obtain structural information on polysaccharides. In study **I**, the barley arabinoxylan samples (5 g/l) in 0.02 M sodium acetate buffer, pH 5.0, were first incubated with Shearzyme, using an endo-(1→4)-β-D-xylanase dosage of 10000 nkat/g xylan, at 40 ºC for 48 h. Hydrolysis was terminated by keeping the samples in a boiling water bath for 10 min. Both samples were further incubated separately with two different α-L-arabinofuranosidases (AXH-m and AXH-d3). The AXH-m dosage was 5000 nkat/g xylan and the AXH-d3 dosage 1000 nkat/g xylan. Reaction conditions for AXH treatments were similar (40 ºC, 24 h), except that 0.02 M sodium acetate buffer, pH 5.0, was used for AXH-m and 0.05 M sodium phosphate buffer, pH 6.5, for AXH-d3. In study **II**, the wheat and rye arabinoxylans were treated with Shearzyme using the above-mentioned conditions.

The HPAEC-PAD equipment consisted of two Waters 515 HPLC pumps (Waters Corp., Milford, MA, USA), a Waters 717 Plus autosampler (Waters Corp., USA), a column and pre-column (CarboPac PA-100, 4 × 250 mm and CarboPac PA-100 Guard, 4 × 50 mm, Dionex, Sunnyvale, CA, USA), a Decade pulse amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), and an SSI pulse equalizer (Scientific Systems, Inc., model LP 21, State College, PA, USA). Gradient elution with 0.1 M NaOH and 1 M NaAc in 0.1 M NaOH was used for separation (Rantanen et al. 2007). Millenium[32] software (Waters Corp., USA) was used for instrument control and data handling.

4.2.4.2 ^{1}H NMR spectroscopy (I-III)

For the ^{1}H NMR spectroscopic analysis, arabinoxylan samples were exchanged three times with D_2O and finally dissolved in pure D_2O (99.96%). Before being analyzed, the samples were filtered with 0.45 μm syringe filters (GHP Acrodisc 13, Pall Corp., Ann Arbor, MI,

USA). The ^{1}H spectra of the samples were obtained on a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 499.84 MHz. The measurements were performed at 50 °C, and the ^{1}H chemical shifts (ppm) were referenced to an internal acetone signal at 2.225 ppm.

4.2.5 Specific enzymatic modification of arabinoxylans and galactomannans (III, V)

In study **III,** the wheat and rye arabinoxylan samples (WAX-HV and RAX-HV, 10 g/l) were modified with two α-L-arabinofuranosidases, AXH-d3 and AXH-m, which differ in their substrate specificities. The commercial arabinoxylans were chosen for specific structure-function studies due to the high purity and lower molecular dispersity compared with alkali-extracted barley arabinoxylans. The AXH-d3 treatments were carried out in 0.05 M sodium phosphate buffer, pH 6.5, and incubated at 40 °C for 24 h, and AXH-m treatments in 0.02 M sodium acetate buffer, pH 5.0, at 40 °C for 48 h. The enzyme dosage for AXH-d3 was 1000 nkat/g of arabinoxylan and for AXH-m 10000 nkat/g of arabinoxylan. After incubation, the samples were kept in a boiling water bath for 10 min to terminate the hydrolysis. The amount of released α-L-Ara*f* was measured with a spectrophotometer using a commercial enzymatic assay (K-LACGAR, Megazyme). In addition, modifications were confirmed with ^{1}H NMR spectroscopy. SEC analyses for modified wheat and rye arabinoxylan samples were accomplished using aqueous and DMSO-based systems as shown in Figure 9. Abbreviations of WAX-HV_{d3}, WAX-HV_{m}, RAX-HV_{d3}, and RAX-HV_{m} are used for the enzymatically modified samples and WAX-HV_{0} and RAX-HV_{0} for the blank samples treated similarly but without added enzymes.

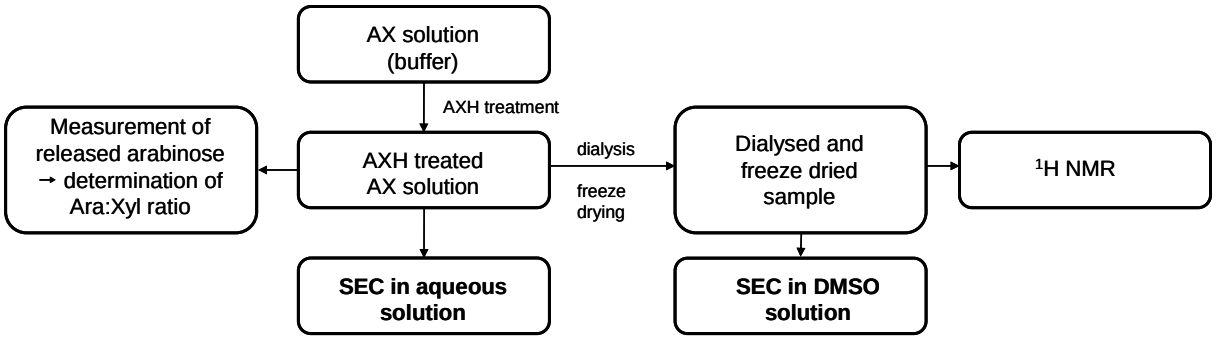


Figure 9. Schematic presentation of the enzymatic modification of arabinoxylans.

Stepwise enzymatic modification for galactomannan in study **V** was performed to achieve a series of samples with four different chain lengths and three different degrees of galactose substitution (Table 9). The endo-(1→4)-β-D-mannanase dosages used were 0, 2, 10, or 50 nkat/g of mannan, and the incubation time was 4 h. After the mannanase treatment, the samples were further incubated with α-D-galactosidase (0, 200, or 500 nkat/g) for 5 h. After the enzymatic modification, the samples were freeze-dried, washed with ethanol to remove monosaccharides and short oligosaccharides, and freeze-dried again. The carbohydrate composition of the enzymatically modified galactomannan samples was determined as alditol-acetate derivatives using GC after complete enzymatic hydrolysis (Mikkonen et al. 2007).

Table 9. Sample codes, enzyme dosages, and galactose-to-mannose ratios of enzymatically modified galactomannans adapted from Mikkonen et al. (2007).

Sample code	Galactosidase/Mannanase dosage (nkat/g)	Gal:Man ratio	Molar mass code
6Gal-H	0/0	0.49	High
6Gal-MH	0/2	0.52	Medium high
6Gal-ML	0/10	0.57	Medium low
6Gal-L	0/50	0.54	Low
4Gal-H	200/0	0.36	High
4Gal-MH	200/2	0.37	Medium high
4Gal-ML	200/10	0.39	Medium low
4Gal-L	200/50	0.36	Low
2Gal-H	500/0	0.23	High
2Gal-MH	500/2	0.23	Medium high
2Gal-ML	500/10	0.20	Medium low
2Gal-L	500/50	0.19	Low

[a] 6, 4, and 2 denote the approximate number of galactose units per 10 mannose units.

4.2.6 Solution characterization

The arabinoxylans extracted from BH and BF were characterized using the DMSO-based SEC system. The commercial wheat and rye arabinoxylans were analyzed using DMSO- and water-based eluent to compare the effect of solvent for arabinoxylans. DMSO is commonly used for dissolution of wood xylans (Goring and Timell 1960) and relatively linear arabinoxylans, such as arabinoglucuronoxylans from wheat straw and oat spelts (Saake et al. 2001), and was thus chosen to characterize BHAX and BFAX. Highly substituted arabinoxylans, on the other hand, are generally regarded as water-soluble polysaccharides and often characterized in aqueous solutions, such as in dilute $NaNO_3$ solution (Dervilly-Pinel et al. 2001a; Dervilly et al. 2000). Only aqueous solution characterization was employed for galactomannans due to their insolubility in DMSO.

Solution characterization of dextrans has usually been accomplished in aqueous solution whereas only in a very few studies have organic solvents, such as DMSO, been used (Alekseeva et al. 2007; Merienne et al. 2000). DMSO, however, has been widely used for dissolution of other α-glucans, namely, amylose, amylopectins, and glycogen, due to its non-aggregative nature (Gilbert 2011). In this study, water and DMSO were used as solvents for dextrans.

4.2.6.1 SEC analysis (I-VI)

The SEC analyses were accomplished in all studies **I-VI** using either aqueous (eluent: H_2O + 0.1 M $NaNO_3$) and/or an organic system (eluent: DMSO + 0.01 M LiBr). The SEC equipment consisted of an integrated autosampler and a pump module (GPCmax, Viscotek Corp., Houston, TX, USA), a UV-detector (Waters 486 Tunable Absorbance Detector, Milford, MA, USA), a combined light-scattering and viscometric detector (270 Dual Detector, Viscotek Corp.), and a refractive index (RI) detector (VE 3580, Viscotek Corp.). The light-scattering detector (λ_0 = 670 nm) included two scattering angles: 7° and 90°. Two OHpak SB-806M HQ columns 8 × 300 mm (Showa Denko, Ogimachi, Japan) with a guard column OHpak SB-6 (4.6 × 10 mm) were used for the aqueous eluent. In the DMSO-based system, two LF-804 columns 8 × 300 mm (Showa Denko, Tokyo, Japan) with a guard column LF-G (4.6 × 10 mm) were used. The flow rate in both SEC systems was 1 ml/min. The SEC data were processed with the OmniSEC 4.5 software (Viscotek Corp.). For the analysis in DMSO, the dry polysaccharide samples were dissolved in eluent and allowed to dissolve at room temperature for 4 days to maximize the solubility. The samples for the water-based system were prepared in the same way, except in study **III** in which the enzymatically tailored arabinoxylans were analyzed directly with SEC after incubation. Before being analyzed, all the samples were filtered with 0.45 μm syringe filters. The concentrations for the polysaccharide samples in the SEC analysis ranged from 0.9 mg/ml to 4 mg/ml depending on the sample and the solvent. The d*n*/d*c* values for different polysaccharides obtained from the literature are listed in Table 10.

Table 10. Refractive index increments (d*n*/d*c*) for studied polysaccharides.

Polysaccharide	**Solvent**	**d*n*/d*c* (ml/g)**	**Reference**
Arabinoxylan	aqueous	0.146	Dervilly et al. 2002
Arabinoxylan	DMSO	0.064	Goring and Timell 1960
Galactomannan	aqueous	0.150	Kapoor et al. 1994
Dextran	aqueous	0.144	Vink and Dahlström 1967
Dextran	DMSO	0.072	Basedow et al. 1978

Pullulan was used to calibrate the RI detector and a combined light-scattering and viscometric detector in SEC. Because the d*n*/d*c* value for pullulan in DMSO was unknown, the d*n*/d*c* was measured using the commercial sample. The purity of the sample was checked with ^{1}H NMR spectroscopy. The d*n*/d*c* of pullulan was determined at 20 °C using an Abbe refractometer (ABBE 60/ED). A He-Ne laser from Spectra-Physics Lasers Inc. operating at λ_0 = 632.8 nm was the light source. Stock solutions of four different pullulan concentrations (20, 30, 40, 50 mg/ml) in DMSO containing 0.01 M LiBr were further diluted, and the refractive indexes of the solutions were measured three times. Then the refractive indexes were plotted versus the pullulan concentration, and the d*n*/d*c* value (0.056 ml/g) was obtained from the slope of the fitted linear function.

4.2.6.2 AsFlFFF analysis (IV-VI)

AsFlFFF experiments were carried out using an AF2000 MT instrument (including software, Postnova Analytics, Landsberg/Lech, Germany) equipped with multi-angle light-scattering (MALS, Brookhaven Instruments Corporation, Holtsville, NY, USA) and refractive index (PN 3150, Postnova Analytics) detectors. The MALS detector contains a 30 mW laser as the light source operating at λ_0 = 660 nm with seven scattering angles (35°, 50°, 75°, 90°, 105°, 130°, 145°). Separation occurred in a rectangular channel consisting of a bottom plate with a ceramic frit, a spacer with a thickness of 350 µm, and a top plate with flow outputs (Figure 10). A membrane was placed on top of the ceramic frit. A regenerated cellulose membrane (cut-off value of 10 000 g/mol) was used for the arabinoxylans, galactomannans, and dextrans. In addition, a polyethersulfone membrane with the same cut-off value was tested for galactomannans.

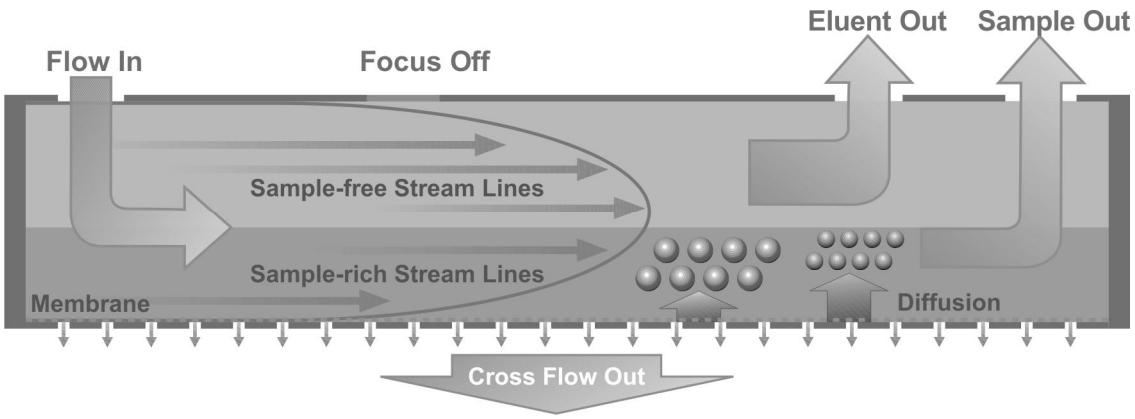


Figure 10. AsFlFFF channel representing the separation of analytes and flow outputs. The excess solvent from the upper part of the channel is pumped to waste to intensify the detector signals. The figure is presented courtesy of Postnova Analytics.

The pump system included two isocratic pumps and a piston pump for maintaining cross-flow. Exponential decay of cross-flow (exponent 0.2) was used for the separation step of the arabinoxylans, galactomannans, and dextran standards starting with a cross-flow of 2 ml/min, and the detector flow was kept constant at 0.5 ml/min during the analysis. Because molecules flow in the channel near the membrane, the excess solvent from the upper part of the channel was pumped to the waste at a flow rate of 0.5 ml/min before the detector outlet to intensify the detector signals (Figure 10). For isolated dextran samples with higher molar mass, the faster decay of cross-flow (exponent 0.1) and a lower cross-flow of 1.5 ml/min were used. The detector flow was 1 ml/min. The elution conditions for different samples are presented graphically in Figure 11. The RI and MALS detectors were calibrated according to instructions from Postnova Analytics (Postnova Analytics 2009) using the bovine serum albumin and polystyrene sodium sulfonate standards. The Berry equation was used for the molar mass and size calculations, and the injection volume was 50 µl. The aqueous eluent of H_2O + 0.1 M $NaNO_3$ was used for elution in all analyses. The samples for AsFlFFF analysis were prepared the same way as for SEC (section 4.2.6).

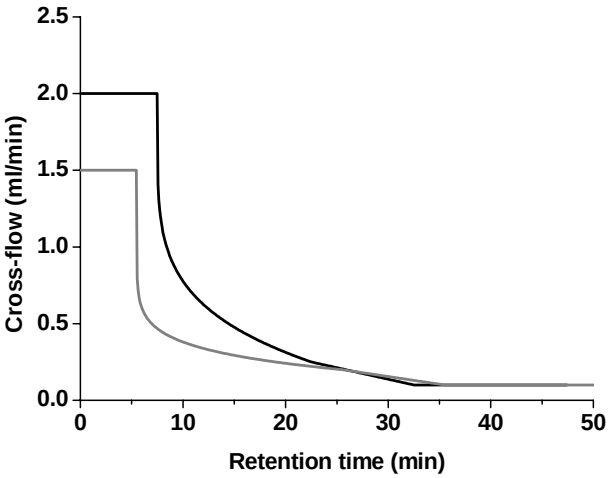


Figure 11. AsFlFFF cross-flow gradients for arabinoxylans, galactomannans, and dextran standards (black line) and for isolated, high-molar-mass dextrans (gray line).

5 Results

5.1 Composition of barley husks and barley fiber (I)

The composition of barley husks (BH) and barley fiber (BF) used for arabinoxylan extraction is presented in Table 11. Both materials consisted mainly of carbohydrates, proteins, and lignin. BH contained much more starch than barley BF, which is most likely due to the endosperm remains in BH. The arabinoxylan content of BF was higher compared with BH.

Table 11. The composition of barley husks (BH) and barley fiber (BF) (% of dry weight ± sd). n = 3

Component (%)	BH	BF
Carbohydrates[a]	72.6 ± 0.5	59.2 ± 0.5
Starch	31.9	9.77
Cellulose + β-glucan	34.0	35.8
Arabinoxylan	32.5	50.0
Proteins	5.4 ± 1.3	12.6 ± 0.1
Lipids	0.9 ± 0.2	2.8 ± 0.1
Ash	4.7 ± 0.1	3.5 ± 0.2
Klason lignin	17.1 ± 0.4	18.6 ± 0.2
Total	100.7	96.7

[a]Carbohydrate composition is depicted as percentage values from the total amount of anhydrous glucose, xylose, and arabinose. Cellulose + β-glucan are calculated by subtracting the starch content from the total amount of anhydroglucose. Arabinoxylan is presented as the sum of anhydro xylose and anhydro arabinose.

The majority of monosaccharides in BH and BF consisted of glucose, xylose, and arabinose, and only a small amount of other sugars was present (Table 12). The xylose content of both barley materials was almost equal, but the arabinose content in BF was double that in BH. The arabinose-to-xylose ratio was 0.25 for BH and 0.49 for BF.

Table 12. The monosaccharide content of barley husks (BH) and barley fiber (BF) (% of dry weight ± sd). n = 3

Monosaccharide (%)	BH	BF
Glc	47.8 ± 0.5	27.0 ± 0.2
Xyl	18.9 ± 0.3	19.8 ± 0.2
Ara	4.7 ± 0.2	9.8 ± 0.2
Man	0.4 ± 0.1	1.1 ± 0.0
Gal	0.7 ± 0.1	1.4 ± 0.0
Rha	0.1 ± 0.0	0.1 ± 0.0
Total	72.6 ± 0.5	59.2 ± 0.5

5.2 Monosaccharide composition of arabinoxylans from barley, wheat, and rye (I-IV)

The monosaccharide composition of extracted barley husk arabinoxylan (BHAX) and barley fiber arabinoxylan (BFAX) was determined using GC after acid methanolysis in study **I**. The data for wheat and rye arabinoxylans used in this study were published previously (Höije et al. 2008; Virkki et al. 2008). The monosaccharide compositions of all studied arabinoxylans and their arabinose-to-xylose ratios are summarized in Table 13. The arabinose-to-xylose ratio of BFAX, WAX-HV, WAX-MV, and RAX-HV were in the same range (0.50-0.56). The arabinose content of BHAX and WAX-LV was lower.

Table 13. The monosaccharide compositions for cereal arabinoxylans and their arabinose-to-xylose ratios.

Sample	Xylose (%)	Arabinose (%)	Glucose (%)	Ara:Xyl
BHAX	73	21	6	0.29
BFAX	62	32	6	0.52
WAX-HV[a]	61	31	8	0.51
WAX-MV[a]	64	36	n.d.	0.56
WAX-LV[a]	72	23	5	0.32
RAX-HV[a]	66	33	1	0.50

[a]Analyzed by Höije et al. (2008) and Virkki et al. (2008).

5.3 Structural differences of cereal arabinoxylans (I-III)

The structures of cereal arabinoxylans from barley, wheat, and rye were analyzed with ^{1}H NMR spectroscopy from polymeric samples and with HPAEC-PAD after selective enzymatic hydrolysis (**I-II**). By using these complementary methods, information on the substitution of arabinoxylans could be obtained. The α-anomeric region of the ^{1}H NMR spectra for barley arabinoxylans is presented in Figure 12. The main signal in both spectra at (A) indicated the

presence of an α-L-Ara*f* (1→3)-linkage to monosubstituted β-D-Xyl*p* residue. The signals B and C originated from the (1→3)-linked and (1→2)-linked α-L-Ara*f* substituents on the doubly-substituted β-D-Xyl*p* unit in the main chain (Hoffmann et al. 1991; Viëtor et al. 1994). The signal originating from α-L-Ara*f* (1→2)-linkage to monosubstituted β-D-Xyl*p* residue was overlapped with that of the (1→3) α-L-Ara*f* residue in the doubly-substituted xylose unit. The signal D indicated the presence of a disaccharide side chain 2-*O*-β-D-Xyl*p*-α-L-Ara*f* attached to *O*-3 of xylopyranosyl residue (Höije et al. 2006). The comparison of signal intensities reveals that BFAX contained more disubstituted β-D-Xyl*p* residues (B, C) than BHAX but at the same time fewer disaccharide side chains (D).

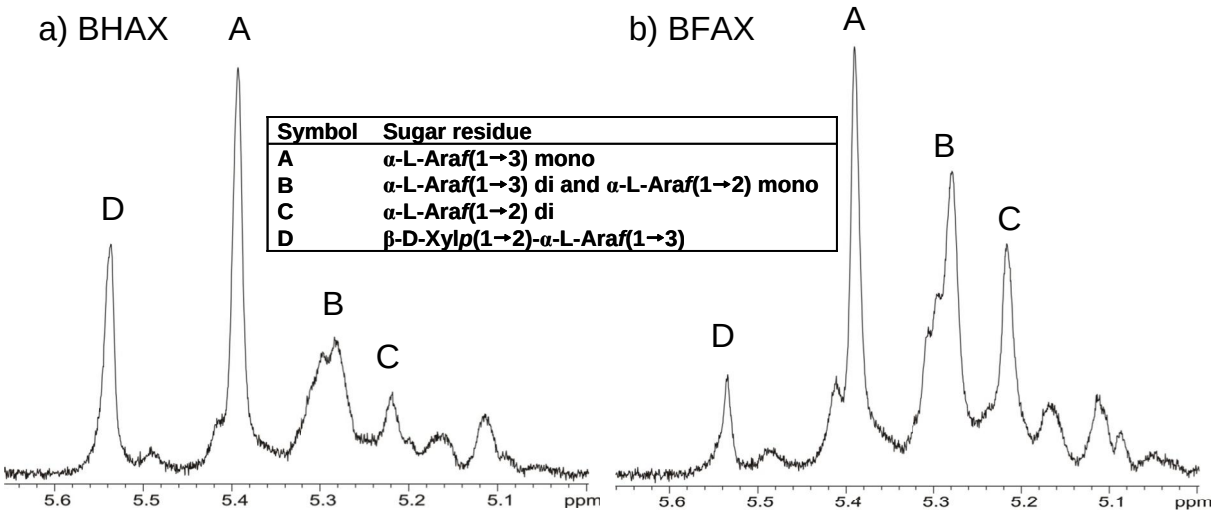


Figure 12. The α-anomeric region of ^{1}H NMR spectra including signal interpretation for a) barley husk arabinoxylan (BHAX) and b) barley fiber arabinoxylan (BFAX).

Further information on the arabinose substitution of barley arabinoxylans was obtained with HPAEC-PAD after selective enzymatic hydrolysis. The samples were treated with endo-(1→4)-β-D-xylanase followed by two α-L-arabinofuranosidases that differ in their substrate specificities: AXH-m and AXH-d3. The formed arabinoxylooligosaccharides were identified (Figure 13) using knowledge of the enzyme specificities and previously published data on arabinoxylooligosaccharides (Rantanen et al. 2007; Pastell et al. 2008). The results from the HPAEC-PAD supported the NMR spectroscopic data. The level of disubstituted β-D-Xyl*p* residues was higher in BFAX than in the BHAX sample (increase in α-L-Ara*f*(1→2)-xylobiose from diarabinosylxylobiose as a result of the AXH-d3 modification of the endoxylanase-treated sample, Figure 13 c, f). The content of β-D-Xyl*p* residues carrying a disaccharide side unit was higher in BHAX than in BFAX seen as a higher peak of β-D-Xyl*p*(1→2)-α-L-Ara*f*-xylobiose in Figure 13 a compared to Figure 13 d. A tetrasaccharide

consisting of xylotriose with an α-L-arabinofuranosyl substituent linked to the middle xylopyranosyl residue via (1→3) linkage eluted together with α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p* (Pastell 2010). That partly explains the formation of xylotriose beside xylobiose after the Shearzyme and AXH-m treatments. Oligosaccharides with disubstituted xylose residues have a much lower response factor in PAD and thus result in significantly smaller peaks (small peaks eluting after the main xylooligosaccharide peaks in Figure 13). HPAEC-PAD analysis confirmed the presence of (1→2)-linked α-L-Ara*f* substituents especially in the BFAX, seen as the peak originating from α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl*p* in the Shearzyme hydrolyzed sample (Figure 13 d).

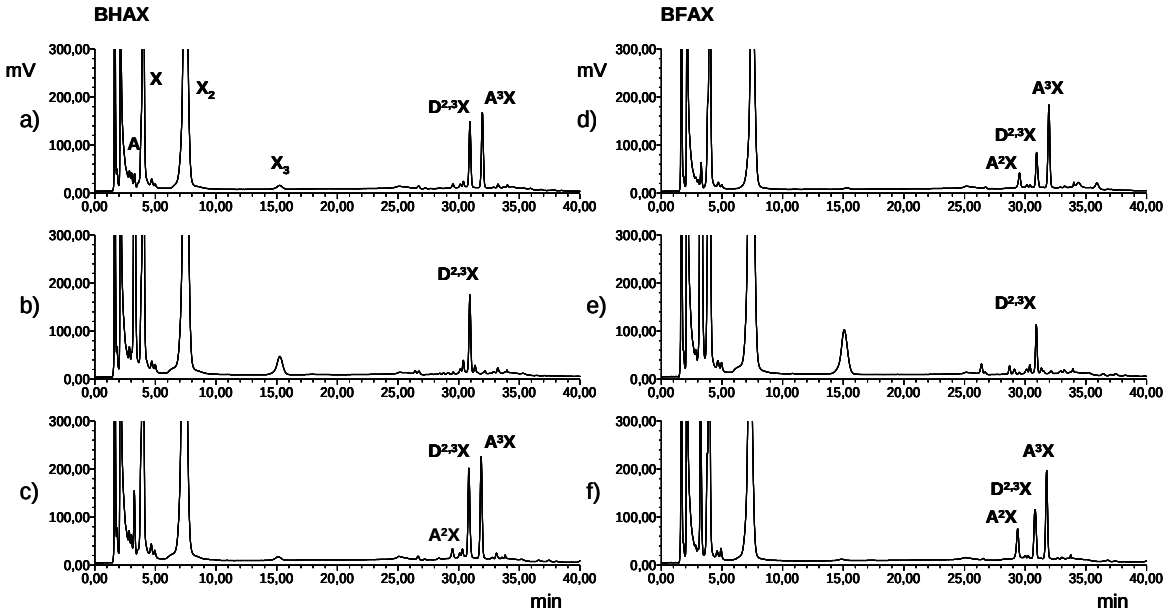
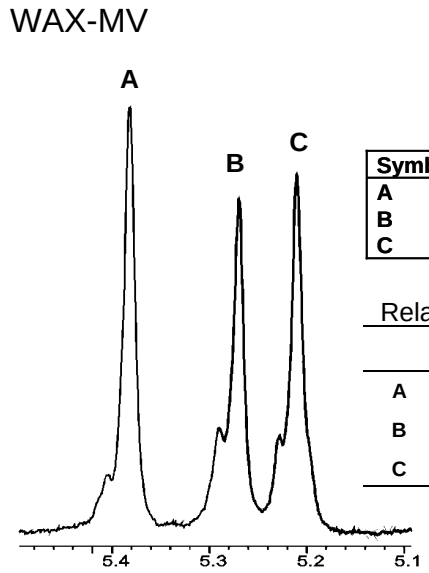


Figure 13. HPAEC-PAD chromatograms of oligosaccharides obtained from BHAX (a-c) and BFAX (d-f) after sequential treatments with Shearzyme (endo-(1→4)-β-D-xylanase) and two α-L-arabinofuranosidases (AXH-m and AXH-d3): Shearzyme (a, d), Shearzyme followed by AXH-m (b, e), Shearzyme followed by AXH-d3 (c, f). A = arabinose, X = xylose, X_2 = xylobiose, X_3 = xylotriose, A^2X = α-L-Ara*f*(1→2)-xylobiose, $D^{2,3}X$ = β-D-Xyl*p*(1→2)-α-L-Ara*f*-xylobiose, A^3X = α-L-Ara*f*(1→3)-xylobiose. The abbreviations for oligosaccharides presented in the chromatograms are adopted from Fauré et al. (2009).

The α-anomeric region of the ^{1}H NMR spectra from the commercial wheat and rye arabinoxylan samples showed three major signals (a spectrum for WAX-MV is presented in Figure 14 as an example). The relative peak intensities for all the wheat and rye samples are also shown in Figure 14. According to the peak intensities, RAX-HV differed from all the WAX samples. It clearly contained more monosubstituted β-D-Xyl*p* residues than the WAX

samples. No difference between WAX-HV and WAX-MV could be observed, and roughly two thirds from their substituted β-D-Xyl*p* residues were disubstituted and one third monosubstituted at the *O*-3 position. In the case of RAX-HV, the situation was reversed. The level of α-L-Ara*f* (1→3)-linked to monosubstituted β-D-Xyl*p* residue (A) in WAX-LV was in accordance with the other WAX samples, but a difference in the signal intensities of the partly overlapped signal from α-L-Ara*f* (1→3)-linked to disubstituted β-D-Xyl*p* residue and α-L-Ara*f* (1→2)-linked to monosubstituted β-D-Xyl*p* residue (B) and the signal originating from α-L-Ara*f* (1→2)-linked to disubstituted β-D-Xyl*p* residue (C) could be seen. The intensity ratio deviating from 1:1 indicates the presence of α-L-Ara*f* side units (1→2)-linked to the monosubstituted β-D-Xyl*p* residue. This was verified with HPAEC-PAD after the endo-(1→4)-β-D-xylanase (Shearzyme) treatment based on α-L-Ara*f*(1→2)-xylobiose formation (**II**: Figure 2).



Symbol	Sugar residue
A	α-L-Ara*f*(1→3) mono
B	α-L-Ara*f*(1→3) di and α-L-Ara*f*(1→2) mono
C	α-L-Ara*f*(1→2) di

Relative peak intensities (%)

	WAX-HV	WAX-MV	WAX-LV	RAX-HV
A	34	35	33	66
B	33	32	40	17
C	33	33	27	17

Figure 14. The α-anomeric region of the ^{1}H NMR spectrum for WAX-MV and the relative intensities (%) for WAX-HV, WAX-MV, WAX-LV, and RAX-HV measured from the peak integrals for the α-anomeric protons of α-L-Ara*f* units.

The hypothetical structures of wheat and rye arabinoxylans, based on the ratio of β-D-Xyl*p* and α-L-Ara*f* together with relative peak intensities of each sugar residue from NMR spectroscopy, are illustrated in Figure 15.

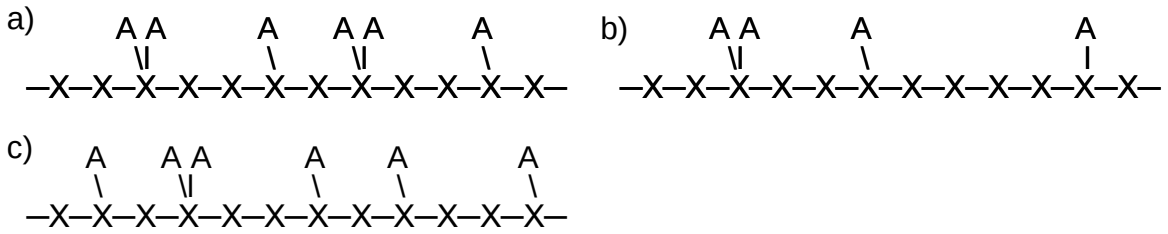


Figure 15. The hypothetical structures for a) WAX-HV and WAX-MV, b) WAX-LV, and c) RAX-HV. A = α-L-Ara*f*, X = β-D-Xyl*p*.

5.4 Effect of enzymatic modifications on the chemical structures of wheat and rye arabinoxylans (III)

The structures of wheat and rye arabinoxylans (WAX-HV and RAX-HV) were specifically altered using two α-L-arabinofuranosidases: AXH-d3 and AXH-m. The enzyme actions are clearly seen in the ^{1}H NMR spectra (Figure 16). When the adjacent α-L-Ara*f* (1→3) substituent is released from the disubstituted β-D-Xyl*p* residue as a result of AXH-d3 modification, the resonance of the remaining (1→2)-linked α-L-Ara*f* substituent (originally signal C at 5.22 ppm) shifts to 5.28 ppm (signal B) (Figure 16 b, e). The intensity of signal C decreased significantly as a result of treatment with the AXH-d3 enzyme, and the modified sample, thus, contained α-L-Ara*f* units in the monosubstituted β-D-Xyl*p* residues. The AXH-m acted on the α-L-Ara*f* units attached to the monosubstituted β-D-Xyl*p* residue, which can be seen as a decrease in signal A originating from the (1→3)-linked α-L-Ara*f* unit in the monosubstituted β-D-Xyl*p* residue (Figure c, f). Modification with AXH-m resulted in a xylose chain with disubstituted xylose units.

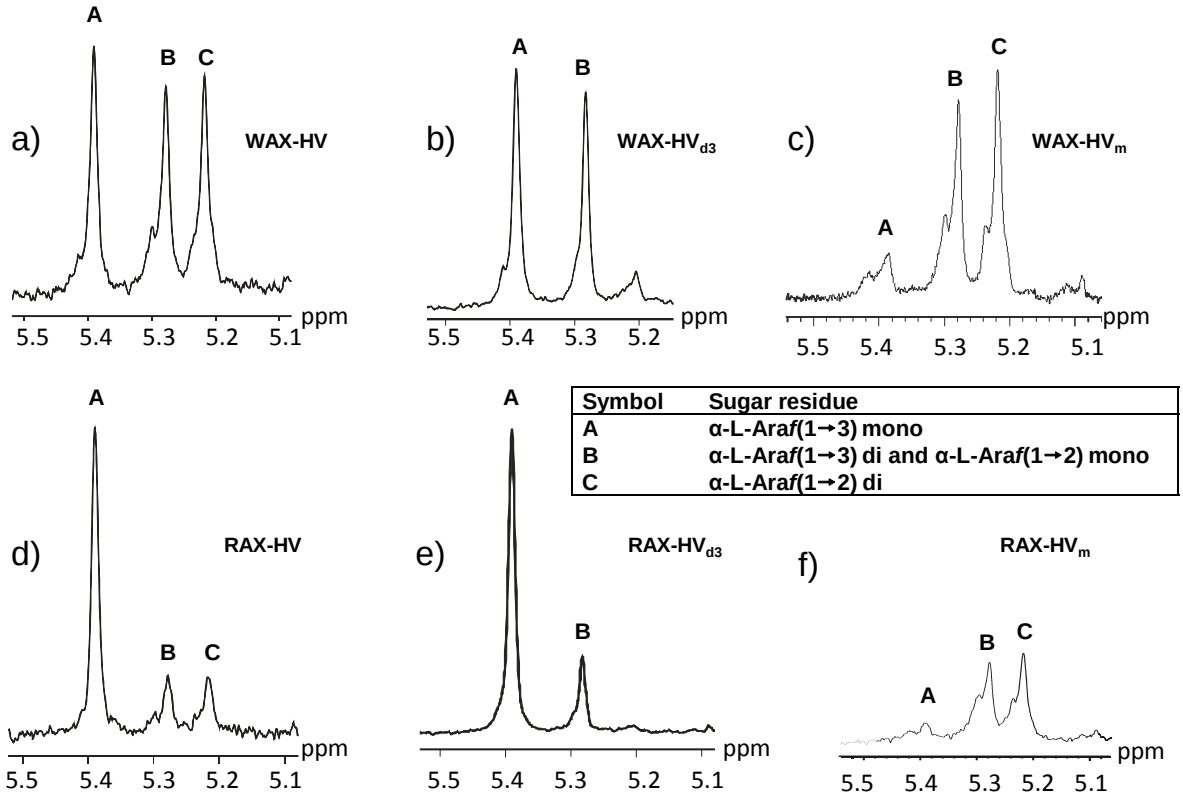


Figure 16. α-Anomeric region of the ^{1}H NMR spectra for unmodified (WAX-HV and RAX-HV) and enzymatically modified (WAX-HV$_{d3}$, WAX-HV$_m$, RAX-HV$_{d3}$, and RAX-HV$_m$) arabinoxylans from wheat and rye.

The change in the arabinose-to-xylose ratios caused by the enzymatic modifications to the wheat and rye arabinoxylans can be seen in Table 14. The proportion of disubstituted β-D-Xyl*p* residues from all substituted β-D-Xyl*p* residues was 66%, and that of monosubstituted was 34% in WAX-HV, whereas the situation was the opposite in RAX-HV (see section 5.3). Thus, the AXH-d3 enzyme worked efficiently, and the amounts of released α-L-Ara*f* units from the initial arabinose content are feasible (Table 14). The AXH-m enzyme was not as efficient as AXH-d3, and some of the α-L-Ara*f* units in monosubstituted β-D-Xyl*p* residues was not released by the enzyme. During the AXH-m treatment, the intensive removal of α-L-Ara*f* resulted in precipitation of modified RAX-HV that may cause restricted accessibility of the residual α-L-Ara*f* units to the enzyme.

Table 14. Amounts of released arabinose after enzymatic treatments calculated from the initial arabinose (% of weight) content, arabinose-to-xylose ratios (Ara:Xyl), and contents of the mono-, di-, and unsubstituted β-D-Xyl*p* residues for the unmodified (WAX-HV and RAX-HV) and enzymatically modified (WAX-HV_{d3}, WAX-HV_m, RAX-HV_{d3}, and RAX-HV_m) wheat and rye arabinoxylan samples.

	Amount of released α-L-Ara*f* from initial arabinose content (%)[a]	**Ara:Xyl[b]**	**Content of mono-, di-, and unsubstituted β-D-Xyl*p* residues (%)[c]**		
			Mono	**Di**	**Un**
WAX-HV		0.51	17	17	66
WAX-HV_{d3}	35	0.33	33	0	67
WAX-HV_m	28	0.37	3	17	80
RAX-HV		0.50	33	9	58
RAX-HV_{d3}	18	0.41	41	0	59
RAX-HV_m	44	0.28	11	9	80

[a]Based on the quantification of released arabinose compared to the arabinose content of unmodified WAX-HV and RAX-HV.
[b]Calculated for enzymatically treated samples by subtracting the content of the released arabinose from the initial arabinose content and assuming that the xylose content remains unaltered in the modifications.
[c] Calculated from the NMR spectroscopic data presented in Figure 14 together with arabinose-to-xylose ratios.

5.5 SEC analysis of arabinoxylans

5.5.1 Barley arabinoxylans (I)

The SEC chromatograms for barley arabinoxylans overlaying the RI and UV signals and molar mass are shown in Figure 17. Both samples, BHAX and BFAX, contained some protein and lignin remains that elute at higher elution volumes according to the UV responses. The peak shapes of the refractometric and viscometric signals (Figures 17 and 18) were asymmetric, which was probably due to the heterogeneous nature of the extracted samples. The heterogeneity of the samples could also be seen as high dispersity index values (Table 15). The molar mass and average intrinsic viscosity for BFAX were higher than for BHAX (Figure 17, Table 15). The higher intrinsic viscosity at lower retention volumes for BHAX may result from the lower degree of branching (and further lower density) compared with BFAX (Figure 18). The recovery value calculated from the RI signal for BHAX was 62% and for BFAX 72%.

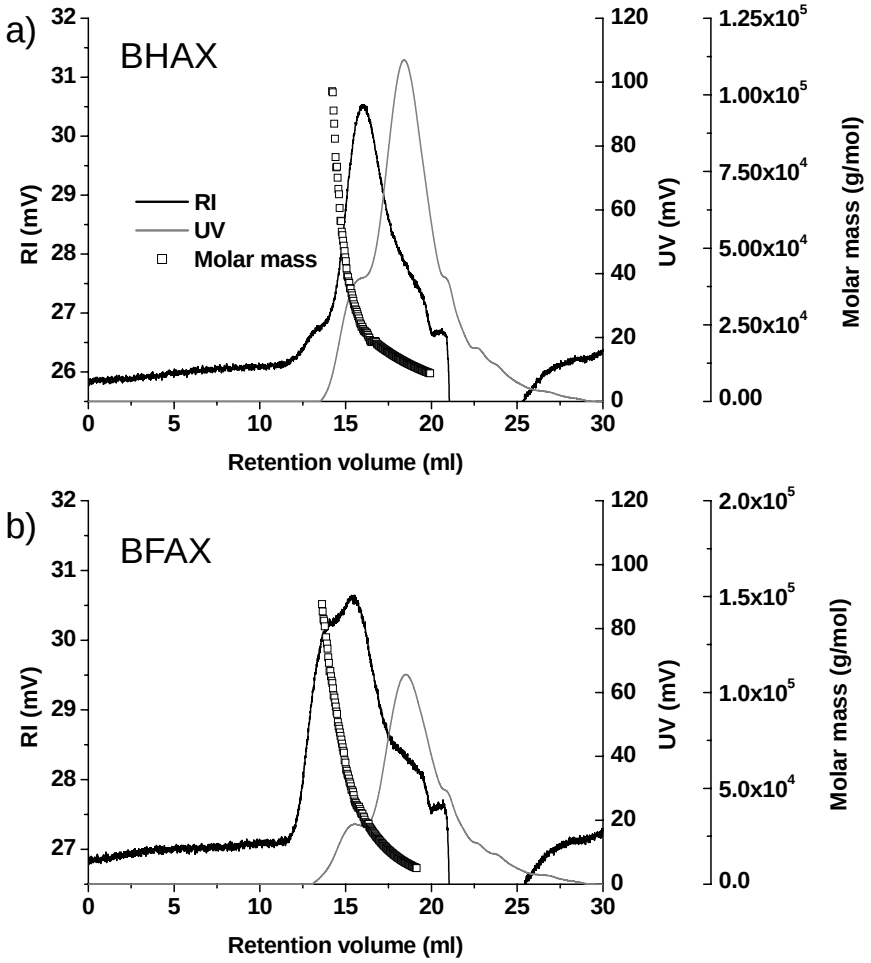


Figure 17. SEC chromatograms for a) barley husk arabinoxylan (BHAX) and b) barley fiber arabinoxylan (BFAX) analyzed in DMSO containing 0.01 M LiBr. RI = black line, UV= gray line, molar mass = open squares.

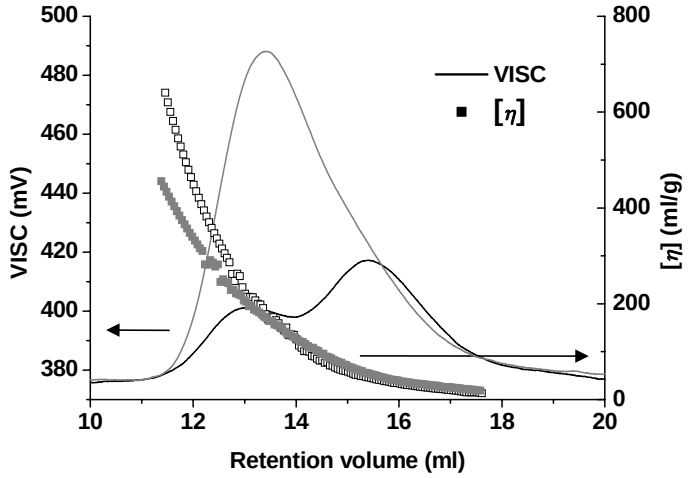


Figure 18. Viscosity signal and intrinsic viscosity ([η]) for barley husk arabinoxylan (black line and black open squares) and barley fiber arabinoxylan (gray line and gray solid squares) from SEC in DMSO containing 0.01 M LiBr.

Table 15. SEC data (molar mass averages, dispersity index, average intrinsic viscosity, average viscometric radius, and recovery) for barley arabinoxylans.

	BHAX	**BFAX**
$M_w \times 10^{-5}$ (g/mol)	0.49	0.81
$M_n \times 10^{-5}$ (g/mol)	0.15	0.34
M_w/M_n	3.3	2.4
[η] (ml/g)	45	73
R_η (nm)	6.0	8.9
Sample recovery (%)	62	72

5.5.2 Unmodified and enzymatically tailored wheat and rye arabinoxylans (II-III)

In study **II**, the detailed solution characterization of three commercial wheat arabinoxylans and one rye arabinoxylan was accomplished using organic and aqueous eluent. The α-L-Ara*f* substitution pattern of one wheat arabinoxylan sample and one rye arabinoxylan sample was selectively modified with enzymes in order to get further information on the role of side units in the solution behavior of arabinoxylans, and those results were reported in study **III**. This section summarizes the main results of studies **II-III**; more data are presented in the original papers.

The average molar masses and recovery values for wheat and rye arabinoxylans are presented in Table 16. The molar mass for the high viscosity sample (HV) of wheat arabinoxylan was

the highest and the low viscosity sample (LV) the lowest as expected. The average intrinsic viscosity for arabinoxylans ranged from 80 ml/g to 350 ml/g and the average viscometric radius from 8 nm to 25 nm. All samples were soluble in both eluents used, seen as SEC recovery values ranging from 66% to 105%.

Table 16. Molar mass averages, dispersity indices, average intrinsic viscosities, average viscometric radii, and recoveries of cereal arabinoxylans from SEC.

Sample	$M_w \times 10^{-5}$ **(g/mol)**	$M_n \times 10^{-5}$ **(g/mol)**	M_w/M_n	$[\eta]$ **(ml/g)**	R_η **(nm)**	**Recovery %**
H_2O + 0.1 M $NaNO_3$						
WAX-HV	3.36	2.10	1.6	350	25	86
WAX-MV	2.35	1.32	1.8	270	21	105
WAX-LV	0.83	0.35	2.4	100	10	73
RAX-HV	2.89	1.85	1.6	370	25	66
DMSO + 0.01 M LiBr						
WAX-HV	3.19	2.68	1.2	320	25	73
WAX-MV	2.14	1.78	1.2	230	19	89
WAX-LV	0.49	0.30	1.6	80	8	69
RAX-HV	2.46	2.10	1.2	310	23	68

In general, all molar masses obtained with the aqueous SEC system were higher compared with values from the DMSO-based system. Especially in the case of WAX-LV, the difference was remarkable, which can also be seen in the overlaid molar mass distributions (Figure 19). The reason for this difference was aggregation. The WAX-LV sample with lower side unit content but low molar mass formed aggregates in aqueous solution, whereas the aggregation tendency seemed to be lower in DMSO.

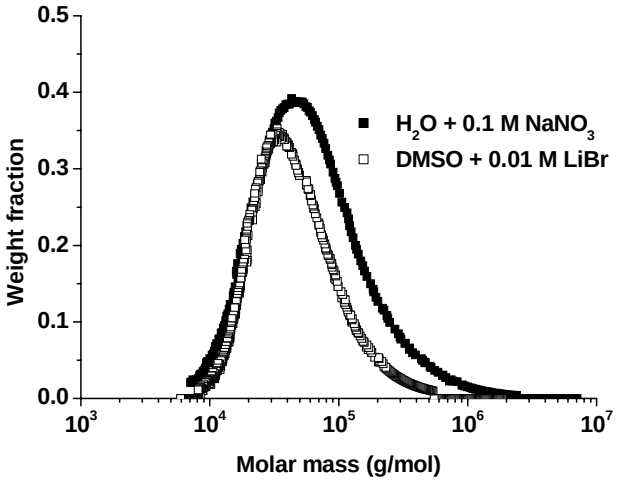


Figure 19. Molar mass distributions for the WAX-LV sample analyzed in water containing 0.1 M $NaNO_3$ (solid squares) and in DMSO containing 0.01 M LiBr (open squares).

Although the arabinose-to-xylose ratio of wheat and rye arabinoxylans (WAX-HV and RAX-HV) was similar (0.5), the α-L-Ara*f* distribution along the β-D-Xyl*p* backbone was different. Wheat arabinoxylan contained more disubstituted β-D-Xyl*p* residues and fewer monosubstituted β-D-Xyl*p* residues (Table 14). The situation in rye arabinoxylan was reversed (see paragraph 5.3). Due to differences in the content of the mono- and disubstituted β-D-Xyl*p* residues between WAX-HV and RAX-HV, the content of the unsubstituted β-D-Xyl*p* residues was different, being lower for RAX-HV than for WAX-HV (Table 14). The conformation plots, in which the viscometric radius (R_η) is plotted against the molar mass, are presented in Figure 20. Only a small difference between the structurally different wheat and rye was observed.

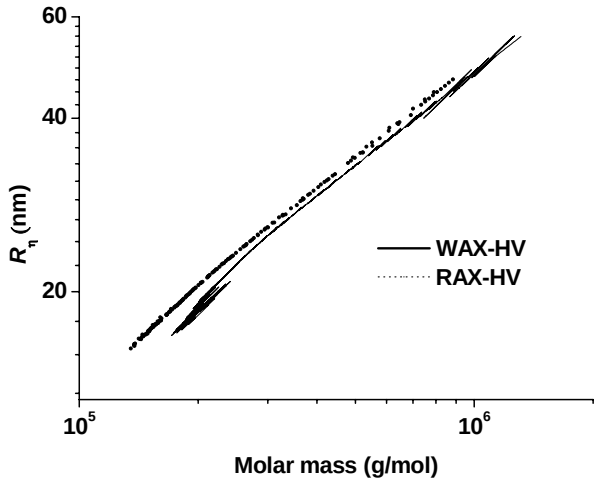


Figure 20. The relationship between the viscometric radius and the molar mass for WAX-HV (conventional line) and for RAX-HV (dotted line).

To get deeper insight into the role of α-L-Ara*f* substituents in the solution properties of arabinoxylans, the WAX-HV and RAX-HV samples were further modified using two α-L-arabinofuranosidases with different substrate specificities. The samples were characterized with SEC directly after modifications in aqueous solution and in DMSO after dialysis, freeze drying, and redissolution. Removing side units decreased the molar mass as expected in the case of AXH-d3 modification (Figure 21, Table 17). In Table 17, the theoretical molar masses are presented for comparison beside the experimental values. The theoretical M_w values for the modified samples were calculated using the M_w of the control sample, the α-L-Ara*f* content (weight %) of a sample before modification, and the content of the released α-L-Ara*f* in the modifications. The experimental and theoretical M_w values for the AXH-d3 modified samples corresponded well. The experimental molar masses for the AXH-m modified samples in DMSO, on the other hand, were lower than expected from the mass of the removed α-L-Ara*f* units. The most likely explanation for this result is the decrease in the molecular density of arabinoxylan chains after the AXH-m treatment and thus coelution of high-molar-mass species with lower-molar-mass molecules. Incomplete resolution of AXH-m treated arabinoxylans can be seen from the intrinsic viscosity, which was constant over the whole peak (Figure 21 f).

Most of the RAX-HV_m sample precipitated in buffer solution during the extensive removal of α-L-Ara*f* units from the monosubstituted β-D-Xyl*p* residues with the AXH-m enzyme. This was also observed as low recovery values in aqueous SEC (Table 17). According to the recovery values, all samples were well soluble in DMSO, including the part that precipitated in an aqueous environment.

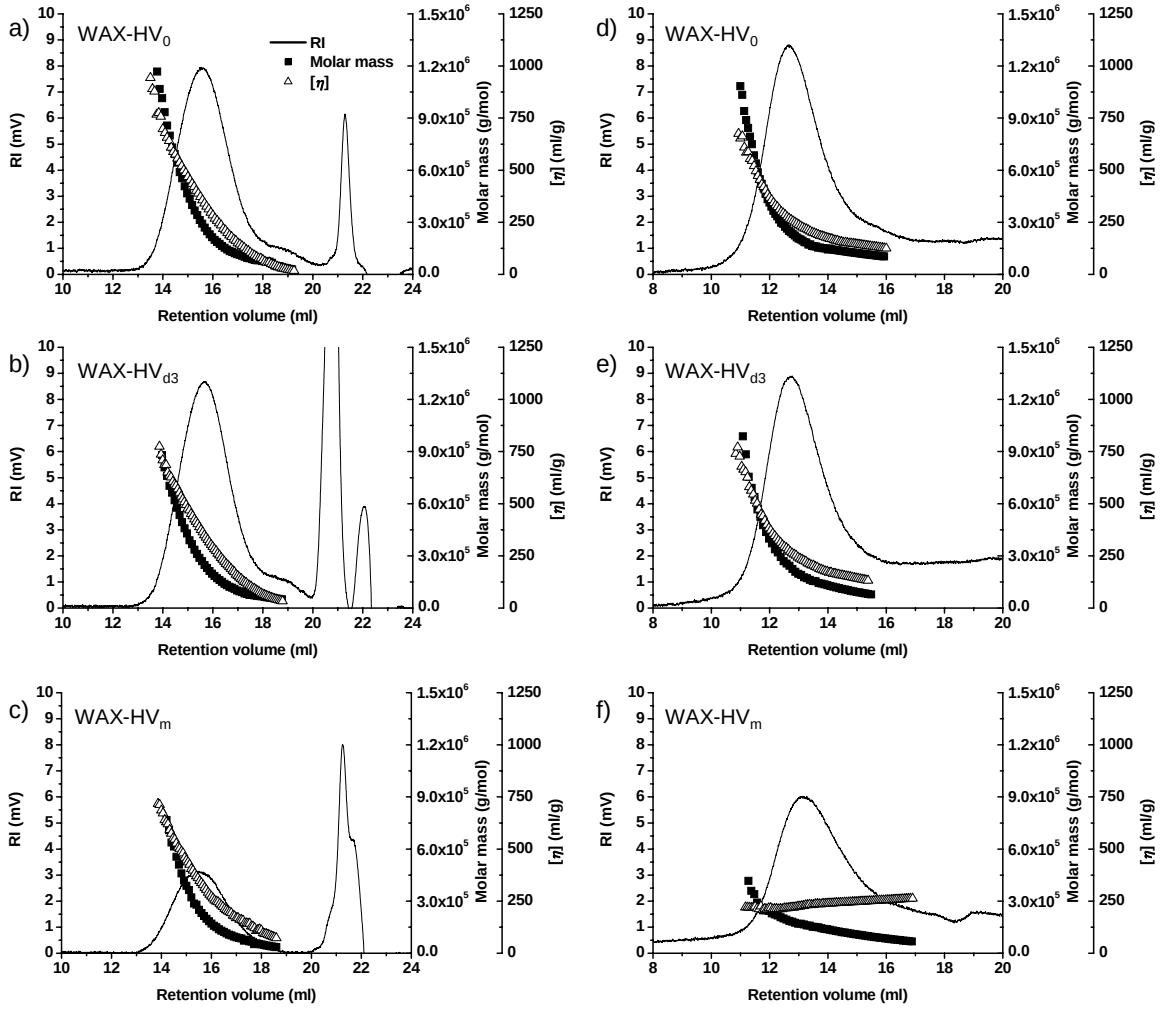


Figure 21. SEC elution profile (RI, black line), molar mass (solid square), and intrinsic viscosity (open triangle) for unmodified and enzymatically treated WAX-HV samples in water containing 0.1 M $NaNO_3$ (a-c) and DMSO containing 0.01 M LiBr (d-f).

Table 17. Weight-average molar masses (M_w), intrinsic viscosities [η], and recoveries obtained from the SEC analyses for the arabinoxylan samples.

	H_2O + 0.1 M $NaNO_3$[a]				DMSO + 0.01 M LiBr			
	$M_w \times 10^{-5}$ (g/mol)	$M_w \times 10^{-5}$ estimated[b]	[η] (ml/g)	recovery[c] %	$M_w \times 10^{-5}$ (g/mol)	$M_w \times 10^{-5}$ estimated[b]	[η] (ml/g)	recovery %
WAX-HV_0	3.59		360	80	2.86		270	85
WAX-HV_{d3}	3.15	3.28	360	80	2.62	2.61	280	80
WAX-HV_m	3.22	3.34	360	33	1.57	2.66	230	87
RAX-HV_0	2.96		340	82	2.55		270	90
RAX-HV_{d3}	2.82	2.80	350	79	2.41	2.41	270	80
RAX-HV_m	2.49	2.58	270	12	1.81	2.22	240	91

[a]Analyzed directly after enzymatic treatment without drying the sample.
[b]Estimated for enzymatically modified samples from the mass of released arabinose units.
[c]Only the polymer peak was taken into account; the recovery values cannot reach 100% because the liberated arabinose units were eluted separately from the polymers.
WAX-HV_0 and RAX-HV_0 are blank samples without added enzymes.

The removal of the (1→3)-linked α-L-Ara*f* units from the disubstituted β-D-Xyl*p* residues had no effect on the solution density of the arabinoxylans (Figure 22). The increase in intrinsic viscosity with increasing molar mass was similar in the unmodified and AXH-d3 treated samples regardless of the solvent. In the case of the AXH-m treated samples, straightforward conclusions were difficult to draw. Most of the sample precipitated during the enzymatic modification, and thus the results obtained in aqueous solution represent only the minor population of the molecules. In DMSO, the incomplete separation clearly affected the results.

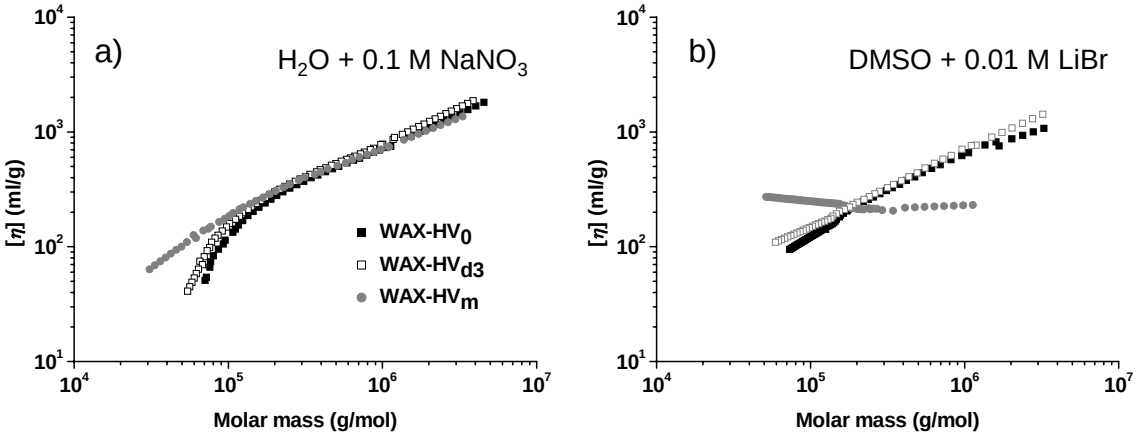


Figure 22. Mark-Houwink plots for WAX-HV samples in a) water containing 0.1 M $NaNO_3$ and b) in DMSO containing 0.01 M LiBr. Solid, black squares represent WAX-HV_0, open squares WAX-HV_{d3}, and gray solid circles the WAX-HV_m sample.

5.6 AsFlFFF analysis of wheat arabinoxylans (IV)

Based on the characterization of wheat and rye arabinoxylans with SEC using aqueous and DMSO-based solvents, the aqueous arabinoxylan solutions were thought to contain aggregates. The same samples were also analyzed with aqueous AsFlFFF to see the behavior of samples in a different separation system.

To find the optimal separation conditions, different cross-flow profiles were tested. Because polysaccharides have low diffusion coefficients (Brandrup et al. 1998), i.e., they diffuse slowly, decaying cross-flow gradients were used. Figure 23 shows the molar mass distribution for the WAX-MV sample obtained with linearly and exponentially decaying cross-flow. When the linear cross-flow gradient was used, the high-molar-mass tail in the distribution was observed. This was due to incomplete separation of aggregates from the individual chains eluting before aggregates. In the fractogram obtained with exponentially decaying cross-flow, two molecular populations were clearly observed especially in the light-scattering signal (Figure 24), and aggregates could be excluded in the molar mass and size calculations.

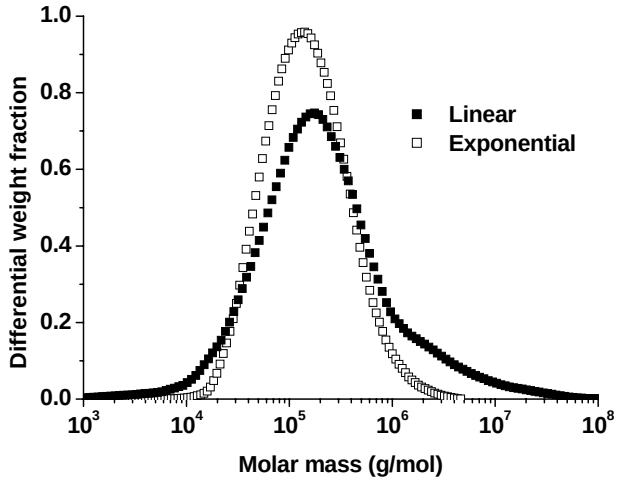


Figure 23. The effect of the cross-flow gradient on the molar mass distribution of the WAX-MV sample. The solid squares represent the distribution obtained with linear decay of cross-flow and open squares with exponentially decaying cross-flow. In the case of exponentially decaying cross-flow, only the first peak from the fractogram presented in Figure 24 has been included in the molar mass distribution.

The M_w value obtained for the first peak in the WAX-MV fractogram was very close to the value obtained for the same sample in DMSO using SEC (Figure 24, Table 16). These

observations confirmed the presence of aggregates in aqueous arabinoxylan solutions and led to the conclusion that in AsFlFFF, arabinoxylan aggregates are resolved better from the completely dissolved individual chains compared with SEC when the exponential decay of cross-flow is used (**IV**: Figure 3).

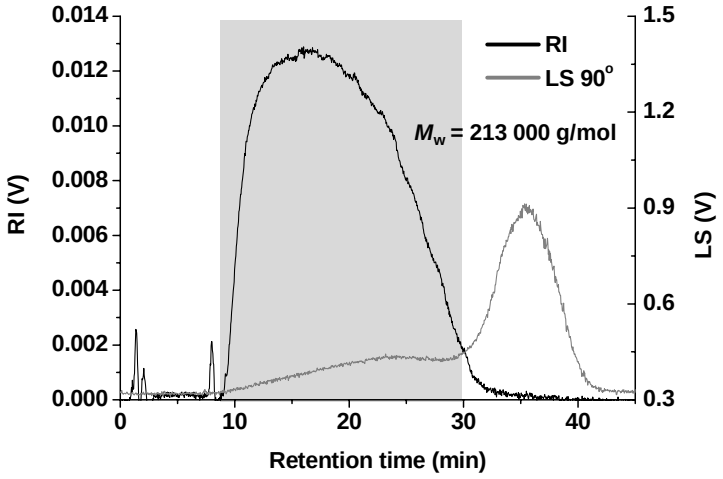


Figure 24. AsFlFFF fractogram for the wheat arabinoxylan sample (WAX-MV) obtained using exponentially decaying cross-flow. RI = black line, LS at 90° = gray line. Weight-average molar mass (M_w) is defined for the first peak of the fractogram shaded in light gray.

5.7 Solution characterization of enzymatically modified galactomannans (V)

The enzymatically modified guar galactomannan sample set was characterized with SEC and AsFlFFF using aqueous eluent in order to study the effect of α-D-Gal*p* side units and mannan chain length on the solution behavior of galactomannans. The commercial galactomannan from guar was treated with endo-(1→4)-β-D-mannanase and further with α-D-galactosidase to form a series of samples with different chain lengths and degrees of substitution. The higher molar mass of guar galactomannan compared with arabinoxylans enabled the modification of DP in addition to modification of DS. The chain length–reducing effect of mannanase was evident accompanied by increased polydispersity when higher enzyme dosages were used (Figure 25). As galactomannans are large, the samples were also analyzed with AsFlFFF, which is especially suitable for separating large polymers and particles, besides SEC. The separation was not, however, affected by the method as seen in the similar molar mass distributions (some lower molar mass material went through the membrane and thus was not seen in the distribution of AsFlFFF) presented for the unmodified 6Gal-H

sample and the 4Gal samples (Figure 24), and both SEC and AsFlFFF could be considered applicable for separating galactomannans. The apparent polymodal nature of distributions obtained with AsFlFFF probably resulted from the low signal intensities (especially RI), that causes the noise to the distributions. One concern in the AsFlFFF analysis was the low recovery values compared with SEC. The recovery values calculated from the RI signal in AsFlFFF were below 60% for all the galactomannan samples using regenerated cellulose (RC) and polyethersulfone (PES) membrane whereas the recoveries in SEC were above 60% for all samples except for the mainly insoluble 2Gal samples (Table 18).

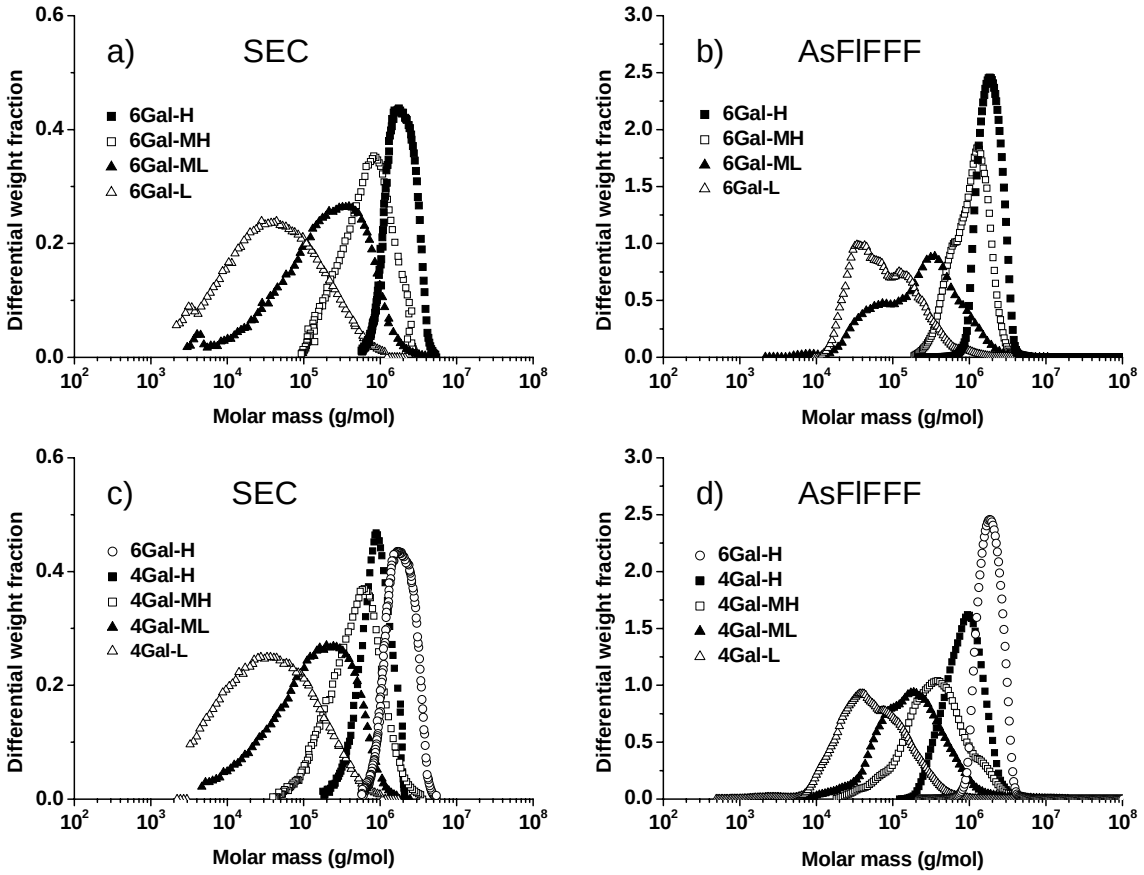


Figure 25. Molar mass distributions for 6Gal samples obtained with a) SEC and b) AsFlFFF and for 4Gal samples obtained with c) SEC and d) AsFlFFF. The unmodified 6Gal-H sample is presented together with the 4Gal samples to show the significant difference between the molar masses of the 6Gal-H and 4Gal-H samples.

Table 18. Comparison of recovery values from SEC and AsFlFFF with different membrane materials, regenerated cellulose (RC) and polyethersulfone (PES).

Sample	Recovery % SEC	Recovery % AsFlFFF RC	Recovery % AsFlFFF PES
6Gal-H	65	61	44
6Gal-MH	78	30	39
6Gal-ML	103	55	47
6Gal-L	86	37	37
4Gal-H	70	38	43
4Gal-MH	73	29	40
4Gal-ML	86	51	41
4Gal-L	79	31	32
2Gal-H	32	16	16
2Gal-MH	31	5	17
2Gal-ML	38	20	21
2Gal-L	68	23	23

Weight-average molar masses and intrinsic viscosities for galactomannan samples obtained with SEC are presented in Table 19. The remarkable difference in molar mass between unmodified (6Gal-H) and the sample with no mannanase treatment and only slight α-galactosidase treatment (4Gal-H) could not be caused by the mass loss of removed α-D-Gal*p* units in the 4Gal-H sample, which was around 9% when calculated from the amount of released α-D-Gal*p*. Because the intrinsic viscosity between 6Gal-H and 4Gal-H differed only slightly, the 6Gal-H sample most probably contained some aggregated molecules. In general, the molar masses for the 6Gal samples were higher than for the 4Gal samples, but the intrinsic viscosities were similar. Thus, partial debranching of galactomannans seemed to prevent aggregate formation.

Table 19. Weight-average molar masses (M_w) and intrinsic viscosities ([η]) obtained with SEC for enzymatically modified galactomannans.

Sample	$M_w \times 10^{-5}$ (g/mol)	[η] (ml/g)
6Gal-H	18.4	1050
6Gal-MH	8.56	600
6Gal-ML	3.45	300
6Gal-L	1.00	110
4Gal-H	9.33	890
4Gal-MH	5.96	610
4Gal-ML	2.41	310
4Gal-L	0.83	130
2Gal-H	3.76	510
2Gal-MH	2.26	340
2Gal-ML	1.30	180
2Gal-L	2.17	120

The aggregation tendency of galactomannans is lower when the mannan chain length is reduced (Cheng et al. 2002). To investigate the effect of α-D-Gal*p* side units on the solution conformation of galactomannans, the radius of gyration of medium low (ML) samples with approximately 6 and 4 α-D-Gal*p* per ten β-D-Man units was plotted against the molar mass (Figure 26). No difference between the samples was observed, indicating the negligible effect of side units on the solution conformation.

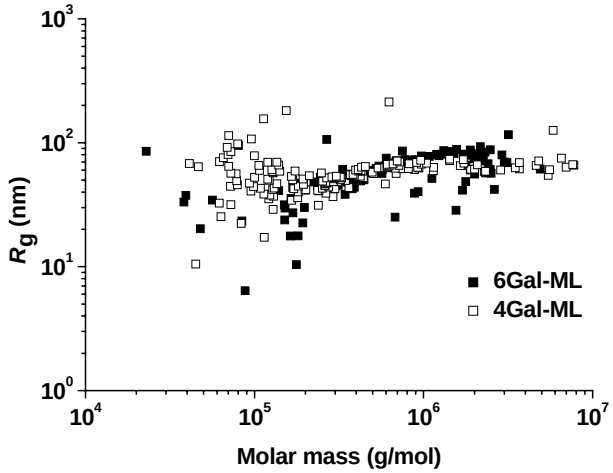


Figure 26. Comparison of the radius of the gyration–molar mass relationships for the 6Gal-ML (filled squares) and 4Gal-ML (open squares) samples. Size and molar data were obtained with AsFlFFF.

5.8 Characterization of dextrans with SEC and AsFlFFF (VI)

Structurally different dextrans from *Weissella confusa* and *Leuconostoc citreum* were characterized with SEC and AsFlFFF in order to better understand their functional properties. The dextran from *Leuconostoc citreum* is more branched than the dextran from *Weissella confusa* containing (1→2) branched linkages in addition to (1→3) branched linkages (Table 2). Most studies on the solution properties of dextrans have been concentrated mainly on characterizing dextrans from different *Leuconostoc mesenteroides* strains. The results for commercially available *Leuconostoc* spp. dextran are presented here for comparison.

The AsFlFFF elution profiles (RI), molar mass, and R_g for the dextran samples are presented in Figure 27. Both molar mass and R_g were higher for the dextran from *L. citreum* than for the dextran from *W. confusa*. Isolated dextran samples were not completely soluble in either water- or DMSO-based solvent, and removing insoluble material with filtration may have lowered the representativeness of the data.

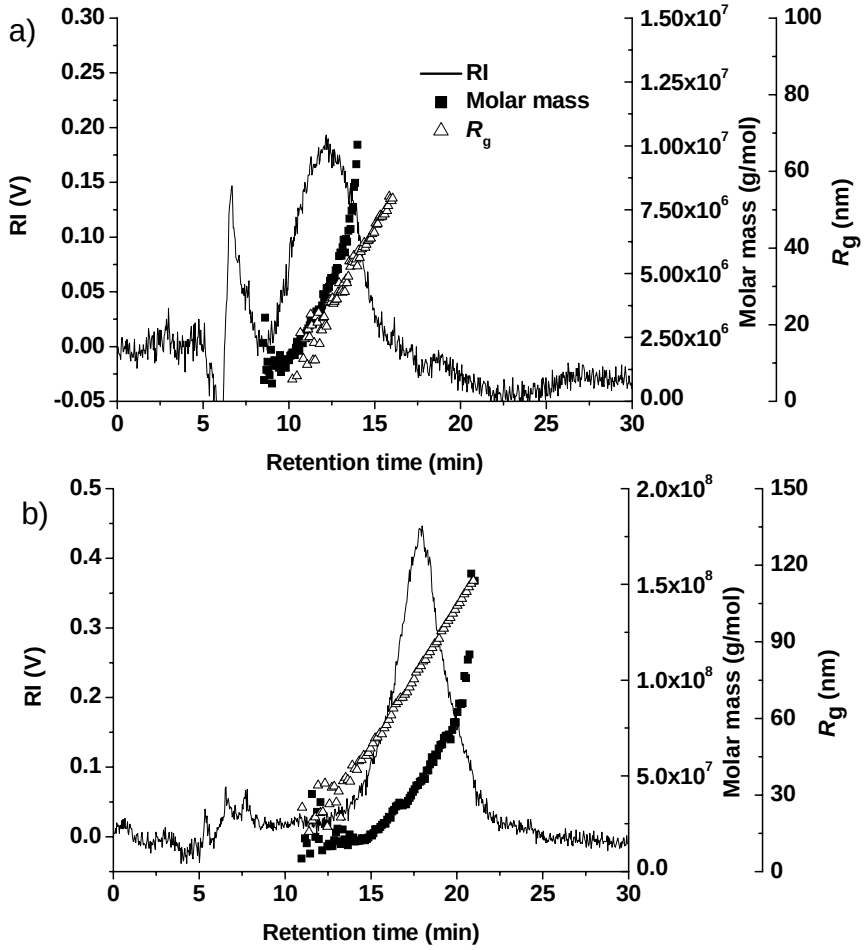


Figure 27. AsFlFFF elution profile (RI), molar mass (solid square), and radius of gyration (R_g, open triangle) for dextrans from a) *W. confusa* and b) *L. citreum*.

The M_w values for the dextrans in DMSO were significantly lower than in water (Table 20). This difference was believed to arise from the stronger aggregation tendency of dextrans in water than in DMSO. Especially for the *L. citreum* dextran, the difference between the molar masses obtained in aqueous and DMSO solution differed exceedingly. Thus, the dextran from *L. citreum* in aqueous solution seemed to be even more aggregated or the interactions between molecules in aggregates were stronger if compared with the more linear *W. confusa* and *Leuconostoc* spp. dextrans.

Table 20. Weight-average molar masses (M_w) and dispersity indices (M_w/M_n) for dextrans.

Dextran sample	DMSO + 0.01 M LiBr		H_2O + 0.1 M $NaNO_3$			
	SEC $M_w \times 10^{-6}$ (g/mol)	M_w/M_n	SEC $M_w \times 10^{-6}$ (g/mol)	M_w/M_n	AsFlFFF $M_w \times 10^{-6}$ (g/mol)	M_w/M_n
W. *confusa*	1.47	1.08	6.37	2.05	6.17	1.84
L. citreum	1.85	1.05	11.3	1.04	62.8	1.63
Leuconostoc spp.[a]	0.54	1.21	1.67	1.66	2.04	5.64

[a]M_w given by the manufacturer 2 000 000 g/mol (method not provided)

Although the molar masses for the dextran samples were lower in DMSO than in water and this difference was most likely due to aggregation of dextrans in aqueous solution, evidence of aggregation was also observed in DMSO. Figure 28 shows the RI elution profiles and molar mass for two consecutive injections of dextran from *W. confusa* analyzed using SEC in DMSO. In the case of the second injection, the molar mass has clearly increased from the first injection. This difference in molar masses suggested an unstable state of the solution.

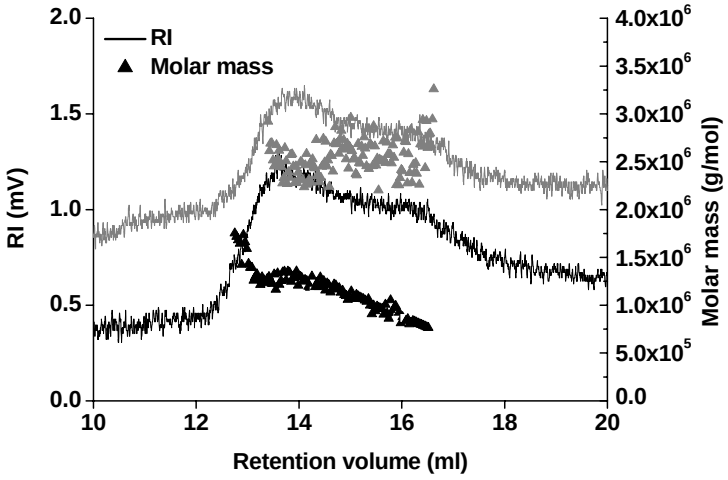


Figure 28. SEC elution profile (RI, conventional line) and molar mass (triangles) for two consecutive injections of the *W. confusa* dextran obtained with SEC in DMSO + 0.01 M LiBr. Black line and symbols correspond to the data from the first injection and the gray line and symbols to the data from the second injection, which reveals the aggregation of a sample.

6 Discussion

6.1 Structural comparison and solution properties of arabinoxylans from two barley fractions

The water-insoluble arabinoxylans from the husks and fiber of barley were isolated using selective bariumhydroxide extraction, and the structures of the extracted arabinoxylans were characterized. The focus was to analyze barley arabinoxylans with SEC and compare their solution properties. The arabinoxylan samples extracted from BF and BH represented well the arabinoxylans originally occurring on the cereal cell-walls as indicated by the similar arabinose-to-xylose ratios of BH and BF (0.25 and 0.49) and the corresponding arabinoxylans BHAX and BFAX (0.29 and 0.52, respectively). Barley husks primarily consist of cellulose, arabinoxylan, and lignin (Viëtor 1992). The main components of barley kernel are starch (49.4-66.2%), dietary fiber including arabinoxylans and (1→3)(1→4)-β-D-glucans (13.6-27.5%), and crude protein (9.3-12.9%) (Oscarsson et al. 1996). Barium hydroxide extraction was found to be specific toward arabinoxylans as reported earlier (Gruppen et al. 1991). Only a low amount of glucose-containing material, i.e., starch, (1→3)(1→4)-β-D-glucan or cellulose was coextracted.

As indicated by the arabinose-to-xylose ratios, BFAX contained significantly more α-L-Ara*f* substituents than BHAX. According to the ^{1}H NMR spectroscopy and enzyme-assisted HPAEC-PAD analysis, there was no significant difference in the content of the monosubstituted α-L-Ara*f*-(1→3)-xylopyranosyl residues between BHAX and BFAX. Thus, the higher content of α-L-Ara*f* in BFAX mainly resulted from the greater amount of doubly-substituted xylopyranosyl residues. Another clear difference identified with ^{1}H NMR spectroscopy and enzyme-assisted HPAEC-PAD analysis was in the content of the longer substituent 2-*O*-β-D-Xyl*p*-α-L-Ara*f*, which was clearly higher in BHAX than in BFAX. This structural unit has previously been identified in arabinoxylan from corn cob (Kusakabe et al. 1983), some milling fractions of rye (Nilsson et al. 1996), and barley husks (Aspinall and Ferrier 1957), but not in arabinoxylan from barley grain. The content of the xylopyranosyl residues carrying a monosubstituted α-L-Ara*f*-(1→2) substituent was low in both samples compared to the content obtained for barley and barley flours (Viëtor et al. 1992; Trogh et al. 2005).

The SEC chromatograms for both barley arabinoxylans were asymmetric, indicating the heterogeneity of the samples. In the BHAX sample, the proportion of the first eluting component was very low according to the RI and viscosity signals, whereas the BFAX sample contained more of that high viscosity component. This could clearly be seen in the molar

masses. The M_w for BHAX was lower (49300 g/mol) than for BFAX (81000 g/mol) as expected. The molar mass of arabinoxylans from the outer parts of the kernel is typically lower than the arabinoxylans from the inner parts. Alkaline-extracted oat spelt xylan, for instance, has been reported to have M_w of 21900 g/mol (Saake et al. 2001). The M_w for BHAX obtained in this study was slightly higher than that recently published for barley husks using the DMSO system and universal calibration (34300-44700 g/mol) (Höije et al. 2005). Molar mass data for alkali-extractable barley flour or whole grain could not be found in the literature. The molar masses for the water-soluble arabinoxylans of barley are higher, in the order of a few hundred thousand (Dervilly et al. 2002). The BFAX extracted in this study, however, does not represent the arabinoxylan preparation from native barley. The water-soluble part was not present in the BF material due to the separation of the water-soluble components (especially starch) in a wet-milling process. Additionally, the β-glucanase preparation, used in the Koskenkorva process to enhance the separation of starch (information from the company), may contain some xylanase side activity that can have a reducing effect on the molar mass of BFAX.

6.2 Structural features of wheat and rye arabinoxylans

The main structural features of wheat and rye arabinoxylans were determined by combining the analysis data obtained for the polymeric arabinoxylans and for the completely or partially degraded samples. The monosaccharide compositions and arabinose-to-xylose ratios of these commercial wheat and rye arabinoxylan samples have been published previously by Höije et al. (2008) and Virkki et al. (2008). Further structural information was obtained with ^{1}H NMR spectroscopy of polymeric arabinoxylans and by characterizing the oligosaccharides formed as a result of specific enzymatic hydrolysis with HPAEC-PAD.

The arabinose-to-xylose ratio for wheat and rye arabinoxylans was almost identical, approximately 0.5. The data found in the literature for extracted wheat and rye arabinoxylans are comparable with the values obtained for the commercial arabinoxylan preparations. The arabinose-to-xylose ratios of water-extractable wheat and rye arabinoxylans from endosperm are reported to range from 0.48 to 0.60 (Izydorczyk and Biliaderis 1995) although with ethanol precipitation even broader range of arabinose-to-xylose ratios can be obtained (Izydorczyk and Biliaderis 1993). According to the manufacturer, WAX-MV has been prepared from WAX-HV using enzyme hydrolysis and WAX-LV using acid hydrolysis (McCleary BV, personal communication). Because the arabinose-to-xylose ratio for WAX-LV was significantly lower (0.32) than for WAX-HV and WAX-MV, acid hydrolysis has also partially debranched WAX-LV.

The proportions of mono- and disubstituted β-D-Xyl*p* residues obtained using NMR spectroscopy corresponds well with the ones previously published for wheat (Ordaz-Ortiz and Saulnier 2005) and rye (Ragaee et al. 2001). Roughly one-third of the β-D-Xyl*p* residues in wheat are monosubstituted, and two-thirds are disubstituted with α-L-Ara*f* units. In rye, the situation is reversed: one-third of the β-D-Xyl*p* residues are disubstituted and two-thirds monosubstituted. In WAX-HV and WAX-MV, all α-L-Ara*f* units attached to the monosubstituted β-D-Xyl*p* residues were (1→3)-linked. According to the NMR spectroscopic and HPAEC-PAD data, however, WAX-LV also contained α-L-Ara*f* residues that are (1→2)-linked to monosubstituted β-D-Xyl*p* residues. As WAX-LV is prepared from WAX-HV using acid hydrolysis, it can be assumed that monosubstituted β-D-Xyl*p* units carrying (1→2)-linked α-L-Ara*f* residues are formed through cleavage of (1→3)-linked α-L-Ara*f* in the doubly substituted β-D-Xyl*p* during the acid hydrolysis and are thus not significantly present in native wheat arabinoxylan. Presumably, all the α-L-Ara*f* units in monosubstituted β-D-Xyl*p* residues in the RAX-HV sample were also (1→3)-linked as in WAX-HV and WAX-MV, since there was no indication of (1→2)-linked α-L-Ara*f*. Minor amounts of (1→2)-linked α-L-Ara*f*, however, has been reported to exist in water-soluble whole meal rye arabinoxylan (Vinkx et al. 1995).

6.3 Role of arabinose substituents in the solution properties of arabinoxylans

One of the main interests in this study was to investigate the effect of α-L-Ara*f* substituents on the solution properties of arabinoxylans using SEC with multiple-detection. For this purpose, commercial wheat and rye arabinoxylans, with high purity and lower molecular dispersity compared with extracted barley arabinoxylans, were used. As already discussed in the previous chapter, the substitution pattern of arabinoxylans from wheat and rye naturally differs. Furthermore, the α-L-Ara*f* content and substitution pattern of wheat and rye arabinoxylans (WAX-HV and RAX-HV) were modified in a controlled manner with specific α-L-arabinofuranosidases (AXH-d3 and AXH-m). The effect of α-L-Ara*f* substituents on the conformation and chain flexibility of wheat arabinoxylans in aqueous solution has been studied by Dervilly et al. (2000). They produced arabinoxylan fractions with different arabinose-to-xylose ratios using graded ethanol precipitation. Using specific enzymes, however, the fine structure of arabinoxylans could be selectively altered without changes in the length of the xylans' backbone and the influence of α-L-Ara*f* units in different positions addressed.

The comparison of unmodified wheat and rye arabinoxylans (WAX-HV and RAX-HV) revealed small differences in the arabinoxylans' solution properties. The viscometric radius/molar mass relationship proposed slightly more extended solution conformation for RAX-HV than for WAX-HV. In study **II**, the L_p values were determined for structurally different wheat and rye arabinoxylans in order to see the effect of the arabinose substitution pattern on the chain flexibility. The L_p values for RAX-HV were higher than for WAX-HV in aqueous and DMSO solution indicating slightly less flexible solution conformation of RAX-HV compared with WAX-HV (**II**: Table 5).

According to the NMR spectroscopic data and measurement of the released arabinose content, the enzymatic modifications of the WAX-HV and RAX-HV samples were successful especially in the case of the AXH-d3 treatments. AXH-d3 efficiently removed the (1→3)-linked α-L-Ara*f* from disubstituted β-D-Xyl*p* residues. Although the content of the released α-L-Ara*f* after the AXH-m treatments was somewhat below the theoretical maximums, the removal of α-L-Ara*f* from the monosubstituted β-D-Xyl*p* residues was significant enough to study the effect of the fine structure on the solution properties. As a result of the enzymatic modifications, the amount of released α-L-Ara*f* from arabinoxylan ranged from 6% to 15% (% of weight).

When the WAX-HV sample was treated with AXH-d3 and AXH-m, the total removal of the α-L-Ara*f* side groups was about the same with both enzymes (30 %). The treatment with AXH-m caused the formation of a longer unsubstituted region to the β-D-Xyl*p* backbone than AXH-d3, which removes only the other α-L-Ara*f* from the disubstituted β-D-Xyl*p* residues. The effect of AXH-d3 treatment on the solution density (intrinsic viscosity) of WAX-HV in both solvents was negligible due to the relatively even distribution of the remaining α-L-Ara*f* along the β-D-Xyl*p* backbone. The interpretation of the results, on the other hand, for the AXH-m treated sample is more difficult. First, most of the arabinoxylan sample precipitated during the modification, leaving the SEC results in aqueous solution open to doubt. Because the samples were soluble in DMSO after modification, the DMSO data can be considered more reliable. However, when the AXH-m treated WAX-HV sample was dialyzed, freeze-dried, and redissolved in DMSO, part of the higher molar mass molecules was not visible in the molar mass distribution (**IV**: Figure 2 c,d). The most likely explanation for this is the decrease in the molecular density of arabinoxylan chains as a result of the treatment. The molecular size of especially high-molar-mass molecules in solution decreased, leading to incomplete resolution of these molecular species from the ones with lower molar mass but similar hydrodynamic volume in SEC. Thus, the solution conformation of the AXH-m treated chains in DMSO seemed to be more compact compared with the untreated chains, but

investigating their properties was hindered by the coelution of molecules with different molar mass in SEC. Enzymatically treated RAX-HV behaved much the same way as WAX-HV.

Although the solution density and solution conformation were not largely affected by the arabinose substitution pattern as already proposed by Dervilly et al. (2000), the effect of the fine structure on the water solubility was more significant. Previous studies have indicated that the water solubility of arabinoxylans is decreased when the number of α-L-Ara*f* side groups is low (Andrewartha et al. 1979; Höije et al. 2008). The present study revealed the significance of the α-L-Ara*f* side group distribution. As already mentioned, both enzymes AXH-d3 and AXH-m released roughly 30% of the α-L-Ara*f* side groups from WAX-HV. After the AXH-d3 treatment, the sample stayed in solution, whereas the AXH-m modification resulted in the precipitation of the most of the sample.

6.4 Macromolecular characterization in different solvents revealed the aggregation of arabinoxylans and dextrans

The aggregation tendency of various polysaccharides in aqueous solution has been known for several decades. Polysaccharides, in general, are known not to form easily molecularly dispersed aqueous solutions, but rather have a tendency for time-dependent aggregation (Kratochvíl 1987; Burchard 2005). Evidence of aggregation of arabinoxylans in aqueous solution has earlier been reported by Ebringerova et al. (1994), but dextrans are regarded as completely water-soluble molecules. Classically, the presence of aggregates can be revealed by analyzing the same sample with different solvents using, e.g., SEC (Gidley et al. 2010).

In this study, the commercial arabinoxylans from wheat and rye were analyzed with SEC using aqueous and DMSO-based systems. Cereal arabinoxylans are typically characterized in aqueous solutions while organic solvents, such as DMSO, have been used only for analyzing xylans from the outer layers of kernels, including oat spelt xylans and arabinoxylan from barley husks with relatively low DS (Saake et al. 2001; Höije et al. 2005). The molar masses obtained in this study for unmodified and arabinose depleted arabinoxylans were systematically higher in aqueous solution than in DMSO solution, indicating the presence of aggregates in aqueous arabinoxylan solutions. The most remarkable difference in the molar masses was observed in the case of the WAX-LV sample (M_w (aq.) = 83000 g/mol and M_w (DMSO) = 49000 g/mol). The arabinose-to-xylose ratio of WAX-LV was lower than in other commercial wheat and rye arabinoxylan samples, being 0.32, and thus, the lowered content of α-L-Ara*f* side groups clearly increased the aggregation tendency. The formation of large aggregates could be also observed as a shoulder at the peak front in the light-scattering signal

of the aqueous SEC chromatogram (**II**: Figure 4 c). The presence of aggregates in the WAX-HV, WAX-MV, and RAX-HV samples could not be observed from the peak or molar mass distribution shapes obtained with aqueous SEC; the aggregation was confirmed only by comparing SEC data in aqueous and DMSO solutions. AsFlFFF analysis gave further proof of the aggregation of wheat arabinoxylans (see chapter 6.6).

In addition to arabinoxylans, the dextrans were also analyzed with SEC using aqueous and DMSO solutions. In the beginning of the study, only aqueous dextran solutions were characterized because in most of the studies found in the literature solution characterization of dextrans has been accomplished in aqueous conditions. The solubility of the dextrans from *W. confusa* and *L. citreum* in water, however, was clearly below 50% (recovery on the RI of SEC), and thus, the samples were analyzed in DMSO for comparison. As for arabinoxylans, the molar masses for the dextrans from *W. confusa* and *L. citreum* as well as for the commercial dextran standards (**VI**: Table 1 and 3) were higher in water than in DMSO, the difference being significant for isolated high-molar-mass dextrans. In principle, three different possibilities might lead to this considerable difference in molar masses. First, and most likely, is the formation of compact aggregates in aqueous solutions of high-molar-mass dextrans. The second explanation is chain degradation during SEC analysis in DMSO solution. The degradation of alternan has recently been proposed during SEC analysis in DMSO (Striegel et al. 2009a). To see the effect of separation, the dextran sample from *W. confusa* was analyzed with off-line DLS in DMSO, and no significant difference between the number-average R_h from DLS (22 nm) and the R_η from SEC (24 nm) was observed, thus indicating that degradation during SEC was not a likely explanation. Finally, some high-molar-mass material might have been retained in the syringe filters and/or in-line filters and thus decreased the observed molar masses. As in the case of arabinoxylans, excluding WAX-LV, the aggregation of dextrans in aqueous solution could not have been predicted from the SEC peak shape or shape of molar mass distribution. Interestingly, even though the aggregation tendency was much higher in water, the SEC results for *W. confusa* showed that aggregation may also occur in DMSO solution of high-molar-mass dextrans. Pullulan standards, which are α-glucans consisting of maltotriose units, were used as the calibration of LS/VISC and RI detectors in SEC, and no difference in molar masses was observed whether the analysis was accomplished in aqueous or DMSO-based solvent.

6.5 Effect of degree of polymerization and degree of substitution on the solution properties of galactomannans

The commercial guar galactomannan sample set, which was produced by treatments with endo-(1→4)-β-D-mannanase and further with α-D-galactosidase to form samples with different chain length and degree of substitution, was characterized using SEC and AsFlFFF. The approximate number of α-D-Gal*p* side units affected the solubility of galactoctomannan. The low recovery values from SEC and AsFlFFF indicated that the samples with only two α-D-Gal*p* units per ten β-D-Man*p* units were mostly insoluble. An exception to this was the 2Gal-L sample with the shortest chain length. Although most of the sample was in solution, it contained large aggregates seen as a sharp pre-peak in the light-scattering signal of the SEC chromatogram (**V**: Figure 2a) and as the higher M_w value when compared to the other low-molar-mass samples. The samples with higher α-D-Gal*p* (the 6Gal and 4Gal samples) content could be regarded as soluble in water containing 0.1 M $NaNO_3$. The evidence of the reduced solubility of galactomannans with low DS was reported several decades ago (Dea and Morrison 1975).

The aggregation of native guar galactomannan has been reported before by Picout et al. (2001) and Cheng et al. (2002). Their data suggest that aggregation tendency is lower when the DP is lowered. The results of this study, however, took into account the DS in addition to the DP, and interestingly, partial debranching of native guar galactomannan was found to decrease the aggregation tendency. The galactomannan samples with approximately four α-D-Gal*p* side units per ten β-D-Man*p* units were clearly less aggregated than the samples with six α-D-Gal*p* side units per ten β-D-Man*p* units. Generally, the higher DS of β-(1→4)-linked polysaccharides, such as galactomannans and arabinoxylans, is related to high water solubility, whereas the debranched backbones are regarded as water-insoluble.

As already discussed in chapter 6.5, the most common way to reveal aggregation is to characterize the sample in different solvents and compare the molar mass data. In addition to water containing 0.1 M $NaNO_3$, the solubility of galactomannans was tested in DMSO and dimethylacetamide (DMAc) containing LiBr, and DMSO:H_2O (90:10) containing LiBr, but they were not soluble in any of the tested solvents except the aqueous $NaNO_3$ solution. Detecting aggregates was thus complicated by the solubility of galactomannans solely in aqueous solution. In fact, the aggregation of the unmodified galactomannan sample was not unambiguously revealed by the SEC and AsFlFFF analyses, but only after the molar mass and $[\eta]$ data for the unmodified and enzymatically modified samples were compared. Aggregates, if present only in low quantities, strongly affect molar mass obtained with light scattering, whereas the contribution to the $[\eta]$ is smaller (Kratochvíl 1987). In this study, the molar

masses for the 6Gal samples were higher than for the 4Gal samples, but the average [η] values for the samples with similar DP varied only slightly. Thus, in addition to the enzymatic modification, an on-line viscometer coupled with SEC proved to be an invaluable tool for revealing aggregation.

The effect of the α-D-Gal*p* side units on solution conformation has not been studied before. According to the R_g/molar mass relationships for the samples with different numbers of side units, solution conformation was not affected by the side unit content. Similar results were obtained for arabinoxylans containing short-chain branches (chapter 6.5).

6.6 Comparison of two separation methods: SEC and AsFlFFF

In this study, commercial wheat arabinoxylans (WAX-MV, WAX-LV), enzymatically modified galactomannans, and structurally different dextrans were characterized in aqueous solution using SEC and AsFlFFF. The use of AsFlFFF was reasonable especially for galactomannans and dextrans with high molar mass. Galactomannans are also large in size. The AsFlFFF could not be used with DMSO due to the lack of DMSO-compatible membrane materials. In addition, very poor signal-to-noise ratios in RI detection have been reported for AsFlFFF analysis of starch in DMSO with LiBr (Gilbert 2011). Polysaccharides are not very "visible" in DMSO, i.e., their d*n*/d*c* values are relatively low. Thus, because the sample dilutes in the AsFlFFF channel, and in some cases, part of the sample may go through the membrane, the poor signal-to-noise ratios can be a serious problem in AsFlFFF compared with SEC when polysaccharide samples are characterized in DMSO solutions.

For the enzymatically modified galactomannans, the SEC and AsFlFFF data were consistent. Differences, on the other hand, were observed for the wheat arabinoxylans and dextrans. The light-scattering signals in the AsFlFFF fractograms of the arabinoxylans were clearly divided into two peaks. The latter peak consisting of large material was attributed to aggregates. A similar aggregate peak was observed in the fractogram of the *W. confusa* dextran. The corresponding peaks in SEC had only one apex. In the case of the dextran from *L. citreum*, the observed molar masses from AsFlFFF were higher than from SEC. Thus, discrepancies in the results depending on the analysis method were seen for arabinoxylans and dextrans.

In SEC, the separation takes place in columns, whereas in AsFlFFF the analytes are separated in an open channel. The pressure is therefore commonly higher in SEC analysis than in AsFlFFF analysis. In addition, undesired interactions between the column matrix and the sample components may occur. Non-ideality effects in SEC might induce coelution of large

aggregates together with single molecules. The entanglement of highly branched polystyrene to the column packing material has been shown to occur, seen as abnormal elution behavior in conformation plots presenting the radius of gyration against molar mass (Podzimek et al. 2001). Retardation of branched molecules caused the plot to turn upwards in the low molar mass range. In the case of the arabinoxylan samples, which contained a small number of large aggregates, the anchoring of aggregates in the column might have caused the differences in the SEC and AsFlFFF elution profiles. The molar mass difference of the aggregated dextran from *L. citreum* was more likely caused by the flow-induced degradation of aggregates (disaggregation) during SEC analysis. Another explanation could be the formation of aggregates during focusing in AsFlFFF.

Comparing the SEC and AsFlFFF data in respect of the separation mechanisms is not unambiguous. In both techniques, the separation is based on the hydrodynamic volume V_h (Jones et al. 2009). The relationship of V_h to the actual molecular structure, however, is different in each separation technique. In SEC, according to the theory of universal calibration, V_h is proportional to the product of the weight-average intrinsic viscosity and M_n (Hamielec and Ouano 1978). In AsFlFFF, the separation is based on the center-of-mass diffusion coefficient and further R_h by the Stokes-Einstein equation (Giddings et al. 1976). Although the definitions for different separation parameters are established, their reciprocal relationship is not well discussed. In addition, publications comparing AsFlFFF and SEC data are few. Rolland-Sabate et al.'s (2011) recent paper describes the characterization of starches with AsFlFFF and the combination of hydrodynamic and size-exclusion chromatography (HDC-SEC). In HDC-SEC, the analytes flow in the interstitial volume of a column packed with very small pore material and elution order is similar to that in SEC (Brewer and Striegel 2009). They discovered that the separation range in AsFlFFF was larger than in HDC-SEC and proposed high local dispersity in HDC-SEC caused by incomplete resolution. Most interestingly, the separation range was larger also at the low-molar-mass region. At the high-molar-mass region, that would be expected due to the limited separation capacity of large molecules in SEC. Any explanation for higher local dispersity in HDC-SEC than in AsFlFFF has not been presented. The differences observed in this study for arabinoxylans and dextrans could be, at least partially, explained by the difference in the separation mechanisms, but any exact explanation based on the separation theories cannot be provided.

6.7 Challenges in the macromolecular characterization of branched polysaccharides

Aggregates were observed in aqueous solutions of cereal arabinoxylans, high-molar-mass dextrans from the *Weissella* and *Leuconostoc* genera, and galactomannan samples. Interestingly, evidence of aggregation was also found for the DMSO solution of the *W. confusa* dextran. Aggregation thus complicates the solution characterization of polymers. In some cases, as with WAX-LV and 2Gal-L containing large aggregates, aggregation could be observed directly from the light-scattering peak shape or molar mass distribution, whereas in the case of the unmodified galactomannan sample (6Gal-H) and the dextrans from *W. confusa* and *L. citreum*, aggregation could not be discovered solely from the aqueous SEC or AsFlFFF data. In principle, the form of polysaccharide aggregates may vary, and due to the separation based on the volume in SEC and AsFlFFF (and not molar mass), the possibility for coelution of aggregates with individual molecules is high. This kind of imperfect resolution has been previously reported to occur in SEC analysis of branched polymers when the relationship between molar mass and size (volume) is not constant (section 2.4.1).

Similar problems of coelution were observed when the arabinoxylans from wheat and rye were modified with α-L-arabinofuranosidase AXH-m. The extensive removal of side units increased the density of especially higher-molar-mass molecules that eluted with the lower-molar-mass molecules. That is one example of a fundamental dilemma of SEC and AFlFFF characterization of (enzymatically) modified heterogeneous sample of biological origin: the chemical structure (molar mass) is altered, but separation techniques recognize differences only as hydrodynamic volumes.

The heterogeneity of chemical structures and poor solubility of branched polysaccharides with high-molar-mass and/or low DP samples complicate the solution characterization. Even the solubility of dextran samples (DMSO, water) decreased heavily as the molar mass increased. The smaller dextran standards were soluble, but the dextrans from *W. confusa* and *L. citreum* and the standard with the highest M_w of 11 900 000 g/mol were significantly less soluble.

7 Conclusions

In this study, the dilute solutions of cereal arabinoxylans, guar galactomannans, and dextrans from LAB were investigated using SEC and AsFlFFF. Arabinoxylans and dextrans were dissolved in water containing 0.1 M $NaNO_3$ and DMSO containing 0.01 M LiBr to see the effect of solvent, whereas the characterization of guar galactomannans was accomplished only in aqueous solution due to their insolubility in DMSO. To study the effect of the structure on the solution properties of polysaccharides, structurally different arabinoxylans and dextrans from different origin were compared. The fine structure of commercial, pure arabinoxylans was further modified with specific enzymes in order to study the effect of side units in detail. In the case of galactomannan from guar, the DS and DP were modified enzymatically to include the effect of chain length on the solution characterization of galactomannan with relatively high molar mass.

Characterization of arabinoxylans and dextrans in different solvents revealed the presence of aggregates in aqueous polysaccharide solutions. The number of aggregates in arabinoxylan solutions was low as confirmed with the AsFlFFF analyses, in which the large aggregates were well resolved from the individual molecules. The aggregation in the case of high-molar-mass dextrans from LAB was more significantly seen as relatively large differences in the molar masses obtained in two solvents. Interestingly, the evidence of aggregation for the dextran from *W. confusa* in DMSO solution was found although the aggregation tendency in aqueous solution was clearly higher. The aggregation of native guar galactomannan was revealed by comparing the molar mass and intrinsic viscosity data of the samples with altered DP and DS. Moderate debranching of native galactomannan decreased the aggregation tendency. When the DS of galactomannan was decreased more extensively, large aggregates were observed in the low DP sample whereas the samples with higher DP were mostly insoluble. The aggregation was more pronounced also for the arabinoxylan with a low number of side units in aqueous solution. The water solubility of polysaccharides was decreased with increasing molar mass and with extensive decrease in DS. The solubility of high-molar-mass dextrans was low in water and in DMSO.

In the case of cereal arabinoxylans, the unmodified arabinoxylans from wheat and rye contained the same number of α-L-Ara*f* side groups but a different substitution pattern. Thus, with arabinoxylans, conclusions on the effect of side units could be drawn by comparing structurally different wheat and rye arabinoxylans. The rye arabinoxylan, with a higher degree of monosubstituted β-D-Xyl*p* residues than in wheat arabinoxylan (i.e., the distribution of α-L-Ara*f* side units is more even along the xylan backbone when monosubstituted β-D-Xyl*p* residues dominate over disubstituted ones and at the same time the total content of the

substituted β-D-Xyl*p* residues is higher), was found to have slightly more extended solution conformation compared with wheat arabinoxylan. The effect of side units on the solution conformation of arabinoxylans and galactomannans could be further studied by the samples with enzymatically altered DS. However, the effect of the α-L-Ara*f* side groups on the solution conformation of water-soluble arabinoxylans was not very significant. In terms of industrial utilization of arabinoxylans, this can be regarded as favorable because the arabinose content in various potential arabinoxylan sources, such as cereal husks and straw, is naturally low. Arabinoxylans from these agricultural side-streams could thus be exploited in different applications regardless of their low DS. Guar galactomannans behaved in a similar way as arabinoxylans: the content of the α-D-Gal*p* side units seemed not to have a role in the solution conformation of galactomannans.

The results of this study clearly showed the power of combining various tools in the dilute solution characterization: SEC with MALS/VISC/RI/UV detection, AsFlFFF with MALS/RI detection, and specific enzymatic treatments. The advantage of SEC is the possibility of using organic solvents, such as DMSO, as an eluent besides aqueous eluents and the compatibility of the viscometric detector. With AsFlFFF, good resolution was obtained especially for arabinoxylan samples with a low number of large aggregates. To better understand the differences in the SEC and AsFlFFF data, however, the differences in the separation mechanisms should be established. Enzymes proved to be invaluable tools particularly in characterizing galactomannan solutions: aggregation of native guar galactomannan was not revealed solely with SEC and AsFlFFF analysis, but only after comparing the molar mass and intrinsic viscosity data of the unmodified and partially debranched samples.

8 References

AACC. 2001. The definition of dietary fibre. Cereal Foods World 46: 112-26.

Alekseeva OV, Eliseeva OV, Noskov AV, Rozhkova OV. 2007. Hydrodynamic and bulk properties of dextrans in liquid media. Russ J Appl Chem 80:482-5.

Andrewartha KA, Phillips DR, Stone BA. 1979. Solution properties of wheat-flour arabinoxylans and enzymically modified arabinoxylans. Carbohydr Res 77:191-204.

Annison G, Choct M, Cheetham NW. 1992. Analysis of wheat arabinoxylans from a large-scale isolation. Carbohydr Polym 19:151-9.

Aspinall GO, Ferrier RJ. 1957. The constitution of barley-husk hemicelluloses. J Chem Soc 4188-4194.

Aspinall GO. 1959. Structural chemistry of the hemicelluloses. Adv Carbohydr Chem 14:429-68.

Aspinall GO. 1980. Chemistry of cell wall polysaccharides. Biochem Plants 3:473-500.

Basedow AM, Ebert KH, Ruland U. 1978. Specific refractive index increments of dextran fractions of different molecular weights. Makromol Chem 179:1351-3.

Bengtsson S, Åman P, Andersson RE. 1992a. Structural studies on water-soluble arabinoxylans in rye grain using enzymic hydrolysis. Carbohydr Polym 17:277-84.

Bengtsson S, Andersson R, Westerlund E, Åman P. 1992b. Content, structure and viscosity of soluble arabinoxylans in rye grain from several countries. J Sci Food Agric 58:331-7.

Bergmans MEF, Beldman G, Gruppen H, Voragen AGJ. 1996. Optimization of the selective extraction of (glucurono)arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. J Cereal Sci 23:235-45.

Biely P, Vrsanska M, Tenkanen M, Kluepfel D. 1997. Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151-66.

Borneman WS, Ljungdahl LG, Hartley RD, Akin DE. 1992. Purification and partial characterization of two feruloyl esterases from the anaerobic fungus *Neocallimastix* strain MC-2. Appl Environ Microbiol 58:3762-6.

Brandrup J, Immergut EH, Grulke EA, Abe A, Bloch DR (Eds.). 2005. Polymer Handbook, Fourth Edition. John Wiley & Sons. 2336 pp.

Brewer AK, Striegel AM. 2009. Particle size characterization by quadruple-detector hydrodynamic chromatography. Anal Bioanal Chem 393:295-302.

Brummer Y, Cui SW. 2005. Understanding carbohydrate analysis. In: Cui SW, Ed. Food Carbohydrates. Boca Raton, FL, USA: Taylor & Francis Group. pp. 67-104.

Burchard W. 1999. Solution properties of branched macromolecules. Adv Polym Sci 143:113-94.

Burchard W. 2005. Light scattering from Polysaccharides. In: Dumitriu S, Ed. Polysaccharides -Structural diversity and functional versatility. Second edition. Marcel Dekker, NY, USA. pp. 189-252.

CAZy - Carbohydrate Active enZymes. www.cazy.org.

Cheng Y, Brown KM, Prud'homme RK. 2002. Preparation and characterization of molecular weight fractions of guar galactomannans using acid and enzymatic hydrolysis. Int J Biol Macromol 31:29-35.

Chmelik J, Chmelikova J, Novotny MV. 1997. Characterization of dextrans by size-exclusion chromatography on unmodified silica gel columns, with light-scattering detection, and capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr A 790:93-100.

Ciucanu I, Kerek F. 1984. A simple and rapid method for the permethylation of carbohydrates. Carbohydr Res 131:209-17.

Cole EW. 1969. Physicochemical properties of a wheat flour hemicellulose in solution. Cereal Chem 46:382-90.

Collins HM, Burton RA, Topping DL, Liao M, Bacic A, Fincher GB. 2010. Variability in fine structures of noncellulosic cell wall polysaccharides from cereal grains: potential importance in human health and nutrition. Cereal Chem 87:272-82.

Côté, GL, Leathers TD. 2005. A method for surveying and classifying *Leuconostoc spp*. glucansucrases according to strain-dependent acceptor product patterns. J Ind Microbiol Biotechnol 32:53-60.

Daas PJH, Schols HA, de Jongh HHJ. 2000. On the galactosyl distribution of commercial galactomannans. Carbohydr Res 329:609-19.

Dea ICM, Morrison A. 1975. Chemistry and interactions of seed galactomannans. Adv Carbohydr Chem Biochem 31:241-312.

Dervilly G, Saulnier L, Roger P, Thibault JF. 2000. Isolation of homogeneous fractions from wheat water-soluble arabinoxylans. Influence of the structure on their macromolecular characteristics. J Agric Food Chem 48:270-8.

Dervilly G, Leclercq C, Zimmermann D, Roue C, Thibault JF, Saulnier L. 2002. Isolation and characterization of high molar mass water-soluble arabinoxylans from barley and barley malt. Carbohydr Polym 47:143-9.

Dervilly-Pinel G, Thibault JF, Saulnier L. 2001a. Experimental evidence for a semi-flexible conformation for arabinoxylans. Carbohydr Res 330:365-372.

Dervilly-Pinel G, Rimsten L, Saulnier L, Andersson R, Åman P. 2001b. Water-extractable arabinoxylan from pearled flours of wheat, barley, rye and triticale. Evidence for the presence of ferulic acid dimers and their involvement in gel formation. J Cereal Sci 34:207-14.

Dhami R, Harding SE, Elizabeth NJ, Ebringerová A. 1996. Hydrodynamic characterization of the molar mass and gross conformation of corncob heteroxylan AGX. Carbohydr Polym 28:113-9.

Ebringerová A, Hromádková Z, Burchard W, Dolega R, Vorwerg W. 1994. Solution properties of water-insoluble rye-bran arabinoxylan. Carbohydr Polym 24:161-9.

Enzyme commission (EC) numbers. http://www.chem.qmul.ac.uk/iubmb/enzyme/.

Fauré R, Courtin CM, Delcour JA, Dumon C, Faulds CB, Fincher GB, Fort S, Fry SC, Halila S, Kabel MA, Pouvreau L, Quemener B, Rivet A, Saulnier L, Schols HA, Driquez H, O´Donohue MJ. 2009. A brief and informationally rich naming system for oligosaccharide motifs of heteroxylans found in plant cell walls. Aust J Chem 62:1-5.

Fishman ML, Doner LW, Chau HK, Hoagland PD. 2000. Characterization of hemicellulose B from corn fiber by high-performance size-exclusion chromatography with on-line molar mass and viscometric detection. Int J Polym Anal Charact 5:359-79.

Gaborieau M, Nicolas J, Save M, Charleux B, Vairon J, Gilbert RG, Castignolles P. 2008. Separation of complex branched polymers by size-exclusion chromatography probed with multiple detection. J Chromatogr A 1190:215-23.

Giddings JC, Yang FJ, Myers MN. 1976. Theoretical and experimental characterization of flow field-flow fractionation. Anal Chem 48:1126-32.

Giddings JC. 1993. Field-flow fractionation: analysis of macromolecular, colloidal, and particulate materials. Science 260:1456-65.

Giddings JC. 2000. The field-flow fractionation family: underlying principles. In: Schimpf M, Caldwell K, Giddings JC, Eds. Field-flow fractionation handbook. John Wiley & Sons, NY, USA, pp 3-30.

Gidley MJ, Hanashiro I, Hani NM, Hill SE, Huber A, Jane J, Liu Q, Morris GA, Rolland-Sabate A, Striegel AM, Gilbert RG. 2010. Reliable measurements of the size distributions of starch molecules in solution: Current dilemmas and recommendations. Carbohydr Polym 79:255-61.

Gilbert RG. 2011. Size-separation characterization of starch and glycogen for biosynthesis-structure-property relationships. Anal Bioanal Chem. 399:1425-38.

Goring DAI, Timell TE. 1960. Molecular properties of six 4-O-methylglucuronoxylans. J Phys Chem 64:1426-30.

Grönwall, AJT, Ingelman BGA. Aktiebolaget Pharmacia, Uppsala. 19.5.1944. Infusion and injection fluids. US patent 2437518.

Grubisic Z, Rempp P, Benoit H. 1996. A universal calibration for gel permeation chromatography. J Polym Sci Part B 34:1707-14.

Gruppen H, Hamer RJ, Voragen AGJ. 1991. Barium hydroxide as a tool to extract pure arabinoxylans from water-insoluble cell wall material of wheat flour. J Cereal Sci 13:275-90.

Gruppen H, Kormelink FJM, Voragen AGJ. 1993. Water-unextractable cell wall material from wheat flour. 3. A structural model for arabinoxylans. J Cereal Sci 18:111-28.

Hamielec AE, Ouano AC. 1978. Generalized universal molecular weight calibration parameter in GPC. J Liq Chromatogr 1:111-20.

Hamielec AE, Ouano AC, Nebenzahl LL. 1978. Characterization of branched poly(vinyl acetate) by GPC and low angle laser light scattering photometry. J Liq Chromatogr 1:527-54.

Hannuksela T, Tenkanen M, Holmbom B. 2002. Sorption of dissolved galactoglucomannans and galactomannans to bleached kraft pulp. Cellulose 9:251-61.

Heinze T, Liebert T, Heublein B, Hornig S. 2006. Functional Polymers Based on Dextran. In Klemm D. Ed. Polysaccharides II. Springer Berlin, Heidelberg, pp. 199-291.

Henry RJ. 1987. Pentosan and (1→3), (1→4)-b-glucan concentrations in endosperm and whole grain of wheat, barley, oats and rye. J Cereal Sci 6:253-8.

Hettrich K, Fischer S, Schroeder N, Engelhardt J, Drechsler U, Loth F. 2006. Derivatization and characterization of xylan from oat spelts. Macromol Symp 232:37-48.

Hoffmann RA, Leeflang BR, de Barse MM, Kamerling JP, Vliegenthart JF. 1991. Characterisation by 1H-n.m.r. spectroscopy of oligosaccharides, derived from arabinoxylans of white endosperm of wheat, that contain the elements →4)[a-L-Ara*f*-(1→3)]-b-D-Xyl*p*-(1→ or →4)[a- L-Ara*f*-(1→2)][a-L-Ara*f*-(1→3)]-b-D-Xyl*p*-(1→. Carbohydr Res 221:63-81.

Hromádková Z, Ebringerová A. 1987. Isolation and characterization of hemicelluloses from rye bran. Nahrung 31:149-56.

Hughes SA, Shewry PR, Li L, Gibson GR, Sanz ML, Rastall RA. 2007. *In vitro* fermentation by human fecal microflora of wheat arabinoxylans. J Agric Food Chem 55:4589-95.

Höije A, Gröndahl M, Tømmeraas K, Gatenholm P. 2005. Isolation and characterization of physicochemical and material properties of arabinoxylans from barley husks. Carbohydr Polym 61:266-75.

Höije A, Sandström C, Roubroeks JP, Andersson R, Gohil S, Gatenholm P. 2006. Evidence of the presence of 2-O-β-D-xylopyranosyl-α-L-arabinofuranose side chains in barley husk arabinoxylan. Carbohydr Res 341:2959-66.

Höije A, Sternemalm E, Heikkinen S, Tenkanen M, Gatenholm P. 2008. Material properties of films from enzymatically tailored arabinoxylans. Biomacromolecules 9:2042-7.

Ioan CE, Aberle T, Burchard W. 2000. Structure properties of dextran. 2. Dilute solution. Macromolecules 33:5730-9.

Isenberg SL, Brewer AK, Côté GL, Striegel AM. 2010. Hydrodynamic versus size exclusion chromatography characterization of alternan and comparison to off-line MALS. Biomacromolecules 11:2505-11.

Izydorczyk MS, Biliaderis CG. 1993. Structural heterogeneity of wheat endosperm arabinoxylans. Cereal Chem 70:641-6.

Izydorczyk MS, Biliaderis CG. 1995. Cereal arabinoxylans: advances in structure and physiochemical properties. Carbohydr Polym 28:33-48.

Izydorczyk MS, Macri LJ, MacGregor AW. 1998a. Structure and physicochemical properties of barley non-starch polysaccharides. I. Water-extractable β-glucans and arabinoxylans. Carbohydr Polym 35:249-58.

Izydorczyk MS, Macri LJ, MacGregor AW. 1998b. Structure and physicochemical properties of barley non-starch polysaccharides. II. Alkali-extractable β-glucans and arabinoxylans. Carbohydr Polym 35:259-69.

Jeanes A, Haynes WC, Wilham CA, Rankin JC, Melvin EH, Austin M, Cluskey JE, Fisher BE, Tsuchiya HM and Rist CE. 1954. Characterization and classification of dextrans from ninety-six strains of bacteria. J Am Chem Soc 76:5041-52.

Jones RG, Wilks ES, Val Metanomski W, Kahovee J, Hess M, Stepto R, Kitayama T, Editors. 2009. Compendium of Polymer Terminology and Nomenclature: IUPAC recommendations 2008. p. 443.

Kapoor VP, Milas M, Taravel FR, Rinaudo M. 1994. Rheological properties of seed galactomannan from *Cassia nodosa* buch.-hem. Carbohydr Polym 25:79-84.

Katina, K, Maina NH, Juvonen R, Flander L, Johansson L, Virkki L, Tenkanen M, Laitila A. 2009. In situ production and analysis of *Weissella confusa* dextran in wheat sourdough. Food Microbiol 26:734-43.

Kratochvíl, P. 1987. Classical light scattering from polymer solutions. Polymer Science Library 5. Elsevier science publishers B. V, Amsterdam, The Netherlands, pp. 334.

Kusakabe I, Ohgushi S, Yasui T, Kobayashi T. 1983. Studies on the xylanase system of Streptomyces. Part X. Structures of the arabinoxylo-oligosaccharides from the hydrolytic products of corncob arabinoxylan by a xylanase from Streptomyces. Agric Biol Chem 47:2713-23.

Köhnke T, Östlund A, Brelid H. 2011. Adsorption of arabinoxylan on cellulosic surfaces: influence of degree of substitution and substitution pattern on adsorption characteristics. Biomacromolecules 12:2633-41.

Lacaze G, Wick M, Cappelle S. 2007. Emerging fermentation technologies: Development of novel sourdoughs. Food Microbiology 24:155-60.

Lawther JM, Sun R, Banks WB. 1995. Extraction, fractionation, and characterization of structural polysaccharides from wheat straw. J Agric Food Chem 43:667-75.

Lee YC. 1990. High-performance anion-exchange chromatography for carbohydrate analysis. Anal Biochem 189:151-62.

Lee YC. 1996. Carbohydrate analyses with high-performance anion-exchange chromatography. J Chromatogr A 720:137-49.

Litzen A. 1993. Separation speed, retention, and dispersion in asymmetrical flow field-flow fractionation as functions of channel dimensions and flow rates. Anal Chem 65:461-70.

Lu ZX, Walker KZ, Muir JG, Mascara T, O'Dea K. 2000. Arabinoxylan fiber, a byproduct of wheat flour processing, reduces the postprandial glucose response in normoglycemic subjects. Am J Clin Nutr 71:1123-8.

Maina NH, Tenkanen M, Maaheimo H, Juvonen R, Virkki L. 2008. NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoc citreum* and *Weissella confusa*. Carbohydr Res 343:1446-55.

Maina NH, Virkki L, Pynnönen H, Maaheimo H, Tenkanen M. 2011. Structural Analysis of Enzyme-Resistant Isomaltooligosaccharides Reveals the elongation of α-(1→3)-linked branches in *Weissella confusa* dextran. Biomacromolecules 12:409-18.

McNeil M, Albersheim P, Taiz L, Jones RL. 1975. Structure of plant cell walls. VII. Barley aleurone cells. Plant Physiol 55:64-8.

Merienne S, Busnel J, Fricoteaux F, Prudhomme J. 2000. Size exclusion chromatography of dextrans in DMSO as eluent. J Liq Chromatogr Rel Technol 23:1745-56.

Mikkonen KS, Rita H, Helén H, Talja RA, Hyvönen L, Tenkanen M. 2007. Effect of polysaccharide structure on mechanical and thermal properties of galactomannan-based films. Biomacromolecules 8:3198-205.

Mikkonen KS, Heikkinen S, Soovre A, Peura M, Serimaa R, Talja RA, Helén H. Hyvönen L, Tenkanen M. 2009a. Films from oat spelt arabinoxylan plasticized with glycerol and sorbitol. J Appl Polym Sci 114:457-66.

Mikkonen KS, Tenkanen M, Cooke P, Xu C, Rita H, Willför S, Holmbom B, Hicks KB, Yadav MP. 2009b. Mannans as stabilizers of oil-in-water beverage emulsions. LWT - Food Science and Technology 42:849-55.

Mikkonen KS, Heikkilä MI, Helén H, Hyvönen L, Tenkanen M. 2010. Spruce galactoglucomannan films show promising barrier properties. Carbohydr Polym 79:1107-12.

Mikkonen KS, Pitkänen L, Liljeström V, Bergström EM, Serimaa, R, Salmén L, Tenkanen M. Arabinoxylan structure affects the reinforcement of films by microfibrillated cellulose. Submitted.

Monchois V, Willemot R, Monsan P. 1999. Glucansucrases: mechanism of action and structure–function relationships. FEMS Microbiol Rev 23:131-51.

Monsan P, Bozonnet S, Albenne C, Joucla G, Willemot R, Remaud-Siméon M. 2001. Homopolysaccharides from lactic acid bacteria. Int Dairy J 11:675-85.

Mourey T. 2004. SEC molecular-weight-sensitive detection. Int J Polym Anal Charact 9:97-135.

Nilsson M, Saulnier L, Andersson R, Åman P. 1996. Water unextractable polysaccharides from three milling fractions of rye grain. Carbohydr Polym 30:229-37.

Nilsson M, Andersson R, Andersson RE, Autio K, Åman P. 2000. Heterogeneity in a water-extractable rye arabinoxylan with a low degree of disubstitution. Carbohydr Polym 41:397-405.

Nilsson L, Leeman M, Wahlund K, Bergenståhl B. 2006. Mechanical degradation and changes in conformation of hydrophobically modified starch. Biomacromolecules 7:2671-9.

Nordmeier E. 1993. Static and dynamic light-scattering solution behavior of pullulan and dextran in comparison. J Phys Chem 97:5770-85.

O'Donoghue EM, Somerfield SD. 2009. Plant cell wall polysaccharides: a commentary on their role as agents for food structure and for health. N Z J For Sci 39:169-85.

Ordaz-Ortiz JJ, Saulnier L. 2005. Structural variability of arabinoxylans from wheat flour. Comparison of water-extractable and xylanase-extractable arabinoxylans. J Cereal Sci 42: 119-25.

Oscarsson M, Andersson R, Salomonsson A, Åman P. 1996. Chemical composition of barley samples focusing on dietary fiber components. J Cereal Sci 24:161-70.

Otte T, Brüll R, Macko T, Pasch H, Klein T. 2010. Optimisation of ambient and high temperature asymmetric flow field-flow fractionation with dual/multi-angle light scattering and infrared/refractive index detection. J Chromatogr A 1217:722-30.

Pastell H, Tuomainen P, Virkki L, Tenkanen M. 2008. Step-wise enzymatic preparation and structural characterization of singly and doubly substituted arabinoxylo-oligosaccharides with non-reducing end terminal branches. Carbohydr Res 343:3049-57.

Pastell H, Virkki L, Harju E, Tuomainen P, Tenkanen M. 2009. Presence of 1→3-linked 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranosyl side chains in cereal arabinoxylans. Carbohydr Res 344:2480-8.

Pastell H. 2010. Preparation, structural analysis and prebiotic potential of arabinoxylo-oligosaccharides, EKT-series 1463, PhD Thesis, University of Helsinki.

Picout DR. Ross-Murphy SB, Errington N, Harding SE. 2001. Pressure cell-assisted solution characterization of polysaccharides. 1. Guar gum. Biomacromolecules 2:1301-9.

Picout DR, Ross-Murphy SB. 2002. On the chain flexibility of arabinoxylans and other β-(1→4) polysaccharides. Carbohydr Res 337:1781-4.

Picout DR, Ross-Murphy SB. 2007. On the Mark-Houwink parameters for galactomannans. Carbohydr Polym 70:145-8.

Podzimek S, Vlcek T, Johann C. 2001. Characterization of branched polymers by size exclusion chromatography coupled with multiangle light scattering detector. I. Size exclusion chromatography elution behavior of branched polymers. J Appl Polym Sci 81:1588-94.

Porsch B, Sundelöf L. 1994. Size-exclusion chromatography and dynamic light scattering of dextrans in water: explanation of ion-exclusion behavior. J Chromatogr A 669:21-30.

Postnova Analytics. 2009. AF2000 Software manual.

Puls J. 1993. Substrate analysis of forest and agricultural wastes. In: Saddler JN, Ed. Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, UK, pp. 13-32.

Ragaee SM, Campbell GL, Scoles GJ, McLeod JG, Tyler RT. 2001. Studies on Rye (*Secale cereale* L.) lines exhibiting a range of extract viscosities. 1. Composition, molecular weight distribution of water extracts, and biochemical characteristics of purified water-extractable arabinoxylan. J Agric Food Chem. 49:2437-45.

Rantanen H, Virkki L, Tuomainen P, Kabel M, Schols H, Tenkanen M. 2007. Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus* endo-1,4-β-D-xylanase. Carbohydr Polym 68:350-9.

Reinhold VN, Reinhold BB, Costello CE. 1995. Carbohydrate molecular weight profiling, sequence, linkage, and branching data: ES-MS and CID. Anal Chem 67:1772-84.

Richardson PH, Willmer J, Foster TJ. 1998. Dilute solution properties of guar and locust bean gum in sucrose solutions. Food Hydrocolloids 12:339-48.

Robinson G, Ross-Murphy SB, Morris ER. 1982. Viscosity-molecular weight relationships, intrinsic chain flexibility, and dynamic solution properties of guar galactomannan. Carbohydr Res 107:17-32.

Robyt JF. 1986. Dextran. In: Kroschwitz JI, Ed. Encyclopedia of polymer science and engineering. Wiley-VCH, NY, USA, pp. 752-767.

Roessner D, Kulicke W. 1994. Online coupling of flow field-flow fractionation and multi-angle laser light scattering. J Chromatogr A 687:249-58.

Roger P, Baud B, Colonna P. 2001. Characterization of starch polysaccharides by flow field-flow fractionation-multi-angle laser light scattering-differential refractometer index. J Chromatogr A 917:179-85.

Rolland-Sabate A, Colonna P, Mendez-Montealvo MG, Planchot V. 2007. Branching features of amylopectins and glycogen determined by asymmetrical flow field flow fractionation coupled with multiangle laser light scattering. Biomacromolecules 8:2520-32.

Rolland-Sabate A, Guilois S, Jaillais B, Colonna P. 2011. Molecular size and mass distributions of native starches using complementary separation methods: asymmetrical flow field flow fractionation (A4F) and hydrodynamic and size exclusion chromatography (HDC-SEC). Anal Bioanal Chem 399:1493-505.

Saake B, Kruse T, Puls J. 2001. Investigation on molar mass, solubility and enzymatic fragmentation of xylans by multi-detected SEC chromatography. Bioresour Technol 80:195-204.

Saake B, Erasmy N, Kruse T, Schmekal E, Puls J. 2004. Isolation and characterization of arabinoxylan from oat spelts. In: Gatenholm P, Tenkanen M, Eds. Hemicelluloses: Science and technology, ACS symposium series 864. American chemical society. pp 52-65.

Saastamoinen M, Plaami S, Kumpulainen J. 1989. Pentosan and β-glucan content of Finnish winter rye varieties as compared with rye of six other countries. J Cereal Sci 10:199-207.

Seymour FR, Knapp RD, Chen ECM, Jeanes A, Bishop SH. 1979. Unusual dextrans, part V. Structural analysis of dextrans containing 2-O-α-D-glucosylated α-D-glucopyranosyl residues at the branch points, by use of carbon-13 nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. Carbohydr Res 71:231–50.

Shiiba K, Yamada H, Hara H, Okada K, Nagao S. 1993. Purification and characterization of two arabinoxylans from wheat bran. Cereal Chem 70:209-14.

Smith F. 1948. Constitution of carob gum. J Am Chem Soc 70:3249-53.

Son M, Jang E, Kwon O, Seo J, Kim I, Lee I, Park S, Lee S. 2008. Characterization of dextran produced from *Leuconostoc citreum* S5 strain isolated from Korean fermented vegetable. Eur Food Res Technol 226:697-706.

Stålbrand H, Siika-aho M, Tenkanen M, Viikari L. 1993. Purification and characterization of two β-mannanases from *Trichoderma reesei*. J Biotechnol 29:229-42.

Sternemalm E, Höije A, Gatenholm P. 2008. Effect of arabinose substitution on the material properties of arabinoxylan films. Carbohydr Res 343:753-7.

Striegel AM, Isenberg SL, Côté GL. 2009a. An SEC/MALS study of alternan degradation during size-exclusion chromatographic analysis. Anal Bioanal Chem 394:1887-93.

Striegel AM, Yau WW, Kirkland JJ, Bly DD. 2009b. Modern size-exclusion liquid chromatography. Second Edition. John Wiley & Sons, NJ, USA, pp. 494.

Sun R, Lawther JM, Banks WB. 1996. Fractional and structural characterization of wheat straw hemicelluloses. Carbohydr Polym 29:325-31.

Sundararajan PR, Rao VSR. 1969. Conformational studies of *β*-D-1,4'-xylan. Biopolymers 8:305-12.

Sundberg A, Sundberg K, Lillandt C, Holmbom B. 1996. Determination of hemicelluloses and pectins in wood and pulp fibers by acid methanolysis and gas chromatography. Nord Pulp Pap Res J 11:216-219, 226.

Tenkanen M. 2004. Enzymatic tailoring of hemicelluloses. In: Gatenholm P, Tenkanen M, Eds. Hemicelluloses: Science and technology, ACS symposium series 864. American chemical society. pp 292-311.

Tieking M, Korakli M, Ehrmann MA, Gänzle MG, Vogel RF. 2003. In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl Environ Microbiol 69:945-52.

Trogh I, Croes E, Courtin CM, Delcour JA. 2005. Enzymic degradability of hull-less barley flour alkali-solubilized arabinoxylan fractions by endoxylanases. J Agric Food Chem 53:7243-50.

van Bruijnsvoort M. Wahlund K-G, Nilsson G, Kok WT. 2001. Retention behavior of amylopectins in asymmetrical flow field-flow fractionation studied by multi-angle light scattering detection. J Chromatogr A 925:171-82.

van Laere KMJ, Beldman G, Voragen AGJ. 1997. A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. Appl Microbiol Biotechnol 47:231-5.

Viëtor, RJ. 1992. Structural characteristics of arabinoxylans from barley, malt and wort, PhD thesis. University of Wageningen. The Netherlands.

Viëtor RJ, Angelino SAGF, Voragen AGJ. 1992. Structural features of arabinoxylans from barley and malt cell wall material. J Cereal Sci 15:213-22.

Viëtor RJ, Hoffmann RA, Angelino SAGF, Voragen AGJ, Kamerling JP, Vliegenthart JFG. 1994. Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*. Carbohydr Res 254:245-55.

Vink H, Dahlström G. 1967. Refractive index increments for polymers in solutions in multicomponent solvents. Makromol Chem 109:249-52.

Vinkx CJA, Delcour JA, Verbrüggen MA, Gruppen H. 1995. Rye water-soluble arabinoxylans also vary in their 2-monosubstituted xylose content. Cereal Chem. 72, 227-8.

Vinkx CJA, Delcour JA. 1996. Rye (*Secale cereale* L.) arabinoxylans: A critical review. J Cereal Sci 24:1-14.

Virkki L, Johansson L, Ylinen M, Maunu S, Ekholm P. 2005. Structural characterization of water-insoluble nonstarchy polysaccharides of oats and barley. Carbohydr Polym 59:357-66.

Virkki L, Maina HN, Johansson L, Tenkanen M. 2008. New enzyme-based method for analysis of water-soluble wheat arabinoxylans. Carbohydr Res 343:521-9.

Viscotek. 2004. Model 270 Series Detectors, Instrument manual.

Wahlund K-G. 2000. Asymmetrical flow field-flow fractionation. In: Schimpf M, Caldwell K, Giddings JC, Eds. Field-flow fractionation handbook. John Wiley & Sons, NY, USA, pp 279-294.

Wang Q, Cui, S.W. 2005. Understanding the physical properties of food polysaccharides. In: Cui SW, Ed. Food Carbohydrates. Boca Raton, FL, USA: Taylor & Francis Group. pp. 161-217.

Westerlund E, Andersson R, Åman P. 1993. Isolation and chemical characterization of water-soluble mixed-linked β-glucans and arabinoxylans in oat milling fractions. Carbohydr Polym 20:115-23.

Wientjes RHW, Duits MHG, Jongschaap RJJ, Mellema J. 2000. Linear rheology of ruar Gum solutions. Macromolecules 33:9594-605.

Williams SKR, Lee D. 2006. Field-flow fractionation of proteins, polysaccharides, synthetic polymers, and supramolecular assemblies. J Sep Sci 29: 1720-1732.

Wittgren B, Wahlund K-G. 1997. Fast molecular mass and size characterization of polysaccharides using asymmetrical flow field-flow fractionation-multiangle light scattering. J Chromatogr A 760:205-18.

Wu Y, Cui W, Eskin NAM, Goff HD. 2009. An investigation of four commercial galactomannans on their emulsion and rheological properties. Food Res Int 42:1141-6.

Yohannes G, Jussila M, Hartonen K, Riekkola M-L. 2011. Asymmetrical flow field-flow fractionation technique for separation and characterization of biopolymers and bioparticles. J Chromatogr A 1218:4104-16.

Zhang G, Hamaker BR. 2010. Cereal carbohydrates and colon health. Cereal Chem 87:331-41.

Zhang Y, Pitkänen L, Douglade J, Tenkanen M, Remond C, Joly C. 2011. Wheat bran arabinoxylans: Chemical structure and film properties of three isolated fractions. Carbohydr Polym 86:852-859.